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Tesis Doctoral

María Dolores Ibáñez Jaime

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Facultat de Farmàcia

Departament de Farmacologia

Programa de Doctorat en Biomedicina i Farmàcia

Commercial essential oils: sustainable alternatives in the agri-food industry

Tesis Doctoral

Presentada por:

María Dolores Ibáñez Jaime

Valencia, Septiembre de 2019

Dirigida por la Catedrática

María Amparo Blázquez Ferrer

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COMMERCIAL ESSENTIAL OILS: SUSTAINABLE ALTERNATIVES IN THE AGRI-FOOD INDUSTRY

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Directora: Catedrática

María Amparo Blázquez Ferrer

Valencia, septiembre 2019

Doctorado en Biomedicina y Farmacia

Dña. María Amparo Blázquez Ferrer, Full Professor of the University of Valencia

CERTIFIES:

that the work submitted by the Graduate **María Dolores Ibáñez Jaime**, entitled **"Commercial essential oils: sustainable alternatives in the agri-food industry"**, to obtain a PhD, has been carried out in the Department of Pharmacology of the University of Valencia, under my direction and advice.

Completed the experimental and bibliographic research, I authorize the presentation of the Thesis to be judged by the assessment panel appointed.

Valencia, September 23, 2019.

Fdo. Dña. María Amparo Blázquez Ferrer.

‘*No one knows what he can do*

until he tries”

Publilius Syrus

Dedicated to my grandparents

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The results obtained in this Doctoral Thesis have resulted in the following publications

- Ibáñez MD, Blázquez MA. Herbicidal value of essential oils from oregano-like flavour species. *Food and Agricultural Immunology* **2017**, 28(6), 1168-1180. Impact Factor (JCR): 2.568. JCR category rank: 37/133 (Q2) in "Food Science and Technology". Impact Factor (SJR): 0.548. SJR category rank: 69/320 (Q2) in "Agronomy and Crop Science".
- Ibáñez MD, Blázquez MA. Post-emergent herbicidal activity of *Eucalyptus globulus* Labill. essential *Nereis. Interdisciplinary Ibero-American Journal of Methods, Modelling and Simulation* **2018**, 10, 25-36.
- Ibáñez MD, Blázquez MA. Phytotoxicity of essential oils from culinary herbs against seed germination and seedling growth of selected weeds. *International Journal of Pharmacognosy and Phytochemical Research* **2018**, 10(4), 123-131. Impact Factor (SJR): 0.121. SJR category rank: 132/152 (Q4) in "Drug Discovery".
- Ibáñez MD, Blázquez MA. Phytotoxicity of essential oils on selected weeds: Potential hazard on food crops. *Plants* **2018**, 7(4), 79. Impact Factor (JCR): 2.632. JCR category rank: 59/228 (Q2) in "Plant Sciences". Impact Factor (SJR): 1.361. SJR category rank: 24/404 (Q1) in "Plant Science".
- Ibáñez MD, Blázquez MA. Ginger and turmeric essential oils for weed control and food crop protection. *Plants* **2019**, 8(3), 59. Impact Factor (JCR): 2.632. JCR category rank: 59/228 (Q2) in "Plant

Sciences". Impact Factor (SJR): 1.361. SJR category rank: 24/404 (Q1) in "Plant Science".

- Ibáñez MD, Blázquez MA. Tea tree and wintergreen essential oils in the management of the invasive species *Cortaderia selloana* and *Nicotiana glauca*. *Journal of Plant Protection Research* **2019** 59(2), 160-169. Impact Factor (SJR): 0.381. SJR category rank: 146/320 (Q2) in "Agronomy and Crop Science".
- Ibáñez MD, Blázquez MA. Phytotoxic effects of commercial *Eucalyptus citriodora*, *Lavandula angustifolia* and *Pinus sylvestris* essential oils on weeds, crops and invasive species. *Molecules* **2019** 24(15), 2847. Impact Factor (JCR): 3.060. JCR category rank: 68/172 (Q2) in "Chemistry Multidisciplinary" Impact Factor (SJR): 0.757. SJR category rank: 29/164 (Q1) in "Pharmaceutical Science".

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ABBREVIATIONS

ABTS	2,2’-Azino-Bis(3-ethylbenzthiazoline-6-sulfonic acid)
β-CD	β-Cyclodextrin
BHA	Butylated Hydroxyanisole
BHT	Butylhydroxytoluene
CAGR	Compound Annual Growth Rate
DPPH	2,2-Diphenyl-1-Picrylhydrazyl
EE	Encapsulation Efficiency
FRAP	Ferric Reduction Antioxidant Power
GC-MS	Gas Chromatography-Mass Spectrometry
MCC	Microcrystalline Cellulose
MIC	Minimum Inhibitory Concentration
Ph. Eur.	European Pharmacopoeia
TBARS	Thiobarbituric Acid-Reactive Substance

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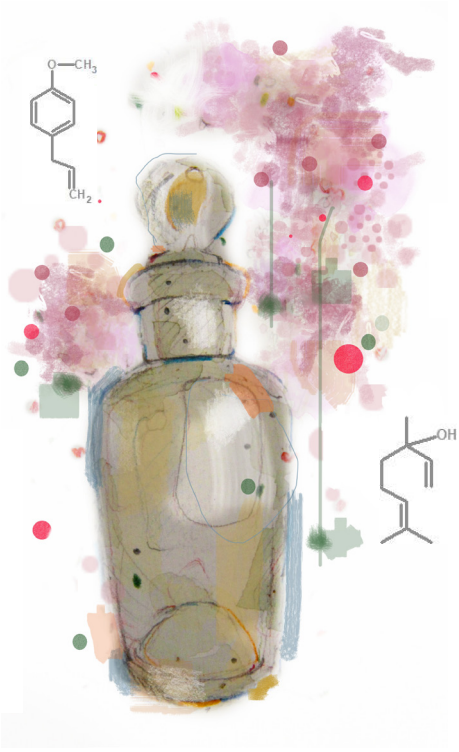
Foreword



Essential oils have enjoyed great popularity from ancient times to today due to agreeable scent and widely known beneficial properties. As a result, nowadays they have become valuable natural ingredients in perfume and cosmetics, food and beverages, agricultural, pharmaceutical and other industries for employment in human health, agriculture and environment, representing potentially safer and more sustainable alternatives to synthetic substances. This Doctoral Thesis has been developed with the aim of corroborating that essential oils are safer and more sustainable alternatives to synthetic products used in the agri-food industry, and consequently promoting their use as "bio" products to maintain and improve the quality of crops and food, as well as human health and environment.

It includes a compendium of scientific articles published in scientific journals indexed in Journal Citation Reports (JCR) and/or Scimago Journal Rank (SJR), positioned mainly in the first and second quartile, as well as other scientific articles indexed in different databases. It consists of a global summary with the main objectives, results and conclusions, followed by eight chapters that include the publications of this Doctoral Thesis. They are distributed on the one hand, in a section with the three articles published in the first quartile of SJR (*Chapters* 1-3), a second section (*Chapters* 4-7) with the other four accepted publications and a final chapter (unpublished data) with a comparative study about the phytotoxic effects of the commercial essential oils on food crops. *Chapter 1* corroborates the previous *in vitro* phytotoxic activity of winter savory and peppermint essential oils by means of *in vivo* assays using a commercial emulsifiable concentrate including these essential oils, representing these products as a natural and environmental-friendly alternative to synthetic herbicides. *Chapter 2* shows that turmeric essential oil represents a promising

alternative in the control of both the invasive species *Cortaderia selloana* and weeds invading cucumber crops. *Chapter 3* assigns lavender essential oil as an effective pre-emergent treatment for the control of *Lolium multiflorum* in cucumber crops. *Chapter 4* specifies the differences in the chemical composition and phytotoxic activity between oregano, marjoram and *Thymus mastichina* essential oils, and the need to include information about their main compounds to standardize their activity. *Chapter 5* demonstrates that tea tree and wintergreen essential oils can be useful in the control of the invasive species *C. selloana* and *N. glauca*, especially against the former that showed higher sensitivity to these essential oils. *Chapter 6* details the phytotoxic activity of rosemary and basil essential oils with remarkable inhibition of the seedling length of weeds tested. *Chapter 7* reports the post-emergent herbicidal effect of *Eucalyptus globulus* against *Echinochloa crus-galli*. *Chapter 8* searches for the possible side effects derived from the application of bioherbicides on food crops.

Summary

1. INTRODUCTION

1.1. AROMATIC PLANTS: SOURCE OF BIOACTIVE COMPOUNDS

"Nature is the best drug factory of all time."

From the beginning, aromatic plants have been widely prized for their beauty enhancing properties, as well as spiritual, aromatic and medicinal values. Humankind has been aware of the grand variety of possibilities that aromatic plants offer and has exploited these natural resources since ancient times for its uncountable advantages: fuel, clothing, shelter, food, health remedies…

Particularly, between all the identified features of aromatic plants, people have always had a continuous and special interest in the search for their medicinal properties and therapeutic uses that comes from the need to relieve and avoid pain and diseases. Ancient documents explaining the beneficial properties for health of ginseng, cinnamon bark, pepper, clove, garlic, juniper, among many others, are found worldwide. Amongst them, several manuscripts and books in which the greatest scholar of all time such as Hippocrates, Galena and Avicenna collected and upgraded the inherited knowledge at that time with their personal and professional experience regarding the valuable properties of these products (1,2). Especially remarkable is the contribution of Dioscorides, considered "the father of Pharmacognosy", who wrote the book ("encyclopaedia") called *De Materia Medica* in which he included plenty of data regarding the medicinal plants he found in his multiple trips, being the complete knowledge of medicinal plants at the time. Concurrently, 657 of 944 drugs that were described had a vegetal origin and he included information like their names in different

languages, descriptions of their appearance, location, mode of collection, making of the medicinal preparations, and their therapeutic effect (1). For instance, in this book, Dioscorides described the medicinal uses of opium poppy and related species:

> *"A little of it (taken with as much as a grain of ervum) is a pain-easer, a sleep-causer, and a digester, helping coughs and abdominal cavity afflictions. Taken as a drink too often it hurts (making men lethargic) and it kills. It is helpful for aches, sprinkled on with rosaceum; and for pain in the ears dropped in them with oil of almonds, saffron, and myrrh. For inflammation of the eyes it is used with a roasted egg yolk and saffron, and for erysipelas and wounds with vinegar; but for gout with women's milk and saffron. Put up with the finger as a suppository it causes sleep".*

Likewise, he differentiated between a number of the species included in the genus *Mentha*, which were used to relieve headache and stomach ache, and he reported on the diuretic effects of parsley. However, the abortive properties of chamomile were later denied.

> *"The flower, root, and the entire plant accelerate menstruation, the release of the embryo, and the discharge of urine and stone, provided that they are used in the form of an infusion and baths".*

Just like that, knowledge concerning the properties of aromatic plants was being discovered, accumulating and growing through time, passing this information from generation to generation. In this way, with the advance of science, the usage of medicinal plants gradually abandoned the empirical basis and began to rely on explanatory facts, so the experience became evidence. Nowadays, the value of medicinal plants in curing and

maintaining health is fully recognized. Furthermore, we know that these plants are a rich source of natural compounds of great structural variety and properties that can be used in drug synthesis (3–5). Concerning this, a continuous increase in the number of monographs on plant substances and semisynthetic substances have been included in the European Pharmacopoeia (Ph. Eur.), from 147 articles in Ph. Eur. 4 (2002) to 377 in Ph. Eur. 8 (2014). Indeed, this edition already included 123 monographs of plant substances and 254 regarding semisynthetic substances derived from plant products (6). Among them are quinine, the widest known antimalarian, obtained from Cinchona bark, the cardiac glycoside digoxin derived from foxglove (*Digitalis lanata* Ehrh.), the anticholinergic atropine from *Atropa belladonna* L., the analgesics morphine and codeine from opium poppy, and even the anticancer drugs such as vincristine and vinblastine from *Catharanthus roseus* (L.) G.Don.*;* and a large number of monographs (31 monographs on essential oil drugs and 33 monographs about essential oils) of essential oils with 5 monographs about some of the main components (eucalyptol, D-camphor, levomenthol, thymol, eugenol) of the essential oils (6).

In general, more than a tenth of the plant species (over 50 000 species) are used in pharmaceutical and cosmetic products. Still in South American, Asiatic and African continents, over 85 % of the populations predominantly rely on traditional medicine, especially on herbal medicines and, without going further, about 100 million people in the European Union and in some countries as high as 90% of the population do it too (7).

Moreover, the total global trade of medicinal aromatic plants has augmented more than two and half times in the past 18 years (8) as a result

of a growing reliability and demand in natural and organic products between consumers. Industry takes advantage of this using them not only in drug development, but also as nutraceuticals for health and wellness, colouring and flavouring agents in food and beverages, perfumes, cosmetics, detergents in cleaning… What is more, scientific research is still being carried out to discover new benefits of active plant compounds and furthermore to produce nature-based products to apply in the future.

1.2. ESSENTIAL OILS

"-¿Dinero o especias?
–Llevo conmigo especias que se cultivan en los campos de Toledo y que no se dan por aquí –dijo el joven ovejero-. A veces la gente prefiere que pague con ellas.
–¿Y cuáles son esas especias? –preguntó muy interesado el físico.
–Orégano, comino, pimienta, tomillo y azafrán.
– ¿Azafrán?
–En mi tierra se dice "onza de azafrán, onza de oro" por el gran valor que tiene –explicó el ovejero sonriendo por primera vez." Martínez de Lezea (9).

As it has been previously commented, an extensive variety of constituents with great structural diversity and functions can be found in aromatic plants. Among them, essential oils have become valuable natural ingredients in perfume, cosmetic, food, beverage, agricultural and pharmaceutical industries (10–12). They are so prized that according to Statistics Market Research Consulting, the Global Essential Oil Market accounted for $5.91 billion in 2016 and is expected to reach $12.85 billion by 2023 growing at a Compound Annual Growth Rate (CAGR) of 11.7% during the forecast period (13,14).

1.2.1. Brief history of the use of essential oils

Essential oils have been very valued products in the most ancient cultures. For instance, they were the protagonists in almost all aspects of Egyptian life as they were included in balms, ointments, powders… to be used in beauty care, religious ceremonies and for medicinal purposes. The Egyptians were important influencers in both Greek and Roman education who also used essential oils for therapeutic aims. In fact, Hippocrates, considered "the father of modern medicine", advised "*the way to health is to have an aromatic bath and scented massage every day*". Also, he strongly believed in the fumigation properties of aromatics and used them to combat plagues.

Similarly, essential oils were a main element of the Indian and Chinese ancient cultures, being principal in their medicine system. Indeed, hundreds of volatile oils, such as cinnamon, ginger, coriander… and their therapeutic properties were collected in leading documents, such as The Vedas, India's most sacred book, and Shennong's Herbal, the oldest medical text.

However, the use of essential oils started to become founded on a scientific basis with the contribution of René-Maurice Gattefossé, a French cosmetic chemist, who coined the term "aromatherapy" between 1920-1930 to specify the treatment of disease and injury using the aromatic essential oils. His fascination for essential oils started when he badly burned his hands during an experiment, as he described "*both my hands were covered with rapidly developing gas gangrene*". He submerged them in a container of lavender essential oil reporting "*just one rinse with lavender essence stopped the gasification of the tissue. This treatment was followed by profuse sweating and healing which began the next day*." After this

experience, he experimented the antiseptic and healing properties of essential oils on soldiers in the military hospitals during the I World War, in which he noted an increase in the rate of restoration. Since then, the medical benefits of essential oils have been studied and have demonstrated that not only have they stood the test of time, but they have proven their efficacy and effectiveness (15).

1.2.2. What are essential oils

The term "essential oil" does not mean that these compounds are essential for human life, instead they refer to the famous Paracelso who named distilled oils from herbs "quintessence" of the plant. The name mostly refers to the pleasant aroma of smell and taste they produce when evaporated, so that these products are so-called "essences".

According to the Ph. Eur. 9.5 edition (07/2018), essential oils are "odorous product, usually of complex composition, obtained from a botanically defined plant raw material by steam distillation, dry distillation, or a suitable mechanical process without heating. Essential oils are usually separated from the aqueous phase by a physical process that does not significantly affect their composition" (16).

Essential oils belong to the secondary metabolism of plants and, although they are present in minimum amounts (often below 1%), they have many important functions. Especially, they represent the closest thing to an immune system inside the plants: their characteristic flavour provides protection against destructive predators, such as herbivores and disease-causing organisms, contributes to the restoration and healing of damaged tissues and wounds, and attracts pollinating agents (12). On the other hand, this characteristic flavour has been appreciated worldwide because of its

pleasant flavour, so that they have been used in beauty, SPA and wellness, and moreover for their numerous pharmacological properties, among them anti-inflammatory, antiviral, repellent, antibacterial, antifungal, or antioxidant (10,11). At the moment, there are more than 3 000 essential oils that are physical and chemically characterized, and about 150 of which are manufactured on an industrial scale (12).

Despite the currently knowledge of essential oils, it is still needed to develop their chemistry, way of application and biological properties as well as the one of their individual components in order to discover other new and valuable applications in human health, agriculture and environment.

1.2.3. Chemical composition of essential oils

The analysis of the chemical composition of essential oils is an important step as the principal characteristics, such as odour and flavour, as well as biological activities of the plant will depend on it. Furthermore, the study of the essential oils' constituents is also significant for the quality control and freshness of the resulting product.

Essential oils are complex mixtures of more than 300 different lipophilic and highly volatile compounds, with a molecular weight below 300 Da. They represent less than 5% of the total plant composition, although each of them can be composed of from only a few up to a complex mixture of far more than 100 substances (17). Minor compounds have a strong impact on the flavour and characteristics of the essential oil. So, any significant blending, rectification and/or adulteration of commercial essential oils should be monitored by their biological activities (18). They are poorly soluble in water and generally less dense than this. They are liquid at room

temperature, with the exception of anise (*Pimpinella anisum* L.) essential oil, and nearly always with rotational and high refractory index (19).

They are biosynthesized and localized in specific parts of the cytoplasm of various plant organs. Substancially, in the secretory hairs/trichomes, epidermal cells, internal secretory cells, and the secretory pockets (20), where complex natural biochemical pathways (19) with different enzymatic reactions happen. In this way, the main phytochemical groups detected in essential oils are formed: terpenes, aromatic compounds and straight-chain compounds (17,21) (Figure 1).

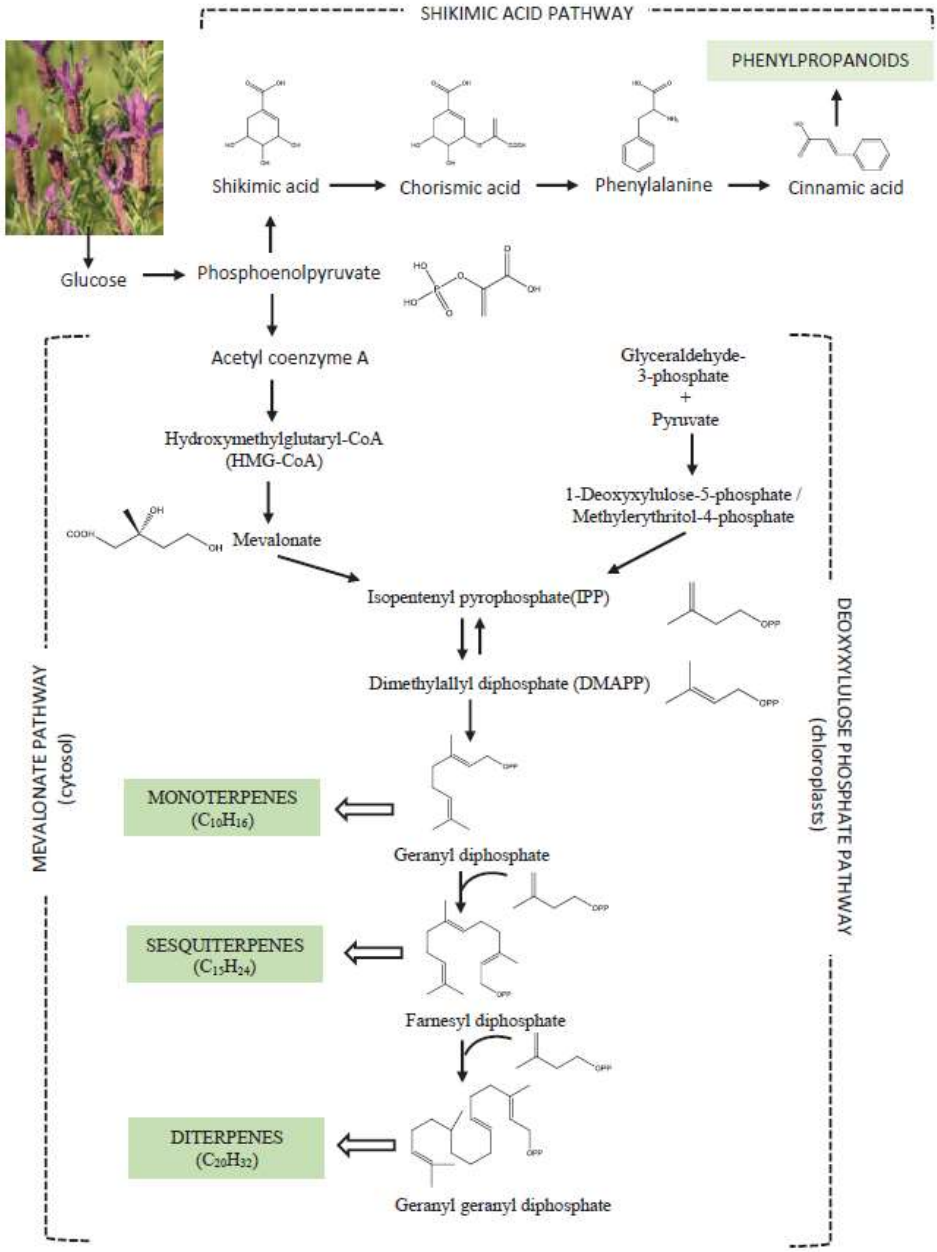


Figure 1. Biosynthetic pathways of terpenes and aromatic compounds in plants (20).

Terpenes

Terpenes are considered the largest class of secondary metabolites of the essential oils. In fact, plants synthesize several hundred distinct types of terpenoids (22). They are synthesized in the herb's cytosol, fungus and in some animals throught mevalonic acid (MVA route), and in plant plastids (chloroplast) and also by bacteria by 1-deoxyxylulose-D-5-phosphate (DXP) also known as methylerythritol phosphate (MEP route) pathways (19,23,24).

Essential oils' terpenes can be rearranged in different cyclic and acyclic assemblies by the action of terpene synthases. These structures can be divided in two major groups: hydrocarbons (Figure 2) and oxygenated (terpenoids) (Figure 3).

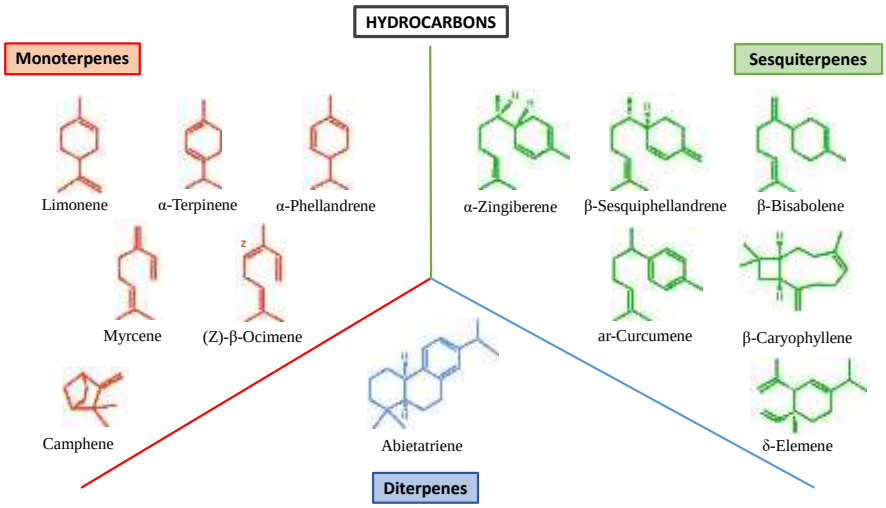


Figure 2. Chemical structures of common terpene hydrocarbons.

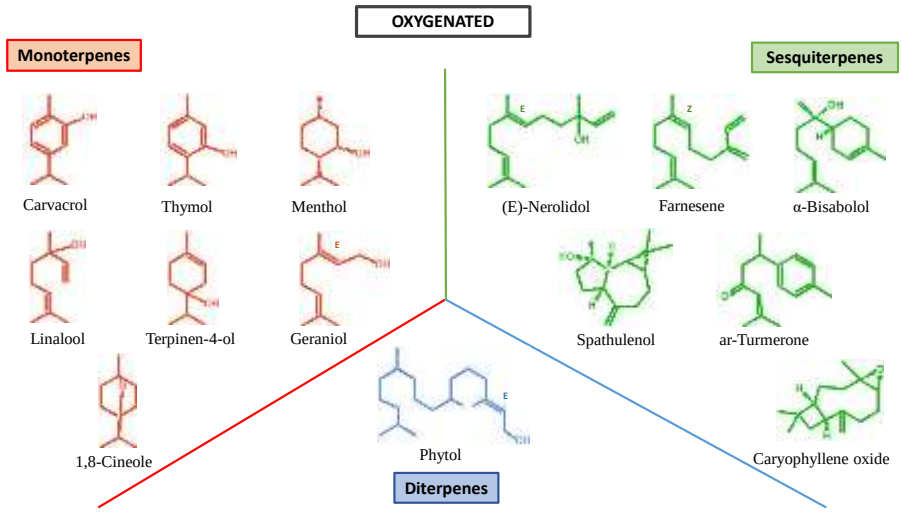


Figure 3. Examples of some oxygenated terpenes found in essential oils of plants.

Among them, monoterpenes are the dominant class of terpenes. They can be aromatic, acyclic and cyclic which are simultaneously subdivided in mono, bi and tricyclic. Their pharmacological properties have been demonstrated, including antifungal (citral, limonene), antibacterial (linalool, thymol), anti-spasmodic (α-pinene), antioxidant, anticancer and anti-inflammatory (limonene, 1,8-cineole). Besides, they have been observed to act as regulators of growth, heat, transpiration, inhibitors of tumour and oxidative phosphorilation, insect repellents, feline and canine attractants, and antidiabetics (25).

Sesquiterpenes are the following dominant group. They are also unsaturated linear, branched or cyclic (mono, bi and tricyclic) terpenes. Especially, certain sesquiterpenes have shown antibacterial and antifungal activities at high levels, such as in the case of cadinene, (Z)-β-farnesene, γ-muurolene, spathulenol and α-selinene (26).

On the other hand, diterpenes are present in low concentrations in essential oils, because they are too heavy to be evaporated. They can also experience rearrangements and/or substitutions.

In general, the roles of terpenes are particularly remarkable in secondary metabolism of plants regarding signal transduction, reproduction, communication, climatic acclimation and principally in defence and development, such as in the case of maize (*Zea mays* L.) in which they contribute to pest resistance (27).

Hydrocarbons

Terpene hydrocarbons are common terpenes in essential oils. They represent a large and structurally diverse class of molecules due to the multiple rearrangements (Figure 2). Despite this, they share as a common building block an isoprene unit (2-methyl-1,3-butadiene) with the general structural formula $(C_5H_8)n$. Regarding this, essential oils include mainly monoterpenes consisting of two isoprene units (2 x C_5) with general molecular formula of $C_{10}H_{16}$, sesquiterpenes containing three isoprene units (3 x C_5) being the formula $C_{15}H_{24}$, and diterpene with four isoprene units (4 x C_5) ($C_{20}H_{40}$) (11); *nor*-terpenes or bigger structures with a higher number of isoprene units are less common in essential oils.

Oxygenated (terpenoids)

Oxygenated compounds constitute a varied group of terpenes as they can be subdivided in aldehydes, ethers, alcohols, esters, ketones, phenols and epoxides (Figure 3). These compounds are usually the responsible for giving the fragrance due to their high odoriferous properties. For instance, thymol and carvacrol contribute to the herbal odour of oregano essential oil,

and piperitone and pulegone provide minty notes to essential oils derived from *Mentha* spp.

Aromatic compounds

Essential oils contain a complex but relatively small part of benzene derivatives, called aromatic compounds (C_6-C_3 and C_6-C_1) (Figure 4). These are synthesized from the aromatic amino acids phenylalanine and L-tyrosine through the shikimic acid pathway. From the primary structure of the aromatic compounds, a series of derivatives can emerge by adding hydroxyl, methoxy or methylene dioxy groups to the aromatic ring together with hydroxyl or carbonyl groups to the propyl side chain (28,29).

They are also involved in the sensorial properties of the plant, being important in flavour and fragrance industry, defence and protection (30,31). In addition, phenolic compounds provide interesting properties to plants: they are well-known by their antioxidant properties contributing in the prevention of cancer, cardiovascular and neurodegenerative diseases (32); as well as for their antimicrobial, anti-inflammatory, antiviral, hypolipidemic and hypoglycemic effects (31).

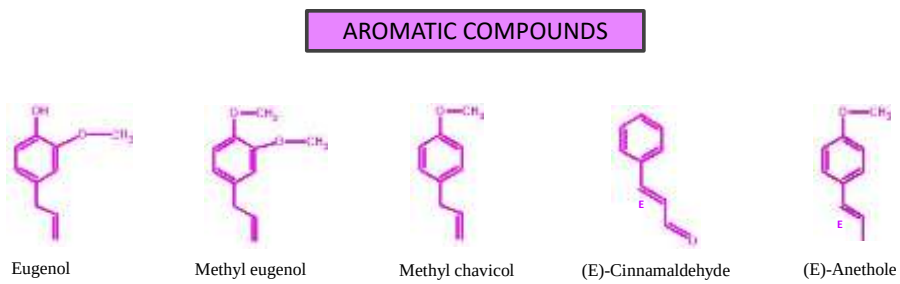


Figure 4. Molecular structures of some examples of aromatic compounds found in essential oils of plants.

Straight-chain compounds

Finally, straight-chain non-terpenoid hydrocarbons and their oxygen derivatives (alcohols, aldehydes, ketones, acids, ethers and esters) have been also identified in essential oils (Figure 5).

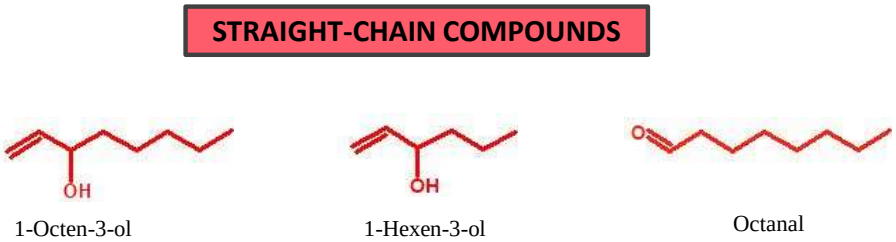


Figure 5. Chemical structures of straight-chain compounds in essential oils of plants.

1.2.4. Methods for extracting and identifying essential oils

Essential oils are localized in various plant organs (flowers, fruits, seeds, leaves, stems, and roots). For instance, ginger essential oil comes from the rhizomes of the plant, rose essential oil from the petals of the rose flower, citric essential oils (orange, mandarin, lemon, lime) are extracted from the peel of the fruits, pine essential oil from the needles and twigs of pine trees.

With the aim of trapping essential oils from aromatic plants, several techniques can be used. These can be classified according to conventional/classical methods and advanced/innovative methods (33) (Figure 6). In the first group, the techniques are based on water distillation by heating to recover the essential oils from the plant (hydrodistillation, entrainment by water stream, cold pressing) what sometimes cause certain chemical alterations (hydrolysis, isomerization, oxidation) in the essential oils due to the high applied temperatures. In the second group new techniques dealing with microwave and ultrasound are used (33). It is

important the careful selection of the extraction method as it will influence both the quality and quantity of the resulting essential oil. The chosen extraction method should maintain the chemical composition and its natural proportion in the essential oil as its original state, as well as provide the maximum yield and biological activity (34). Together with the extraction method, both the quality and the quantity of the resulting yield is also highly variable in essential oils depending on many factors. These can be intrinsicly related to the plant and its maturity stage, as well as extrinsicly associated with the environment (soil type and climate, etc.) and the harvest time (35–38).

Figure 6. Comparison between a conventional method of extraction (hydrodistillation, left (39)) and an innovative one (microwave dry diffusion and gravity process, right (33)).

Regarding the chemical composition of the essential oils, chromatographic techniques coupled with different systems are the most common identification methods employed, with gas chromatography coupled with mass spectrometry (GC-MS) as the best standard technique for their analysis, being used for more than 90% of research (11). According to this, the qualitative characterization of essential oils is carried out by comparing the resulting mass spectrum with those contained in a mass spectra library.

On the other hand, the relative quantitative characterization is performed throught the peak area of each identified component (Figure 7) in the total essential oil.

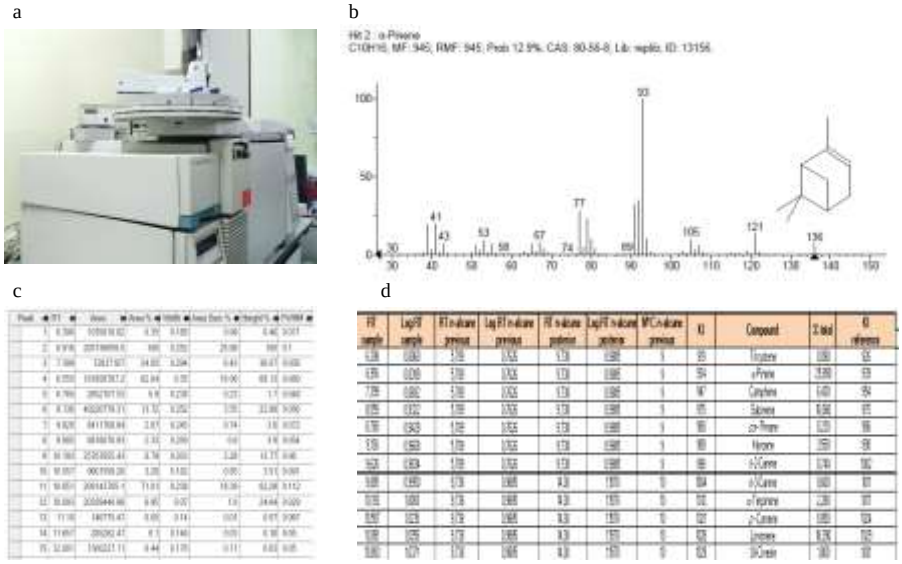


Figure 7. GC-MS equipment (a). The resulting mass spectrum (b) gives us information for the identification of the compound. Retention Times (c) are used to calculate Kovat's Indeces (d) which help us to identify the component by comparison with reference ones.

1.2.5. Uses of essential oils: from human health to food and crop preservation

As it has been previously commented on, the traditional uses of essential oils in medicine have been popularly known since ancient times, and even today they are gaining importance especially as complementary medicine in aromatherapy and phytotherapy because of their well-known pharmacological activities (40,41) (Annex I). On the other hand, other different properties and applications of essential oils have been studied and demonstrated, consequently becoming valued products not only in

pharmaceutical industry, but also in food and beverage, perfume and cosmetic, chemical and agri-food, in order to respond to today's demands with natural substances safe for humans and environment.

Antimicrobial activity

The antiseptic properties of essential oils have been popularly-recognized for many years, being useful in fighting infections. For years, many studies have highlighted the broad antimicrobial activity spectrum of essential oils, which is especially interesting because they would mean an alternative to overcome the microbial resistance problem. Furthermore, this potent antimicrobial activity of essential oils is not only useful in medical applications, but also in other areas, such as food and crop preservation (42). Nowadays, the consumers are concerned about the synthetic and harmful products used as antimicrobials and preservatives in agri-food industry. In response to this, there is an increasing interest in natural antimicrobial products which could avoid pest attack in crops, as well as food spoilage pathogens and to extend their shelf-life (43,44).

In particular, essential oils represent safer and “greener” alternatives to commercial synthesized antimicrobial products whose safety in environment and human health is disputed. It is especially relevant nowadays when 137 pathogens and pests have been associated with yield losses in the basic crops worldwide - wheat, rice, maize, potato and soybean, being especially remarkable in food-deficit regions with fast-growing populations, and frequently with emerging or re-emerging pests and diseases (45). These microorganisms have detrimental effects on the shelf-life, physical characteristics and quality of the food products; also causing serious economic losses. Therefore, the prevention and/or

inhibition of microbial contamination of crops and food products is an important challenge for the global agri-food industry.

Specifically, eucalypt (*Eucalyptus globulus* L.), peppermint (*Mentha* x *piperita* L.) and rose-scented geranium (*Pelargonium graveolens* L'Hér) essential oils are some examples of essential oils that have exhibited antimicrobial efficiency in the control of pre- and post-harvest rot. They have also showed a remarkable *in vitro* antimicrobial effect against Gram positive food-spoiling bacteria, such as *Bacillus subtilis* and *Staphylococcus aureus*, and against fungi and yeasts, *Aspergillus flavus*, *A. niger*, *Mucor* spp., *Fusarium oxysporum* and *Candida albicans*. This activity is especially noticeable in vapour phase, being suitable alternatives for use in the food industry as natural antimicrobial agents (46–48). Many other essential oils have revealed antimicrobial activity against phytopathogenic microoganisms: *Zataria multiflora* Boiss., *Thymus vulgaris* L. and *T. kotschyanus* Boiss. & Hohen. essential oils completely inhibited the growth of the phytopathogenic fungi *Pythium apanidermatum*, *Rhizoctonia solani*, *F. graminearum* and *Sclerotinia sclerotiorum* at 200 µL/L (49); *Thuja occidentalis* essential oil inhibited the growth of the most economic plant pathogenic bacteria *Agrobacterium tumefaciens* and *Erwinia carotovora* var. *carotovora* at Minimum Inhibitory Concentrations (MIC) values of 400 and 350 mg/L and *Artemisia monosperma* Delile essential oil did with the phyopathogenic fungi *Alternaria alternata*, *Botrytis cinerea*, *F. oxysporum* and *F. solani* with effective concentration (EC_{50}) between 106-148 mg/L (50).

Recently, the *in vivo* antifungal activity of peppermint essential oil has been demonstrated against the spoilage yeasts, *C. albicans*, *C. tropicalis*, *Pichia*

anomala and especially *Saccharomyces cerevisiae* in cashew, guava, mango and pineapple juices, affecting its membrane permeability and potential, enzymatic activity and efflux pump at the minimum dose assayed (1.875 µL/mL) (51). Finally, other *in vivo* tests have shown the significant improvement of potato slices infected with *A. niger*, *M. wutungkiao*, *Penicillium funiculosum* and *Rhizopus oryzae* after the application of 2.00 $\mu L/mL_{air}$ of the essential oil obtained from the peel of navel orange (*Citrus sinensis* (L.) Osbeck) (52).

Antoxidant activity

Stored food products are subject to free radical generation due to oxidative stress. Natural preservatives to combat this deterioration, represent natural alternatives to synthetic phenolic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and propyl gallate, safer for human health and environment (53). According to this, the antioxidant properties of essential oils against the degeneration of food have been studied, demonstrating their usefulness in overcoming storage losses and enhancing shelf-life (54). In many of these cases, the essential oils have been incorporated in films and coatings which contain the protected food. In this sense, oregano essential oil, which exhibited effective antioxidant properties in retarding lipid peroxidation in fatty foods and scavenging free radicles, has been incorporated in edible pectin coatings increasing the antioxidant activity in coated tomatoes without negative effects on aroma acceptability of the food (55,56). Furthermore, thymol and carvacrol, the main components of oregano essential oil, have also demonstrated pronounced antioxidant activity when improving the oxidative stability of walnut oil triacylglycerols (57).

Similarly to oregano, *Z. multiflora* essential oil was incorporated in chitosan nanoparticles and its antioxidant activity was evaluated over cucumber showing higher levels of DPPH-radical (2,2-Diphenyl-1-picrylhydrazyl) scavenging activity and longer shelf-life during storage (58).

In addition to the deterioration of fruits and vegetables, essential oils are also incorporated to coatings to protect other foodstuff, such as meat. Coating with black pepper essential oil was tested against lipid degeneration of dry-cured meat, showing potential to suppress their deterioration and improve the overall aroma of the product during storage (59). Likewise, *Pimpinella saxifraga* L. essential oil was incorporated in sodium alginate coating showing an improvement in coated cheese during refrigerated storage by reducing the weight loss, preserving the pH and color, as well as enhancing oxidative stability without unpleasant flavour for consumers (60).

The antioxidant activity of essential oils has been compared with the one exerted by commonly-used antioxidant standards. For instance, the antioxidant activity of *Xylopia sericea* A. St.-Hil. essential oil was evaluated by DPPH scavenging, Ferric reduction antioxidant power (FRAP), β-carotene/linoleic acid bleaching and phosphomolybdenum and thiobarbituric acid-reactive substance (TBARS) assays, showing greater antioxidant activity than the reference compounds α-tocopherol, ascorbic acid, quercetin and BHT. Thus, in the TBARS assays *X. sericea* essential oil caused 80% of oxidation inhibition whereas BHT presented only 13.7% of inhibition (61). Other essential oils such as *Vetiveria nigritana* (Benth.) Stapf also showed, similar antioxidant activity to BHT and low compared to that of quercetin and ascorbic acid in FRAP test (62) whereas *Lantana*

camara L. essential oil showed better radical scavenging power than quercetin, ascorbic acid and BHT (63).

Herbicidal activity

The control of weeds and other unwanted plants in a cost-effective way is crucial to agriculture and related industries as they have produced the highest potential loss of productivity (34%) in comparison to animal pests and pathogens (18 and 16%, respectively) (64,65). This fact is especially remarkable in the basic crops worldwide, such as rice (*Oryza sativa* L.), in which weeds are known to cause one-third of the total losses of rice in India reducing the grain yield by 75.8, 70.6 and 62.6% in dry seeded rice, wet seeded rice and transplanted rice, respectively (66); soybean (*Glycine max* (L.) Merr.), with a grain yield reduction due to weeds between 31-84% during the first six weeks after planting (67); and wheat (*Triticum aestivum* L.), in which weeds account for between 30 and 50% loss of potential yield (68,69).

As a response, there are more than 200 herbicidal compounds commercially available worldwide (70). In fact, herbicides represent the widest pesticide product traded accounting for 47.6% of global pesticide sales followed by insecticide (29.4%), fungicide (17.5%) and others (5.5%) (71). In addition, according to Statistics MRC (Market Research Companies), the Global Herbicides Market accounted for $27.45 billion in 2016 and still the demand for herbicides in agriculture is expected to increase around the world reaching $44.56 billion by 2023 growing at a CAGR of 7.1% during the forecast period (72). These numbers are expected to continue increasing to supply the demand of 9 billion people by the year 2050 (73). However, constant and indiscriminate use of synthetic herbicides have led to a rapid

spread of herbicide resistant weeds (74): these have been reported in 93 crops in 70 countries being currently 500 unique cases (species x site of action) of herbicide resistant weeds globally, with 256 species (149 dicots and 107 monocots). Furthermore, weeds have evolved resistance to 23 of the 26 known herbicide sites of action and to 167 different herbicides (75).

According to this, emerging trends in technology and innovation offer a sustainable weed management for the future and more concretely the research in natural products, such as extracts of plants, leads to the discovery of new herbicides as well as new modes of action (73,76). Although only a small fraction of the world's plant biodiversity has been screened for herbicidal activity until now, interesting herbicidal compounds with novel mechanisms of action have been discovered. Among them, 350 plant extracts of which some of them have become successful commercial herbicides (73).

Specifically, essential oils have extensively demonstrated their phytotoxic properties, representing potential sources of efficient and safer herbicides for human health and environment. Particularly, Lamiaceae, Myrtaceae, Asteraceae, Pinaceae, Lauraceae, Poaceae, Cupressaceae, Apiaceae and Anacardiaceae are some of the families with compounds like α-pinene, limonene, 1,8-cineole, carvacrol, camphor and thymol with high phytotoxic activity (77,78). Some examples are peppermint essential oil with menthol (35%), mentone (17.48%) and menthofuran (11.7%) as main components that significantly affected the seed germination and seedling growth of field blindweed (*Convolvulus arvensis* L.) and purslane (*Portulaca oleracea* L.), and jungle rice (*Echinochloa colonum* L.) at 1800 and 1200 µL/L, respectively (79). Other examples are *Artemisia annua* L. and *Xanthium*

strumarium L. essential oils, which inhibited the seed germination of the weeds *Setaria viridis* (L.) P. Beauv. and *Amaranthus retroflexus* L. at 100 and 10 µg/L, respectively, under laboratory conditions, and the development of cotyledons and the third true leaf between 10-1000 mg/L *in vivo* (80). Also, high concentration of lemongrass (*Cymbopogon citratus* (D.C.) Stapf) essential oil decreased the germination and seedling growth of *E. crus-galli* under laboratory conditions and furthermore, it caused leaf wilt of the leaves of *E. crus-galli* after 6 hours of treatment with 1.25, 2.5, 5 and 10% (v/v) in glass house bioassay (81). In a screening of the phytotoxic potential of 82 essential oils samples, 11 of them strongly inhibited the seedling growth of rapeseed (*Brassica napus* L.), among them cinnamon, citronella, clove, palmarosa and lemongrass. These essential oils mainly contained oxygenated monoterpenes which had different potency inhibiting the growth of *E. crus-galli* and *Aeschynomene indica* L. between 29-1000 µg/mL, being especially remarkable, the activity of citral and geraniol, main components of lemongrass and palmarosa, respectively (82).

Many essential oils exhibited remarkable inhibitory effects against weeds and minimal phytotoxicity against food crops. That is the case of *Eremanthus erythropappus* (DC.) MacLeish essential oil that significantly inhibited the seed germination of field mustard (*Brassica rapa* L.) and the radicle length of hairy beggaticks (*Bidens pilosa* L.) causing minimal effects on lettuce and tomato (83). Another example is *Hyptis suaveolens* (L.) Poit. essential oil which caused a reduction in chlorophyll content and cell viability of *E. crus-galli* until finally producing its complete wilting at 2 mg/mL, without reaching the same level of inhibition on rice (84). Equally, rosemary (*Rosmarinus officinalis* L.) essential oil inhibited the seed germination of *A. retrofexus* in 91.3% at 400 µL/L, whereas it caused

a reduction of 26.7% in tomato at the same dose (85). The same volatile with 1,8-cineole (54.6%), camphor (12.27%) and α-pinene (7.09%) as main compounds inhibited the seed germination of the weeds *Trifolium incarnatum* L., *Silybum marianum* (L.) Gaertn. and *Phalaris minor* Retz. at 5 mM *in vitro* and equally affected the weeds *in vivo* (86).

Due to these numerous examples that corroborate the effective phytotoxicity of essential oils, commercial products for weed management including essential oils as active components are being marketed nowadays. For instance, Avenger© Weed Killer including citrus oil (17.5%), Weed Zap© and Weed Blitz© with clove/cinnamon (45%/45%) and pine oils (13.6%), respectively, and EcoExempt HC© with a mixture of clove and 2-phenethyl propionate (24%/24%) that causes membrane disruption (87).

1.2.6. Formulation of essential oils

Essential oils are unstable and fragile volatile compounds so they can be degraded if they are not protected from external factors like oxygen, temperature and light. Regarding this, the current trend is to find the most suitable material to encapsulate essential oils with the aim of increasing their action duration and providing a controlled release as well as improving the activity of essential oils (88).

For this purpose, many techniques and materials to encapsulate the essential oils have been described. Some examples are (fluidised) spray-drying, granulation/agglomeration/compaction, cyclodextrin, microspheres, silica particles, co-extrusion, among others (89,90).

Among the excipients employed in these techniques, chitosan represents an interesting material to combine with essential oils. In fact, the incorporation of a reasonable amount of essential oils in chitosan films has shown an

interesting improvement of the physical properties of these films without affecting the integrity. *Thymus capitatus* (L.) Hoffmanns and Link essential oil showed remarkable antimicrobial activity against fish-spoiling microorganisms when included in chitosan-based coatings at 1.0%. At this amount, the addition of the essential oil to chitosan films improved the physical properties of the complex with a significant decrease in the permeability, as well as an equilibrium between tension strength and elasticity (91).

Similarly, the formation of inclusion complexes of essential oils and β-cyclodextrines (β-CD) also represents an interesting way to improve the physical properties of the material, as well as to reduce the volatility, increase the release time of essential oils, and improve their overall activity, in general (92,93). In this sense, the incorporation of *Pimenta dioica* (L.) Merr. essential oil in β-CD by kneading method has shown complete inhibition of the mycelial growth of the food-spoiling mold *Byssochlamys nivea* dispersed in agar. Kneading method represents a better manner of essential oil encapsulation with β-CD, by means of freezing for instance, as the interaction between both compounds are weaker so the release of the essential oil is easier (94).

Another attractive material for improving food quality is alginate. Previous studies have reported that films containing gelatin-alginate together with 1.5% of oregano essential oil represent a suitable packaging to avoid the deterioration of fish, being able to delay this process during refrigerated storage in 15 days (95). Equally, gelatin mixed with gum arabic by a process of complex coacervation represents another interesting method to make microcapsules containing essential oils. Specifically, this combination

together with boldo (*Peumus boldus* Molina) essential oil represents a useful tool against the deterioration of stored peanuts by food spoiling fungi (96).

In particular, the technique of extrusion has been widely described for encapsulation, in general. It is a common process in pharmaceutical, chemical, cosmetic, food and printing industries to envelope bioactive molecules within a physical barrier (wall material) (Figure 8). This wide use is a consequence of its multiple advantages: it improves stability of the final product, increases the shelf-life, prevents reactions with other molecules such as oxygen or water and it is suitable for heat sensitive active agents avoiding evaporation and degradation (97). For instance, pumpkin (*Cucurbita pepo* L.) seed oil was encapsulated by extrusion method achieving a 91.9% encapsulation efficiency and 63.0% loading capacity without affecting the antibacterial activity of the oil (98). Furthermore, previous authors have reported an increase in flavour after extrusion process (99). However, extrusion has a limited range of wall materials to be used. In this way, microcrystalline cellulose (MCC) represents the most suitable material for this technique because of its appropriate rheological properties, cohesiveness and plasticity to provide apt particles (100) (Figure 8).

According to the Ph. Eur. 9.5, MCC is a "purified, partially depolymerized cellulose prepared by treating alpha-cellulose, obtained as a pulp from fibrous plant material, with mineral acids" (16). It is a fine or granular, white or almost white, odourless, free flowing crystalline powder widely used in pharmaceutic, cosmetic, and principally food industry in which it acts as functional ingredient in food, such as meat, emulsions, beverages, dairy products, bakery, confectionary and filling (101). Until now, MCC

has been included as reinforcement material in gelatin- and hydroxypropyl methyl cellulose (HPMC)-based films, showing an improvement in the physico-chemical characteristics of the films, as well as a maintencance of the properties of the active agent incorporated (102,103). Particularly, in previous work, matrices including MCC have shown to preserve the aroma of retained oils better (104). This fact represents a starting point to investigate its possible use in the encapsulation of essential oils.

Figure 8. Example of a extruder (left) with the hooper or feeding zone (a), followed by the barrel with a screw and the dying zone (c) through which the MCC extrudates are obtained (right).

2. OBJECTIVES

Main objective

To take advantage of the numerous biological properties of essential oils to promote their use as "bio" products in a sustainable, environmentally friendly agriculture that allows us to maintain and improve the quality of crops and food, as well as human health and environment.

Specific objectives

1. To determine the chemical composition of commercial essential oils with known pharmacological activity, through GC-MS technique, in order to know the major and minor compounds, and consequently verify their identity.
2. To study the *in vitro* phytotoxic activity of these essential oils at four concentrations (0.125, 0.25, 0.5 and 1 µL/mL) against the seed germination and seedling growth of weeds that affect the cultivation and production of rice, maize and tomato and other crops of considerable importance globally, and against invasive plant species, which represent an ecological hazard in the invaded ecosystems.
3. To measure the *in vitro* influence of these essential oils at the same concentrations in the seed germination and seedling growth of food crops, in order to discard a potential phytotoxicity of the volatiles on the crops to be protected.
4. To assay the *in vivo* phytotoxic activity through the use of emulsionable concentrates at two doses (5 and 10 µL/mL) of the essential oils with remarkable *in vitro* phytotoxicity against weeds and less harmful on food crops, with the purpose of determining if these essential oils are in fact a natural alternative, cheaper and

without the corresponding hazards for the environment and human health than synthetic herbicides. Simultaneously, the inactivity of the commercial excipients added to the emulsionable concentrate will be observed.

5. To evaluate the antioxidant activity of these essential oils using DPPH method and its comparison *versus* the established antioxidant potential of the natural antioxidants quercetin and ascorbic acid, and the synthetic one BHT.
6. To determine the antibacterial and antifungal capacity of essential oils by means of disk diffusion technique on phytopathogenic bacteria and fungi affecting the selected food crops.
7. To study the characteristics and properties of MCC as wall material and extrusion technique as a way to encapsulate essential oils with the intention of finding the most efficient and sustainable technique to apply the essential oils *in vivo*.

3. MATERIAL AND METHODS

3.1. MATERIAL

Commercial essential oils and reference standard were purchased and stored at 4ºC until chemical analysis and biological studies were carried out.

3.1.1. Essential oils

Table 1. Information about commercial essential oils used.

Common name	Plant part	Batch	Sell-by-date
	Guinama		
Anise	Ripe dried fruit	0059857	06/2017
Cinnamon	Leaves	0072188	30/11/2018
Clove	Leaves	0065709	22/08/2018
Eucalypt	Leaves & stems	0065901	28/02/2019
Marjoram	Leaves & flowers	0042773	13/11/2016
Oregano	Flowers	0042451	31/05/2016
Peppermint	Leaves	0058567	25/11/2017
Rosemary	Leaves	0037337	30/04/2016
Scots pine	Needles	0065144	08/08/2018
Tea tree	Leaves	0051451	30/09/2019
Winter savory	Whole plant	0054366	18/02/2017
	Planalto Dourado		
Spanish marjoram	Leaves & flowers	TM010711	07/2017
	Pranarôm		
Basil	Flowering top	0F22144	08/2020
Ginger	Rhizome	0F26093	04/2022
Lavender	Flowers	0082842	30/11/2020
Lemon eucalypt	Leaves	OF25830	02/2022
Turmeric	Root	0F27683	10/2021
Wintergreen	Leaves	0F18989	11/2020
	Plantis		
Chamomile	Flowers	725	11/2017
	Carobels		
Green tea	Leaves	26903	09/2015

3.1.2. Reference standard

Table 2. Information about reference standard used.			
	Company	Batch	Sell-by-date
Carvacrol	Sigma-Aldrich	MKBN3724V	01/2018

3.2. SEEDS OF WEEDS, FOOD CROPS AND INVASIVE PLANT SPECIES

Table 3. Seeds of weeds, food crops and invasive plant species studied.		
	Plant species	Source
Weeds	Common purslane (*Portulaca oleracea* L.)	Herbiseed (website: www.herbiseed.com)
	Italian ryegrass (*Lolium multiflorum* Lam.)	
	Barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.)	
Food crops	"Muchamiel"/"Huevo de toro" tomato (*Solanum lycopersicum* L.)	Intersemillas S.A. and/or inner part in Utiel (Tomato) (Valencia, Spain).
	Cucumber (*Cucumis sativus* L.)	
	"Perseo-type" maize (*Zea mays* L.)	Piensos Alfonso (15/01/2014)
	"Albufera-type" rice (*Oryza sativa* L.)	Copsemar Sueca (Valencia, Spain)
Invasive plant species	Tree tobacco (*Nicotiana glauca* Graham)	Botanical Garden of Valencia
	Pampas grass (*Cortaderia selloana* (Schult. & Schult. f.) Asch. & Graebn.)	

3.3. DETERMINATION OF THE CHEMICAL COMPOSITION

3.3.1. Gas chromatography–Mass spectrometry analysis

GC-MS analysis was carried out using a 5977A Agilent mass spectrometer and a gas chromatograph (Agilent 7890B) apparatus equipped with an Agilent HP-5MS (30 m long and 0.25 mm i.d. with 0.25 µm film thickness) capillary column (95% dimethylpolysiloxane, 5% diphenyl). The column temperature program was 60 ºC for a duration of 5 min, with 3 ºC/min increases up to 180 ºC, then 20 ºC/min increases up to 280 ºC, which was maintained for 10 min. The carrier gas was helium at a flow rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were collected over the *m/z* range 30-650 with an ionizing voltage of 70 eV. The resulting individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index, calculated using co-chromatographed standard hydrocarbons relative to C_8-C_{32} *n*-alkanes and mass spectra with reference samples or with data already available in the NIST 11 mass spectral library and in the literature (105).

3.4. HERBICIDAL ACTIVITY

3.4.1. In vitro assays

Sets of 20 seeds each with five replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No.1). The lower filter papers were moistened with 4 mL of distilled water and the upper ones with 0 (control), 0.125, 0.250, 0.5, and 1 µL/mL of the essential oils, homogeneously distributed in the filter paper with a micropipette. So, the seeds were in contact directly with moistened filter papers and indirectly with the vapours of the essential oils. The Petri dishes were sealed with parafilm and incubated in an Equitec EGCS 301 3SHR model germination chamber, according to previous assays (106)

alternating between 30.0 ± 0.1 ºC 16 h of light and 20.0 ± 0.1 ºC 8 h of darkness, with (*E. crus-galli, C. selloana, N. glauca,* cucumber, rice, maize) and without humidity (*P. oleracea, L. multiflorum,* tomato). To evaluate the herbicidal activity of the essential oils, the number of germinated seeds was counted and compared with that of untreated seedlings. The emergence of the radicle (≥1 mm) was used as an index of germination, and seedling length (hypocotyl and/or radicle) data were recorded after 3, 5, 7, 10, and 14 days in each replicate.

3.4.2. In vivo assays

Ten seeds of each species (*P. oleracea, L. multiflorum, E. crus-galli*, maize, rice, and tomato) with ten replicates per treatment were randomly chosen and placed in pots (9 cm diameter) with 40 g of substrate. They were placed less than 1 cm below Substrate Projar Professional containing coir and peat make, fertilizer N-P-K: 14 + 16 + 18 + micronutrients, and dolomitic limestone with a sorption capacity of 183 g/10 min. A set of 10 pots was watered on the first day with 20 mL of water (control), 20 mL commercial products (Nosbur OE 12 NS (32% *w*/*w*), Emulson AG/CAL/E (7% *w*/*w*), Emulson CO 36 (13% *w*/*w*) or Emulson AG/CAL/E (2.2% *w*/*w*), Alpicare 410H (21.7% *w*/*w*), Emulson AG/7720/A (2.6% *w*/*w*) respectively) without essential oils (blank), and 20 mL of emulsifiable concentrate with winter savory (48% *w*/*w*) or peppermint essential oil (73.5% *w*/*w*) at 5 and 10 µL/mL. A tray was used every five pots to hold and separate them when watering. In order to prevent leaching, the pots were covered with plastic film. Over a period of 33 or 20 days (winter savory or peppermint), each tray was watered with 250 mL of water every two days. The greenhouse conditions were: 23.3 °C average indoor temperature, 18.1 °C minimum indoor temperature, 29.7 °C maximum indoor temperature, 57.2% average

humidity, 80.9 µmol/m^2/s PAR (Photo Active Radiation), and 135.6 W/m^2 intensity of radiation.

To evaluate the herbicidal effect, the number of germinated seeds in 5 µL/mL and 10 µL/mL pot trays was counted and compared with those of control and blank samples. Emergence of the hypocotyl (≥1 mm) was used as an index of germination and seedling length data were recorded every two days, coinciding with watering days over 33 or 20 days.

3.4.3. Statistical analysis

Experiments were performed *in vitro* with five replicates. Data were subjected to one-way analysis of variance (ANOVA) using SPSS statistics 24 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* test was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq 0.05$.

3.5. ANTIOXIDANT ASSAY

The free radical scavenging activity of the essential oils was evaluated by DPPH method. Briefly, 1 mL of ethanol was taken as blank and 750 µL added in 250 µL of 0.5 mM DPPH solution was taken as control (A_0). Reaction mixture (A_1) was prepared by taking 740 µL of ethanol mixed well with 250 µL of 0.5 mM DPPH and 10 µL of essential oils and reference standard (Section 3.1.1. and 3.1.2.) and positive control. Control mixture was incubated in the dark. The reaction mixtures were allowed to stand at room temperature for 30 minutes. The absorbance was measured at 517 nm using a UV-Visible spectrophotometer Ultrospec® 100E (Pharmacia Biotech). The results were compared with the positive control: the natural flavonoid quercetin and the synthetic antioxidant BHT (0.5, 5 and 25 mM),

as well as the ascorbic acid (1 and 2.5 mM). The antioxidant activity (%) was expressed in percentage of inhibition of the DPPH radical by using the following formula:

$$\text{DPPH scavenging effect (\%) inhibition} = \frac{A_0 - A_1}{A_0} \cdot 100$$

Where, A0 is the absorbance of the control reaction (without test compound), and A1 is the absorbance in presence of all of the essential oils and positive control. All the tests were performed in triplicates and the results were averaged.

3.6. ANTIBACTERIAL ASSAY

Bactericidal activity tests were performed by Kirby-Bauer method (disk diffusion technique) using King B Agar as a culture medium (Table 4).

Table 4. Composition and preparation of 200 mL of King B Agar (x2).

Compound	Quantity
King B agar (King B Medium Pseudomonas F Agar USP) (Pronadisa)	7.4 g
Glycerol	2 g
Distilled H_20	Until 200 mL
AUTOCLAVE (121 ºC, 15 min)	
Rifampicin (10 mg/mL)	1 mL

Under laminar flow conditions, 1 mL of the bacterium *Pseudomonas syringae* pv. *tomato* with an Optical Density (OD) of 0.1 at 600 nm was inoculated in King B Agar medium once it was sterilized and cooler. The medium containing the bacterium was distributed in plastic Petri dishes that were allowed to stand until solidifying. Then, five cotton discs were placed on the medium. These discs were impregnated by the positive (tetracycline 0.3925% (p/v)) and negative controls (methanol), and essential oils and

reference standard (Section 3.1.1. and 3.1.2.) at different doses (1, 5, 10 and 20 µL). The test dishes were incubated at 28 ºC in darkness during 24 h. The results were expressed as the mean of the diameter of the halo of bacterial growth inhibition (mm).

3.7. ANTIFUNGAL ASSAY

Fungicidal activity was evaluated against the phytopathogenic fungus of tomato *Fusarium oxysporum* f. sp. *lycopersici*. For this purpose, the method of inclusion of the spores in the culture medium (Potato Dextrose Agar, PDA) was carried out for the posterior application of the samples by means of discs.

First of all, a solution of purified spores was prepared at a concentration of 10^5 spores/mL from the fungus growth in liquid medium. This solution was filtered through a double sterile gauze to remove the mycelium formed. The spores were sedimented by centrifugation at 3600 rpm for 5 minutes. The supernatant was discarded and re-dissolved in sterile water three times until becoming transparent, indicating that all spores were deposited in the bottom of the container. After purification of the spores, their count was performed in a cytometry chamber in the microscope using the following formula:

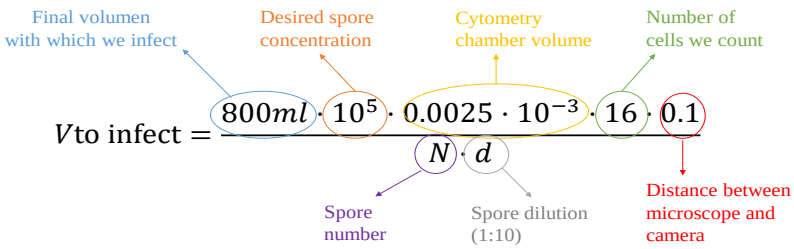


Figure 9. Calculation of the volume of the spore solution of Fusarium oxysporum f. sp. lycopersici for distribution in Petri dishes. Adapted from Hernández (107).

The volume of the spore solution obtained by the formula was added to PDA which was distributed in Petri dishes under laminar flow conditions. They were allowed to solidify and five cotton discs were placed on the medium. These discs were impregnated by positive (tebuconazole) and negative (methanol) controls and the essential oils and reference standard (Sections 3.1.1. and 3.1.2.) at different doses (1, 5, 10 and 20 µL). The test dishes were incubated at 28 ºC in darkness for three days. The results were expressed as the mean of the diameter of the halo of fungus growth inhibition (mm). This experiment was repeated in triplicate.

3.8. ENCAPSULATION OF ESSENTIAL OILS

3.8.1. Material and equipment

Table 5. Material used for encapsulation of essential oils.		
Material	Company	Batch
Microcrystalline cellulose (MCC) PH 101	JR Pharma	66101186740

Table 6. List of equipment employed for encapsulation of essential oils.	
Equipments	Models and Manufacturers
Balance	Mettler AE 260 DeltaRange®
Drying oven (40ºC)	1758 WTC Binder Precision Oven DF0010
Extruder	Extruder *à vis* (Pharmex 35T, Allemagne)
Mixer	Kenwood Professional PM900
Centrifuge	Jouan BR4i Centrifuge
Moisture analyzer	Sartorius MA160 Moisture Analyzer
UV-Visible spectrophotometer	Specord® 200 Plus
Helium pycnometer	1305 (Micromeritics, USA)

3.8.2. Preparation of reference MCC and MCC-essential oil extrudates

On the one hand, for the preparation of reference MCC extrudates, 100 g of MCC were subjected to dry mixing for one minute at minimum velocity in a Kenwood Professional PM900 mixer. Half of the container of 120 g of ultrapure water was added, increasing velocity in one level for one minute. Then, the rest of the container was added and the wet mass was left to agitate for five minutes at minimum velocity again. During this wet massing and at the end of the process, the material was repeatedly scraped from the mixing bowl walls to ensure uniform water distribution.

The wet mass was extruded at an extrusion speed of 17 rpm using an extruder *à vis* (Pharmex 35T, Allemagne) equipped with an axial discharge in a single bench-top unit including an extraction screen with perforation diameters of 1.5 mm. The total mass obtained of extrudates was measured in order to know the yield of the extrusion process.

The extrudates were dried in the 1758 WTC Binder Precision Oven DF0010 at 40 ºC for 24 h. After the desiccation process, the mass of the dried extrudates was weighed in order to know the yield after the loss of water. The extrudates were stored in sealed plastic containers in a room under controlled temperature (20±2 ºC) and humidity (45±5%) conditions before testing.

On the other hand, for the preparation of MCC-essential oil extrudates, two dilutions of pine essential oil (1 and 10 µL/mL) were kept in magnetic agitation for 30 min in 120 g of ultrapure water each one. At the end of the process, these mixtures were added and mixed with 100 g of MCC, as well as extruded as described previously. The total mass obtained of impregnated extrudates was recorded. Then, the wet impregnated

extrudates were dried in oven at 40 ºC for 24 h. After that, the mass of the dried impregnated extrudates was also measured. These extrudates were also stored under the same conditions as the reference ones until assays were carried out.

3.8.3. Measurement of the relative humidity

As the moisture content is of critical importance in encapsulated oils, the relative humidity of the 24h-dried extrudates was measured. For this purpose, three grams of each sample were taken and subjected to moisture analysis at a heating programming of 100 ºC. The measurements were carried out in triplicate.

3.8.4. Encapsulation efficiency (EE) and homogeneity test

In order to estimate in which concentration the essential oil is found in the final extrudate and to ensure a homogeneous distribution of the essential oil among MCC, the encapsulation efficiency was calculated.

The amount of pine essential oil encapsulated in MCC was determined spectrophotometrically at 286 nm in a UV-Visible Specord® 200 Plus spectrophotometer. For this, one gram of it was dissolved in 10 mL of absolute ethanol and ultrapure water and was left for 15 h under magnetic agitation, enough time for all entrapped essential oil to be in solution, and with constant conditions of temperature and humidity.

After 15 h of agitation, the centrifugation of the samples was made with a Jouan BR4i Centrifuge at the following conditions: 2500 rev, 10 min 25 ºC. Then, the absorbance of each sample was measured. This experiment was carried out in triplicate.

The EE was calculated as:

$$\mathrm{EE} = \frac{\text{Amount of encapsulated essential oil}}{\text{Initial amount of essential oil}} * 100$$

where "amount of encapsulated essential oil" is the compound amount present in the extrudate, and "initial amount of essential oil" indicates the compound amount initially used to produce the extrudates.

3.8.5. Essential oil release studies

A known quantity of each of the impregnated extrudates (0.05 g) was placed into a known volume of absolute ethanol (5 mL). The absorbance was measured by UV-Visible Specord® 200 Plus spectrophotometer every hour until 7 hours of release. This experiment was carried out in triplicate.

3.8.6. Extrudate size measuring

The length (cm) of 50 impregnated extrudates were measured in triplicate with ImageJ. The average length and standard deviation were calculated.

3.8.7. Density measurement

True density of the extrudates was measured using a helium pycnometer 1305 (Micromeritics, USA) and the required mass of material for each measurement was about three grams. Measurements were done in triplicate for each sample.

4. RESULTS AND DISCUSSION

4.1. CHEMICAL COMPOSITION OF ESSENTIAL OILS

The chemical composition of the commercial essential oils sold in Pharmacy with guaranteed safety in humans has been determined by GC-MS analysis. The main compounds identified in the selected essential oils are shown in Table 7.

Table 7. Main compounds in the analysed essential oils.

Essential oil	Main compounds
Anise (*Pimpinella anisum* L.)	*trans*-Anethole (99.5±0.1%).
Basil (*Ocimum basilicum* L. ssp. *basilicum*)	Methyl chavicol (79.1±0.3%) and linalool (14.6±0.3%).
Eucalypt (*Eucalyptus globulus* Labill.)	1,8-Cineole (76.4±0.4%) and α-pinene (14.6±0.3%).
Ginger (*Zingiber officinale* Rosc.)	α-Zingiberene (24.9±0.8%), β-sesquiphelladrene (11.9±0.3%), camphene (11.6±0.3%), ar-curcumene (10.7±0.2%) and β-bisabolene (10.5±0.3%).
Lavender (*Lavandula angustifolia* Mill.)	Linalool (38.7±0.1%), 1,8-cineole (26.5±0.1%) and camphor (14.2±0.1%).
Lemon eucalypt (*Eucalyptus citriodora* Hook)	Citronellal (88.0±0.8%).
Marjoram (*Origanum majorana* L.)	1,8-Cineole (59.6±0.9%), α-terpineol (3.4±0.1%), β-pinene (4.4±0.4%) and α-pinene (4.1±0.5%).
Oregano (*Origanum vulgare* L.)	Carvacrol (60.4±0.1%) and *p*-cymene (15.5±0.0%).
Peppermint (*Mentha piperita* L.)	Menthol (48.2±0.4%), menthone (23.3±0.6%) and *iso*-menthone (16.3±0.0%).
Rosemary (*Rosmarinus officinalis* L.)	1,8-Cineole (25.0±0.1%), camphor (20.5±0.1%) and α-pinene (16.7±0.1%).

Scots pine (*Pinus sylvestris* L.)	α-Pinene (25.6±0.2%), limonene (18.5±0.2%) bornyl acetate (17.9±0.0%), and β-pinene (15.9±0.1%).
Spanish marjoram (*Thymus mastichina* L.)	1,8-Cineole (49.5±0.4%), linalool (5.7±0.0%) and α-terpineol (5.6±0.0%).
Tea tree (*Melaleuca alternifolia* Maiden & Betche ex Cheel)	Terpinen-4-ol (28.4±0.1%), 1,8-cineole (15.8±0.1%), γ-terpinene (15.6±0.0%) and α-pinene (10.9±0.1%).
Turmeric (*Curcuma longa* L.)	ar-Turmerone (38.7±0.8%), β-turmerone (18.6±0.6%) and α-turmerone (14.2±0.9%).
Wintergreen (*Gaultheria procumbens* L.)	Methyl salicylate (99.6±0.0%).
Winter savory (*Satureja montana* L.)	Carvacrol (43.3±0.1%), thymol (23.2±0.1%) and *p*-cymene (11.41±0.0%).

Although as a general rule major compounds in essential oils contribute to distinguish different chemotypes, minor compounds play an important role in the differentiation between the chemical groups (108,109). Furthermore, the interaction between major and minor compounds is responsible for the characteristic properties of the essential oil (110,111). Regarding this, the minor compounds linalool and geranyl acetate detected in lemongrass oil had a synergistic or additive antibacterial effect with the major components citral, geraniol and myrcene. In this way, the whole essential oil has greater antibacterial activity than its major components (112). Therefore, the analysis of chemical composition of essential oils is a fundamental step in the quality control of essential oils.

Monoterpenes have been the main class of compounds detected in most of the essential oils analysed, and more specifically the oxygenated ones; with

the exceptions of *P. sylvestris* essential oil, in which monoterpene hydrocarbons was the main phytochemical group, and ginger and turmeric essential oils with sesquiterpene compounds, both hydrocarbons and oxygenated sesquiterpenes, respectively, as the principal phytochemical group. Basil, anise and wintergreen essential oils were the only ones in which aromatic compounds predominated (Chapters 1-8). The greater presence of one or the other represents the fingerprint of the essential oil as it provides it with a characteristic flavour and properties (111).

However, the qualitative and quantitative chemical composition of essential oils is not constant among the same plant species, because it is influenced by genetic and epigenetic, as well as environmental factors, such as mineral nutrition, water, light, temperature and soil type (abiotic), and pathogen, pest and herbivore attack (biotic) (113). Other factors, such as the dryness and extraction method also influence in the yield and chemical profile of the resulting essential oils (34,114). Therefore, to obtain the maximum yield as well as the richest qualitative and quantitatively chemical composition, a careful selection of the steps ranging from the cultivation of the plant to the method to obtain the essential oil is needed.

Regarding the genetic factor, there was a great variability in the chemical composition of the essential oils obtained from the hydrodistillation of nine different cultivars of *R. officinalis* grown under homogeneous environmental conditions in the glasshouse, which reflects the genotypic influence in the final characteristics of the essential oil. In certain cases, the main compounds were 1,8-cineole and camphor, as in rosemary essential oil analysed, whereas in other cases borneol, linalool or camphene were the

major ones. This fact affected the characteristics of the essential oil so that they could be distinguished even by the smell (115).

On the other hand, according to the environmental effect, the phenological stage also influences the yield and chemical composition of essential oils. This fact was observed in the volatile extracted from the aerial parts of Tunisian *E. globulus* in which two chemotypes were detected according to the growth stage: 1,8-cineole (13.23%) was the main compound identified at the vegetative stage, as eucalypt essential oil analysed, while *p*-cymene was the main one at full flowering (32.19%) and fructification (37.82%) stages (116). Similarly, the essential oil content of winter savory was also significantly affected by the plant age and growth stage, as well as the interaction between them, the two-year-old plants in full flowering stage being the ones that accumulate more essential oil (117). Furthermore, the cropping season in which the plant is cultivated also influences the content and composition of essential oils. In this way, the predominant compound in the essential oil of basil was different depending on a dry or rainy season, defining different chemotypes. Especially, methyl chavicol, main compound in *O. basilicum* essential oil studied, was highly influenced by the planting season, being predominant in the rainy one (118). In addition, the harvest time is one of the environmental factors that usually affects the chemical profile and characteristics in general of essential oils, although it depends on the species. For instance, the characteristics of *T. mastichina* essential oil have been less affected by the year of harvest than other Labiateae species as *Salvia lavandulfolia* Vahl and *Lavandula latifolia* Medik., collected in full bloom during 3 years (119). In the case of Tunisian oregano essential oil, the yield varied from one year to another reaching the

highest value when they were collected in the harvest time with rainfall and flowering period (120).

Even the treatment to prepare the aromatic plants prior to obtain the essential oil affects the result. For instance, the yield and composition of peppermint essential oil obtained from leaves varied according to the drying method. Drying of peppermint leaves at ambient air as well as hot air temperatures of 50 °C, 60 °C, and 70 °C can be recommended for high oil yield and to obtain a better quality product in terms of menthofuran, neoisomenthol and 1,8-cineole (114).

Sometimes, the variability in the chemical composition of essential oils occurs naturally; however, other times the yield and chemical profile are manipulated in order to satisfy the consumers' demand. Specifically, it consists of the addition of synthetic and/or natural compounds, related or not with the authentic composition of essential oils, in order to accomplish the requirements or increase the benefits. It is estimated that approximately 80% of commercially available, so-called "natural" essential oils are adulterated in some way (121). For this reason, authentication of commercial essential oils is fundamental to give consumers reliable information about the essential oil purchased (Chapter 4). In this study, essential oils were principally purchased from Guinama and Pranarôm, two companies addressed to the pharmaceutical sector and particularly specialized in the distribution of raw materials, and in scientific and medical aromatherapy, respectively. In general, both trademarks offered a wide range of details of commercialized essential oils, including the batch and expiration date, main compounds, security warnings, use… In general, this data coincides with the results obtained, meaning that the essential oil

extracted was pure, free of adulteration, but in some other cases, mainly in Guinama company, more information about the main compounds as well as the scientific name instead of the common name is needed (Chapter 4).

4.2. PHYTOTOXIC ACTIVITY OF THE ESSENTIAL OILS AGAINST THE WEEDS, FOOD CROPS AND INVASIVE PLANT SPECIES

The *in vitro* phytotoxic effect of the essential oils against the seed germination and seedling growth of the weeds, food crops and invasive plant species was determined at different doses (0.125, 0.25, 0.5 and 1 µL/mL). After that, the *in vivo* phytotoxic activity of those essential oils with more remarkable herbicidal activity and less injury for food crops *in vitro* was studied at two doses (5 and 10 µL/mL).

Taking all data into account, oregano and winter savory essential oils completely inhibited the seed germination of the three weeds tested, *P. oleracea*, *L. multiflorum* and *E. crus-galli*, at all doses applied (Chapter 1), being the most potent herbicidal products of all the essential oils assayed (Figures 10 and 11). Indeed, both essential oils have by far demonstrated their strong phytotoxic activity (77,122), for which natural herbicide compositions are based on oregano essential oil (123) and particularly winter savory essential oil has been proposed by Institut Technique de l'Agriculture Biologique (ITAB) as "basic substance" with value for plant protection (124). This potent activity may be due to their main components carvacrol and thymol that have demonstrated their remarkable activity not only as herbicides (125), but also as antimicrobials for which they have already been included in antimicrobial compositions (126).

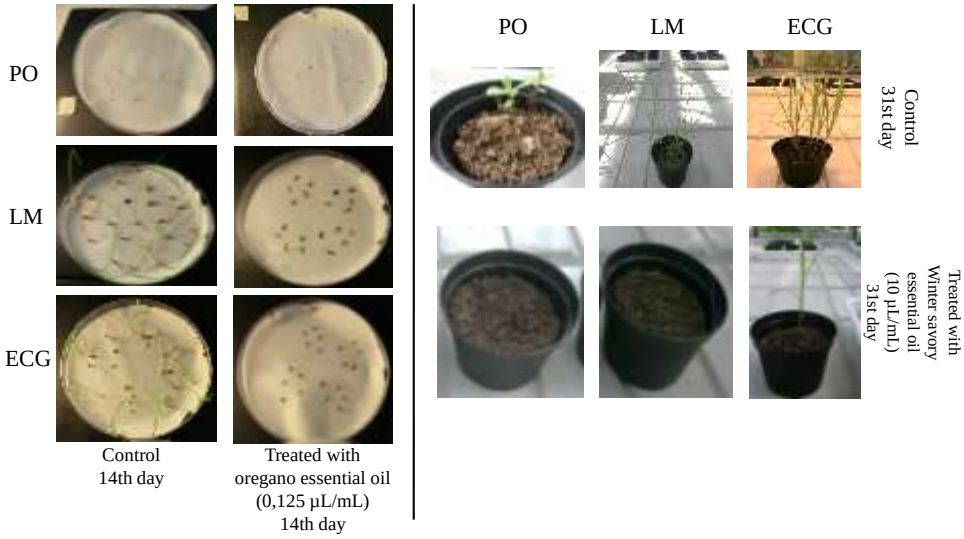


Figure 10. Left: In vitro control growth of P. oleracea, L. multiflorum and E. crus-galli compared with their development with oregano essential oil at 0.125 µL/mL in the 14th day of experimente. Right: In vivo control growth of the same weeds compared with their development with the emulsionable concentrate containing winter savory at 10 µL/mL in the 31st day of treatment.

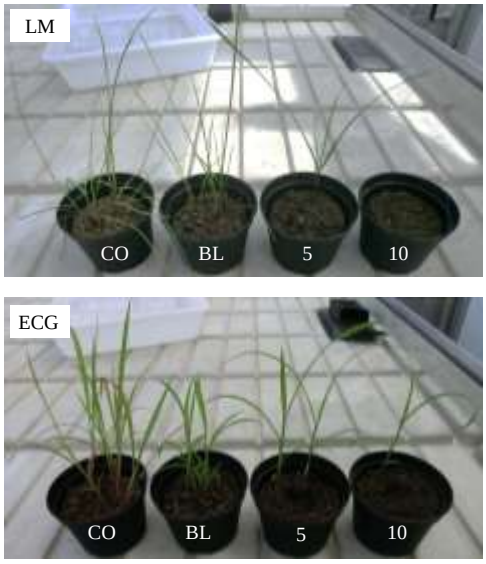


Figure 11. L. multiflorum (up) and E. crus-galli (down) in vivo growth control (CO), with only excipients of the emulsionable concentrate (BL) and treated with the emulsionable concentrate containing winter savory essential oil at 5 and 10 µL/mL (5 and 10, respectively).

However, the phytotoxic effect of these essential oils is not selective as they also affected the seed germination and seedling growth of food crops. On the one hand, oregano essential oil exhibited an *in vitro* dose-dependent phytotoxic activity against the seed germination and seedling growth of both cucumber and tomato, reaching major inhibition percentages against this last one until achieving a complete inhibition at the highest dose (1 µL/mL) assayed (Chapter 8, unpublished data) (Figure 12).

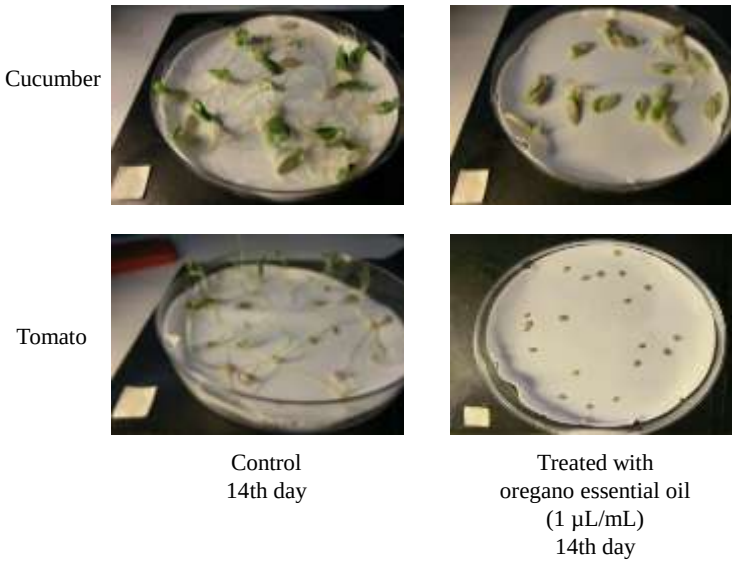


Figure 12. In vitro control growth of cucumber and tomato compared to the one with oregano essential oil at 1 µL/mL in the 14th day of experiment.

On the other hand, winter savory volatile oil applied *in vivo* as active principle in an emulsifiable concentrate totally inhibited the seed germination of maize and rice, and between 80 and 98% of tomato at 5 and 10 µL/mL, respectively (Chapter 1) (Figure 13).

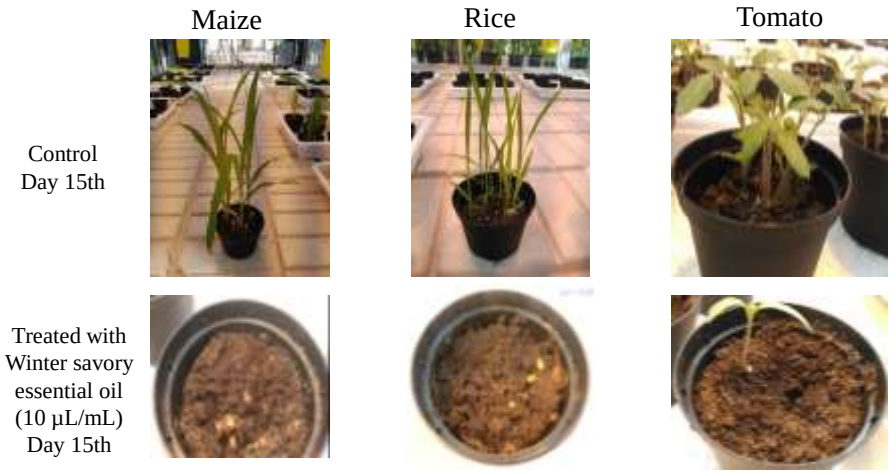


Figure 13. In vivo control growth of maize, rice and tomato compared to the one with emulsionable concentrate containing winter savory essential oil at 10 µL/mL in the 15th day of experiment.

Regarding this, oregano and winter savory essential oils could be used as bioherbicides in the control of weeds in non-agricultural fields. Especially, the wide-spectrum phytotoxic effect of winter savory essential oil has already been reported: it has exhibited high inhibition of seed germination and root and shoot growth of other weeds as *Vicia sativa* L. and *Chenopodium album*, with the drawback of causing serious injuries to crops *Z. mays*, *Triticum durum* L., *Pisum sativum* L., *Lactuca sativa* L., *Raphanus sativus* L. and *Capsicum annuum* L.(127,128).

The total phytotoxic effect of oregano and winter savory essential oils is especially important against *P. oleracea*, which has been the most sensitive weed to the effect of essential oils studied, in general, coinciding with other authors (79). This weed not only affects agricultural fields, but also lawns, driveways, dunes, beaches, salt marshes, waste areas, eroded slopes, bluffs and riverbanks due to its wide tolerance to changes in photoperiod, light intensity, temperature, moisture and soil type, as well as a rapid growth.

Therefore, it is considered very troublesome worldwide (129). According to our results, both oregano and winter savory essential oils are natural alternatives for the control of *P. oleracea*.

It is also remarkable the fact that peppermint essential oil completely inhibited the seed germination of *L. multiflorum* both *in vitro* and *in vivo* (Chapter 1). This result is especially outstanding for the pre-emergent control of this weed in rice paddies: peppermint essential oil showed a lower phytotoxic effect over rice with an inhibition percentage of seed germination between 16.30-18.48% at 0.125- 0.5 µL/mL, concentrations at which there was a complete inhibition of the weed (Figure 14).

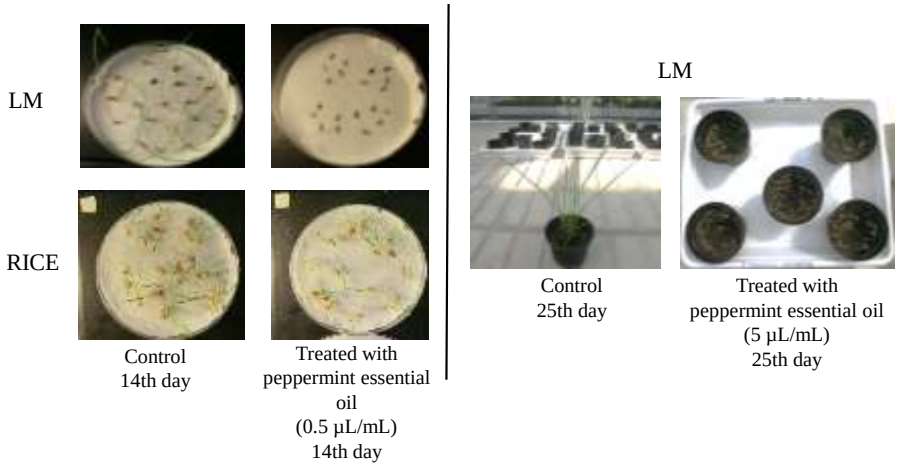


Figure 14. Left: In vitro growth of L. multiflorum and rice. Control and treated with peppermint essential oil at 0.5 µL/mL the 14th of experiment. Right: In vivo development of L. multiflorum. Control and treated with the emulsionable concentrate including peppermint essential oil at 5 µL/mL the 25th day of experience.

Likewise, lavender essential oil also represents a promising candidate for the development of bioherbicides for the control of *L. multiflorum* in cucumber crops as it decreased the hypocotyl and radicle by 87.8% and 76.6%, respectively, without causing a significant reduction in the seed

germination and hypocotyl growth of the crop (Chapter 3). In general, the seed germination and seedling growth of *L. multiflorum* were sensitive to the effect of assayed essential oils. These findings are especially useful nowadays due to increasing cases of resistance of this weed to glyphosate and many other synthetic herbicides (130–132), representing one of the greatest challenges to global agriculture because resistant *L. multiflorum* drastically reduces the yield of food crops (133).

Furthermore, rosemary and eucalypt essential oils could be employed at the tested doses, as post-emergent treatment in the control of particularly *E. crus-galli* in cucumber and tomato orchards (Chapters 6-8) as these essential oils strongly inhibited both the seed germination and its seedling growth of the weed in a dose-dependent manner, whereas they caused minimum injury to both crops. Other authors have corroborated that rosemary essential oil could be used as pre-emergence agent in other weeds with *in vivo* assays in which rosemary essential oil included in starch-based microencapsulates has been able to inhibit the germination of seeds and growth of the tested species for longer time and in a more sustainable way (134). And, according to our results, as post-emergence bioherbicide at concentrations higher than 20 mM, in the control of the weeds *T. incarnatum*, *S. marianum* and *P. minor* (86). In both cases, in order to obtain the maximum inhibitory effects of rosemary essential oil on weeds and not to crops, a careful harvesting of rosemary plant at full ripened fruit stage should be done (135).

On the other hand, the phytotoxicity against *E. crus-galli* seems to be a common feature between *Eucalyptus* spp.: *E. tereticornis* essential oil also affected the seed germination and seedling growth of the weed at 100-250

µg/mL. Besides, it also reduced the respiratory activity in 60% and affected the macromolecules at the highest dose (136). Furthermore, *E. globulus* essential oil has been effective against other weeds such as *Amaranthus blitoides* S.Wats. and *Cynodon dactylon* (L.) Pers. causing a significant decrease in the germination percentage, germination rate, radicle length, plumule length, seedling height, primary root length and primary pedicle length (137).

According to the rest of essential oils assayed, they showed significant effects in hypocotyl and/or hypocotyl + radicle length depending on the weed and dose, some of them being useful as post-emergent treatment of weeds. For instance, turmeric essential oil showed significant inhibition of the hypocotyl growth of *P. oleracea*, *L. multiflorum* and *E. crus-galli* at all the tested doses (0.125-1 µL/mL) assayed, whereas it did not affect either the seed germination or seedling growth of tomato, cucumber and rice (Chapter 2).

Regarding food crops sensitivity, cucumber was the most resistant food crop *versus* almost all essential oils assayed. Particularly, the seed germination and hypocotyl development were the less affected at all applied doses of the essential oils (Chapters 1, 2, 3, 8). However, other essential oils, such as clove essential oil showed herbicidal effects on cucumber seedling growth through a different mechanism of paraquat (138). The results obtained are interesting because the major part of these essential oils was injurious at the same doses for weeds. On the contrary, tomato was the most sensitive food crop to the application of essential oils, especially its seedling growth. The sensitivity of tomato has been widely described with

Curcuma zedoaria, *R. officinalis* and *Satureja* spp. *S. kuzestanica*, *S. rechingeri* and *S. hortensis* essential oils (79,85,139,140).

In relation to invasive plant species, *C. selloana* showed higher sensitivity to essential oils than *N. glauca* (Chapters 2, 5), obtaining interesting results at the highest dose (1 µL/mL) assayed. Ginger, turmeric, tea tree and wintergreen essential oils significantly affected the seed germination of *C. selloana* at 1 µL/mL Specifically, it was turmeric essential oil which showed noteworthy phytotoxicity against this invasive species reaching high inhibition percentages of the seed germination (81.71%) and hypocotyl and radicle growth (97.83 and 99.74%, respectively) at the higher dose (1 µL/mL) assayed. Also high values in seedling growth up to 96.38 and 96.65% of reduction of both hypocotyl and radicle elongation, respectively, were obtained with wintergreen essential oil. These results are particularly interesting in the management of these invasive plant species with alarming fast and wide expansion, representing an ecological hazard nowadays.

Despite these plants being native to South America (141–143), their continuous use and trade as ornamental plants around the world have favoured their spread along non-native systems (144), such as Australia, Hawaiian islands, the Pacific coast of the USA and Southern Europe (141,142,145), having an especially remarkable impact on the Mediterranean Basin (146,147). In their new habitat, invasive species compete with the natural flora and produce changes in the soil properties affecting the native species and life diversity (148,149). So, the use of these commercial essential oils with short soil half-life and without toxicity for human health represents a sustainable alternative in the restoration of affected ecosystems.

4.3. ANTIOXIDANT ACTIVITY OF ESSENTIAL OILS

Antioxidants are compounds that help to delay or inhibit the oxidation of other molecules through the inhibition of either initiation or propagation of oxidative chain reactions (150). In this study, the ability of 21 essential oils to donate hydrogen atoms or electrons was evaluated spectrophotometrically by DPPH method. The essential oils assayed which reduced DPPH to the yellow coloured product and decreased the absorbance at 517 nm possessed antioxidant activity.

These results were compared with the reference ones (quercetin, BHT and ascorbic acid) at different concentrations to estimate the antioxidant potency (Figure 15).

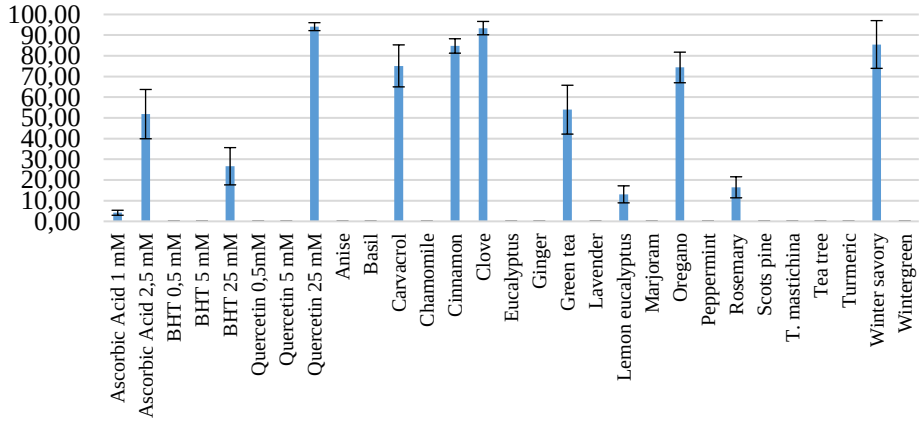


Figure 15. Antioxidant activity of essential oils studied and comparison with references, quercetin, ascorbic acid and BHT at different concentrations.

Particularly, 10 µL of clove essential oil reduced DPPH in 93.4%, being the essential oil with the highest antioxidant activity of all assayed. Indeed, this result was comparable to the most potent antioxidant reference at 25 mM quercetin (94.11%). The fact that clove essential oil had more analogous antioxidant activity than quercetin is especially interesting as quercetin,

natural antioxidant belonging to flavonoids, is considered a powerful free radical scavenger, even more potent than others such as curcumin, commonly found in foods and widely-known for its many beneficial effects on health (151–153). In previous studies, clove essential oil has also demonstrated the highest percentage of inhibition of DPPH radical, above other essential oils like oregano, thyme, rosemary and sage (154), and even higher than combinations between them (155). Furthermore, clove essential oil has not only shown DPPH radical reduction, but also of ABTS (2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) and superoxide anion, besides hydrogen peroxide scavenging, and metal chelating activities compared with reference antioxidants, including α-BHA, BHT, tocopherol and trolox (156). As a consequence, clove essential oil represents an alternative and natural preservative of foodstuff, such as meat products, without affecting the organoleptic properties (157). In fact, clove essential oil incorporated in an active packaging for sausages showed an antioxidant effect at the end of a 15 day refrigeration period without causing significant variations in pH and water content of the product (158).

In our study, winter savory and cinnamon essential oils also exhibited a relevant antioxidant activity, with values of 85.49 and 84.77%, respectively. Both essential oils have previously shown significant higher persistent antioxidant activity (159). Winter savory essential oil has already demonstrated a retardation of lipid oxidation in cured meat (160,161). Cinnamon essential oil has revealed remarkable *in vitro* DPPH radical scavenging activity in comparison to α- tocopherol, BHA and BHT mainly attributed to cinnamaldehyde and eugenol (162,163). Due to this activity, cinnamon essential oil has been included in packaging containing pre-harvest and resulting foodstuff with the purpose of extending the shelf-life

of food. In this way, biodegradable polyester nets with cinnamon were observed to maintain the quality of tomatoes during storage (164) and carboxymethyl cellulose (CMC)-polyvinyl alcohol (PVA) based films containing cinnamon demonstrated a great improvement of the antioxidant properties for bread preservation (165).

Furthermore, the antioxidant potential of oregano essential oil was compared to the one exerted by its main compound, carvacrol. Analogous antioxidant activity was shown between both of them (74.39 and 75.14%, respectively). However, although carvacrol showed higher antioxidant activity than oregano essential oil in this study, previous authors have reported the contrary, maybe as a consequence of a synergistic effect between the different compounds present in oregano (166). Anyway, the strong antioxidant activity of both the essential oil and the oxygenated monoterpene compound have been generally demonstrated, even higher than BHT in accordance with our results (166). In relation to this, oregano essential oil has been combined with BHT showing a synergistic and consequently higher antioxidant activity avoiding lipid oxidation in food, specifically sunflower oil (167). Similar to our findings, oregano essential oil and carvacrol have also demonstrated their remarkable antioxidant effect by other means, such as the chelating effect (168). Specific, carvacrol has shown such antioxidant potential that it has already been incorporated in materials like gelatin edible films and potato starch nanofibers for its application in food preservation (169,170). Nevertheless, different isolated components of other essential oils have improved the quality of food more than carvacrol, for instance *trans*-anethole and eugenol (171). Combined with other components as thymol, it has had an additive effect at lower doses but antagonistic at higher concentrations (2.50 or 2.66 mM) (172).

On the other hand, the antioxidant activity of green tea essential oil with *cis*-methyl dihydrojasmonate (15.82%) as the main compound (173) was relatively high with 53.99%, analogous to the one of 2.5 mM ascorbic acid with a percentage of 51.83%.

Finally, rosemary (1,8-cineole, 25.0%; camphor, 20.5%) and lemon eucalypt (citronellal, 88.0%) essential oils showed low DPPH reducing power (16.46 and 13.04%, respectively), values between the one of the synthetic antioxidant BHT (25 mM) and ascorbic acid (1 mM). Previous studies have confirmed the moderate antioxidant activity of rosemary essential oil (174) with a wide range of DPPH radical scavenging activity (8.16-51.80%) (175). In the same way, lemon eucalypt essential oil has also displayed medium-low antioxidant capacity, even smaller than other essential oils with lower antioxidant activity in this investigation, such as wintergreen essential oil (176,177).

The other essential oils studied here showed negligible antioxidant potential, such as eucalypt (1,8-cineole, 76.4%) essential oil whose weak antioxidant capacity has also been reported by other authors (178). In contrast, other essential oils that have shown negative antioxidant activity similar to eucalypt essential oil under this investigation, have demonstrated free radical scavenging activity in previous studies. It may be due to a diverse chemical composition, both qualitatively and quantitatively. For instance, wintergreen essential oil also with methyl salicylate as main component (96.90%) but considerable amount of limonene (2.17%) different from our sample (limonene, 0.01%), exhibited moderate antioxidant activity with a dose-dependent DPPH-radical-scavenging (179). Likewise, turmeric essential oil with higher percentage of α-

turmerone (42.6%) than ar-turmerone (12.9%) unlike in our case (ar-turmerone, 38.7%; α-turmerone (14.2%), possessed good antioxidant activity in ABTS and DPPH methods between 0.54-10.03 mg/mL, respectively (180).

4.4. ANTIMICROBIAL ACTIVITY OF ESSENTIAL OILS

In this study, the antibacterial and antifungal activities of 21 essential oils at different doses (1, 5, 10 and 20 µL) were evaluated against the growth of the Gram-negative bacterium *P. syringae* pv. *tomato*, the causative agent of the bacterial speck disease producing important economical loses on tomato (181), and the fungus *F. oxysporum* f. sp. *lycopersici*, casual agent of the vascular wilt in tomato (182) (Figures 16 and 19).

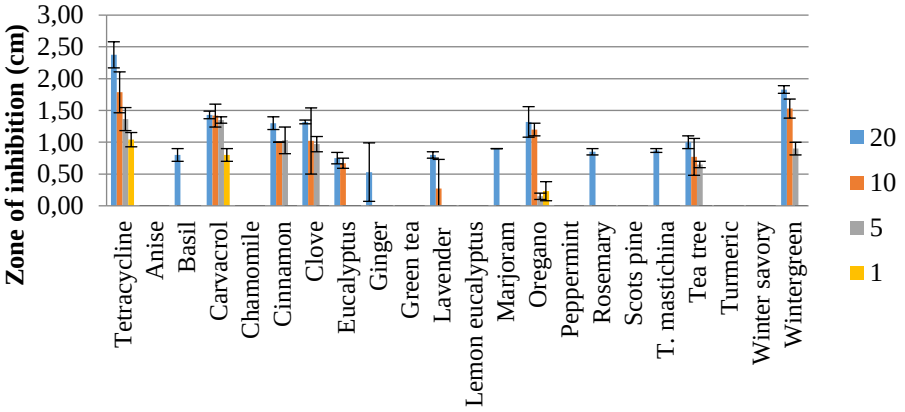
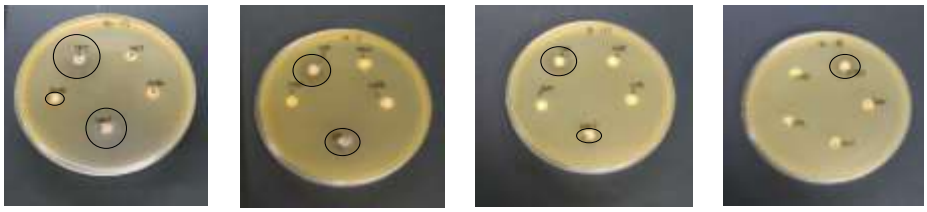


Figure 16. Antibacterial activity of essential oils studied by Kirby-Bauer method. The lenght of inhibition (cm) of P. syringae by the essential oils at several doses (1, 5, 10 and 20 µL) was measured and compared with the reference one (tetracycline).

Wintergreen essential oil showed the major values of growth inhibition of *P. syringae* at the highest doses (20 and 10 µL) applied (1.83 and 1.53 cm, accordingly) (Figure 17). The antimicrobial activity of this essential oil has

already been reported, having effect on a wide-spectrum of Gram-positive and Gram-negative food spoiling bacteria and fungi (179).



20 µL 10 µL 5 µL 1 µL

Figure 17. Dose-dependent antibacterial effect of wintergreen essential oil against P. syringae in comparison to tetracycline.

Followed by this, it was carvacrol, principal component of *O. vulgare* essential oil, the one with the best antibacterial activity. Althoug it did not inhibit the bacterial growth completely at the highest dose (20 µL) assayed (1.43 cm *vs.* 1.83 cm by wintergreen), it showed elevated reduction of bacterial development in the rest of the treatments (1.42 and 1.35 cm at 10 and 5 µL, respectively). It even reached considerable antibacterial effect at the lowest one (1 µL) (0.80 cm) (Figure 18). However, in other studies, carvacrol has exhibited negligible activity against *P. syringae* in comparison to other components of essential oils, such as eugenol, when incorporated in films at doses between 1 and 4 mg/cm^2 (183). Anyway, the antimicrobial properties of carvacrol have been confirmed in general, being considered a natural alternative antimicrobial agent for future application in food preservation (184). In fact, the following essential oil with more antibacterial effect against *P. syringae* was oregano as, analogous to its main component carvacrol, it achieved inhibition of the bacterial growth even at 1 µL, reaching a halo of inhibition of 0.23 cm (Figure 18). Indeed, oregano essential oil containing carvacrol as the main compound, has

shown inhibitory activity against different strains of *P. syringae* better than the antibiotic streptomycin (185).

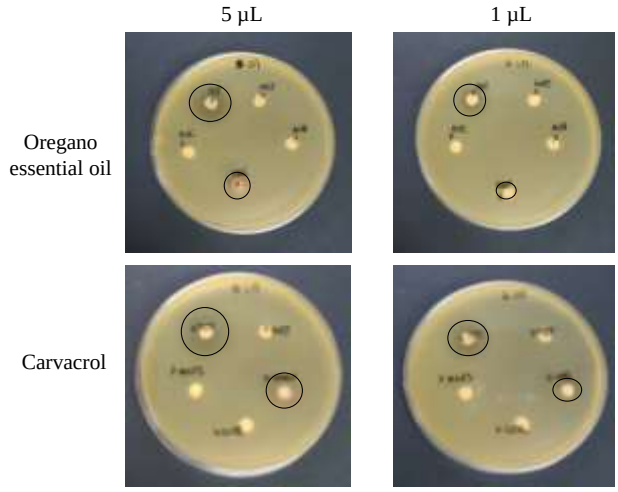


Figure 18. Effectiveness of oregano essential oil and carvacrol at the lowest doses (5 and 1 µL) assayed against P. syringae.

The antibacterial activity of cinnamon and clove essential oils with eugenol as the principal component (56.34% and 89.37%, respectively) was also noticeable and comparable between each other. However, differently to the previous essential oils, they did not inhibit the bacterial growth at the lowest dose (1 µL) assayed. Equally, cinnamon essential oil with cinnamaldehyde as the main compound (~70%) has also already demonstrated highly effective and specific antibacterial activity against *P. syringae*, being able to inhibit the bacterial growth after 24 h at a concentration as low as 0.016% (v/v) (186). This effect was even improved when encapsulated into mesoporous silica nanoparticles (MSNPs) eliminating 99.9% of the bacterial growth (186). Conversely, different results have been obtained from these essential oils with the same main compound against *P. syringae* (187).

Similarly, tea tree essential oil, whose antimicrobial properties have been extensively described (188), showed under this investigation antibacterial activity only at the three major doses (20, 10 and 5 µL) applied, reaching reduction levels half of the tetracycline ones.

Eucalypt and lavender essential oils only showed antibacterial effect at the highest doses (20 and 10 µL) assayed. Although the reduction at the highest dose was comparable between both essential oils (0.75 and 0.80 cm, respectively), the values were quite different at 10 µL with 0.67 cm of growth inhibition zone with eucalypt essential oil and 0.27 cm with lavender one. Also in previous reports, eucalypt essential oil has observed to be highly effective against *P. syringae* pv. *tomato* (189). Furthermore, together with cinnamon and clove essential oils, it has caused inhibition zones of *P. syringae* pv. *tomato* at a concentration of 1% (90).

Ginger, basil, rosemary, *T. mastichina* and marjoram essential oils only exhibited antibacterial activity at the highest dose (20 µL) tested. In previous works, *P. syringae* has shown certain resistance to essential oils, the lack of activity of both rosemary and ginger essential oils being documented against *P. syringae* pv. *tomato* and *P. syringae* pv. *syringae,* respectively (189,190). Finally, basil and rosemary essential oils have been effective against other harmful plant pathogenic bacteria of agricultural importance *Erwinia amylovora* and *Xanthomonas campestris* pv. *campestris*, respectively (190).

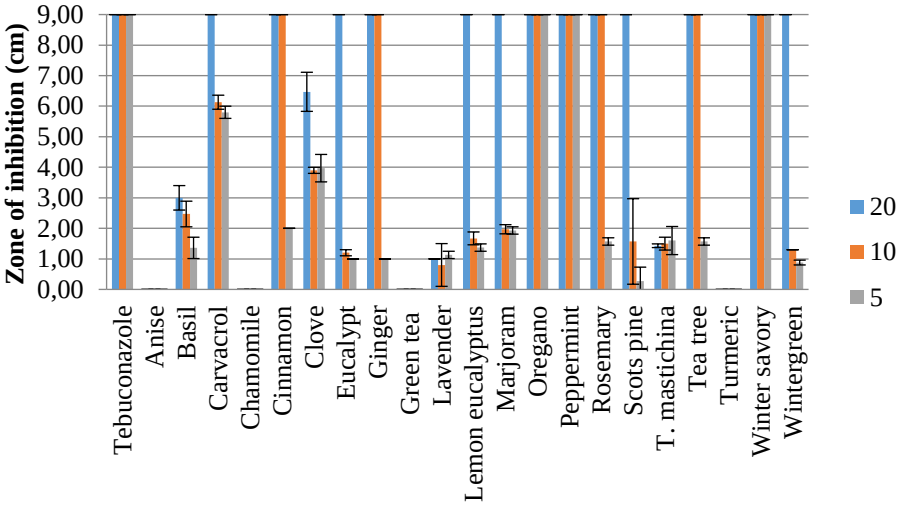


Figure 19. Antifungal activity of essential oils studied. The lenght of inhibition (cm) of F. oxysporum by the essential oils at several doses (5, 10 and 20 µL) was measured.

Regarding the antifungal properties, the essential oils of oregano, winter savory as well as peppermint were the ones with the most remarkable antifungal activity as they completely inhibited the mycellial growth at all doses (20, 10 and 5 µL) assayed (Figure 19). Other authors have also described the strong antifungal activity of oregano essential oil against other fungi, such as of *Penicillium corylophilum* at MIC 0.6250 µL/mL (191). Its potent antifungal activity makes it suitable to be incorporated in active films for post-harvest conservation against phytopathogenic fungi including *A. alternata*, *Geotrichum candidum* and *R. stolonifer* (192). Besides, peppermint essential oil has already shown effective inhibitory effect of *F. oxyspourm*, among other important horticultural phyto- and mycopathogens (193).

Tea tree, cinnamon, ginger and rosemary essential oils totally inhibited the fungal growth at the highest doses (20 and 10 µL) with low activity at 5 µL

(Figure 19). Particularly, tea tree essential oil is considered a natural antimicrobial. In many other studies, its wide-spectrum of antifungal activity has been described against the phytopathogenic fungi *Ascochyta rabiei*, *Colletotrichum lindemuthianum*, *F. graminearum*, *F.culmorum*, *Drechslera avenae*, *A. radicina*, *A. dauci* and *A. ochraceus*, which can be attributed to its main compounds (194,195). Also, the antifungal activity of ginger essential oil against *F. oxysporum* has been previously notified even at lower dose of only 0.3 % (v/v) (196). For its part, rosemary essential oil has also been especially damaging for *S. sclerotiorum* (193).

Wintergreen, eucalypt, scots pine, lemon eucalypt and marjoram essential oils absolutely inhibited the growth of *F. oxysporum* only at the maximum dose (20 µL), whereas the fungal reduction was low at the rest of the treatments. Carvacrol, principal component of *O. vulgare* analysed, also inhibited the growth of *F. oxysporum* at 20 µL and together with clove essential oil, both showed a relevant activity not only at the maximum doses, but also at 10 and 5 µL (Figure 19). Related to this, other authors have already reported the moderate antifungal and mycotoxin inhibitory activity of clove, also against *F. oxysporum*, which can be improved by encapsulating in nanoemulsions (193,197).

Basil, *T. mastichina* and lavender essential oils showed low antifungal potential at the three doses. The lack of antifungal activity of lavender essential oil has been previously demonstrated against *F. oxysporum* f. sp. *lycopersici*, as well as other pathogens of agricultural interest as *A. alternata*, *A. brassicae*, *B. spicifera*, *B. cinerea*, *R. solani*, *Cladobotryum mycophilum*, *C. gloeosporoides*, *Curvularia hawaiiensis*, *F. equiseti*, *F. graminearum*, *Phytophthora parasitica*, *Pythium aphanidermatum*, *P.*

expansum, P. italicum, S. sclerotiorum and *Trichoderma aggressivum* f.sp. *europaeum* (193,198). However, *T. mastichina* essential oil with also 1,8-cineole (43.26%) and a higher amount of linalool (36.72%) than in our study (5.7%) has shown remarkable antifungal activity against other phytopathogenic fungi, such as *S. sclerotiorum* (193).

Turmeric, anise, chamomile and green tea did not exhibit antifungal activity at neither dose (20, 10 and 5 µL) assayed (Figure 19).

4.5. ENCAPSULATION OF PINE ESSENTIAL OIL IN MCC

4.5.1. Encapsulation efficiency (EE)

MCC was chosen for this investigation due to its numerous benefits as wall material for encapsulation in general. Especially, it represents the most suitable material for extrusion technique as when wetted it has the appropriate rheological properties, cohesiveness and plasticity to yield strong extrudates (100). Also, it is the right excipient for compressibility enhancement, binding in wet and dry granulation, thickening and viscosity building in liquid dosage forms and free flowing in solid dosage forms (199). These characteristics ensure a rapid disintegration and release of the active principle (200).

However, little has been reported about encapsulation of specifically essential oils in MCC until now. It is necessary to study the behaviour of both MCC and essential oil together and observe if it represents an effective wall material to improve the stability and prolong the shelf life of the essential oil.

In this study, the EE reflects the percentage of protection of scots pine essential oil at 1 and 10 µL/mL embedded within MCC. It was calculated by using the formula shown in Section 3.8.4., which demonstrated that the

EE of prepared extrudates with 1 and 10 µL/mL was 63.19 and 41.59%, respectively.

These results are comparable with other studies in which different materials have been also used to encapsulate essential oils. For instance, β-CD has been repeatedly used as entrapping material for essential oils and their components. Particularly, it showed a high encapsulation efficiency of 34.8% when combined with 2-nonanone, major component of the rue (*Ruta chalepensis* L.) essential oil (201), in a ratio of 1:0.5 (202). On the other hand, liposomes made of soy lecithin, cholesterol and anhydrous ethanol entrapped 4 mg/mL of *E. citriodora* essential oil with an efficiency of 22.47±1.03% (203). Another material, alginate microbeads, incorporated basil essential oil with a high encapsulation efficiency of 60% (204).

In this study, there was a decreasing tendency in EE as increasing the initial concentration of essential oil. Other studies have also reported this fact; for example, the maximum percentage of EE of clove and oregano essential oils in chitosan was obtained at the minimum concentration of the essential oils (205,206).

4.5.2. Oil release studies

The study of the release profile of scots pine essential oil at 1 and 10 µL/mL from the extrudates made of MCC was carried out with the aim of evaluating the effectiveness of the encapsulation, that is to say the retention capacity of the essential oil by MCC, as well as observing the release tendency along time (Figures 20 and 21).

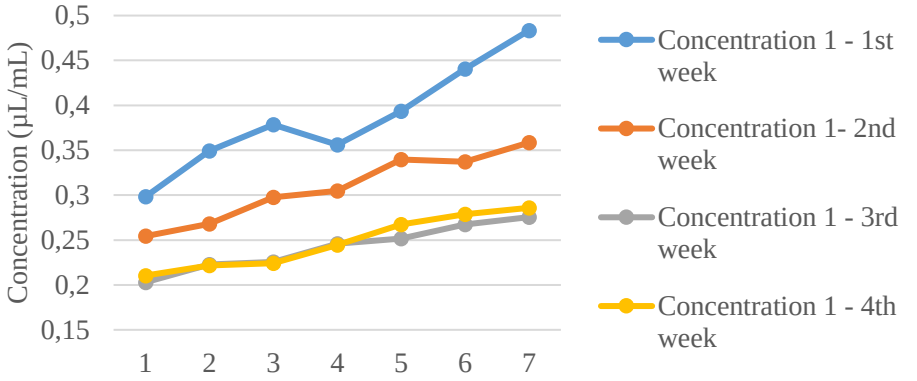


Figure 20. Variation in the concentration of scots pine essential oil at initially 1 uL/mL along seven hours in four weeks.

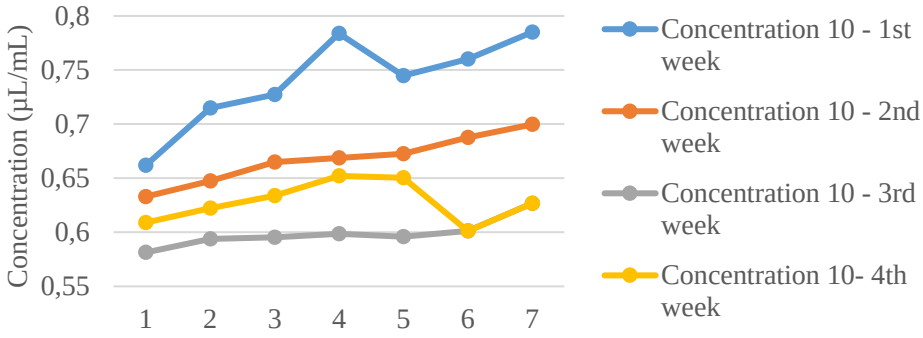


Figure 21. Variation in the concentration of scots pine essential oil at initially 10 uL/mL along seven hours in four weeks.

As it can be observed in Figure 20, the initial concentration of scots pine essential oil (1 µL/mL) in MCC decreased to almost 0.30 µL/mL after the first week of storage, meaning a loss percentage of 70.17% of the essential oil. This fact may be as a consequence of the primary liberation of the most superficial fractions of scots pine essential oil adsorbed in MCC.

In the second week of measurement, 0.25 µL/mL of scots pine essential oil still remained entrapped by MCC (Figure 20). Following this, scots pine essential oil showed a similar release pattern in both measurements, third and fourth weeks of measurement (Figure 20). Data obtained in these weeks

would represent the subsequent release of scots pine essential oil more deeply trapped in MCC until reaching a ceiling of release in which the concentration of the essential oil remained constant. So, if the oil release were to be measured one more week, the tendency of the resulting curve would be the same as the previous one probably. As a result, MCC achieved in retaining an average of 24.15% of the essential oil during storage in the four weeks of experience.

Furthermore, an increasing release of scots pine essential oil along the seven hours of measurement can be observed in the four curves represented in Figure 20. Specifically, a cumulative release of 18.51, 10.42 and an average of 7.4% were recorded after seven hours of measurement in the first, second and third-fourth weeks, respectively. These percentages decreased over the weeks due to a reduced initial concentration of the essential oil.

It is important to value that scots pine essential oil was still present in MCC extrudates after four weeks of storage at constant conditions of temperature and humidity. Although the initial concentration was reduced by a considerable percentage, a quantity of 0.28 µL/mL was still released in the fourth week of storage. At this and even lower concentrations, *P. sylvestris* essential oil caused a significant reduction in the seedling growth of *P. oleracea*, as well as a remarkable inhibition of the radicle development of *L. multiflorum* without affecting the growth of the food crops cucumber and tomato (Chapter 3).

On the other hand, the release profile of scots pine essential oil contained in MCC in a concentration of 10 µL/mL is represented in Figure 21. In this case, the release rate is more sustained than in the previous situation (Figure 20) and the four curves are closer to each other.

On this occasion, the initial concentration of scots pine essential oil (10 µL/mL) in MCC decreased to 0.66 µL/mL after the first week of storage, which represents a loss percentage of 93.4% of the essential oil. The amount was reduced to 0.63 and 0.61 µL/mL in the next two weeks of measurement, accordingly, until reaching a final concentration of 0.58 µL/mL in the final measurement (Figure 21). As a whole, an average of 6.2% of the essential oil was retained during storage in the four weeks of experience.

In comparison with the results obtained from the lowest dose (1 µL/mL) previously assayed, it would mean that at a higher concentration, faster volatility of the essential oil is produced and lower EE (205,206). Despite this, the final concentration of scots pine essential oil in extrudates with initial concentration of 10 µL/mL was still higher at the end of 7h of the experience than in those with 1 µL/mL (Figure 21).

4.5.3. Extrudate size measuring

The size of the resulting extrudates was measured in order to ensure homogeneity between them.

The shape of the extrudates was cylindric with rounded ends (Figure 22). The average length of 50 of these dried cylindric extrudates containing scots pine essential oil was 0.56±0.03 cm. It is possible that this result was smaller in comparison to the length of freshy-prepared extrudates. Related to this, previous authors have notified a contraction of the extrudates after the drying process due to water evaporation (204).

Figure 22. Appearance of extrudates containing scots pine essential oil.

4.5.4. Density measurement

The density of the extrudates represents an important parameter as it can vary depending on the formulation and/or process, and therefore affect other aspects, such as the potency of the finished product and the batch size determinations in the coating equipment (207).

In this investigation, the average density of the extrudates containing 1 and 10 µL/mL of scots pine essential oil was 1.58 g/cm^3. Previous authors have reported that the true density of MCC is expected to be lower than 1.582 g/cm^3 (208). So, the presence of scots pine essential oil at either dose (1 and 10 µL/mL) assayed did not alter the density of MCC.

5. CONCLUSIONS

1. Nineteen commercial essential oils from three trademarks have been analysed by Gas Chromatography-Mass Spectrometry analysis in order to know the chemical composition responsible for their biological activities assayed.
2. Oregano and winter savory essential oils with carvacrol (60.4±0.1% and 43.3±0.1%, respectively) as the main compound, were the most potent herbicides of the tested essential oils completely inhibiting the seed germination of *Portulaca oleracea*, *Lolium multiflorum* and *Echinochloa crus-galli* at all doses (0.125, 0.25, 0.5 and 1 μL/mL) applied. They also affected the seed germination and seedling development of food crops. However, they represent effective broad-spectrum herbicides less pernicious to glyphosate in weed control, mainly in non-agricultural fields.
3. Peppermint essential oil with menthol (48.2±0.4%), menthone (23.3±0.6%) and *iso*-menthone (16.3±0.0%) as principal components inhibited *in vitro* and *in vivo* the seed germination of *L. multiflorum*, showing lower phytotoxicity *versus* rice at the same doses. So, it constitutes a valid pre-emergent treatment for the control of *L. multiflorum* in rice paddies.
4. The majority of the studied essential oils could be employed as post-emergent treatment against *P. oleracea, L. multiflorum* and *E. crus-galli* in food crops. Particularly remarkable is the effectivity of lavender essential oil against *L. multiflorum* in cucumber, and turmeric essential oil against the three infesting cucumber, tomato and rice crops.
5. Essential oils represent a sustainable alternative in the management of the invasive species *C. selloana* and *N. glauca. Gaultheria procumbens*

(methyl salicylate 99.63%) and *Curcuma longa* (ar-turmerone 38.7%, β and α-turmerone 18.6% and 14.3%, respectively) essential oils could be potential alternatives to control (pre- and post-emergent) the high invasiveness of *C. selloana*; whereas *Eucalyptus citriodora* (citronellal 88.0%) essential oil represents an effective pre- and post-emergent management of the invasive species *N. glauca*.

6. In general, the studied essential oils showed more remarkable antifungal than antibacterial and antioxidant activity. Sixteen essential oils exhibited antifungal potential against *F. oxysporum* f. sp. *lycopersici* with respect to thirteen and nine essential oils that presented antibacterial and antioxidant properties, respectively. Among these sixteen essential oils, 12 had antimicrobiological properties in general as they also revealed antibacterial potential against *P. syringae*. Especially, four essential oils, cinnamon, oregano and its main component carvacrol, rosemary and eucalypt essential oils shared both antimicribiological and antioxidant properties, being the most active essential oils.
7. Microcrystalline cellulose represents an interesting wall material for the encapsulation of essential oils by means of the extrusion technique. Although the encapsulation efficiency diminishes as the initial concentration of essential oil was increased, *P. sylvestris* essential oil was still entrapped inside MCC until the 4th week of experiments with 20.29 and 5.8 % at 1 and 10 μL/mL, respectively, values at which the essential oil still shows herbicidal activity. Further work would be necessary in order to follow studying potential interactions between MCC and essential oils, as well as characteristics of the resulting extrudates.

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Publications

CHAPTER 1.

Phytotoxicity of essential oils on selected weeds: Potential hazard on food crops

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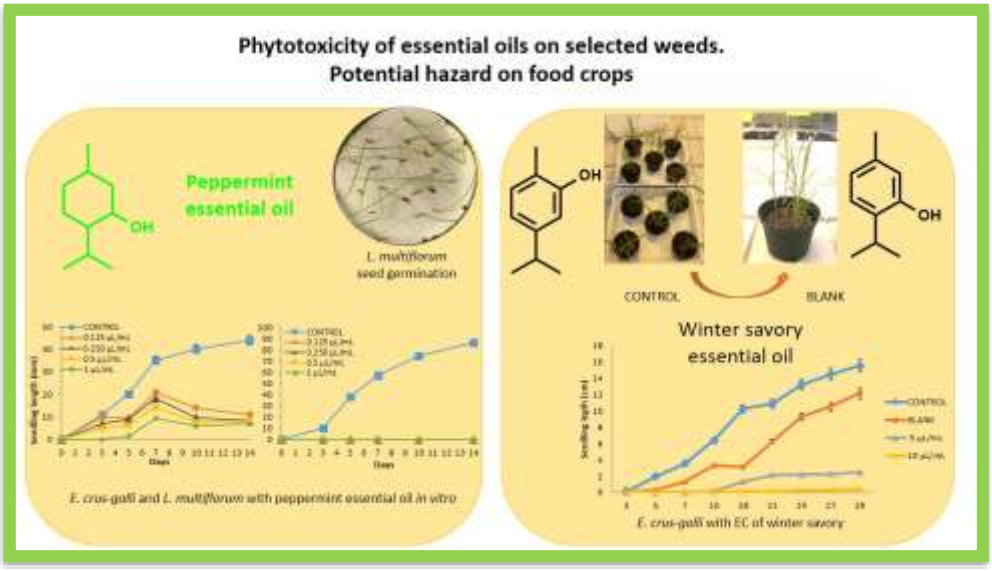

ABSTRACT

The chemical composition of winter savory, peppermint, and anise essential oils, and *in vitro* and *in vivo* phytotoxic activity against weeds (*Portulaca oleracea*, *Lolium multiflorum*, and *Echinochloa crus-galli*) and food crops (maize, rice, and tomato), have been studied. Sixty-four compounds accounting for between 97.67-99.66% of the total essential oils were identified by Gas Chromatography-Mass Spectrometry analysis. Winter savory with carvacrol (43.34%) and thymol (23.20%) as the main compounds produced a total inhibitory effect against the seed germination of tested weed. Menthol (48.23%), menthone (23.33%), and *iso*-menthone (16.33%) from peppermint only showed total seed germination inhibition on *L. multiflorum*, whereas no significant effects were observed with *trans*-anethole (99.46%) from anise at all concentrations (0.125-1 µL/mL). Low doses of peppermint essential oil could be used as a sustainable alternative to synthetic agrochemicals to control *L. multiflorum*. The results corroborate that *in vivo* assays with a commercial emulsifiable concentrate need higher doses of the essential oils to reproduce previous *in vitro* trials. The higher *in vivo* phytotoxicity of winter savory essential oil constitutes an eco-friendly and less pernicious alternative to weed control. It is possible to achieve a greater *in vivo* phytotoxicity if less active essential oil like peppermint is included with other active excipients.

Keywords: winter savory; peppermint; essential oils; food crops; weed control; phytotoxicity.

1. INTRODUCTION

The potential hazard to the environment and human health, as well as the emergence of resistant weeds, are still the main problems of the overuse of synthetic herbicides used to improve global crop productivity. The continuous use of glyphosate, marketed in 1974 as a highly effective broad-spectrum herbicide [1], has made particular populations, such as the annual ryegrass (*Lolium rigidum* L.) in Australia [2] or barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) in cotton fields of the midsouthern United States [3], become glyphosate-resistant [4]. Recently, glyphosate resistance has been described in many world-wide species, like common ragweed (*Ambrosia artemisiifolia* L.) in several row crops of the south-eastern USA, following other still unknown mechanisms of action [5]. Together, resistant problems; potential health risks including skin irritancy, muscle atrophy, and nerve axons damage with prolonged exposure; and the use of a surfactant to enhance penetration [6] can even induce acute poisoning in humans, as well as chronic and sub-chronic toxicity, which has been reported in mammals after the consumption of contaminated food [7]. As these problems have appeared, some other weed killers have been synthesised as alternative solutions. However, the latest generation of the synthetic triketone herbicide family (sulcotrione, mesotrione, and tembotrione) also has negative impacts in microbial edaphic communities or plants, and even their degradation products can be more toxic than those of the parent, leading to similar environmental problems to glyphosate [8].

Resistance to other agrochemicals has also been reported in *Lolium* spp., exhibiting resistance to ALS-inhibiting herbicides that hinder acetolactate synthase (ALS), the enzyme common to the biosynthesis of the branch-

chain amino acids (valine, leucine, and isoleucine) and ACCase (acetyl-coenzyme A carboxylase) inhibitors [9]. Similarly, common purslane (*Portulaca oleracea* L.) has developed resistance against linuron, a selective pre- and early post-emergent herbicide, in carrot (*Daucus carota*) fields [10]. In summary, weeds have evolved resistance in most of the known herbicide sites of action, being reported in 75 crops of 69 countries [11]. So, it is necessary to find more eco-friendly and less hazardous natural alternatives than synthetic herbicides, without promoting the emergence of resistance. In terms of natural compounds, essential oils are well-known for their multiple biological properties: anti-inflammatory, anticancer, antiviral, repellent, antibacterial, antifungal, or antioxidant, and have been widely used in the perfumery, cosmetics, pharmaceutical, and food industry, also being investigated to control crop pests [12]. In this sense, *Satureja montana* L. (Lamiaceae) was the highest effective larvicide of the essential oils tested against *Culex quinquefasciatus* [13] and was particularly active against several of the most damaging phytopathogenic fungi (*Fusarium*, *Alternaria*, *Rhizoctonia*, *Phytophthora*, and *Botrytis* spp.) that are able to destruct plant tissues, mainly cereals [15].

According to their phytotoxic capacity, *Satureja* spp. have potential as natural herbicides due to their main components, carvacrol and thymol, which are able to decrease in vitro germination and the growth of lambsquarters, common purslane, and barnyardgrass [15].

Another interesting bioresource is peppermint (*Mentha piperita* L.) essential oil, because it is able to exert a higher antimicrobial effect against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*, as well as upper antioxidant activity in DPPH free radical scavenging and β-

carotene/linoleic acid systems, compared to other plant species, such as *Myrtus communis* [16]. These characteristics make peppermint essential oil a possible suitable bio-preservative to prevent post-harvest food decay. In this sense, it could be used to delay mold formation and reduce the incidence of infections when included as part of a coating as a previous experiment with only low amounts of the volatile that were enough to control fungal rot affecting *Vitis labrusca* L. maintained fruit quality during storage [17]. Regarding our topic, it is one of the most phytotoxic essential oils of 12 aromatic species, including *Thymus vulgaris* and *Salvia officinalis* against *Amaranthus retroflexus*, *Avena fatua*, *Bromus secalinus*, and *Centaurea cyanus* [18]. In fact, a dose-dependent inhibition of seed germination percentage, root and shoot lengths, and dry weight of field bindweed (*Convolvulus arvensis* L.), purslane, and jungle rice (*E. colonum* L.) has been observed at different concentrations (0, 300, 600, 900, 1200, 1500, and 1800 µL/L) of peppermint essential oil, whereas horticultural crops such as tomato (*Lycopersicon esculentum* Mill.) and radish (*Raphanus sativus* L.) were even more susceptible [19].

On the other hand, it is also interesting to expand the research with anise (*Pimpinella anisum* L.) essential oil since it is an annual medicinal plant belonging to the Apiaceae family and popularly known for its widespread use in the food and drink industry [20]. Its essential oil has shown higher antioxidant activity in in vitro models than the synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), being possibly used for protecting fat-containing foods [21]. Antibacterial effects [22], as well as antifungal capacity, against *Saccharomyces cerevisiae*, *Aspergillus niger* [23], *Bipolaris/Dreschslera sorociniana*, *Fusarium subglutinans*, *Fusarium vertricilioides*, *Fusarium oxysporum*,

Fusarium tricinctum, *Fusarium sporotrichioides*, *Fusarium equiseti*, *Fusarium incarnatum*, *Fusarium proliferatum*, and *Macrophomina phaseolina* have been also demonstrated [24], especially against *Saccharomyces cerevisiae*, which was effectively inhibited by anethole, the main component of aniseed, with an MFC value of 200 µg/mL [25]. In addition, it has been able to exert an insecticidal effect against young larvae of the Colorado potato beetle [26]. According to its phytotoxic activity, it has been described as one of the least active Mediterranean essential oils: it has shown a lower in vitro inhibitory effect in the seed germination of garden cress (*Lepidium sativum*), even promoting its germination and/or radicle elongation, as well as in food crops, such as lettuce (*Lactuca sativa*) [27]. Despite its low phytotoxic potential, it has exhibited an effective competitive ability on common purslane, common lambsquarters, black nightshade, and barnyardgrass, being more suitable in low-input agricultural systems [28]. However, due to the harmful capacity of herbicides to remain inactivated for months in the soil and food products later consumed, anise essential oil has been recently included as one of the volatiles able to decompose and/or inhibit the function of the herbicide, together with ginger, peppermint, juniper, and lemongrass essential oils, through the dissolution and alteration of its chemical structure. In this case, the primary degradation product after the application of these essential oils is aminomethylphosphonic acid (AMPA), which is detected in much lower amounts in soil than glyphosate [29].

So, the aims of this work are firstly to test the in vitro phytotoxic activity (of previously analysed commercial essential oils, winter savory, peppermint, and anise, in order to assure their main compounds by GC/MS) against seed germination and seedling growth of *P. oleracea*, a

cosmopolitan annual weed of tropical and subtropical climates; *L. multiflorum*, because *Lolium* spp. has been ranked as one of the specimens most frequently exhibiting herbicide resistance in many countries [30,31,32]; and *E. crus-galli*, a serious weed of irrigation crops, especially rice. Secondly, we aim to corroborate in vivo the previous in vitro phytotoxic effect using a commercial emulsifiable concentrate with the more phytotoxic essential oils and finally, we will study their potential hazard against maize (*Zea mays* L.), rice (*Oryza sativa* L.), and tomato (*Solanum lycopersicum* L.) seeds in order to obtain selective bioherbicides for food crops.

2. RESULTS

2.1. Chemical composition of winter savory, peppermint, and anise essential oils

Sixty-four compounds accounting for 97.67-99.66% of the total commercial winter savory, peppermint, and anise essential oils were identified by GC/MS analysis. Components are clustered (Table 1) in homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, diterpene hydrocarbons, aromatic compounds, and others, and are listed according to Kovat's retention index calculated in GC on an apolar HP-5MS column.

Table 1. Chemical composition of commercial *S. montana*, *M. piperita* and *P. anisum* essential oils.

RI	Compound	*S. montana*	*M. piperita*	*P. anisum*
Monoterpene hydrocarbons		23.16±0.33	-	-
931	α-Thujene	0.88±0.01	-	-
939	α-Pinene	0.77±0.00	-	-
953	Camphene	0.32±0.00	-	-
979	β-Pinene	0.11±0.08	-	-
993	Myrcene	1.39±0.01	-	-
1005	α-Phellandrene	0.21±0.00	-	-
1012	δ-3-Carene	0.08±0.00	-	-
1019	α-Terpinene	1.62±0.01	-	-
1029	*p*-Cymene	11.41±0.01	-	-
1033	Limonene	0.35±0.26	-	-
1053	*trans*-Ocimene	0.04±0.00	-	-
1063	γ-Terpinene	5.78±0.01	-	-
1090	Terpinolene	0.21±0.01	-	-
Oxygenated monoterpenes		71.90±0.08	94.77±0.07	-
1035	1,8-Cineole	0.07±0.00	-	-
1070	*cis*-Sabinene hydrate	0.20±0.01	-	-
1098	*trans*-Sabinene hydrate	0.07±0.01	-	-
1101	Linalool	2.34±0.01	-	-
1146	Camphor	0.02±0.00	-	-
1150	Isopulegol	-	0.80±0.02	-
1155	Menthone	-	23.33±0.59	-
1164	*iso*-Menthone	-	16.33±0.03	-
1169	Borneol	0.71±0.02		-
1176	Menthol	-	48.23±0.36	-
1179	Terpinen-4-ol	1.04±0.01		-
1184	*iso*-Menthol	-	0.52±0.03	-
1186	*p*-Cymen-8-ol	0.02±0.01		-
1188	*neo*-*iso*-Menthol	-	0.22±0.01	-
1191	α-Terpineol	0.41±0.01	0.26±0.01	-
1203	*trans*-Dihydrocarvone	0.03±0.01	0.09±0.01	-
1237	Methyl ether Thymol	0.26±0.01	-	-
1242	Pulegone	-	0.85±0.06	-
1246	Neral	0.06±0.01	-	-
1249	Carvone	0.06±0.01	-	-
1256	Piperitone	-	0.68±0.06	-

1297	Thymol	23.20±0.06	-	-
1298	Menthyl acetate	-	3.38±0.26	-
1307	*iso*-Menthyl acetate	-	0.06±0.07	-
1314	Carvacrol	43.34±0.09	-	-
1374	Carvacryl acetate	0.08±0.01	-	-
Sesquiterpene hydrocarbons		3.11±0.02	2.49±0.04	0.09±0.00
1338	δ-Elemene	-	0.13±0.01	-
1351	α-Cubebene	-	0.08±0.02	-
1388	β-Bourbonene	-	0.34±0.02	-
1390	β-Elemene	-	0.14±0.01	-
1416	α-*cis*-Bergamotene	-	-	0.01±0.00
1420	β-Caryophyllene	2.81±0.01	1.26±0.04	-
1437	α-*trans*-Bergamotene	-	-	0.08±0.00
1454	α-Humulene	0.11±0.01	-	-
1495	Viridiflorene	0.05±0.01	-	-
1500	α-Muurolene	-	0.11±0.00	-
1509	β-Bisabolene	0.06±0.00	-	-
1514	γ-Cadinene	0.03±0.01	0.12±0.00	-
1524	δ-Cadinene	0.06±0.00	0.30±0.01	-
Oxygenated sesquiterpenes		0.35±0.02	0.26±0.01	-
1565	E-Nerolidol	-	0.09±0.01	-
1578	Spathulenol	0.04±0.01	0.09±0.00	-
1583	Caryophyllene oxide	0.31±0.01	0.09±0.01	-
Diterpene hydrocarbons		0.06±0.01	-	-
2067	Abietatriene	0.06±0.01	-	-
Aromatic compounds		0.05±0.00	-	99.57±0.05
1197	Methyl Chavicol	-	-	0.04±0.00
1253	*p*-Anis aldehyde	-	-	0.04±0.00
1255	*cis*-Anethole	-	-	0.03±0.00
1286	*trans*-Anethole	-	-	99.46±0.05
1359	Eugenol	0.05±0.00	-	-
1406	Methyl Eugenol	-	-	-
Others		0.09±0.01	0.15±0.02	-
980	1-Octen-3-ol	0.09±0.01	-	-
1275	*n*-Decanol	-	0.15±0.02	-
	Total	98.73±0.40	97.67±0.08	99.66±0.05

RI, retention index relative to C_8-C_{32} *n*-alkane on HP-5MS column; values are mean relative area (%) ± standard deviation of three samples.

In winter savory essential oil, the monoterpene compounds (95.06%), both oxygenated (71.90±0.08%), with 16 compounds identified and hydrocarbons (23.16±0.33%) including 13 components, were the main qualitative and quantitative fractions found. The phenolic compounds carvacrol (43.34±0.09%) and thymol (23.20±0.06%), followed by their biogenetic precursors *p*-cymene (11.41±0.01%) and γ-terpinene (5.78±0.01%), were the main compounds of winter savory essential oil. Together, the oxygenated monoterpenes, linalool, became the next major constituent of this fraction, although at a far lower percentage (2.34±0.01%). Other compounds were detected in lower quantities, such as terpinen-4-ol (1.04±0.01%) and *cis*-sabinene hydrate (0.20±0.01%). Among the sesquiterpene fraction (3.46%), only relatively large amounts of the sesquiterpene hydrocarbon β-caryophyllene (2.81±0.01%) were found, while the rest ranged from 0.03% for γ-cadinene to 0.31% for the oxygenated sesquiterpene caryophyllene oxide. Abietatriene (0.06%) and eugenol (0.05%) were the only diterpene hydrocarbon and phenylpropanoid detected in winter savory essential oil, respectively.

Regarding peppermint essential oil, oxygenated monoterpenes (94.77%) with 12 compounds identified were the main qualitative and quantitative phytochemical group found. Sesquiterpene hydrocarbons at a far lower percentage (2.49%) constituted the next phytochemical group. Oxygenated sesquiterpenes and others were found at percentages lower than 1% (0.26 and 0.15%, respectively). Finally, neither monoterpene hydrocarbons nor aromatic compounds were detected in the commercial peppermint essential oil analysed here. Between the 24 identified compounds in *M. piperita*, the oxygenated monoterpene menthol (48.23±0.36%), followed by menthone (23.33±0.59%) and its diasteromer *iso*-menthone (16.33±0.03%), were the

main compounds. Among the sesquiterpene hydrocarbons, only β-caryophyllene (1.26±0.04%) reached a percentage higher than 1%. E-nerolidol, spathulenol, and caryophyllene oxide were the only oxygenated sesquiterpenes identified, with each one reaching 0.09%.

Finally, in anise essential oil, the main phytochemical group was by far the aromatic fraction (99.57±0.05%), with four compounds identified, in which the leading component was *trans*-anethole (99.46±0.05%). The rest contained within this fraction did not reach percentages higher than 0.1%: methyl chavicol (0.04%), *p*-anis aldehyde (0.04%), and *cis*-anethole (0.03%). Only two more compounds, the sesquiterpene hydrocarbons, α-*cis*-bergamotene and α-*trans*-bergamotene, were identified in *P. anisum* essential oil.

2.2. Seed germination and seedling growth inhibition of P. oleracea, L. multiflorum, and E. crus-galli, and maize, rice, and tomato with essential oils

The phytotoxic effect of winter savory, peppermint, and anise essential oils was evaluated in vitro against three known harmful herbs: *P. oleracea*, *L. multiflorum*, and *E. crus-galli*. In this set of trials, the high phytotoxicity of winter savory essential oil (Table 2) was highly remarkable, exhibiting a total inhibitory effect against the seed germination of the tested weeds at all doses (0.125, 0.25, 0.50, and 1 µL/mL) assayed.

Table 2. *In vitro* effects of peppermint, anise and winter savory essential oils against *Portulaca oleracea*, *Lolium multiflorum* and *Echinochloa crus-galli* seed germination.

Seed germination (% ± s.e)			
Concentration	*Portulaca oleracea*		
(µL/mL)	Winter savory	Peppermint	Anise
Control	85.00±2.74 a	85.00±2.74 a	85.00±2.74 a
0.125	0.00±0.00 b	81.00±2.45 a,b	82.00±3.74 a
0.25	0.00±0.00 b	80.00±3.54 a,b	85.00±5.24 a
0.5	0.00±0.00 b	75.00±3.87 a,b	82.00±4.34 a
1	0.00±0.00 b	70.00±3.16 b	81.00±1.87 a
Concentration	*Lolium multiflorum*		
(µL/mL)	Winter savory	Peppermint	Anise
Control	67.00±5.15 a	67.00±5.15 a	67.00±5.15 a
0.125	0.00±0.00 b	0.00±0.00 b	65.00±6.89 a
0.25	0.00±0.00 b	0.00±0.00 b	64.00±4.30 a
0.5	0.00±0.00 b	0.00±0.00 b	62.00±4.34 a
1	0.00±0.00 b	0.00±0.00 b	60.00±3.54 a
Concentration	*Echinochloa crus-galli*		
(µL/mL)	Winter savory	Peppermint	Anise
Control	86.00±3.32 a	86.00±3.32 a	86.00±3.32 a
0.125	0.00±0.00 b	82.00±3.74 a,b	89.00±1.87 a
0.25	0.00±0.00 b	82.00±2.55 a,b	88.00±1.23 a
0.5	0.00±0.00 b	80.00±1.58 a,b	83.00±2.55 a
1	0.00±0.00 b	72.00±2.00 b	85.00±4.47 a

Values are mean of five replications ± standard error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p > 0.05$ according to T3 Dunnet and Tukey tests.

Furthermore, it was also noteworthy that the complete inhibition of the seed germination of *L. multiflorum* by peppermint essential oil was exhibited at all doses (0.125, 0.25, 0.50, and 1 µL/mL) applied (Table 2). Besides, significant differences between the control and the highest dose (1 µL/mL) of peppermint essential oil tested were found in the seed germination of both *P. oleracea* and *E. crus-galli*, although no significant effect at lower

doses on the seed germination of *P. oleracea* and *E. crus-galli* was observed (Table 2).

In addition, a stronger phytotoxic effect was found with peppermint essential oil against the seedling growth (hypocotyl and radicle) of *P. oleracea* and *E. crus-galli*, so it could be employed as a potential post-harvest treatment. According to *P. oleracea* seedling growth, significant differences were found between the control and the higher doses (0.50 and 1 µL/mL) tested (Table 3, Figure 1). Lower doses of peppermint essential oil (0.125 and 0.25 µL/mL) assayed did not cause a significant reduction in hypocotyl growth of *P. oleracea* seeds (37.25%), whereas a moderate (50.98%) inhibitory effect was observed when the highest dose (1 µL/mL) was applied. A similar result was found in radicle elongation, with a percentage inhibition of 43.48% at the two higher concentrations (Table 3). *E. crus-galli* seedling growth was more sensible to peppermint essential oil, experiencing a significant reduction in both hypocotyl and radicle development with respect to the control (Table 3, Figure 1b). There was no major difference in either hypocotyl or radicle enlargement when comparing concentrations (0.125, 0.25, 0.50, and 1 µL/mL), achieving between 75.40-86.64% and 71.13-82.10% inhibition of hypocotyl and radicle expansion, respectively.

Table 3. *In vitro* effects of peppermint essential oil against *P. oleracea* and *E. crus-galli* seedling growth.

Seedling growth (mm ± s.e.)		
Concentration (µL/mL)	Peppermint	
	P. oleracea	
	Hypocotyl	Radicle
Control	10.20±0.58 a	13.80±2.04 a
0.125	6.40±0.25 a,b	11.80±1.39 a,b
0.25	6.40±0.25 a,b	10.20±1.39 a,b
0.5	5.80±0.20 b	7.80±0.37 b
1	5.00±0.00 c	7.80±0.97 b
Concentration (µL/mL)	*E. crus-galli*	
	Hypocotyl	Radicle
Control	23.66±3.80 a	20.78±1.78 a
0.125	5.82±0.71 b	6.00±1.03 b
0.25	4.56±0.37 b	3.86±0.44 b
0.5	3.56±0.72 b	4.34±0.52 b
1	3.16±0.69 b	3.72±0.67 b

Values are mean of five replications ± standard error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p>0.05$ according to T3 Dunnet and Tukey tests.

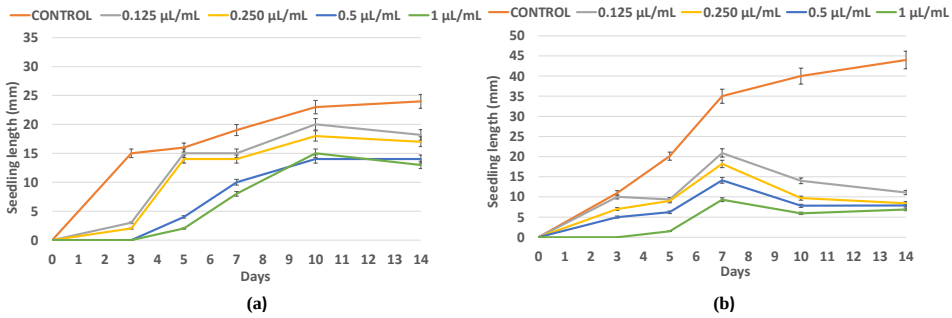

Figure 1. (a) P. oleracea and (b) E. crus-galli seedling growth with peppermint essential oil. Control and treated with peppermint essential oil at 0.125, 0.25, 0.50 and 1 µL/mL.

Anise essential oil showed the absence of a significant herbicidal effect for both the doses and weed species tried (Table 2). It displayed no significant phytotoxic activity against seed germination (Table 2) of weeds affecting

food crops; however, the seedling growth (hypocotyl and radicle) of *L. multiflorum* was significantly inhibited in a dose-dependent manner (Table 4), as well as the hypocotyl development of *E. crus-galli* that was depleted (63.23%) at the highest (1 µL/mL) dose and the radicle elongation at all concentrations applied with a percentage inhibition between 36.29 to 65.40% (Table 4, Figure 2c).

Table 4. *In vitro* effects of anise essential oil against *P. oleracea* and *E. crus-galli* seedling growth.

Seedling growth (mm ± s.e.)		
Concentration (µL/mL)	Anise	
	P. oleracea	
	Hypocotyl	Radicle
Control	10.20±0.58 a	13.80±2.04 a
0.125	10.00±0.89 a	13.40±2.58 a
0.25	9.60±0.68 a	13.60±2.36 a
0.5	8.20±0.37 a	14.60±1.72 a
1	7.60±1.60 a	13.40±1.60 a
Concentration	*L. multiflorum*	
(µL/mL)	Hypocotyl	Radicle
Control	48.50±3.35 a	39.14±2.14 a
0.125	26.21±0.94 b	27.65±1.25 b
0.25	23.07±1.17 b,c	21.29±2.05 b,c
0.50	19.71±2.45 c	18.72±1.11 c
1	12.66±0.61 d	16.66±1.11 c
Concentration	*E. crus-galli*	
(µL/mL)	Hypocotyl	Radicle
Control	23.66±3.80 a	20.78±1.46 a
0.125	19.82±0.95 a	13.24±0.30 b
0.25	18.64±1.17 a	12.90±0.27 b
0.5	14.44±0.30 a,b	12.70±0.27 b
1	8.68±2.24 b	7.19±1.35 b

Values are mean of five replications ± standard error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p>0.05$ according to T3 Dunnet and Tukey tests.

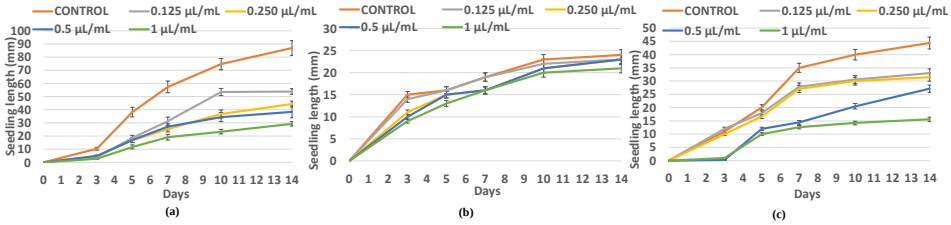

Figure 2. (a) L. multiflorum, (b) P. oleracea and (c) E. crus-galli seedling growth. Control and treated with anise essential oil at 0.125, 0.25, 0.50 and 1 µL/mL.

The previous selective inhibitory effect at the concentrations applied displayed by peppermint essential oil against the three assayed weeds with a total *L. multiflorum* seed germination inhibition, was not observed against the three selected crops (Table 5). Tomato was the most sensible crop, with an almost complete seed germination inhibition (96.84%) at the highest dose assayed (1 µL/mL), followed by maize (79.31%) and rice (36.96%). Peppermint essential oil significantly affected the growth of both the hypocotyl and radicle of rice and tomato (Table 5). The maize radicle was also significantly disturbed by these treatments in comparison to the control, whereas the volatile oil did not affect the hypocotyl growth of maize. It is interesting to note the results of peppermint essential oil against rice and *L. multiflorum*, which show a lower phytotoxic effect in rice, with a seed germination inhibition percentage between 18.48% to 16.30% at the doses of 0.125, 0.25, and 0.50 µL/mL (Table 5); concentrations that cause a total seed germination inhibition of *L. multiflorum*, one of the principal weeds that affect this crop (Table 2).

Table 5. *In vitro* seed germination and hypocotyl and radicle growth of maize, rice and tomato seeds with peppermint essential oil.

	Seed germination (%± s.e)	Seedling growth (mm± s.e)	
Concentration	Maize		
(µL/mL)	Germination	Hypocotyl	Radicle
Control	29.00±4.20 a	5.10±1.49 a	17.65±3.24 a
0.125	15.00±3.25 b	2.29±0.83 a	4.85±1.38 b
0.25	13.50±3.08 b	2.31±0.36 a	3.95±0.89 b
0.5	7.50±2.27 b	1.54±0.74 a	2.06±0.86 b
1	6.00±2.21 b	1.72±0.64 a	2.15±0.74 b
Concentration	Rice		
(µL/mL)	Germination	Hypocotyl	Radicle
Control	92.00±2.55 a	22.29±5.72 a	33.52±5.90 a
0.125	75.00±3.16 b	5.64±1.43 b	19.33±2.30 b
0.25	75.00±3.16 b	5.47±1.74 b	16.48±1.69 b
0.5	77.00±6.44 b	6.85±1.68 b	12.46±1.75 b
1	58.00±2.00 c	2.86±0.23 b	7.25±0.47 b
Concentration	Tomato		
(µL/mL)	Germination	Hypocotyl	Radicle
Control	95.00±1.58 a	21.84±2.00 a	33.14±3.71 a
0.125	39.00±12.59 b	4.30±3.32 b	9.61±5.23 b
0.25	31.00±16.08 b	4.23±1.68 b	7.70±2.67 b
0.5	14.00±4.30 c	1.48±0.63 b	3.92±1.34 b
1	3.00±3.00 c	0.20±0.20 b	1.12±1.12 b

Values are mean of five replications ± standard error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests.

2.3. Seed germination and seedling growth inhibition of P. oleracea, L. multiflorum, and E. crus-galli, and maize, rice, and tomato with an emulsifiable concentrate including winter savory or peppermint essential oils

The phytotoxic effect exhibited by winter savory and peppermint essential oils in in vitro trials were corroborated by in vivo conditions using two different emulsifiable concentrates elaborated by SEIPASA, a pioneer

Spanish Company in the development, manufacturing, and marketing of environmentally friendly agro-inputs in order to produce healthy food.

The results of the emulsifiable concentrate containing a final dose of 5 or 10 µL/mL of essential oil were compared with a control watered with water and a blank without the corresponding essential oils.

The emulsifiable concentrate of winter savory succeeded in inhibiting the seed germination of *P. oleracea* within 33 days at both concentrations, corroborating the previous in vitro results in which there was also total inhibition of the weed germination of common purslane (Table 2).

Despite *L. multiflorum* and *E. crus-galli* being more tolerant species in *in vivo* conditions, the emulsifiable concentrate of winter savory also significantly inhibited their seed germination, with percentages of 95-100% and 82-99% at 5 and 10 µL/mL, and without a significant effect with the blank (Figure 3). Similar results were found for hypocotyl growth (Figure 4), making the formulate with winter savory essential oil an ecological alternative to synthetic herbicides, which have already demonstrated a detrimental influence on the environment, crops, and human health.

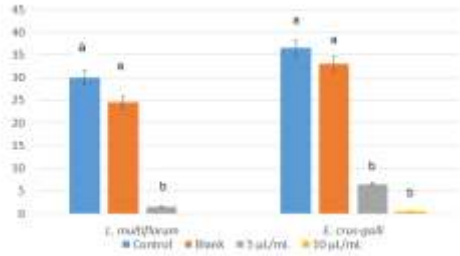

Figure 3. Values of seed germination (%) of L. multiflorum and E. crus-galli control and blank, and treated with the emulsifiable concentrate of winter savory essential oil at 5 and 10 µL/mL. Means followed by different letters in each column indicate that are significantly different at p < 0.05 according to T3 Dunnet and Tukey tests.

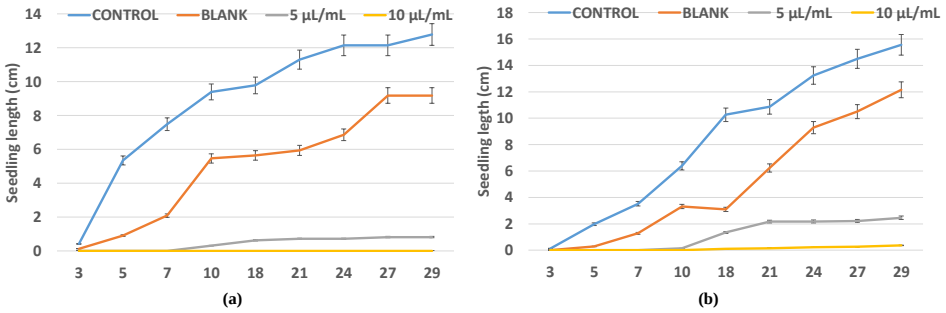

Figure 4. Hypocotyl growth (cm) of (a) L. multiflorum and (b) E. crus-galli control and blank, and treated with winter savory essential oil at 5 and 10 µL/mL.

Regarding peppermint essential oil, due to the less phytotoxical effect, it was emulsified with other herbicidal compounds (blank use by SEIPASA Company) and the formulate was able to inhibit the seed germination of the three weeds in a dose-dependent manner, with percentages between 77-100% for *P. oleracea*, 90-95% against *E. crus-galli*, and total inhibition over *L. multiflorum*, corroborating the in vitro results in which peppermint essential oil was more active against *L. multiflorum* than *P. oleracea* and *E. crus-galli*.

According to the herbicidal effect of the emulsifiable concentrate of winter savory, a new set of trials was carried out with this formulate and three food crops. Unfortunately, a total seed germination inhibition was obtained with maize and rice, and between 80 and 98% of the tomato was inhibited at 5 and 10 µL/mL, respectively, with no significant effect with the blank (Figure 5).

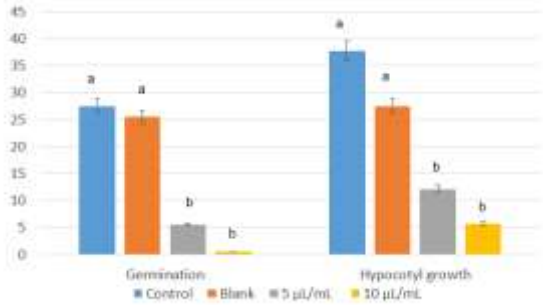

Figure 5. In vivo effect of the emulsifiable concentrate of winter savory essential oil over the germination and hypocotyl growth of tomato seeds. Values are mean of ten replications ± standard error deviation after 30 days of incubation. Means followed by different letters in the same column indicate that are significantly different at p < 0.05 according to T3 Dunnet and Tukey tests.

3. DISCUSSION

The phenolic compounds carvacrol (43.34±0.09%) and thymol (23.20±0.06%) were the main compounds identified in the winter savory essential oil analysed here. It is well-known that the strong spicy flavour of winter savory is determined by the prevailing carvacrol/thymol chemotype [33], which is variable in relation to the stage of development of the plant, harvesting time, and field environment conditions, including circumstances such as a variation in altitude: in fact, a higher content of linalool and other compounds found here in lower quantities (Table 1), such as terpinen-4-ol (1.04±0.01%) and sabinene hydrate (0.20±0.01%), has been detected in higher amounts in *S. montana* essential oil at a higher altitude, while both the major ones identified here, carvacrol and thymol, were quantified in lower percentages [34].

Apart from that, these phenolic compounds found in winter savory essential oil are considered the main bioactive monoterpenes that provide *S. montana* with a wide range of pharmacological and biological properties, such as

natural antimicrobial activity [35] against gram-positive (*Staphylococcus aureus* and *Bacillus cereus*) and gram-negative bacteria (*Salmonella infantis* and *Escherichia coli* O157:H7) [36] that is useful in the treatment of foodborne diseases, as well as anti-inflammatory activity for certain transcription factors [37]. Furthermore, recently, some new thymol and carvacrol derivatives, including the carbamate moiety, have been synthesized with stronger inhibitory effects on acetylcholinesterase [38]. Related to their phytotoxicity, both carvacrol and thymol have shown total suppression of the seed germination and seedling growth of *Amaranthus retroflexus*, *Chenopodium album*, and *Rumex crispus* [39], coinciding with our authors [27] and also with previous [40] studies, in which oregano essential oil with 60.42% of carvacrol exhibited a total inhibition *P. oleracea*, *L. multiflorum*, and *E. crus-galli* at the same *in vitro* doses assayed. These results indicate that winter savory is an effective broad-spectrum herbicide as it occurs with glyphosate that also exerts inhibitory effects on the seed germination of crops, such as wheat (*Triticum durum* L.), pea (*Pisum sativum* L.), lettuce (*Lactuca sativa* L.) [41], and even trees, for instance *Pinus pinaster*, in which it is able to induce in vitro shoot chlorosis and drooping [42]. So, this formulate could be an alternative to glyphosate with less environmental and human health problems.

Several researches corroborate that the biological properties of peppermint essential oil are due to its chemical composition, especially its major components menthol, menthone, and *iso*-menthone [43,44], conferring the high percentage of menthol to peppermint essential oil immunostimulant effects in animals [45] and the herbicidal effect in Mediterranean weed, such as *Amaranthus retroflexus* L., *Solanum nigrum* L., and *P. oleracea* under controlled conditions [46]. The results obtained convert peppermint

essential oil into a sustainable alternative that can solve the recent *L. multiflorum* resistance to glyphosate on rice paddy leaves [47]. Not only do our results match with other studies in reference to larger compounds, but also in reference to smaller ones, such as E-nerolidol, spathulenol, and caryophyllene oxide, which were the only oxygenated sesquiterpenes identified in peppermint essential oil [48,49].

Finally, although *trans*-anethole, the main compound in commercial anise essential oil (99.46±0.05%) analysed here and in other previous works [50] has demonstrated strong antifungal activity through the inhibition of the mycelial growth of a wide range of fungi [51] and could be used as a preservative in food preparation and processing [49], our results together with other research [52] revealed no significant phytotoxic activity against seed germination of selected weed.

4. MATERIALS AND METHODS

4.1. Essential oil

Commercial samples of winter savory (*Satureja montana* L.) (Batch 0054366), peppermint (*Mentha piperita* L.) (Batch 0058567), and anise (*Pimpinella anisum* L.) (Batch 0059857) essential oils supplied by Guinama (Valencia, Spain) were stored at 4 °C until chemical analysis and phytotoxic assays.

With the purpose of decreasing volatility, winter savory and peppermint essential oils were included in an emulsifiable concentrate industrially prepared by Seipasa Company that was stable at room temperature and adequate for *in vivo* weed control assays.

4.2. Seeds

Mature seeds of annual weeds of *Portulaca oleracea* L., *Lolium multiflorum* Lam., and *Echinochloa crus-galli* (L.) Beauv., were purchased from Herbiseed (website: www.herbiseed.com).

Mature seeds of 'Perseo-type' maize (*Zea mays* L.) and 'Albufera-type' rice (*Oryza sativa* L.) were obtained from the cereals in Sueca (Valencia, Spain). 'Huevo de toro-type' tomato (*Solanum lycopersicum* L.) seeds were directly acquired from the fruit found in the inner part in Utiel (Valencia, Spain).

4.3. Gas chromatography- Mass spectrometry

GC-MS analysis was carried out with a 5973N Agilent apparatus, equipped with a capillary column (95 dimethylpolysiloxane - 5% diphenyl), Agilent HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 µm film thickness). The column temperature program was 60 °C for 5 min, with 3 °C/min increases to 180 °C, and then 20 °C/min increases to 280 °C, which was maintained for 10 min. The carrier gas was Helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the *m/z* 30-500 range with an ionizing voltage of 70 eV.

4.4. Identification

The individual compounds were identified by MS and their identity was confirmed by a comparison with their Kovat's retention index calculated using co-chromatographed standard hydrocarbons relative to C_8-C_{32} *n*-alkanes, and mass spectra with reference samples or with data already available in the NIST 2005 mass spectral library and in the literature [53].

4.5. In vitro assays: P. oleracea, L. multiflorum, E. crus-galli, maize, rice, and tomato seed germination and seedling growth with essential oils

Sets of 20 seeds (10 for maize), each with five replicates (ten replicate in maize) per treatment, were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No.1) moistened with 4 mL of distilled water and with 0 (control), 0.125, 0.250, 0.5, and 1 µL/mL of winter savory, peppermint, and anise essential oils. Petri dishes were sealed with parafilm and incubated in a germination chamber Equitec EGCS 301 3SHR model, according to previous assays [54], alternating between 30.0±0.1 °C 16 h in light and 20.0±0.1 °C 8 h in dark, with (*E. crus-galli*, maize, and rice) and without (*P.oleracea*, *L. multiflorum*, and tomato) humidity.

To evaluate the herbicidal activity of the essential oils, the number of germinated seeds was counted and compared with those of untreated seedlings.

Emergence of the radicle (≥1 mm) was used as an index of germination and seedling length (hypocotyl and/or radicle) data were recorded after 3, 5, 7, 10, and 14 days in each replicate.

4.6. In vivo assays: P. oleracea, L. multiflorum, E. crus-galli, maize, rice, and tomato with an emulsifiable concentrate of winter savory or peppermint

Ten seeds of each species (*P. oleracea*, *L. multiflorum*, *E. crus-galli*, maize, rice, and tomato) with ten replicates per treatment were randomly chosen and placed in pots (9 cm diameter) with 40 g of substrate. They were place less than 1 cm below Substrate Projar Professional containing coir and peat make, fertilizer N-P-K: 14 + 16 + 18 + micronutrients, and dolomitic limestone with a sorption capacity of 183 g/10 min. A set of 10 pots was watered on the first day with 20 mL of water (control), 20 mL commercial products (Nosbur OE 12 NS (32% *w*/*w*), Emulson AG/CAL/E (7% *w*/*w*),

Emulson CO 36 (13% *w*/*w*) or Emulson AG/CAL/E (2.2% *w*/*w*), Alpicare 410H (21.7% *w*/*w*), Emulson AG/7720/A (2.6% *w*/*w*) respectively) without essential oils (blank), and 20 mL of emulsifiable concentrate with winter savory (48% *w*/*w*) or peppermint essential oil (73.5 % *w*/*w*) at 5 and 10 µL/mL. A tray was used every five pots to hold and separate them when watering. In order to prevent leaching, the pots were covered with plastic film. Over a period of 33 or 20 days (winter savory or peppermint), each tray was watered with 250 mL of water every two days. The greenhouse conditions were: 23.3 °C average indoor temperature, 18.1 °C minimum indoor temperature, 29.7 °C maximum indoor temperature, 57.2% average humidity, 80.9 µmol/m^2/s PAR (Photo Active Radiation), and 135.6 W/m^2 intensity of radiation.

To evaluate the herbicidal effect, the number of germinated seeds in 5 µL /mL and 10 µL /mL pot trays was counted and compared with those of control and blank samples. Emergence of the hypocotyl (≥1 mm) was used as an index of germination and seedling length data were recorded every two days, coinciding with watering days over 33 or 20 days.

4.7. Statistical analysis

Experiments were conducted with five replicates and ten replicates in vitro and in vivo, respectively. Data were subjected to one-way analysis of variance (ANOVA) with SPSS statistics 22 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* one was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq 0.05$.

5. CONCLUSIONS

The results *in vitro* showed that winter savory and peppermint essential oils can be effective bioherbicides. Peppermint essential oil at lower doses could be used to control *L. multiflorum* in rice. The emulsifiable concentrate based on winter savory essential oil tested in *in vivo* assays corroborates that this effective broad-spectrum herbicide constitutes an eco-friendly and less pernicious alternative to glyphosate in weed control.

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CHAPTER 2.

Ginger and turmeric essential oils for weed control and food crop protection

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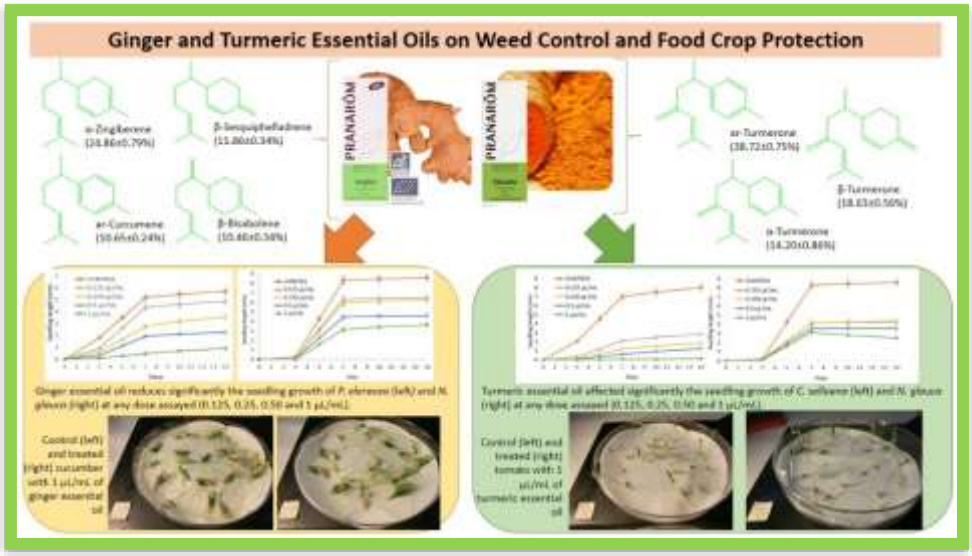

ABSTRACT

Ginger and turmeric are two food ingredients that are in high demand due to their flavor and positive effects on health. The biological properties of these spices are closely related to the aromatic compounds they contain. The chemical compositions of their essential oils and their *in vitro* phytotoxic activity against weeds (*Portulaca oleracea*, *Lolium multiflorum*, *Echinochloa crus-galli*, *Cortaderia selloana*, and *Nicotiana glauca*) and food crops (tomato, cucumber, and rice) were studied. Forty-one compounds, accounting for a relative peak area of 87.7% and 94.6% of turmeric and ginger essential oils, respectively, were identified by Gas Chromatography-Mass Spectrometry analysis. Ginger essential oil with α-zingiberene (24.9±0.8%), β-sesquiphelladrene (11.7±0.3%), ar-curcumene (10.7±0.2%), and β-bisabolene (10.5±0.3%) as the main compounds significantly inhibited the seed germination of *P. oleracea*, *L. multiflorum*, and *C. selloana* at the highest dose (1 µL/mL) assayed, as well as the hypocotyl and radicle growth of the weeds. Turmeric essential oil with ar-turmerone (38.7±0.8%), β-turmerone (18.6±0.6%), and α-turmerone (14.2±0.9%) as principal components significantly inhibited the seed germination of *C. selloana* and hypocotyl and radicle growth of weeds (the latter in particular) at the highest dose, whereas it did not affect either the seed germination or seedling growth of the food crops. Turmeric essential oil can be an effective post-emergent bioherbicide against the tested weeds without phytotoxicity to crops.

Keywords: ginger; turmeric; essential oils; gas chromatography-mass spectrometry; weed control; food crops; phytotoxicity.

1. INTRODUCTION

Human consumption of herbs and spices began in 5000 BC [1] and has continued until today due to the fact that these products are added to a great variety of food, especially ready-to-eat foods [2]. The world production of spices increased from 424.3 tons in 1961 to 2,413,284 tons in 2016 [3].

Herbs and spices offer a wide range of flavors that increase sensory variety in food and beverages without additional energy [4] while providing health benefits, due mainly to their antioxidant properties [5]. Several spices are dietary agents with anticancer properties due to containing compounds like curcumin, gingerol, anethole, or zerumbone, which are powerful inhibitors of nuclear factor κB (NF-κB), protein complex involved in DNA transcription [6].

Ginger (*Zingiber officinale* Rosc.) and turmeric (*Curcuma longa* L.), two powerful spices, have been widely used for both culinary and medical purposes. Ginger is an underground stem (rhizome) of a perennial herb and is used as a spice for pickles, candies, and as a preserve [7], while turmeric, popularly called "Indian saffron" [8], is also a dried rhizome of a herbaceous plant that imparts a distinctive flavor and orange color to food.

Ginger is able to exhibit antioxidant properties comparable to those of the standard synthetic antioxidants butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), indicating that it may reduce or delay the progression of diseases related with oxidative stress [9]. Ginger constituents can relieve arthritic pain by interfering in the inflammatory cascade and the vanilloid nociceptor [10]. Furthermore, *in vitro*, *in vivo*, and epidemiological studies have corroborated that ginger and its active compounds are effective against a wide variety of human cancers, like

gastric, pancreatic, liver, and colorectal cancer, as well as cholangiocarcinoma [11]. These facts, together with its antidiabetic [12], lipid-lowering, anti-obesity, and cardioprotective effects [10], make ginger an excellent nutraceutical among spices.

Beneficial health effects of turmeric and especially of curcumin-an orange-yellow-colored, lipophilic polyphenol substance-have been reported [13]. Curcumin is able to effectively modulate molecular targets that have a role in many phases of cancer development [13,14]. It also has a beneficial effect on inflammation, diabetes, and neurodegenerative diseases [15]. In relation to this, it has been observed that curcumin alleviates airway inflammation and ameliorates the expression of pro-inflammatory cytokines through the phosphorilation of nuclear factor-erythroid 2 related factor 2 together with the expression of heme oxygenase-1 (Nrf2/HO-1 signaling pathway) [16]; curcumin, being an amyloid-binding probe, reduces chronic inflammation, facilitates resolution of inflammation, and reduces lipid peroxidation that is correlated with synapse loss, causing it to have beneficial effects in Alzheimer's disease [17].

Essential oils of these spices also have interesting pharmacological activities, for instance, both essential oils are *in vivo* antimutagenic and anticarcinogenic substances. Ginger essential oil is able to significantly increase the levels of phase II carcinogen-metabolizing enzymes uridine 5′-diphospho-glucuronyl transferase and glutathione-S-transferase [18], and turmeric essential oil inhibits enzymes (p450) such as the cytochromes CYP1A1, CYP1A2, CYP2B, CYP2A, CYP2D, and CYP3A involved in the activation of carcinogens [18]. Furthermore, ginger essential oil might be an effective dietary supplement to ameliorate non-alcoholic fatty liver

disease and related metabolic diseases throughout the regulation of hepatic lipid synthesis, antioxidant enzymes, and inflammatory factors, which involves modulation of the hepatic sterol regulatory element binding the protein SREBP-1c and CYP2E1-mediated pathway [19].

Further investigation is necessary in order to know about other potential activities of these essential oils, not only in medicine but also in other remarkable areas like harvest and post-harvest protection of food and crops. Regarding this, turmeric essential oil in edible coatings has been found to improve the shelf-life of cherry tomatoes and raw poultry milk [20,21]. It has shown toxic and fumigant activity against stored grain insects *Sitophilus oryzae* L. and *Rhyzopertha dominica* F. [22] and antifungal and antimycotoxigenic activities against *Fusarium verticillioides* and *F. graminearum*, as well as fumonisins (B1 and B2) and zearalenone production [23,24]. Ginger essential oil was also found to be effective against fungi such as *Aspergillus flavus*, completely inhibiting conidial germination at 10 µg/mL of ginger essential oil as well as aflatoxin production at 15 µg/mL [25]. Finally, 0.3% (v/v) ginger essential oil exhibited complete inhibition against the phytopathogenic fungi *Alternaria panax*, *Botrytis cinerea*, *Cylindrocarpon destructans*, *F. oxysporum*, *Sclerotinia sclerotiorum*, and *S. nivalis* responsible for ginseng root rot disease [26].

These studies corroborated the insecticidal and antifungal properties of ginger and turmeric essential oils and their beneficial effects on food crops. However, weeds are also responsible for lost production of food crops. Regarding this, weed management in ginger as well as the herbicidal activity against *Parthenium hysterophorus* of both hexane and aqueous

extracts from ginger has been studied [27,28]. The phytotoxic effects of *Curcuma* spp., like *C. zedoaria* essential oil with 1,8-cineole (15.8%) and *epi*-curzerenone (18.2%) as the main compounds, has also been demonstrated against both lettuce and tomato [29]; *C. longa* extracts with curcuminoids are able to inhibit the germination and growth of *Bidens pilosa* [30]. Therefore, the aims of this study were as follows: firstly, to determine through Gas Chromatography-Mass Spectrometry analysis the chemical composition of commercial ginger and turmeric essential oils in order to know their main constituents; secondly, to observe their *in vitro* herbicidal effects against the seed germination and seedling growth of common ragweed (*Portulaca oleracea* L.), Italian ryegrass (*Lolium multiflorum* Lam.), barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.), pampas grass (*Cortaderia selloana* (Schult. & Schult. f.) Asch. & Graebn.), and tree tobacco (*Nicotiana glauca* Graham); and finally, to determine whether these essential oils have phytotoxic effects on food crops like tomato (*Solanum lycopersicum* L.), cucumber (*Cucumis sativus* L.), and rice (*Oryza sativa* L.).

2. RESULTS AND DISCUSSION

2.1. Chemical composition of ginger and turmeric essential oils

Forty-one compounds in commercial ginger and turmeric essential oils accounting for 94.60% and 87.67% of the total composition, respectively, were identified by Gas Chromatography-Mass Spectrometry analysis. The components were clustered (Table 1) as homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, and others and listed according to Kovat's retention index calculated in GC on an apolar HP-5MS column.

Table 1. Chemical composition of commercial ginger and turmeric essential oils.

RI_{Cal}	RI_{Ref}	Compound	Ginger	Turmeric	Technique
Monoterpene hydrocarbons			19.8±0.5	5.4±0.7	
919	926	Tricyclene	0.2±0.0	-	RI, MS
932	939	α-Pinene	2.7±0.0	0.2±0.0	RI, MS
948	954	Camphene	11.6±0.3	-	RI, MS
973	979	β-Pinene	0.2±0.0	-	RI, MS
987	990	Myrcene	1.3±0.04	0.1±0.0	RI, MS
998	1002	α-Phellandrene	0.2±0.0	4.3±0.4	RI, MS
1004	1011	δ-3-Carene	-	0.1±0.0	RI, MS
1013	1017	α-Terpinene	-	0.1±0.0	RI, MS
1021	1024	*p*-Cymene	-	0.5±0.1	RI, MS
1026	1029	Limonene	3.2±0.1	0.2±0.0	RI, MS
1056	1059	γ-Terpinene	-	0.2±0.0	RI, MS
1083	1088	Terpinolene	0.3±0.0	0.2±0.0	RI, MS
Oxygenated monoterpenes			11.8±0.2	1.0±0.0	
1029	1031	1,8-Cineole	3.0±0.1	1.0±0.0	RI, MS
1095	1094	Linalool	0.8±0.0	-	RI, MS
1137	1146	Camphor	0.2±0.0	-	RI, MS
1149	1153	Citronellal	0.2±0.0	-	RI, MS
1171	1177	Terpinen-4-ol	0.2±0.0	-	RI, MS
1188	1188	α-Terpineol	0.7±0.1	-	RI, MS
1236	1238	Neral	2.1±0.1	-	RI, MS
1267	1267	Geranial	3.2±0.0	-	RI, MS
1279	1288	Bornyl Acetate	0.9±0.0	-	RI, MS
1378	1381	Geranyl Acetate	0.6±0.0	-	RI, MS
Sesquiterpene hydrocarbons			59.6±0.1	7.2±0.0	
1383	1390	β-Elemene	0.6±0.1	-	RI, MS
1414	1419	β-Caryophyllene	-	0.3±0.0	RI, MS
1427	1434	α-*trans*-Bergamotene	0.2±0.1	-	RI, MS
1450	1456	(E)-β-Farnesene	1.0±0.1	-	RI, MS
1479	1480	ar-Curcumene	10.7±0.2	1.4±0.1	RI, MS
1492	1493	α-Zingiberene	24.9±0.8	2.6±0.1	RI, MS
1502	1505	β-Bisabolene	10.5±0.3	0.6±0.0	RI, MS
1523	1522	β-Sesquiphelladrene	11.9±0.3	2.2±0.0	RI, MS
Oxygenated sesquiterpenes			1.0±0.2	73.9±1.4	
1576	1583	ar-Turmerol	-	0.9±0.0	RI, MS
1629	1628	1-*epi*-Cubenol	0.9±0.2	-	RI, MS

1649	1646	Cubenol	0.2±0.0	-	RI, MS
1677	1669	ar-Turmerone	-	38.7±0.8	RI, MS
1681	-	α-Turmerone	-	14.2±0.9	MS
1709	-	β-Turmerone	-	18.6±0.6	MS
1742	1742	Bisabolone	-	0.7±0.0	RI, MS
1778	1778	E-α-Atlantone	-	0.7±0.0	RI, MS
Others			2.4±0.1	-	
984	984	6-Methyl-5-Hepten-2-one	2.1±0.1	-	RI, MS
1087	1087	2-Nonanone	0.1±0.0	-	RI, MS
1287	1287	2-Undecanone	0.2±0.0	-	RI, MS
		Total	94.6±2.0	87.7±0.7	

RI_{Cal}: retention index relative to C_8-C_{32} *n*-alkane on HP-5MSi column; $RI_{Ref:}$ retention index reported in Adams, 2007; values are mean relative area (%) ± standard deviation of three samples. Identification based on retention index (RI) and Mass spectra (MS) reported in NIST 11, Wiley 7n and literature.

Sesquiterpene compounds represented the main phytochemical group found in both ginger and turmeric essential oils, of which sesquiterpene hydrocarbons (59.6±0.3%) with seven compounds identified were the major set in ginger essential oil, while oxygenated sesquiterpenes (73.9±1.4%) were the principal ones in turmeric essential oil with six components recognized (Table 1). It is well known that hydrocarbons and oxygenated sesquiterpenes not only have a higher structural diversity than monoterpene, but also contribute to a noteworthy extent to the special aroma and flavor of essential oils. [31].

The sesquiterpene hydrocarbons α-zingiberene (24.9±0.8%), β-sesquiphelladrene (11.9±0.3%), ar-curcumene (10.7±0.2%) and β-bisabolene (10.5±0.3%), detected in lower percentages in turmeric essential oil (2.6±0.1, 2.2±0.0, 1.4±0.1, and 0.6±0.0%, respectively), were the main compounds in ginger essential oil. The results obtained were similar to those of recent research [32] in which zingiberene (16.3%), curcumene (12.4%), sesquiphellandrene (11.4%), and β-bisabolene (4.2%) were also

found to be the major components of ginger essential oil from Ankara (Turkey) or with samples from Ecuador, in which α-zingiberene (17.4%) and β-sesquiphelladrene (6.7%) were between the main sesquiterpene hydrocarbons [33].

Although zingiberene was the major compound in essential oils coming from both fresh and dried ginger rhizomes from Trivandrum (India), fresh ginger essential oil contained more oxygenated sesquiterpenes compared to the dried one which contained large amounts of the sesquiterpene hydrocarbons ar-curcumene (11.0%), β-bisabolene (7.2%), sesquiphellandrene (6.6%), and δ-cadinene (3.5%) [34].

Zingiberene, the chief component of the *Z. officinale* essential oil here analyzed, is a monocyclic sesquiterpene hydrocarbon with natural antioxidant and cytotoxic activities: it is capable of protecting against H_2O_2-induced cytotoxicity and oxidative DNA damage in neuronal cells [35] as well as inhibiting the growth of lymphocytic cells in a dose-dependent manner [36]. Furthermore, high zingiberene content in tomato plants provides resistance against arthropod pests including spider mite (*Tetranychus urticae*) and whitefly (*Bemisia tabaci*) [37,38]. On the other hand, β-sesquiphelladrene, the main isomer of zingiberene and second main compound in the ginger essential oil here analyzed, has antiviral and antifertility effects [38] as well as anticancer potential by inducing apoptosis through mitochondrial pathways [39].

However, different freezing rates and thawing methods can significantly affect the composition of ginger essential oil: gingerol (3.6%) and zingerone (18.3%), the main spicy compounds of fresh ginger, reached maximum percentages when ginger was thawed by an infrared method

(gingerol, 7.3%) or after thawing ginger using an infrared-microwave (zingerone, 38.3%) method [40]. These results indicated that the essential oil here analyzed and employed in phytotoxic assays was not obtained from ginger rhizome by infrared or infrared-microwave methods.

On the other hand, ar-turmerone (38.7±0.8%), β-turmerone (18.6±0.6%), and α-turmerone (14.20±0.86%), which were not found in ginger oil, were the leading components of turmeric essential oil. The rest of the sesquiterpenes did not reach 1% in either essential oil analysed (Table 1). These results coincide with those of previous studies in which ar-turmerone, α-turmerone, and β-turmerone were also found to be the leading compounds in turmeric essential oil [41]. However, similarly to ginger essential oil, other studies have reported changes in the chemical composition of turmeric essential oil depending on the biological raw material (fresh or dried) employed, with ar-turmerone (24.4%), α-turmerone (20.5%) and β-turmerone (11.1%), or ar-turmerone (49.1±3.5%) and β-turmerone (16.8±0.4%) [42] in fresh *C. longa* rhizome and ar-turmerone (21.4%) and the sesquiterpene hydrocarbons α-santalene (7.2%) and ar-curcumene (6.6%) in turmeric essential oil obtained from dry rhizome [42]. Higher percentages of the sesquiterpene hydrocarbons ar-curcumene (7.8%), zingiberene (4.2%), and β-sesquiphelladrene (22.8%) were found in turmeric essential oil obtained by hydrodistillation from *C. longa* leaves [43], confirming the GC-MS analysis [44] that our essential oil was obtained from fresh rhizomes by hydrodistillation.

The therapeutic potential of ar-turmerone has been extensively studied due to its numerous beneficial effects such as anti-inflammatory and cytotoxic effects in the treatment of various neurodegenerative disorders [45,46].

Regarding pest control, ar-turmerone has also been observed to protect against insect and mite infestation; consequently, it has been incorporated into packaging material in order to avoid pest penetration of packaged products [47]. Specially, ar-turmerone has been observed to be highly toxic against maize weevil (*Sitophilus zeamais*) and fall armyworm (*Spodoptera frugiperda*) at low doses [48].

Monoterpene hydrocarbons were the following main phytochemical group with eight (19.8±0.1%) and nine (5.4±0.7%) compounds identified in ginger and turmeric essential oils, respectively (Table 1). Camphene (11.6±0.3%), followed by limonene (3.2±0.1%), α-pinene (2.7±0.0%), and myrcene (1.3±0.0%), was the main compound in ginger essential oil, while α-phellandrene (4.3±0.4%) was the principal component in turmeric essential oil (Table 1).

1,8-Cineole (1.0±0.0%) was the only oxygenated monoterpene detected in turmeric essential oil. In contrast, this fraction, with ten oxygenated monoterpenes identified, was qualitatively the main phytochemical group found in ginger essential oil. 1,8-Cineole (3.0±0.1%), followed by geranial (3.2±0.0%) and neral (2.1±0.1%), were the main compounds (Table 1).

Recent studies [49] showed that essential oils containing 1,8-cineole are toxic against the tick species *Rhipicephalus* (Boophilus) *microplus*, and neral and geranial have exhibited anti-inflammatory activity through significant and similar inhibition of the gene NLRP-3 inflammasome-mediated IL-1β secretion, showing use as functional food ingredients [50].

Finally, other compounds such as 6-methyl-5-hepten-2-one (2.1±0.1%), 2-nonanone (0.1±0.0%), and 2-undecanone (0.2±0.0%) were only identified in ginger essential oil (Table 1).

2.2. Seed germination and seedling growth inhibition of P. oleracea, L. multiflorum, E. crus-galli, C. selloana, and N. glauca with ginger and turmeric essential oils

As several studies have indicated that essential oils may be promising herbicides [51], the effects of ginger and turmeric essential oils were tested (Table 2 and Table 3 and Figure 1 and Figure 2) against the seed germination and seedling growth of *P. oleracea*, *L. multiflorum*, *E. crus-galli*, *C. selloana*, and *N. glauca*.

Turmeric essential oil had no phytotoxic effects on the seed germination of *P. oleracea*, *L. multiflorum*, *E. crus-galli*, and *N. glauca* at all doses (0.125, 0.25, 0.50, and 1 µL/mL) assayed; however, significant inhibition of the seed germination of *C. selloana* was achieved in a dose-dependent manner, reaching 81.71% of reduction at the highest dose (1 µL/mL) tested (Table 2).

Previous studies showed that *P. oleracea*, *L. multiflorum*, and *E. crus-galli* were sensitive to winter savory (*Satureja montana* L.), which exerted a total inhibitory effect on the seed germination of the three weeds at all doses (0.125, 0.25, 0.50, and 1 µL/mL) tested, and peppermint (*Mentha piperita* L.), which completely inhibited the seed germination of *L. multiflorum* and significantly affected the seed germination of *P. oleracea* and *E. crus-galli* at the highest dose (1 µL/mL) applied [52].

Regarding ginger essential oil, although there was no significant inhibitory effect on the seed germination of *E. crus-galli* and *N. glauca*, a remarkable decrease in the seed germination of *P. oleracea*, *L. multiflorum*, and *C. selloana* was observed at the highest dose-reductions of 45.35%, 46.67%, and 43.91%, respectively- in relation to the control (Table 2).

Table 2. *In vitro* inhibitory effect of ginger and turmeric essential oils against *Portulaca oleracea (*PO), *Lolium multiflorum* (LM), *Echinochloa crus-galli* (ECG), *Cortaderia selloana* (CS) and *Nicotiana glauca* (NG) seed germination.

Dose *	Ginger essential oil				
	PO	LM	ECG	CS	NG
0	86.00±2.92 a	60.00±2.74 a	86.00±6.00 a	82.00±3.74 a	94.00±4.00 a
0.125	81.00±4.30 a	50.00±2.74 a,b	79.00±3.67 a	85.00±2.74 a	85.00±5.48 a
0.25	77.00±5.15 a	47.00±5.61 a,b	73.00±4.90 a	81.00±3.32 a	83.00±6.63 a
0.5	82.00±2.55 a	47.00±4.64 a,b	69.00±5.79 a	67.00±6.04 a	79.00±11.34 a
1	47.00±2.55 b	32.00±8.89 b	68.00±6.63 a	46.00±6.21 b	73.00±2.55 a
	Turmeric essential oil				
0	86.00±2.92 a	60.00±2.74 a	75.00±7.01 a	82.00±3.74 a	94.00±4.00 a
0.125	75.00±5.00 a	50.00±3.87 a	74.00±3.67 a	46.00±15.12 a,b	85.00±6.52 a
0.25	71.00±2.45 a	49.00±4.30 a	71.00±2.92 a	43.00±10.68 b	86.00±2.92 a
0.5	70.00±5.24 a	55.00±3.54 a	71.00±1.87 a	32.00±6.82 b	87.00±2.55 a
1	73.00±4.06 a	49.00±6.40 a	68.00±2.55 a	15.00±2.24 b	85.00±2.24 a

Values are mean percentage of five replications ± standard error after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests. *Dose: µL/mL.

In the seedling evolution, ginger essential oil caused a significant dose-dependent inhibition of the hypocotyl development of *P. oleracea*, *L. multiflorum*, *C. selloana*, and *N. glauca*, reaching high reduction percentages of 82.74%, 66.85%, 73.68%, and 63.77%, respectively, at the highest dose (1 µL/mL) in comparison to the control (Table 3). However, no significant reduction in *E. crus-galli* hypocotyl growth was observed at any dose assayed (0.125, 0.25, 0.50, and 1 µL/mL) with respect to the control (Table 3, Figure 1c).

Ginger essential oil also considerably influenced the radicle progress of the five selected weeds. The radicle development of *P. oleracea* was significantly reduced by 57.22% and 86.06% relative to the control after the application of ginger essential oil at 0.5 and 1 µL/mL, respectively; this was similar to *L. multiflorum*, whose radicle enlargement was decreased at these doses between 60.23% and 72.36% (Table 3, Figure 1a,b). The radicle elongation of *E. crus-galli* significantly declined at these doses between 39.95% and 50.61% (Table 3, Figure 1c). A noteworthy reduction in radicle development was achieved in *C. selloana*, which experienced a decline percentage of 75.26% at the highest dose (1 µL/mL) assayed (Table 3, Figure 1d); finally, a significant inhibition of 48.32% of the radicle growth of *N. glauca* was observed at the highest dose (1 µL/mL) applied (Table 3, Figure 1e).

Table 3. ***In vitro*** **effects of ginger and turmeric essential oils on seedling length (hypocotyl and radicle) of** ***P. oleracea*** **(PO),** ***L. multiflorum*** **(LM),** ***E. crus-galli*** **(EC),** ***C. selloana*** **(CS) and** ***N. glauca*** **(NG).**

*Dose			Control	0.125 µL/mL	0.25 µL/mL	0.5 µL/mL	1 µL/mL
GINGER	PO	Hyp	3.65±0.22 a	2.80±0.28 b	2.01±0.12 c	1.39±0.16 c,d	0.63±0.09 d
		Rad	2.08±0.26 a	2.07±0.11 a	1.57±0.21 a	0.89±0.13 b	0.29±0.09 b
	LM	Hyp	25.76±0.90 a	19.65±1.52 a,b	16.39±3.58 b,c	12.46±2.79 b,c	8.54±3.16 c
		Rad	16.82±1.93 a	10.67±1.51 a,b	10.13±2.12 a,b	6.69±1.33 b	4.65±1.85 b
	EC	Hyp	16.96±1.22 a	12.91±0.33 a	12.88±0.97 a	12.33±1.82 a	12.27±1.66 a
		Rad	13.24±0.92 a	10.47±0.89 a,b	9.01±0.75 b	7.95±1.30 b	6.54±0.90 b
	CS	Hyp	4.14±0.56 a	3.92±0.70 a	2.74±0.52 a,b	1.59±0.71 b	1.09±0.78 b
		Rad	3.88±0.36 a	3.68±0.50 a	2.63±0.31 a,b	1.56±0.21 b,c	0.96±0.26 c
	NG	Hyp	4.72±0.30 a	3.26±0.40 a,b	2.99±0.48 a,b	1.86±0.57 b	1.71±0.22 b
		Rad	3.87±0.23 a	3.22±0.24 a,b	3.37±0.53 a,b	2.74±0.70 a,b	2.00±0.15 b
TURMERIC	PO	Hyp	3.65±0.22 a	1.97±0.21 b	1.76±0.13 b	1.51±0.06 b	1.59±0.04 b
		Rad	2.09±0.26 a	2.32±0.20 a	1.62±0.18 a	1.53±0.29 a	1.44±0.12 a
	LM	Hyp	25.76±0.90 a	15.34±2.96 b	16.99±1.41 b	16.85±1.01 b	17.20±1.62 b
		Rad	16.82±1.93 a	11.60±1.62 b	10.31±1.14 b	10.70±1.10 b	10.640.64 b
	EC	Hyp	16.96±1.22 a	11.35±1.42 b	11.19±1.01 b	10.37±0.58 b	10.29±0.86 b
		Rad	13.24±0.92 a	9.80±0.97 b	9.62±0.60 b	8.27±0.50 b	7.36±0.82 b
	CS	Hyp	4.14±0.56 a	1.57±0.65 b	1.12±0.47 b	0.69±0.23 b	0.09±0.05 b
		Rad	3.88±0.36 a	0.88±0.48 b	0.72±0.29 b	0.54±0.16 b	0.01±0.01 b
	NG	Hyp	4.72±0.30 a	1.82±0.48 b	1.31±0.24 b	1.15±0.16 b	0.65±0.17 b
		Rad	3.87±0.23 a	2.55±0.34 b	2.86±0.09 b,c	2.40±0.16 b,c	1.88±0.12 c

Values are mean of five replications ± standard error after 14 days of incubation. Means followed by different letters in the same row indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests. *Dose: µL/mL; Hyp: Hypocotyl (mm); Rad: Radicle (mm).

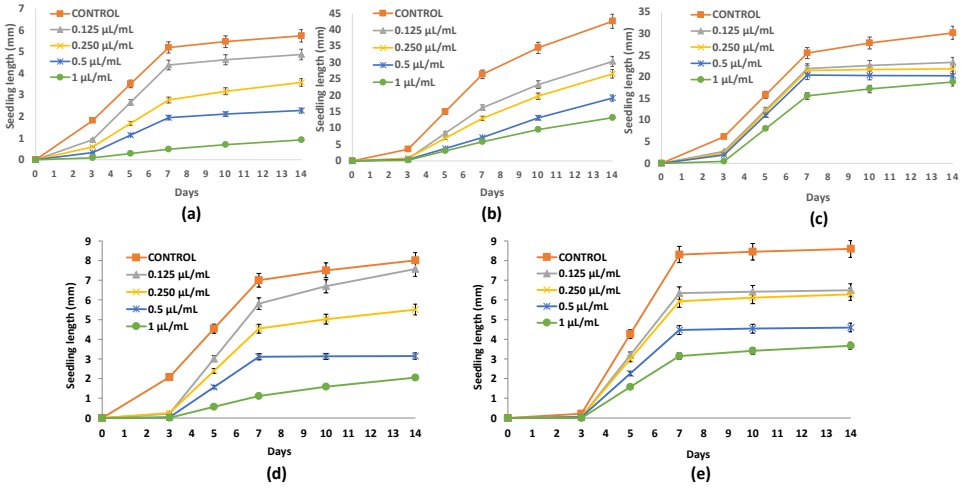

Figure 1. Values of seedling lenght (mm) (mean ± SE) of Portulaca oleracea (a), Lolium multiflorum (b), Echinochloa crus-galli (c), Cortaderia selloana (d) and Nicotiana glauca (e) control and treated with ginger essential oil at 0.125, 0.25, 0.5 and 1 µL/mL.

Furthermore, other *Zingiber* spp. have also shown phytotoxicity against different weeds; for instance, *Z. zerumbet* Smith, with zerumbone (74.82%) as its major compound, affected the seedling growth of *Philaris minor* Retz. in a concentration-dependent manner, achieving inhibition of both the hypocotyl and radicle development at 1000 ppm and showing less or no effect on the germination of seeds of *Triticum aestivum* L. [53].

Turmeric essential oil, with the exception of the radicle elongation of *P. oleracea*, significantly inhibited both hypocotyl and radicle growth of the selected weeds at all doses (0.125, 0.25, 0.50, and 1 µL/mL) assayed. The hypocotyl development was reduced without significant differences between doses applied to reach percentages of 56.55% (*P. oleracea*), 40.45% (*L. multiflorum*), 39.33% (*E. crus-galli*), 97.83% (*C. selloana*), and 86.23% (*N. glauca*) (Table 3). The radicle elongation of *L. multiflorum* and *E. crus-galli* was significantly reduced at all doses of turmeric essential oil,

reaching 36.74% and 44.41%, respectively, at the highest dose tested. *C. selloana* was again the most sensitive species to turmeric essential oil with percentages of radicle growth inhibition of 77.32%, 81.44%, 86.08%, and 99.74% at the doses of 0.125, 0.25, 0.50, and 1 µL/mL, whereas *N. glauca* reached a percentage of 51.42% at the highest dose applied.

Ginger and turmeric essential oils are not suitable as a potent pre-emergent treatment in the control of *P. oleracea*, *E. crus-galli*, and *L. multiflorum* because other essential oils such as oregano essential oil with carvacrol (60.4±0.1%), *p*-cymene (15.5±0.0%), and γ-terpinene (5.2±0.0%) or winter savory essential oil with carvacrol (43.3±0.1%) and thymol (23.2±0.1) as main compounds can completely inhibit the germination of these three weeds at all doses (0.125-1 µL/mL) applied [52,54].

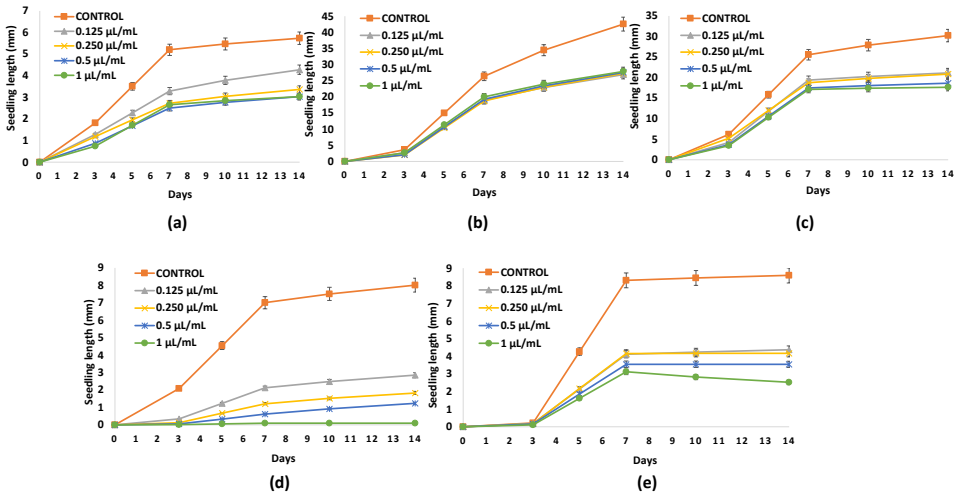

Figure 2. Values of seedling lenght (mm) (mean ± s.e.) of Portulaca oleracea (a), Lolium multiflorum (b), Echinochloa crus-galli (c), Cortaderia selloana (d) and Nicotiana glauca (e) control and treated with turmeric essential oil at 0.125, 0.25, 0.5 and 1 µL/mL.

These essential oils have similar herbicidal potential to *Thymus mastichina* essential oil with 1,8-cineole (49.5±0.4%), linalool (5.7±0.0%), and α-terpineol (5.6±0.0%), which showed significant effects in seedling length depending on the weed and dose [54]. In addition, turmeric essential oil could be used as a bioherbicide in the control of the invasive species *C. selloana.* Their use as promising post-emergent alternatives will depend on the phytotoxicity of these essential oils in food crops.

2.3. Seed germination and seedling growth effect of ginger and turmeric essential oils in tomato, cucumber, and rice

Seed germination of tomato, cucumber, and rice was not affected at any dose (0.125, 0.25, 0.50, and 1 µL/mL) applied of ginger essential oil (Table 4). Phytotoxic effects observed at 1 µL/mL of ginger essential oil in *P. oleracea* (45.35%) and *L. multiflorum* (46.67%) (Table 2)-weeds commonly affecting tomato crops [55]-were not reproduced in tomato germination, but, unfortunately, both hypocotyl and radicle development were significantly inhibited (Table 4, Figure 3a). These results agree with those of previous work in which seed germination of soybean was not inhibited by the aqueous extract of ginger rhizome at the doses assayed, whereas the hypocotyl and radicle length were reduced at the higher doses applied [56]. On the other hand, neither seed germination nor the hypocotyl growth of cucumber and rice were affected by ginger essential oil at any dose (0.125, 0.25, 0.50, and 1 µL/mL) assayed. The radicle elongation of cucumber was decreased in a dose-dependent manner up to a percentage of 21.44% at the highest dose (Table 4, Figure 3c). Slight differences in the radicle lengths of rice among the measurements were observed, but the data are not presented due to the difficulty of accurately measuring curved radicles (Image 1).

Table 4. *In vitro* seed germination and hypocotyl and radicle growth of tomato (TO), cucumber (CU) and rice (RI) with ginger and turmeric essential oils.

	Dose		Control	0.125 µL/mL	0.25 µL/mL	0.5 µL/mL	1 µL/mL
GINGER	TO	Ger	70.00±5.48 a	69.00±6.60 a	66.00±7.97 a	56.00±5.79 a	54.00±3.32 a
		Hyp	12.13±0.80 a	8.76±1.19 a,b	7.60±1.37 b	3.32±0.40 c	2.85±0.57 c
		Rad	13.64±1.41 a	10.88±1.04 a,b	8.67±1.56 b,c	6.12±0.94 c,d	3.41±0.37 d
	CU	Ger	98.00±1.23 a	95.00±2.74 a	97.00±2.00 a	96.00±2.45 a	91.00±2.45 a
		Hyp	10.34±0.33 a	10.48±0.17 a	10.10±0.52 a	11.23±0.78 a	11.75±1.09 a
		Rad	18.61±0.29 a	16.16±0.54 a,b	16.57±0.85 a,b	14.77±0.74 b	14.62±1.19 b
	RI	Ger	97.00±2.00 a	91.00±1.87 a	94.00±2.45 a	92.00±1.23 a	91.00±1.87 a
		Hyp	19.75±2.58 a	21.78±1.99 a	25.07±1.31 a	20.05±1.05 a	19.01±1.02 a
	Dose		Control	0.125 µL/mL	0.25 µL/mL	0.5 µL/mL	1 µL/mL
TURMERIC	TO	Ger	93.00±1.23 a	85.00±5.24 a	85.00±5.24 a	78.00±5.39 a	78.00±5.15 a
		Hyp	12.64±1.58 a	9.91±1.92 a	8.62±0.58 a	7.03±0.93 a	8.77±1.61 a
		Rad	18.13±1.01 a	14.52±1.81 a	14.35±0.26 a	15.66±3.23 a	10.11±1.77 a
	CU	Ger	98.00±1.23 a	92.00±2.55 a	96.00±1.87 a	100.00±0.00 a	97.00±2.00 a
		Hyp	10.34±0.33 a	10.38±0.55 a	10.42±0.71 a	9.57±0.76 a	9.67±0.08 a
		Rad	18.61±0.29 a	17.61±0.94 a	17.67±0.28 a	17.00±0.83 a	16.12±0.51 a
	RI	Ger	97.00±2.00 a	92.00±1.23 a	94.00±2.92 a	94.00±2.45 a	96.00±1.87 a
		Hyp	19.75±2.58 a	25.18±1.12 a	26.83±1.64 a	22.15±1.92 a	21.19±2.06 a

Values are mean of five replications ± standard error after 14 days of incubation. Means followed by different letters in the same row indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests. Hyp: Hypocotyl (mm); Rad: Radicle (mm).

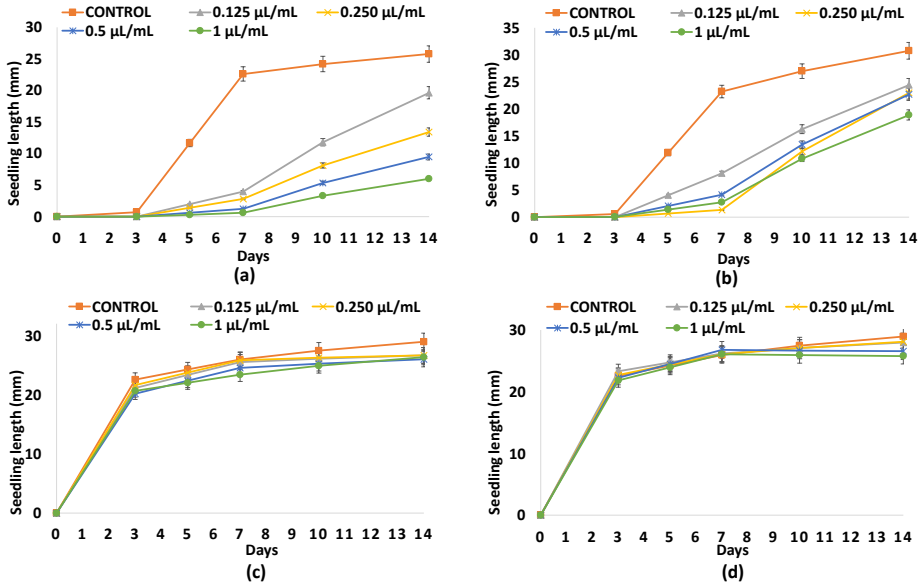

Figure 3. Values of seedling lenght (mm) (mean ± s.e.) of tomato control and treated with ginger (a) and turmeric (b) essential oils and cucumber control and treated with ginger (c) and turmeric (d) essential oils at 0.125, 0.25, 0.5 and 1 µL/mL.

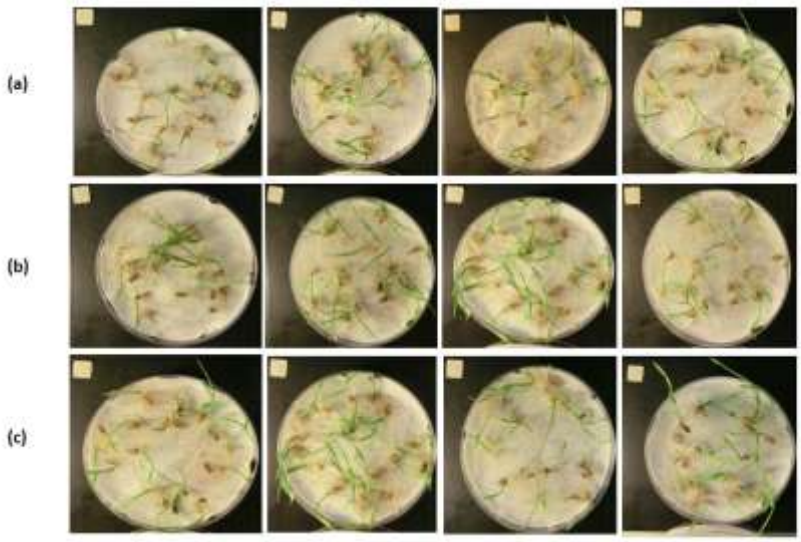

Image 1. Rice control (a) and treated with ginger (b) and turmeric (c) essential oils at 0.125, 0.25, 0.5 and 1 µL/mL in day 14 of treatment.

Promising results were obtained with turmeric essential oil against the food crops tested. Neither seed germination nor the hypocotyl growth of tomato, cucumber, and rice were significantly affected by the application of turmeric essential oil at any dose (0.125, 0.25, 0.50, and 1 µL/mL) (Table 4, Figure 3b,d). The radicle elongation of tomato, cucumber (Table 4), and rice (Figure 4) was also not affected by turmeric essential oil. Previous studies have also reported the harmlessness of turmeric essential oil *versus* other food crops: for instance, chickpea, in which no adverse effect was observed in either seed germination or seedling growth [57]. However, other *Curcuma* spp. Like *C. zedoaria* have been shown to inhibit the seed germination of lettuce and tomato in a dose-dependent manner (0.00%, 0.25%, 0.50%, 0.75%, and 1.00%) as well as to delay their growth, damaging the root in particular. These results are due to the different chemical compositions of these essential oils, with ar-turmerone (38.7±0.8%), β-turmerone (18.6±0.6%), and α-turmerone (14.2±0.9%) being the main compounds in the turmeric essential oil here analysed (Table 1), and 1,8-cineole (15.8%) and *epi*-curzerenone (18.2%) being those in *C. zedoaria* essential oil [29].

It is interesting to note that at the highest dose assayed, turmeric essential oil was able to significantly reduce the hypocotyl development of *P. oleracea* (Table 3) as well as both the hypocotyl and radicle growth of *L. multiflorum*, *E. crus-galli*, *C. selloana*, and *N. glauca*, without the phytotoxic effects shown by other essential oils such as rosemary (*Rosmarinus officinalis* L.), winter savory (*Satureja hortensis* L.), and bay (*Laurus nobilis* L.) in tomato [58].

3. MATERIAL AND METHODS

3.1. Essential oils

Commercial samples of ginger (*Zingiber officinale* Rosc.) (Batch 0F26093; Exp. date 04/2022; 1016 Indonesia) and turmeric (*Curcuma longa* L.) (Batch 0F27683; Exp. date 10/2021; 0516 India) essential oils obtained from rhizome and root, respectively, were supplied by Pranarôm S.A. Both were stored at 4 ºC until chemical analysis and phytotoxic assays were carried out.

3.2. Weed and food crop seeds

Mature seeds of the weeds common ragweed (*Portulaca oleracea* L.), Italian ryegrass (*Lolium multiflorum* Lam.), and barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) were purchased from Herbiseed (website: www.herbiseed.com), and those of pampas grass (*Cortaderia selloana* (Schult. & Schult. f.) Asch. & Graebn.) and tree tobacco (*Nicotiana glauca* Graham) were supplied by the Botanical Garden of Valencia.

Mature seeds of the food crops "Muchamiel" tomato (*Solanum lycopersicum* L.) and cucumber (*Cucumis sativus* L.) were obtained from Intersemillas S.A. "Albufera-type" rice (*Oryza sativa* L.) seeds were acquired from Copsemar in Sueca (Valencia, Spain).

3.3. Gas chromatography-Mass spectrometry analysis

GC–MS analysis was carried out using a 5977A Agilent mass spectrometer and a gas chromatograph (Agilent 7890B) apparatus equipped with an Agilent HP-5MSi (30 m long and 0.25 mm i.d. with 0.25 µm film thickness) capillary column (95% dimethylpolysiloxane - 5% diphenyl). The column temperature program was 60 ºC for a duration of 5 min, with 3 ºC /min

increases to 180 ºC, then 20 ºC /min increases to 280 ºC, which was maintained for 10 min. The carrier gas was helium at a flow rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the *m/z* range 30-650 with an ionizing voltage of 70 eV. The resulting individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index calculated using co-chromatographed standard hydrocarbons relative to C_8-C_{32} *n*-alkanes and mass spectra with reference samples or with data already available in the NIST 11 mass spectral library and in the literature [59].

3.4. In vitro assays: P. oleracea, L. multiflorum, E. crus-galli, C. selloana, N. glauca, tomato, and rice seed germination and seedling growth with essential oils

Sets of 20 seeds each with five replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No.1) moistened with 4 mL of distilled water and with 0 (control), 0.125, 0.250, 0.5, and 1 µL/mL of ginger and turmeric essential oils. Petri dishes were sealed with parafilm and incubated in an Equitec EGCS 301 3SHR model germination chamber, according to previous assays [60], alternating 30.0±0.1 ºC 16 h in light and 20.0±0.1 ºC 8 h in dark and with (*E. crus-galli*, *C. selloana*, *N. glauca*, cucumber, and rice) and without (*P. oleracea*, *L. multiflorum*, tomato) humidity. To evaluate the herbicidal activity of the essential oils, the number of germinated seeds was counted and compared with that of untreated seedlings. Emergence of the radicle (≥ 1 mm) was used as an index of germination and seedling length (hypocotyl and/or radicle) data were recorded after 3, 5, 7, 10, and 14 days in each replicate.

3.5. Statistics

Experiments were performed with five replicates. Data were subjected to one-way analysis of variance (ANOVA) using SPSS statistics 22 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* test was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq 0.05$.

4. CONCLUSIONS

Essential oils from ginger and turmeric, two health-promoting spices, could be used in weed control. Ginger essential oil with high contents of the sesquiterpene hydrocarbons α-zingiberene (24.9±0.8%), β-sesquiphelladrene (11.9±0.3%), ar-curcumene (10.7±0.2%), and β-bisabolene (10.5±0.3%) may be used as a pre-emergent bioherbicide in the control of *P. oleracea* and *L. multiflorum* in tomato, cucumber, and rice crops, whereas turmeric essential oil with the oxygenated sesquiterpenes ar-turmerone (38.7±0.8%), β-turmerone (18.6±0.6%), and α-turmerone (14.2±0.9%) can be applied as a post-emergent substance against the weeds tested since no significant phytotoxic effects in tomato, cucumber, or rice were observed. Turmeric essential oil could be a promising alternative in the management of the invasive species *C. selloana*. More weeds and higher doses of turmeric essential oil must be tested in order to determine any selective herbicide effect.

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CHAPTER 3.

Phytotoxic effects of commercial *Eucalyptus citriodora*, *Lavandula angustifolia* and *Pinus sylvestris* essential oils on weeds, crops and invasive species

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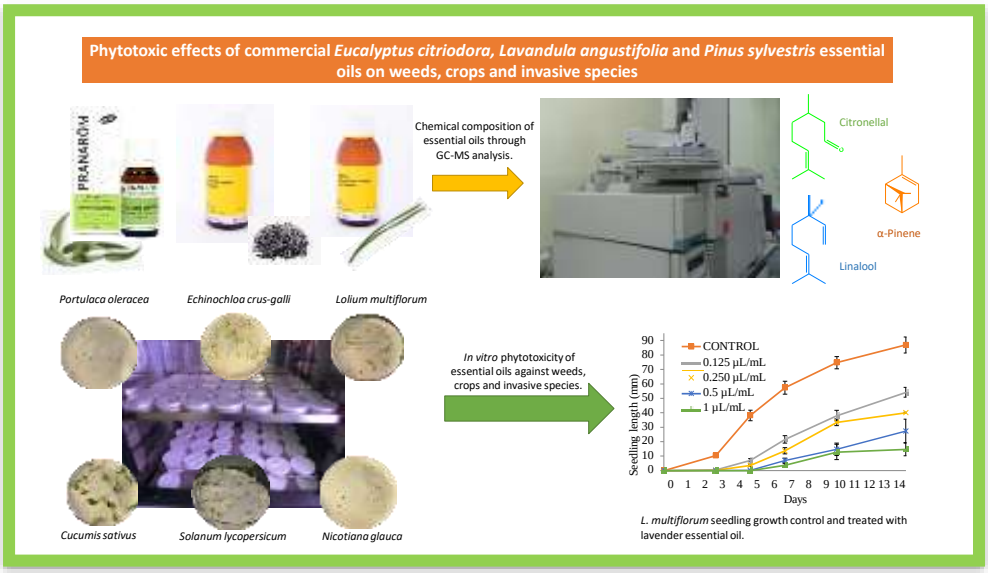

ABSTRACT

Background: essential oils are well known for their pharmacological effectiveness as well as their repellent, insecticide, and herbicide activities. The emergence of resistant weeds, due to the overuse of synthetic herbicides, makes it necessary to find natural alternatives for weed control. The aim of this study was to evaluate the phytotoxic effects of *Eucalyptus citriodora*, *Lavandula angustifolia*, and *Pinus sylvestris*, three common commercial essential oils, on weeds (*Portulaca oleracea*, *Lolium multiflorum*, and *Echinochloa crus-galli*), food crops (tomato and cucumber), and the invasive species *Nicotiana glauca*. Methods: to determine herbicidal effects, essential oils were tested at different concentrations (0.125–1µL/mL). The index of germination and seedling length data were recorded over 14 days. Results: the *in vitro* assays showed that *L. angustifolia* with linalool (38.7±0.1%), 1,8- cineole (26.5±0.1%), and camphor (14.2±0.1%) as the main compounds showed the most phytotoxic effects affecting seed germination in weeds and tomato, and the aforementioned invasive species. *L. multiflorum* was the most sensitive weed, particularly to lavender essential oil, which decreased the growth of its hypocotyl and radicle by 87.8% and 76.7%, respectively, at a dose of 1 µL/mL. Cucumber was the most resistant food crop, with no significant reduction observed in seed germination and hypocotyl growth with *E. citriodora* and *L. angustifolia* essential oils. Conclusions: lavender essential oil represents a promising candidate for the development of effective and safe herbicides in the management of *L. multiflorum* affecting cucumber crops.

Keywords: *E. citriodora; L. angustifolia*; *P. sylvestris*; essential oils; GC-MS; phytotoxicity.

1. INTRODUCTION

The particular characteristics of essential oils-natural mixtures of volatile compounds- provide them with certain pharmacological properties, including their well-known antibacterial, antioxidant, anti-inflammatory, and cancer chemoprotective effects, as well as their repellent, herbicidal, and insecticidal biological activities [1-4], which have led to valuable applications in human health, food, and cosmetics industries, and in environment and agriculture. Certain essential oils have already demonstrated their influence on both the seed germination and seedling growth of weeds [5,6]. In this regard, origanum (*Origanum vulgare* L.) essential oil with carvacrol as its main compound has exhibited a significant inhibitory effect against seed germination and seedling growth of common purslane, Italian ryegrass, and barnyardgrass at a range of concentrations (0.125–1 µL/mL), as well as against *Sinapsis avensis* at 2 µL/mL and also Johnson grass (*Sorghum halepense* L.) [7-9]. *P. sylvestris* exhibited some inhibition of the early root growth of *Cassia occidentalis* (L.) Link. [10], and *E. citriodrora* essential oil affected the development of certain weeds, particularly the seed germination of *Amaranthus viridis* L. [11]. Among weeds, the following deserve special attention: (i) common purslane (*Portulaca oleracea* L.), an annual weed commonly affecting cultivated land, protected agriculture, forests, plantations, and orchards, where it competes for resources with many field crops, including cruciferous crops, potato, and tomato, among others [12]; (ii) Italian ryegrass (*Lolium multiflorum* Lam.), an annual to biennial poaceous species largely spread globally due to its cultivation as a pasture grass [13], which has developed considerable resistance against glyphosate and other synthetic herbicides as an acetolactate synthase (ALS) inhibitor [14,15]; and (iii) barnyardgrass

(*Echinochloa crus-galli* (L.) Beauv.), considered one of the world's worst weeds, affecting agricultural land and grasslands as well as irrigation channels and wetlands, being, in fact, a very serious weed in rice crops [16]. In addition, other species, such as *Nicotiana glauca* Graham, native to South America and naturalized in several countries, have a high invasion potential to disturb ecosystems and reduce native biodiversity, growing on roadsides and lakeshores and becoming a problem in relatively dry areas [17,18]. Several studies are necessary to find efficient and sustainable alternatives to synthetic herbicides, whose persistent use has led to the arousal of multiple problems [5], such as the appearance of resistant weeds and toxicity in humans and other living organisms, as well as the persistence of residues in the environment that affect soil, air, the surrounding environment, ground water and crops [19-21]. The development of natural herbicides based on essential oils could decrease these negative impacts, mainly by counteracting resistant weeds, since it is difficult to develop resistance using mixtures of natural components with different mechanisms of action. In this sense, agricultural compositions, including oregano essential oil together with others also belonging to the Lamiaceae family such as *Lavandula*, *Mentha*, *Rosmarinus*, and *Salvia* species, have been elaborated as natural pesticides [22]. Similarly, lemongrass essential oil (*Cymbopogon citratus*, Poaceae) has been included as a principal ingredient in a natural herbicide invention to control the germination and growth of weeds [23].

On the other hand, it is interesting to demonstrate the selectivity of these eco-friendly active components against weeds and/or invasive species, thereby confirming their harmlessness over food crops. Previous studies demonstrated that winter savory (*Satureja montana* L.) essential oil is

effective in the management of *P. oleracea*, *L. multiflorum*, and *E. crus-galli*, without being pernicious to the food crops maize, rice, and tomato. Similarly, peppermint (*Mentha piperita* L.) essential oil could be used to control *L. multiflorum* in rice (*Oryza sativa* L.) [24]. *E. citriodrora* essential oil affected seed germination in *Amaranthus viridis* L., without harming the food crops commonly affected by the weed *Triticum aestivum* L., *Zea mays* L., and *Raphanus sativus* L. [11]. However, this essential oil also produced a cytotoxic effect against food crops such as *Lactuca sativa* L. [25], and other essential oils, such as wintergreen (*Gaultheria procumbens* L.) essential oil with methyl salicylate (99.6%) as the main compound, could be employed in the control of the invasive species *Cortaderia seollana* (Schult. & Schult. f.) Asch. & Graebn, and *Nicotiana glauca* Graham [26]. The tested essential oils have been selected for their pharmacological or biological properties as well as for their chemical profile. Regarding this, *Eucalyptus citriodora* L. essential oil showed moderate antioxidant action, potent antimicrobial activity against bacteria and yeasts [27,28], and insect-repellent capacity when included in insect-repellent compositions [29]. Recently, citronellal, the main component of *E. citriodora* essential oil, has been encapsulated individually in different types of cyclodextrins to maintain its properties for longer [30]. Lavender (*Lavandula angustifolia* Mill.) essential oil and its main compounds 1,8-cineole and linalool have also shown antimicrobial potential, with synergistic effects with other common antimicrobial agents [31-33]. The antibacterial activity of *L. angustifolia* essential oil can be improved by being embedded with cyclodextrin, because this increases its water solubility and reduces its volatility [34]. The antimicrobial activity of *Pinus sylvestris* L. essential oil has been also well-established. In addition, *P. sylvestris* essential oil has

shown a higher insect larvicidal potential against *Drosophila melanogaster* Meigen than other *Pinus* species, such as *P. peuce*, *P. nigra* subsp. *nigra* and *P. musco* subsp. *musgo* [35,36]. The high antimicrobial activity may be due to α- and β-pinene, the major compounds in *P. sylvestris* essential oil, which have already shown their antibacterial and antifungal potential. Indeed, both pinenes have been combined with commercial antimicrobials resulting in a reduction of their minimum inhibitory concentration and toxicity [37].

Since the phytotoxic effects differ remarkably with the chemical composition and the chemical composition of an essential oil depending on certain intrinsic and extrinsic factors [38] such as the extraction method [39], geographic location [40-42], temperature, and drying period, as well as harvesting time [43,44], the aims of this study were: (i) to determine through Gas Chromatography-Mass Spectrometry analysis the chemical composition of commercial *Eucalyptus citriodora* Hook, *Lavandula angustifolia* Mill., and *Pinus sylvestris* L. essential oils; (ii) to test the *in vitro* phytotoxic activity of these essential oils against the seed germination and seedling growth of the weeds *P. oleracea*, *L. multiflorum* and *E. crus-galli*, to evaluate their herbicidal activity, as well as on food crops such as tomato (*Solanum lycopersicum* L.) and cucumber (*Cucumis sativus* L.), to know its harmful effects on crops; and (iii) to test the same against the invasive species *Nicotiana glauca* Graham, potential reservoir of important viruses, including cucumber mosaic virus and tomato infectious chlorosis virus, which causes economic losses for commercial tomato production.

2. RESULTS AND DISCUSSION

2.1.Chemical composition of E. citridora, L. angustifolia, and P. sylvestris essential oils

Twenty-seven (98.6%), 60 (97.6%) and 38 (99.1%) compounds in commercial *E. citriodora*, *L. angustifolia*, and *P. sylvestris* essential oils, respectively, were identified by Gas Chromatography– Mass Spectrometry analysis. Components were clustered (Table 1) in a homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, oxygenated diterpenes, aromatic compounds, and others, and listed according to Kovat's retention index [45] calculated in GC on an apolar HP-5MS column.

Citronellal was the major component in *E. citriodora* essential oil (88.0±0.8%), whereas linalool (38.7±0.1%) together with 1,8-cineole (26.5±0.1%) and camphor (14.2±0.1%) were the main components in *L. angutifolia* essential oil (Table 1). Citronellal was also the main compound in *E. citriodora* essential oils of different origins and light conditions [46,47], making *E. citriodora* distinct from other *Eucalyptus* species, such as *E. camaldulensis* Dehnh [48].

However, qualitative and quantitative differences in the chemical composition of lavender essential oil have been reported depending on the biological raw material, level of dryness, extraction method, and origin. Previous studies showed that the drying process reduced the concentrations of the principal components in *L. angustifolia* essential oil obtained from flowers and aerial parts [49]. Similarly, the extraction method employed varied the content of linalool, detected in a much higher content using hydrodistillation in comparison to supercritical CO_2 and hexane extraction [50].

Table 1. Chemical compositions of commercial *E. citriodora*, *L. angustifolia* and *P. sylvestris* essential oils

RI_{Cal}	RI_{Ref}	Compound	*E. citriodora*	*L. angustifolia*	*P. sylvestris*
Monoterpene hydrocarbons			1.5±0.1	7.8±0.1	74.4±0.3
924	926	Tricyclene	-	t	0.1±0.0
926	930	α-Thujene	t	-	-
939	939	α-Pinene	0.2±0.0	2.5±0.0	25.6±0.2
953	954	Camphene	-	0.7±0.0	6.4±0.1
977	975	Sabinene	t	0.3±0.0	-
985	979	β-Pinene	0.5±0.0	2.4±0.0	15.9±0.1
980	987	3-*p*-Menthene	-	-	0.2±0.0
998	990	Myrcene	0.1±0.0	0.5±0.0	3.5±0.0
1012	1011	δ-3-Carene	-	-	0.6±0.0
1020	1017	α-Terpinene	t	0.1±0.0	2.3±0.0
1021	1024	*p*-Cymene	t	0.5±0.0	0.9±0.0
1028	1029	Limonene	t	-	18.5±0.2
1043	1037	*cis*-Ocimene	-	0.1±0.1	-
1053	1050	*trans*-β-Ocimene	0.1±0.0	0.1±0.0	-
1056	1059	γ-Terpinene	0.2±0.0	0.3±0.0	0.1±0.0
1090	1088	Terpinolene	0.3±0.0	0.3±0.0	0.3±0.0
Oxygenated monoterpenes			94.7±1.2	85.5±0.1	23.4±0.3
1029	1031	1,8-Cineole	0.3±0.0	26.5±0.0	2.1±0.2
1051	1056	Bergamal	0.1±0.0	-	-
1070	1070	*cis*-Sabinene Hydrate	-	0.2±0.0	-
1076	1072	*cis*-Linalool Oxide	-	0.1±0.0	-
1095	1096	Linalool	0.1±0.0	38.7±0.1	t
1098	1099	α-Pinene Oxide	-	-	0.1±0.0
1104	1108	*cis*-Rose Oxide	0.1±0.0	-	-
1122	1125	*trans*-Rose Oxide	t	-	-
1129		Plinol C	-	0.4±0.1	-
1144	1146	Camphor	-	14.2±0.1	0.5±0.0
1150	1149	Isopulegol	4.3±1.1	-	-
1154	1153	Citronellal	88.0±0.8	-	-
1158	1159	*iso*-Isopulegol	0.5±0.1	-	-
1159	1160	Isoborneol	-	0.4±0.0	-
1168	1166	δ-Terpineol	-	0.3±0.0	-
1170	1169	Borneol	-	1.3±0.0	-
1179	1177	Terpinen-4-ol	-	0.3±0.0	t
1184	1182	*p*-Cymen-8-ol	-	0.1±0.0	-

1187	1185	Cryptone	-	t	-
1188	1188	α-Terpineol	-	1.6±0.0	0.1±0.0
1196	1195	Myrtenal	-	0.1±0.0	-
1197	1199	γ-Terpineol	-	0.2±0.0	-
1212	1220	α-Fenchyl Acetate	-	-	0.1±0.0
1231	1229	Nerol	-	0.1±0.0	-
1256	1252	Piperitone	-	t	-
1258	1252	Geraniol	-	0.2±0.0	-
1260	1257	Linalool Acetate	-	0.5±0.0	t
1287	1288	Bornyl Acetate	-	0.1±0.0	17.9±0.0
1311	1313	Citronellic Acid	0.1±0.0	-	-
1325		β-Terpinyl Acetate	-	-	0.1±0.0
1345	1349	α-Terpinyl Acetate	-	-	2.6±0.0
1348	1352	Citronellyl Acetate	1.3±0.1	-	-
1368	1361	Neryl Acetate	-	0.2±0.0	-
1468	1468	Linalool Isovalerate	-	0.1±0.0	-
1512	1511	Lavandulyl 2-Methyl Butanoate	-	0.1±0.0	-
Sesquiterpene hydrocarbons			2.1±0.2	3.3±0.0	0.7±0.0
1330	1338	δ-Elemene	-	-	t
1377	1376	α-Copaene	-	t	-
1381	1381	Daucene	-	t	-
1383	1388	β-Bourbonene	-	0.1±0.0	-
1385	1390	β-Elemene	-	-	t
1391	1391	7-*epi*-Sesquithujene	-	0.1±0.0	-
1403	1405	Sesquithujene	-	0.1±0.0	-
1407	1407	Longifolene	-	-	0.1±0.0
1409	1409	α-Gurjunene	-	0.1±0.0	-
1410	1411	α-Cedrene	-	-	0.1±0.0
1420	1419	β-Caryophyllene	2.0±0.2	1.8±0.0	0.4±0.0
1427	1434	α-*trans*-Bergamotene	-	0.1±0.0	-
1435	1436	γ-Elemene	-	-	t
1454	1454	α-Humulene	-	0.1±0.0	t
1460	1456	*trans*-β-Farnesene	-	0.2±0.0	-
1470	1472	Dauca-5,8-diene	-	t	-
1481	1479	γ-Muurolene	-	0.3±0.0	-
1495	1500	Bicyclogermacrene	0.1±0.0	-	-

1500	1500	α-Muurolene	-	-	t
1510	1505	β-Bisabolene	-	0.2±0.0	-
1514	1513	γ-Cadinene	-	0.2±0.0	t
1524	1522	*trans*-Calamenene	-	t	-
1525	1523	δ-Cadinene	-	t	0.1±0.0
		Germacrene B	-	-	t
Oxygenated sesquiterpenes			t	0.3±0.0	0.3±0.0
1582	1583	Caryophyllene Oxide	t	0.2±0.0	t
1599	1600	Cedrol	-	-	0.1±0.0
1641	1640	*epi*-α-Cadinol	-	0.1±0.0	-
1684	1685	α-Bisabolol	-	t	-
Oxygenated Diterpenes			-	-	0.1±0.0
1985	1987	Manool Oxide	-	-	0.1±0.0
Aromatic compounds			0.1±0.0	t	0.3±0.0
1247	1250	*p*-Anis Aldehyde	-	-	0.3±0.0
1351	1359	Eugenol	0.1±0.0	-	-
1434	1434	Coumarin	-	t	-
Others			0.1±0.0	0.5±0.1	-
868	870	*n*-Hexanol	-	t	-
910		Isobutyl Isobutyrate	0.1±0.0	-	-
983	979	1-Octen-3-ol	-	t	-
1008		Isoamyl Isobutyrate	t	-	-
1194	1192	Hexyl Butanoate	-	0.1±0.0	-
1234	1332	Hexyl Tiglate	-	0.1±0.0	-
1244	1244	Hexyl Isovalerate	-	0.3±0.0	-
		Total	98.6±1.2	97.6±0.2	99.1±0.0

RI_{Cal}: retention index relative to C_8-C_{32} *n*-alkane on HP-5MS column; RI_{Ref}: retention index reported in Adams 2007 [45]; t: trace amounts <0.05. Values are means relative area (%) ± standard deviation of the three samples.

Furthermore, *L. angustifolia* essential oil hydrodistilled from aerial parts coming from Yazd (Iran) had a dissimilar chemical composition to our results, with 1,8-cineole, camphor, and borneol as its main components. This was also dissimilar to a sample from lavender essential oil obtained from the inflorescences of *L. angustifolia* "Sevtopolis" cultivated in western

Romania, which had linalyl acetate (40.7%), linalool (22.5%), caryophyllene (8.9%), and lavandulyl acetate (7.5%) as its principal components [51]. In addition, it has been recently observed that the chemical composition of *L. angustifolia* essential oil can be modified by the application of gold and silver metals as elicitors, decreasing lower-molecular-weight compounds such as α- and β-pinene, camphene, δ-3-carene, *p*-cymene, 1,8-cineole, pinocarveol, etc., which are replaced by higher-molecular-weight compounds such as α-cadinol 9-cedranone, cadalene, α-bisabolol, and (E,E)-farnesol, varying the biological properties [52]. Other presentations, such as hydrolates, produced a reduction in volatile compound content and a reduction in antioxidant activity [53].

On the other hand, α-pinene (25.6±0.2%), limonene (18.5±0.2%), and β-pinene (15.9±0.1%) were the main components (Table 1) in *P. sylvestris* essential oil. The predominance of monoterpene hydrocarbons is a characteristic feature of essential oils obtained from the Pinaceae family, e.g., monoterpene hydrocarbons were the major fraction of *P. nigra* var. *italica* essential oil (63.4%), with α-pinene as its most abundant compound (49.0%) [54], as well as in the essential oil obtained from the hydrodistillation of *P. armandii*, *P. nigra* and *P. halepensis* cones with α-pinene, limonene, and β-pinene as principal components [55,56]. The remarkable concentration of limonene in *P. sylvestris* essential oil may also contribute to the antimicrobial properties. In fact, a limonene emulsion has been effectively stabilized by *Ulva fasciata* Delile polysaccharide to be applied to food to avoid foodborne pathogen contamination and consequently prolong shelf-life [57,58].

In contrast to our results, sesquiterpene hydrocarbons have been described as one of the most representative phytochemical groups in the *Pinus* genus together with monoterpene hydrocarbons, with germacrene D or β-caryophyllene being the most characteristic compounds within the group [59]. Thus, essential oils obtained from the needles of other *Pinus* species such as *P. roxburghaii* contain large amounts of α-pinene (29.3%) and β-caryophyllene (21.9%), whereas α-pinene (35.4%) and germacrene D (28.1%) were the main components of the *P. nigra* subsp. *nigra* essential oil [36,60].

2.2. Seed germination inhibition of P. oleracea, L. multiflorum, E. crus-galli, tomato, cucumber and N. glauca with E. citriodora, L. angustifolia and P. sylvestris essential oils

The *in vitro* phytotoxic potential of *E. citriodora, L. angustifolia*, and *P. sylvestris* essential oils was evaluated against seed germination in weeds (*P. oleracea, L. multiflorum*, and *E. crus-galli*), as well as against two Mediterranean food crops (tomato and cucumber), and the invasive species *N. glauca*, at several doses (0.125, 0.25, 0.50, and 1 µL/mL) (Table 2, 3).

Regarding the phytotoxic effects of the selected essential oils against weeds, variability at the intraindividual level was observed in the seed germination percentage, without statistical significance. *E. citriodora* and *P. sylvestris* did not cause a significant inhibition of seed germination in either *P. oleracea, L. multiflorum*, or *E. crus-galli* at any assayed dose (0.125, 0.25, 0.5, and 1 µL/mL) (Table 2). However, citronellal, the main compound in *E. citriodora* essential oil analyzed here, showed seed germination inhibition against other weeds including *Ageratum conyzoides* L., *Chenopodium album* L., *Parthenium hysterophorus* L., *Malvastrum*

coromandelianum (L.) Garcke, *Cassia occidentalis* L., and *Philaris minor* Retz. at 100 µg/g [61]. In relation to food crops, citronellal was able to inhibit seed germination in *L. sativa*, reaching 49-15% of the control [62], as well as seed germination in tomato at a percentage of 64.8% at the highest tested dose (1µL/mL). *P. sylvestris* with α-pinene (25.6%) as the main compound showed phytotoxic effects in seed germination in cucumber at all applied doses (0.125, 0.25, 0.50, and 1 µL/mL), while another *Eucalyptus* species (*E. tereticornis*), which contained principally α-pinene (34.5%), produced selective toxicity against the seed germination of *E. crus-galli* without affecting the rice crop to the same extent [63].

By contrast, although *L. angustifolia* essential oil did not exhibit a significant inhibition of seed germination in *P. oleracea*, it achieved a remarkable reduction of seed germination in both *L. multiflorum* and *E. crus-galli*. This fact may be because *L. angustifolia* essential oil, among the essential oils analyzed here, contains the largest number (27 *vs.* 12 and 14) of oxygenated compounds, especially oxygenated monoterpenes (1,8-cineole, linalool, camphor, borneol, α-terpineol) that have shown higher herbicidal properties [64]. *L. multiflorum* showed more susceptibility to the phytotoxic effect of *L. angustifolia* essential oil, which decreased the percentage of seed germination in a dose-dependent manner, reaching increasing percentages of inhibition of 44.6% and 63.1% at the highest applied doses (0.5 and 1 µL/mL, respectively) (Table 2). The fact that *L. multiflorum* showed a certain sensitivity to *L. angustifolia* essential oil could be interesting in the research of essential oils as natural alternatives to synthetic herbicides used against *L. multiflorum*, which have caused the emergence of resistance in this weed [65-67].

Table 2. *In vitro* phytotoxic effect of different doses of *E. citriodora, L. angustifolia* and *P. sylvestris* essential oils on *Portulaca oleracea, Lolium multiflorum, Echinochloa crus-gallli,* tomato and cucumber seed germination.

	Seed Germination (% ± s.e.)				
*Dose	*E. citriodora* essential oil				
	P. oleracea	*L. multiflorum*	*E. crus-galli*	*Tomato*	*Cucumber*
Control	74.0±4.6 a	65.0±6.9 a	69.0±2.9 a	71.0±2.5 a	99.0±1.0 a
0.125	80.0±2.2 a	67.0±4.4 a	74.0±3.7 a	71.0±4.3 a	98.0±1.2 a
0.25	76.0±2.9 a	52.0±2.0 a	72.0±2.6 a	73.0±3.4 a	95.0±2.2 a
0.5	74.0±4.3 a	58.0±2.6 a	61.0±4.6 a	61.0±3.7 a	97.0±1.2 a
1	81.0±6.2 a	57.0±7.2 a	72.0±3.7 a	25.0±11.3 b	96.0±1.8 a
Dose	*L. angustifolia* essential oil				
Control	74.0±3.7 a	65.0±6.9 a	71.0±4.3 a	71.0±2.5 a	99.0±1.0 a
0.125	69.0±5.3 a	65.0±3.2 a	71.0±2.8 a	73.0±4.4 a	97.0±1.2 a
0.25	67.0±2.0 a	50.0±2.7 a,b	72.0±2.6 a	58.0±4.1 a,b	98.0±2.0 a
0.5	66.0±5.8 a	36.0±8.4 b,c	72.0±3.4 a	41.0±13.2 b,c	97.0±1.2 a
1	69.0±3.7 a	24.0±7.0 c	58.0±2.6 b	22.005.8 c	94.0±1.9 a
Dose	*P. sylvestris* essential oil				
Control	75.0±7.1 a	67.0±2.0 a	74.0±3.3 a	68.0±3.4 a	100.0±0.0 a
0.125	74.0±3.7 a	65.0±8.8 a	69.0±7.0 a	67.0±4.4 a	94.0±2.9 a,b
0.25	71.0±2.9 a	65.0±5.0 a	74.0±1.9 a	67.0±4.1 a	94.0±1.9 a,b
0.5	71.0±1.9 a	58.0±5.2 a	74.0±4.6 a	66.0±3.7 a	95.0±1.6 a,b
1	68.0±2.6 a	51.0±12.8 a	75.0±5.0 a	64.0±3.7 a	90.0±2.3 b

Values are the mean percentage of five replications ± standard error, after 14 days of incubation. Means followed by different letters in the same column indicate significant difference at $p < 0.05$, according to T3 Dunnet and Tukey tests. *Dose: µL/mL.

In other studies, peppermint (*Mentha piperita* L.) essential oil caused a total inhibition of seed germination in *L. multiflorum* at a range of concentrations between 0.125 and 1 µL/mL, and caused inhibition in food crops (maize, rice, and tomato). In our study, *L. angustifolia* essential oil produced less phytotoxic effects in food crops. The seed germination of tomato was reduced at the highest dose tested, at a percentage of 69.02% (*vs.* 99.97% with peppermint essential oil) with respect to the control [24], while the seed germination of cucumber was not significantly inhibited at any assayed dose (0.125, 0.25, 0.50, and 1 µL/mL).

Seed germination in *E. crus-galli* also showed a certain weakness to exposure to *L. angustifolia* essential oil at the highest tested dose (1 µL/mL), with a percentage of inhibition of 18.3% (Table 2).

Tomato was more sensitive to *E. citriodora* and *L. angustifolia* essential oils with similar remarkable reduction at the highest applied dose (1 µL/mL), reaching 64.8 and 69.0% reduction, respectively (Table 2).

In general, cucumber was more resistant than tomato to the phytotoxic effects of the three commercial essential oils applied, without inhibitory effect at any assayed dose (0.125, 0.25, 0.5, and 1 µL/mL) with *E. citriodora* and *L. angustifolia* essential oils, and only a low percentage of inhibition (10.00%) at the highest tested dose (1 µL/mL) with *P. sylvestris* essential oil (Table 2).

In addition, the two essential oils richest in oxygenated monoterpenes, *E. citriodora* and *L. angustifolia* (94.7% and 85.5%, respectively), showed similar significant phytotoxic effects against seed germination in the invasive species *N. glauca*, but with a lower percentage in relation to weeds (27.5% and 29.7%) at the highest tested dose (1 µL/mL) (Table 3).

Therefore, the various main compounds of an essential oil can produce similar phytotoxic effects against different species.

Table 3. *In vitro* phytotoxic effect of different doses of *E. citriodora* and *L. angustifolia* essential oils on the seed germination and seedling growth of *N. glauca*

	Seed germination (% ± s.e.)	Seedling growth (mm ± s.e.)	
Concentration	*E. citriodora*		
(µL/mL)	Germination	Hypocotyl	Radicle
Control	91.0±3.3 a	2.5±0.2 a	3.1±0.3 a
0.125	72.00±6.8 a	1.4±0.3 b	2.5±0.4 a
0.25	68.0±9.0 a	1.4±0.3 b	2.5±0.4 a
0.5	67.003.4 a	1.3±0.2 b	2.5±0.3 a
1	66.0±4.7 b	0.4±0.1 c	1.0±0.3 b
Concentration	*L. angustifolia*		
(µL/mL)	Germination	Hypocotyl	Radicle
Control	91.0±3.3 a	2.5±0.3 a	3.1±0.3 a
0.125	81.0±4.0 a	2.6±0.4 a	2.8±0.3 a,b
0.25	81.0±2.9 a	2.6±0.2 a	2.9±0.3 a,b
0.5	78.0±3.7 a	1.8±0.1 a,b	2.4±0.2 a,b
1	64.0±3.7 b	1.2±0.2 b	2.0±0.1 b

Values are the mean of five replications ± standard error deviation, after 14 days of incubation. Means followed by different letters in the same column indicate significantly difference at $p < 0.05$, according to T3 Dunnet and Tukey tests.

2.3.Seedling growth inhibition of P. oleracea, L. multiflorum, E. crus-galli, tomato, cucumber and N. glauca with E. citriodora, L. angustifolia and P. sylvestris essential oil

The hypocotyl growth of *P. oleracea* was not significantly reduced by *E. citriodora* essential oil at any applied dose (0.125, 0.25, 0.5, and 1 µL/mL); however, this essential oil was able to reduce radicle development at the highest assayed doses (0.5 and 1 µL/mL), reaching 36.4% and 43.2% reduction compared to the control, respectively (Figure 1a). The root growth of *P. oleracea* was more sensitive than shoot growth to citronellal, according to previous studies, due to the mitotic activity of growing root tip

cells [61]. However, other mechanisms would have been present with other essential oils because the roots were not significantly affected at doses that produced toxic effects in the hypocotyl. Therefore, the hypocotyl elongation of *P. oleracea* was remarkably reduced from the lowest applied dose (0.125 µL/mL) of *L. angustifolia* (Figure 1b) and *P. sylvestris* (Figure 1c) essential oils with respect to control, reaching a decrease of 30.6% and 39.3%, respectively, at the highest tested dose (1 µL/mL), whereas radicle development was not significantly affected by *L. angustifolia* essential oil (Figure 1b), yet *P. sylvestris* essential oil achieved a significant reduction in radicle development (26.0-44.4%) from the lowest to the highest applied dose (0.125-1 µL/mL) (Figure 1c).

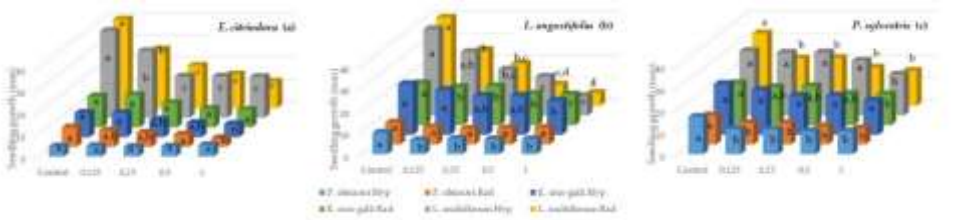

Figure 1. Fitotoxic effect of E. citriodora (a), L. angustifolia (b) and P. sylvestris (c) essential oils on the seedling growth (hypocotyl and radicle) of P. oleracea, L. multiflorum and E. crus-galli. Values are mean percentages of five replications, after 14 days of incubation. Doses 0.125-1 µL/mL. Different letters indicate significant difference at $p < 0.05$, according to T3 Dunnet and Tukey tests.

Regarding the seedling evolution of *E. crus-galli* after the application of the essential oils, it was observed that *L. angustifolia* essential oil was the most harmful for *E. crus-galli* seedling growth, as it decreased its hypocotyl in a high percentage (76.7%) at the highest assayed dose (1 µL/mL), as well as the radicle, in a dose-dependent manner, also reaching a considerable percentage (69.9%) at the highest applied dose (1 µL/mL) (Figure 1b). Although *E. citriodora* essential oil did not influence radicle elongation at

any applied dose (0.125, 0.25, 0.5, and 1 µL/mL), hypocotyl growth was significantly affected at 1 µL/mL, reaching a reduction percentage of 46.1% in comparison to control (Figure 1a). *P. sylvestris* essential oil was the least phytotoxic essential oil, with no reduction in the hypocotyl development of *E. crus-galli* at any dose (0.125, 0.25, 0.5, and 1 µL/mL), and a low percentage of radicle elongation reduction (26.5%) at the highest dose (Figure 1c). However, other studies demonstrated that α-pinene exhibited a certain inhibition of the early root growth of other weeds such as *Cassia occidentalis* (L.) Link., as well as oxidative damage in root tissue [10]. Similarly, the compound β-pinene was shown to be responsible for the disruption of membrane integrity, the enhancement of peroxidation and electrolyte leakage in *Phalaris minor* and particularly in *E. crus-galli* [68].

Both the hypocotyl and radicle development of *L. multiflorum* were significantly inhibited by *E. citriodora* (Figure 1a) and *L. angustifolia* (Figure 1b), which caused a strong dose-dependent reduction, reaching 52.3-53.0% and 60.6-75.4% at 0.25-1 µL/mL, and 55.1-77.5 and 80.1-87.8% at 0.5-1 µL/mL, respectively (Figure 1a,b). *P. sylvestris* essential oil did not significantly affect hypocotyl growth, but it did inhibit the radicle development of *L. multiflorum* in the range of 0.125 to 1 µL/mL without distinction between doses, reaching 51.67% reduction at the highest dose assayed (Figure 1c). With *L. multiflorum*, it was corroborated that α-pinene, the main compound of *P. sylvestris* essential oil analyzed here, affects root development to a greater extent than hypocotyl, as it was also able to inhibit the radicle growth of other weed species such as *Amaranthus viridis* L., *Triticum aestivum* L., *Pisum sativum* L., *Cicer arietinum* L., and especially *C. occidental*, which demonstrated solute leakage, lipid peroxidation and the generation of reactive oxygen species upon α-pinene exposure [10].

Regarding the sensitivity of the seedling growth of food crops to essential oils, it was observed that tomato was more susceptible than cucumber to *E. citriodora*, *L. angustifolia*, and *P. sylvestris* essential oils (Table 4, Figures 2 and 3). Both the hypocotyl and radicle development of tomato were significantly reduced in a dose-dependent manner, reaching elevated reduction percentages at the highest applied dose (1 µL/mL) of *E. citriodora* (89.7 and 79.4%) and *L. angustifolia* (93.2% and 83.4%) essential oils (Figure 2a,b). *L. sativa* was another food crop that showed high sensitivity to the application of citronellal [62], and *E. citriodora* essential oil affected meristematic cells, decreasing the germination and seedling growth of this food crop [25]. Again, *P. sylvestris* was the least phytotoxic essential oil, but also showed a significant inhibition of hypocotyl and radicle development, measuring 72.2% and 62.9%, respectively, at the dose of 1 µL/mL (Table 4).

On the other hand, none of the assayed essential oils significantly affected the hypocotyl growth of cucumber (Table 4). However, the radicle development of cucumber was significantly reduced, up to a percentage of 42.4%, 37.8%, and 28.0% at the highest applied doses of *E. citriodora*, *L. angustifolia*, and *P. sylvestris* essential oils (Table 4).

Finally, *E. citriodora* essential oil showed more phytotoxic effects than *L. angustifolia* essential oil in both the hypocotyl and radicle elongation of the invasive species *N. glauca*, reaching percentage reductions of 85.8% and 69.4% *versus* 51.8% and 37.6%, respectively (Table 3).

Table 4. *In vitro* phytotoxic effect of different doses of *E. citriodora* (EC), *L. angustifolia* (LA) and *P. sylvestris* (PS) essential oils on tomato (TO) and cucumber (CU) seedling growth.

			Seedling growth (mm ± s.e.)				
Dose			Control	0.125 µL/mL	0.25 µL/mL	0.5 µL/mL	1 µL/mL
EC	TO	Hyp	7.3±1.4 a	6.8±1.8 a	5.0±1.2 a,b	2.7±0.5 a,b	0.8±0.4 b
		Rad	16.7±1.5 a	14.1±1.7 a	15.1±1.5 a	6.3±1.3 b	3.4±1.7 b
	CU	Hyp	8.4±0.1 a	8.3±0.4 a	8.4±0.2 a	8.4±0.1 a	8.5±0.9 a
		Rad	23.1±1.5 a	20.2±0.5 a	15.3±0.5 b	15.1±0.9 b	13.3±0.4 b
LA	TO	Hyp	7.3±1.4 a	7.2±0.7 a	4.9±0.9 a,b	2.1±0.8 b,c	0.5±0.3 c
		Rad	16.7±1.5 a	16.4±0.9 a	11.6±1.1 a,b	7.3±2.1 b,c	2.8±2.0 c
	CU	Hyp	8.4±0.1 a	8.3±1.0 a	8.1±0.9 a	8.3±0.9 a	8.3±.04 a
		Rad	23.1±1.5 a	18.9±0.5 b	17.1±0.5 b,c	15.6±1.0 b,c	14.4±0.7 c
PS	TO	Hyp	12.6±1.6 a	3.8±1.2 b	4.0±0.7 b	3.2±0.7 b	3.5±0.3 b
		Rad	18.1±1.0 a	9.4±1.0 b	10.6±0.5 b	6.8±1.7b	6.7±0.4 b
	CU	Hyp	8.5±0.9 a	8.6±0.2 a	8.4±0.3a	8.4±0.8 a	7.7±0.9 a
		Rad	21.2±1.0 a	17.8±0.6 a,b	16.3±1.1 b	16.5±0.8 b	15.2±0.8 b

Values are the mean percentage of five replications ± standard error, after 14 days of incubation. Means followed by different letters in the same row indicate significantly difference at $p < 0.05$, according to T3 Dunnet and Tukey tests. Hyp: Hypocotyl (mm); Rad: Radicle (mm).

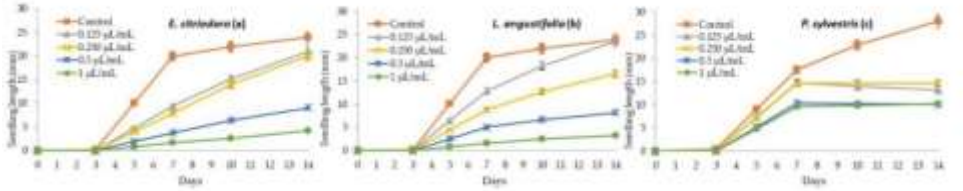

Figure 2. Phytotoxic effect of E. citriodora (a), L. angustifolia (b) and P. sylvestris (c) essential oils at 0.125, 0.25, 0.5 and 1 µL/mL on the seedling growth (hypocotyl + radicle) of tomato.

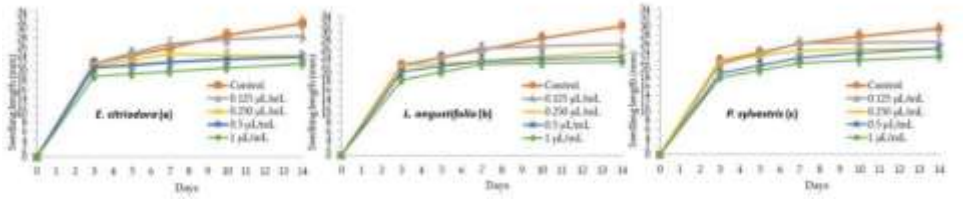

Figure 3. Phytotoxic effect of E. citriodora (a), L. angustifolia (b) and P. sylvestris (c) essential oils at 0.125, 0.25, 0.5 and 1 µL/mL on the seedling growth (hypocotyl + radicle) of cucumber.

3. MATERIAL AND METHODS

3.1.Essential oils

Commercial samples of *Eucalyptus citriodora* Hook (Batch: OF25830; Exp. Date: 02/2022), *Lavandula angustifolia* Mill. (Batch: 0082842; Exp. Date: 30/11/2020), and *Pinus sylvestris* L. (Batch: 0065144; Exp. Date: 08/08/2018) essential oils obtained from the hydrodistillation of leaves, flowers, and needles, respectively, were supplied by Pranarôm S.A. (*E. citriodora*) and Guinama (Valencia, Spain). The essential oils were stored at 4 °C until chemical analysis and phytotoxic assays were carried out.

3.2.Weeds, food crops, and invasive species seeds

Mature seeds of the weeds common purslane (*Portulaca oleracea* L.), Italian ryegrass (*Lolium multiflorum* Lam.), and barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) were purchased from Herbiseed

(website: www.herbiseed.com). Mature seeds of the food crops "Muchamiel" tomato (*Solanum lycopersicum* L.) and cucumber (*Cucumis sativus* L.) were obtained from Intersemillas S.A. Mature seeds of the invasive species tree tobacco (*Nicotiana glauca* Graham) were supplied by the Botanical Garden of Valencia.

3.3. Gas chromatography-Mass spectrometry analysis

Gas Chromatography-Mass Spectrometry analysis was carried out using a 5977A Agilent mass spectrometer and a gas chromatograph (Agilent 7890B, Valencia, España) apparatus equipped with an Agilent HP-5MS (30 m long and 0.25 mm i.d. with 0.25 µm film thickness) capillary column (95% dimethylpolysiloxane - 5% diphenyl). The column temperature program was 60 °C for a duration of 5 min, with 3 °C/min increases up to 180 °C, then 20 °C/min increases up to 280 °C, which was maintained for 10 min. The carrier gas was helium at a flow rate of 1 mL/min. Split-mode injection (ratio 1:30) was employed. Mass spectra were collected over the *m/z* range 30–650 with an ionizing voltage of 70 eV. The resulting individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index, calculated using co-chromatographed standard hydrocarbons relative to C_8-C_{32} *n*-alkanes and mass spectra with reference samples or with data already available in the NIST 11 mass spectral library and in the literature [45].

3.4. In vitro assays: P. oleracea, L. multiflorum, E. crus-galli, tomato, cucumber, and N. glauca seed germination and seedling growth with essential oils

Sets of 20 seeds each with five replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper

(Whatman No.1). The lower filter papers were moistened with 4 mL of distilled water and the upper ones with 0 (control), 0.125, 0.250, 0.5, and 1 µL/mL of *E. citriodora*, *L. angustifolia*, and *P. sylvestris* essential oils, homogeneously distributed in the filter paper with a micropipette (Merck®, Valencia, España). Therefore, the seeds were in contact directly with moistened filter papers and indirectly with the vapors of the essential oils. Petri dishes were sealed with parafilm and incubated in an Equitec EGCS 301 3SHR model germination chamber, according to previous assays [69], alternating between 30.0±0.1 °C 16 h of light and 20.0±0.1 °C 8 h of darkness, with and without humidity. To evaluate the herbicidal activity of the essential oils, the number of germinated seeds was counted and compared with that of untreated seedlings. The emergence of the radicle (≥1 mm) was used as an index of germination, and seedling length (hypocotyl and/or radicle) data were recorded after 3, 5, 7, 10, and 14 days in each replicate.

3.5. Statistics

Experiments were performed *in vitro* with five replicates. Data were subjected to one-way analysis of variance (ANOVA) using SPSS statistics 24 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* test was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq 0.05$.

4. CONCLUSIONS

In this study, the potential of *E. citriodora*, *L. angustifolia*, and *P. sylvestris* essential oils as ecofriendly alternatives to synthetic herbicides was investigated. *L. angustifolia* essential oil, with a high content of the

oxygenated monoterpenes linalool (38.7±0.1%), 1,8-cineole (26.5±0.1%), and camphor (14.2±0.1%), affected seed germination and development of *L. multiflorum*, *E. crus-galli*, and *N. glauca* without any significant phytotoxic effect on cucumber seed germination. *E. citriodora*, with a high content of the oxygenated monoterpene citronellal (88.0±0.8%), showed more phytotoxic effects than *L. angustifolia* on the control of *N. glauca*. Lavender essential oil represents an effective pre-emergent treatment for *L. multiflorum* affecting cucumber crops, and *E. citriodora* essential oil could be used in both pre- and post-management of the invasive species *N. glauca.*

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Other publications

CHAPTER 4.

Herbicidal value of essential oils from oregano-like flavour species

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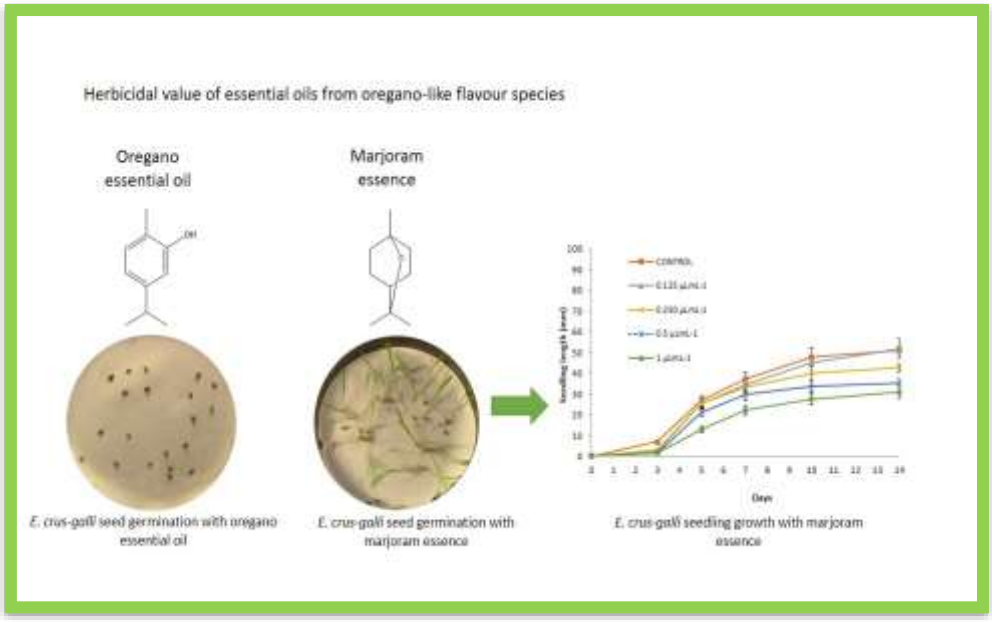

ABSTRACT

Chemical composition and phytotoxicity of commercial oregano, marjoram and *Thymus mastichina* essential oils against *Portulaca oleracea* L., *Lolium multiflorum* Lam. and *Echinochloa crus-galli* (L.) Beauv., has been investigated. Seventy-seven compounds accounting between 97.3-99.4% of the total commercial oils were identified by Gas Chromatography-Mass Spectrometry. Carvacrol (60.42±0.07%), *p*-cymene (15.52±0.02%) and γ-terpinene (5.19±0.02%) were the main compounds in oregano essential oil whereas large amounts of 1,8-cineol (59.59±0.85%, 49.49±0.37%), linalool (13.05±0.04%, 5.66±0.01%) and α-terpineol (3.36±0.10%, 5.59±0.01%), followed by β-pinene (4.35±0.39, 5.54±0.01%) and α-pinene (4.11±0.53, 4.28±0.01%) were found, respectively, in marjoram and *T. mastichina* essential oils. Oregano essential oil completely inhibited seed germination and seedling growth at all concentrations assayed (0.125-1 μL/mL), whereas marjoram and *T. mastichina* essential oils only showed significant effects in hypocotyl and/or hypocotyl+radicle length depending on the weed and dose.

Keywords: Carvacrol; 1,8-cineole; essential oils; seed germination; seedling growth.

1. INTRODUCTION

The commercial value of certain herbs and spices, widely employed in Mediterranean diets as well as their essential oils as natural ingredients in beverages, medicines and cosmetics, plays an important role in their adulteration. Variations in chemical composition of herbs detected in a quite common way do not necessarily reveal adulteration and may be due to satisfy supply and demand (Sprink & Moyer, 2013), such as it occurs with the essential oils. In this case, these changes are due to aging, storage, as well as by the use of species with different common names according to place of origin. Thus, authentication or standardization of commercial essential oils by means of selected main compounds is a fundamental subject for consumers.

In this sense, marjoram (*Origanum majorana* L.) is a species of remarkable economic and industrial importance because both its fresh and dried highly aromatic leaves and flowering tops are widely used as spice and condiment to flavour many foods, with marjoram essential oil also employed in the food industry as flavouring in foodstuff and beverages, in perfumeries for its spicy and herbaceous notes or in pharmaceutical and industrial products due to their antimicrobial and antioxidant properties. However, marjoram is usually confused with other aromatic species, especially oregano (*Origanum vulgare* L.), the most traded and consumed spice, and well-known culinary herb commonly associated with pizzas and other Mediterranean dishes, and even with *Thymus mastichina* L., because this latter species is also known as Spanish marjoram. All species belong to the Lamiaceae family and their confusion may be due to the fact that all bear oregano-like flavour.

Oregano identity is complicated by both the large heterogeneity of *Origanum* genus and the grouping of different botanical genera, *Origanum* from the Mediterranean and *Lippia* from Mexico. The European Pharmacopoeia and the European Spice Association only allow *O. vulgare* L. ssp. *hirtum* and *Origanum onites* L. to be marketed as true oregano. However, the International Organization for Standardization (ISO7925) allows leaves of all *Origanum* genus, species and subspecies, except *O. majorana*, to be marketed as oregano (Black, Haughey, Chevallier, Galvin-King, & Elliott, 2016). European *O. vulgare* essential oil shows a great variability in both yield and chemical composition. Plants from the Mediterranean climate usually exhibit an active/efficient cymyl- and/or linalool pathway, whereas in plants from regions with Continental climate the essential oils are comparatively poor in monoterpenes and geranyl pyrophosphate (GPP) is mainly converted by the sabinyl pathway (Lukas, Schmiderer, & Novak, 2015).

In this way, high-quality plant material from Mediterranean area with high content of phenolic monoterpenes, mainly carvacrol and/or thymol and their biosynthetic precursors γ-terpinene and *p*-cymene with pungent oregano flavour, have a wide commercial potential. So, qualitative and quantitative analyses are needed to ensure quality, consumer safety (Salgueiro, Martins, & Correia, 2010) and fair trade of herbs and spices widely used as culinary seasoning and especially when this species or theirs essential oils are present in cosmetic products or pharmaceutical specialties by their pharmacological activity.

Several studies have described that essential oils obtained from *Origanum* spp. show a wide range of biological activities, such as antifungal,

antibacterial, antioxidant, antiinflammatory, insecticidal, cytotoxic and anti-acetylcholinesterase (Hajlaoui, Mighri, Aouni, Gharsallah, & Kadri, 2016; Revajová, Pistl, Levkut, Marcin, & Levkutová, 2010). In this sense, a supplementation of oregano essential oil increases proliferation of lymphocites, suggesting higher immune defense ability of the body (Revajová et al., 2010). According to their antifungal activity, they have been demonstrated to be very active against numerous pathogens and spoilage fungi affecting worldwide crops and post-harvest products, such as *Verticillium dahlia*, *Penicillium aurantiogriseum* (Rus et al., 2016), as well as in humans and animals mycosis, like *Candida glabrata* isolated from patients (Khosravi et al., 2011). In addition, oregano essential oil denotes a promising natural additive in foodstuff due to its capacity to prevent bacterial contamination and consequently improving food preservation (Huang, Lin, & Chuang, 2010; Revajová et al., 2010).

On the other hand, *Thymus mastichina* essential oil also has been reported by their antimicrobial activity against *Candida* spp. *Staphylococcus aureus*, *Staphylococcus epidermidis* and *Streptococcus agalactiae* (Silva, Gomes, & Palmeira-de-Oliveira, 2014), as well as by their antioxidant, anti-inflammatory and anti-hyperglycaemic activities. In this way, *Thymus caespititius* and *T. mastichina* were the main scavengers of nitric oxide radicals between the six *Thymus* species assayed (Aazza et al., 2016).

As the biological activity is closely related with the chemical composition of the essential oils, and these mixtures of natural compounds represent at the same time usefulmarkers for the evaluation of quality and authenticity of the final products widely demanded in perfumery, cosmetic, food, beverage, agricultural and pharmaceutical industries, commercial essential

oils need more quality control because in several companies only the common name appears in the label of these biological materials. Known main compounds can provide useful information to discriminate between species of different geographical origin and to reveal frauds if substituted by others of different botanical origin.

So, the aims of this work are firstly to standardize through Gas Chromatography-Mass Spectrometry (GC-MS) analysis the essential oils from oregano-like flavour species purchased from a Spanish company dedicated to the supply of raw materials (pharmaceuticals, cosmetics and food supplements), packaging and laboratory material in Pharmacy and Hospitals and from a Portugal company in order to establish the importance of including in the label, take into account the variability in chemical composition, not only the scientific or common name, but also the main components in order to ensure its use, and secondly to compare the phytotoxic activity of these related commercial essential oils against seed germination and seedling growth of *Portulaca oleracea*, a cosmopolitan annual weed of tropical and subtropical climates, *Lolium multiflorum*, a grass distributed along temperate climates affecting mostly cereals and *Echinochloa crus-galli*, an annual plant seriously influencing irrigation crops, especially rice.

2. MATERIAL AND METHODS

2.1.Plant material

Commercial samples of oregano essential oil (Batch 0042451) and natural marjoram (Batch 0042773) essence, purchased from Guinama TM (Valencia, Spain), and *T. mastichina* essential oil (Batch TM010711),

supplied by Planalto Dourado TM (Freixedas, Portugal), were stored at 4°C until chemical analysis and phytotoxic studies.

2.2. Weeds

Mature seeds of annual weeds of *P. oleracea* L., *L. multiflorum* Lam. and *E. crus-galli* (L.) Beauv. were purchased from Herbiseed TM (website: www.herbiseed.com).

2.3. Gas chromatography-Mass spectrometry

GC-MS analysis was carried out with a 5973N Agilent apparatus, equipped with a capillary column (95 dimethylpolysiloxane - 5% diphenyl), Agilent HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 μm film thickness). The column temperature programme was 60 °C for 5 min, with 3 °C/min increases to 180 °C, then 20 °C/min increases to 280 °C, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the *m/z* 30-500 range with an ionizing voltage of 70 eV. The individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index calculated using co-chromatographed standard hydrocarbons relative to C_8-C_{32} *n*-alkanes, and mass spectra with reference samples or with data already available in the NIST 2005 mass spectral library and in the literature (Adams, 2007).

2.4.Herbicidal activity

Sets of 20 seeds each with 5 replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No.1) moistened with 4 mL of distilled water and with 0 (control), 0.125, 0.250, 0.5 and 1 μL/mL of marjoran, oregano and *T.*

mastichina essential oils. Petri dishes were sealed with parafilm and incubated in a germination chamber Equitec EGCS 301 3SHR model, according to previous assays (Blázquez & Carbó, 2015) alternating 30.0±0.1 °C 16 h in light and 20.0±0.1 °C 8 h in dark and with (*E. crus-galli*) and without (*P. oleracea*, *L. multiflorum*) humidity. To evaluate the herbicidal activity of the essential oils, the number of germinated seeds was counted and compared with those of untreated seedlings. Emergence of the radicle (≥1 mm) was used as an index of germination and seedling length (hypocotyl and/or radicle) data were recorded after 3, 5, 7, 10 and 14 days in each replicate.

2.5.Statistical analysis

Experiments were made with five replicates. Data were subjected to one-way analysis of variance with SPSS statistics 22 software. Tukey's test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* test was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq .05$.

3. RESULTS

3.1. Essential oil composition

Seventy-seven compounds reaching between 97.3% and 99.4% of the total commercial oregano, marjoram and *T. mastichina* essential oils were identified by GC/MS analysis.

Components are clustered (Table 1) in homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, aromatic compounds (phenylpropanoids) and others and listed according to Kovat's retention index calculated in GC on an apolar HP-5MS column.

Table 1. Chemical composition of oregano, marjoram and *T. mastichina* essential oils.

RI	Compounds	Oregano	Marjoram	*T. mastichina*
Monoterpene hydrocarbons		27.00 ± 0.10	12.44±1.30	18.65±0.32
931	α-Thujene	-	0.09±0.02	0.14±0.10
939	α-Pinene	1.39±0.01	4.11±0.53	4.28±0.01
953	Camphene	0.19±0.00	0.81±0.10	0.37±0.00
978	Sabinene	-	0.88±0.09	4.24±0.01
980	β-Pinene	0.30±0.01	4.35±0.39	5.54±0.01
994	Myrcene	1.95±0.02	1.34±0.12	1.76±0.00
1002	α-Phellandrene	0.22±0.01	-	0.05±0.00
1004	*p*-Mentha-1(7),8-diene	-	0.08±0.01	-
1018	α-Terpinene	1.13±0.01	-	0.19±0.01
1025	*p*-Cymene	15.52±0.02	-	1.43±0.28
1029	Limonene	1.12±0.02	-	-
1052	*trans*-Ocimene	-	0.37±0.02	0.19±0.01
1060	γ-Terpinene	5.19±0.02	0.41±0.02	0.34±0.01
1090	Terpinolene	-	-	0.13±0.00
Oxygenated monoterpenes		66.05±0.08	84.49±1.12	67.71±0.33
1031	1,8-Cineole	0.62±0.01	59.59±0.85	49.94±0.37
1070	*cis*-Sabinene hydrate	-	0.11±0.01	0.37±0.01
1076	*cis*-Linalool oxide	-	0.26±0.01	0.04±0.00
1086	*trans*-Linalool oxide	-	0.36±0.01	-
1100	Linalool	2.07±0.01	13.50±0.04	5.66±0.01
1098	Dehydrolinalool	-	0.18±0.01	-
1102	α-Thujone	0.05±0.00	-	-
1116	α-Fenchol	-	0.03±0.00	-
1122	*cis*-*p*-Menth-2-en-1-ol	-	-	0.06±0.00
1133	1-Terpineol	-	0.12±0.01	-
1140	*trans*-Pinocarveol	-	0.06±0.01	0.10±0.01
1141	*trans*-*p*-Menth-2-en-1-ol	-	-	0.04±0.01
1146	Camphor	0.05±0.00	1.04±0.01	-
1163	Isoborneol	-	0.39±0.01	0.69±0.01
1167	δ-Terpineol	-	0.40±0.03	1.86±0.01
1169	Borneol	0.48±0.00	1.06±0.01	-

1178	Terpinen-4-ol	0.44±0.00	0.41±0.01	0.83±0.01
1187	*p*-Cymen-8-ol	-	-	0.05±0.01
1191	α-Terpineol	0.12±0.01	3.36±0.10	5.59±0.01
1199	γ-Terpineol	-	0.43±0.01	-
1203	*trans*-Dihydrocarvone	-	-	0.15±0.01
1205	Verbenone	-	0.02±0.01	-
1247	Carvacrol methyl ether	-	-	0.63±0.00
1258	Linalool acetate	-	2.71±0.14	0.04±0.01
1267	Geranial	0.03±0.00	-	-
1286	Isobornyl acetate	-	-	0.03±0.00
1288	Bornyl acetate	-	0.20±0.01	-
1293	Thymol	1.77±0.01	-	-
1302	Carvacrol	60.42±0.07	0.02±0.01	1.63±0.01
1349	α-Terpinyl acetate	-	0.20±0.01	-
1361	Neryl acetate	-	0.01±0.00	-
1477	Geranyl propanoate	-	0.04±0.00	-
Sesquiterpene ydrocarbons		5.76±0.01	1.40±0.12	8.51±0.05
1338	δ-Elemene	-	-	0.15±0.01
1376	α-Copaene	0.05±0.00	-	0.04±0.01
1385	β-Bourbonene	-	0.03±0.01	0.38±0.00
1392	β-Elemene	-	0.04±0.01	0.26±0.01
1409	α-Gurjunene	-	-	0.08±0.01
1419	β-Caryophyllene	5.66±0.01	0.86±0.07	0.62±0.00
1439	Aromadendrene	-	-	0.06±0.00
1454	α-Humulene	0.05±0.00	0.08±0.01	0.05±0.01
1461	*allo*-Aromadendrene	-	0.06±0.01	0.28±0.02
1481	Germacrene D	-	-	1.27±0.01
1486	β-Selinene	-	-	0.03±0.01
1492	Valencene	-	-	0.08±0.00
1496	Bicyclogermacrene	-	0.23±0.02	2.70±0.01
1500	α-Muurolene	-	-	0.07±0.01
1510	β-Bisabolene	-	-	2.09±0.01
1514	γ-Cadinene	-	0.04±0.00	0.09±0.01
1524	δ-Cadinene	-	0.07±0.00	0.28±0.01
Oxygenated sesquiterpenes		0.46±0.01	0.55±0.08	2.41±0.01
1548	Hedycaryol	-	0.14±0.01	-
1577	Spathulenol	-	0.07±0.01	0.96±0.01

1583	Caryophyllene Oxide	0.46±0.01	0.10±0.02	0.22±0.00
1591	Globulol	-	-	1.03±0.01
1592	Viridiflorol	-	0.09±0.01	-
1602	Ledol	-	0.02±0.01	-
1619	*epi*-γ-Eudesmol	-	-	0.03±0.00
1632	γ-Eudesmol	-	0.03±0.01	-
1642	*epi*-α-Cadinol	-	-	0.09±0.00
1650	β-Eudesmol	-	0.04±0.01	-
1653	α-Eudesmol	-	0.05±0.01	-
1655	α-Cadinol	-	-	0.08±0.00
Aromatics			0.01±0.00	
1361	Eugenol	-	0.01±0.00	-
Others		0.17±0.01		
982	1-Octen-3-ol	0.17±0,00	-	-
	Total	99.43±0.01	98.89±0.05	97.28±0.05

RI, retention index relative to C_8-C_{32} *n*-alkane on HP-5MS column; values are mean relative area (%) ±standard deviation of three samples.

In oregano essential oil, highest quantities of monoterpene compounds (93.05%) were found. Both hydrocarbons (27.00%) and oxygenated monoterpenes (66.05%) with 9 and 10 identified compounds, respectively, were also qualitatively the principal phytochemical group. The oxygenated monoterpene carvacrol (60.42±0.07%), followed by their biogenetic precursors, the monoterpene hydrocarbons *p*-cymene (15.52±0.02%) and γ-terpinene (5.19±0.02%), were the main compounds. Among the sesquiterpene hydrocarbons, large quantities of β-caryophyllene (5.66±0.01%) with small amounts of α-copaene (0.05%) and α-humulene (0.05%) were found. Caryophyllene oxide (0.46±0.01%) and 1-octen-3-ol (0.17%) were, respectively, the only oxygenated sesquiterpene and low molecular weight aliphatic compound identified. Finally, phenylpropanoid compounds were not detected in commercial oregano essential oil analysed here.

Also large quantities of monoterpene compounds (96.93% and 86.36%) both hydrocarbons (12.44% and 18.65%) and oxygenated monoterpenes (84.49% and 67.71%) were found in marjoram and *T. mastichina* essential oils. 1,8-cineol (59.59±0.85%, 49.49±0.37%), linalool (13.15±0.04%, 5.66±0.01%) and α-terpineol (3.36±0.10%, 5.59±0.01%), followed by the monoterpene hydrocarbons β-pinene (4.35±0.39%, 5.54±0.01%) and α-pinene (4.11±0.53%, 4.28±0.01%), were the main compounds.

No higher percentages than 0.14% were found between the 16 sesquitepene compounds identified in marjoram essential oil; however, among the sesquiterpene fraction of *T. mastichina* essential oil with 17 sesquiterpene hydrocarbons (8.51%) and six oxygenated sesquiterpenes (2.41%) identified, germacrene D (1.27±0.01%), bicyclogermacrene (2.70±0.01%), β-bisabolene (2.09±0.01%) and globulol (1.03±0.01%) reached percentages higher than 1%. Finally, the aromatic compound eugenol (0.01%) only was detected in marjoram essential oil.

3.2. Seed germination and seedling growth against P. oleracea, L. multiflorum and E. crus-galli

The effect of oregano, marjoram and *T. mastichina* essential oils against seed germination and seedling growth of *P. oleracea, L. multiflorum* and *E. crus-galli* is shown in Tables 2 and 3 and Figures 1-3, respectively.

Oregano essential oil was the most effective, completely inhibiting the seed germination of the three weeds at all doses (0.125, 0.25, 0.50 and 1 μL/mL) applied (Table 2).

Table 2. Effects of oregano, marjoram and *Thymus mastichina* essential oils *on Portulaca oleracea, Lolium multiflorum and Echinochloa crus-galli* seed germination.

Seed germination (% ± s.e.)			
Concentration (µL/mL)	*Portulaca oleracea*		
	Oregano	Marjoram	*T. mastichina*
Control	73.00±3.74 a	74.00±4.60 a	73.00±3.74 ab
0.125	0.00±0.00 b	80.00±1.60 a	66.00±5.10 a
0.25	0.00±0.00 b	76.00±5.30 a	81.00±1.87 ab
0.5	0.00±0.00 b	83.00±4.40 a	81.25±5.54 ab*
1	0.00±0.00 b	84.00±4.50 a	84.00±3.67 b
	Lolium multiflorum		
Control	73.00±3.39 a	73.00±3.39 a	73.00±3.39 a
0.125	0.00±0.00 b	67.00±5.83 a	71.00±2.45 a
0.25	0.00±0.00 b	65.00±4.47 a	63.00±2.55 a
0.5	0.00±0.00 b	65.00±4.18 a	69.00±3.67 a
1	0.00±0.00 b	61.00±5.10 a	54.00±2.92 a
	Echinochloa crus-galli		
Control	71.00±4.30 a	71.00±4.30 a	71.00±4.30 a
0.125	0.00±0.00 b	75.00±1.58 a	79.00±4.85 a
0.25	0.00±0.00 b	78.00±5.61 a	80.00±3.54 a
0.5	0.00±0.00 b	61.00±4.30 a	73.00±3.00 a
1	0.00±0.00 b	71.00±6.78 a	81.00±7.48 a

Values are mean of five replications ± standard error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests. (* four replications).

Marjoram and *T. mastichina* essential oils did not show any effect against *P. oleracea*, *L. multiflorum* and *E. crus-galli* seed germination. No significant differences were found between control and all concentrations of marjoram and *T. mastichina* essential oil tested (Table 2). However, despite germination not being inhibited, the germinated seed did not develop normally compared with the control (Figures 1-3).

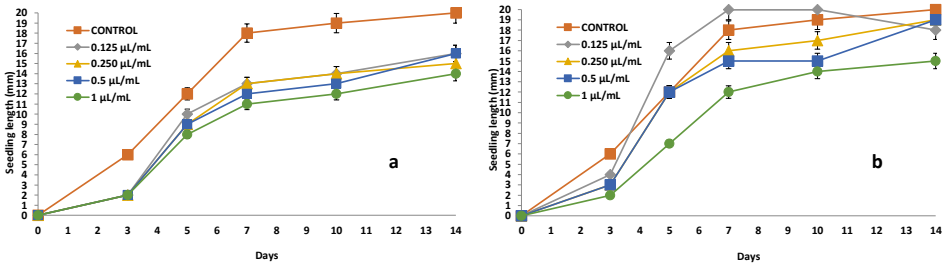

Figure 1. Values of seedling length (mm) (mean ± s.e.) of P. oleracea control and treated with natural marjoram essence (a) and T. mastichina essential oil (b) at 0.125, 0.25, 0.5 and 1 µL/mL *measured over 14 days.*

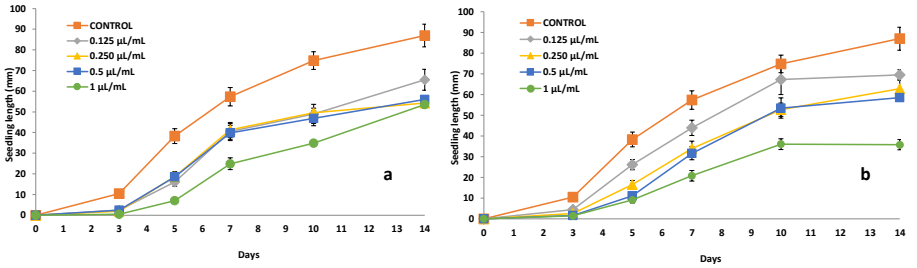

Figure 2. Values of seedling length (mm) (mean ± s.e.) of L. multiflorum control and treated with natural marjoram essence (a) and T. mastichina essential oil (b) at 0.125, 0.25, 0.5 and 1 µL/mL *measured over 14 days.*

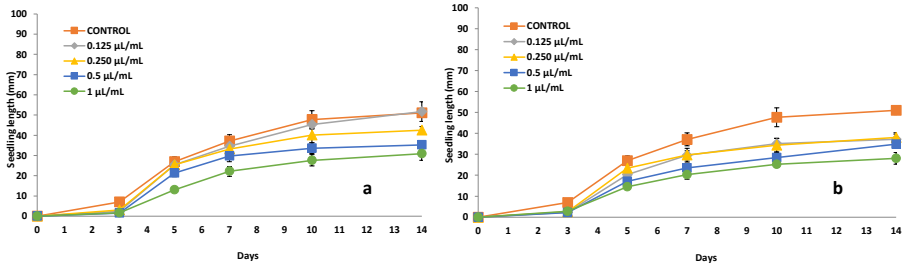

Figure 3. Values of seedling length (mm) (mean ± s.e.) of E. crus-galli control and treated with natural marjoram essence (a) and T. mastichina essential oil (b) at 0.125, 0.25, 0.5 and 1 µL/mL *measured over 14 days.*

Regarding seedling growth, due to strong oregano phytotoxic activity, no seedling length was measured in the three weeds. Marjoram essential oil significantly inhibited hypocotyl of *P. oleracea* and *L. multiflorum* at all doses assayed without any significant effect in radicle elongation (Table 3).

With respect to *E. crus-galli*, noteworthy alterations in both hypocotyls and radicle were detected between control and the highest dose applied (Table 3).

Table 3. Effects of oregano, marjoram and *T. mastichina* essential oils on seedling length (hypocotyl and radicle) of *Portulaca oleracea*, *Lolium multiflorum* and *Echinochloa crus-galli.*

Dose*	*Portulaca oleracea*			
	Marjoram		*T. mastichina*	
	Hyp	Rad	Hyp	Rad
0	9.80±0.92 a	10.00±1.58 a	9.80±0.92 a	10.00±1.58 a
0.125	6.40±0.25 b	9.20±0.74 a	8.00±0.71 a	10.60±0.68 a
0.25	6.60±0.60 b	8.80±0.86 a	9.00±1.58 a	9.60±1.36 a
0.5	6.80±0.37 b	9.00±1.58 a	9.00±1.58 a	9.60±1.36 a
1	6.60±0.25 b	7.00±0.84 a	6.40±1.86 a	8.40±2.25 a
	Lolium multiflorum			
0	48.50±3.35 a	39.20±2.14 a	48.50±3.35 a	39.20±2.14 a
0.125	28.79±2.27 b	36.77±3.33 a	34.74±1.25 b	34.72±1.83 ab
0.25	22.69±1.44 b	31.57±1.29 a	26.89±2.23 c	35.98±2.34 ab
0.5	23.64±1.26 b	32.38±0.67 a	26.85±0.93 c	31.69±0.32 b
1	21.63±1.25 b	31.88±0.80 a	15.47±1.08 d	20.27±1.47 c
	Echinochloa crus-galli			
0	30.01±1.47 a	21.06±1.54 ab	30.01±1.47 a	21.06±1.54 a
0.125	25.69±3.10 abc	26.06±1.94 a	23.45±1.85 b	16.14±2.74 ab
0.25	27.26±0.52 ab	15.28±1.37 bc	19.26±1.21 bc	18.82±1.07 a
0.5	20.17±2.16 bc	15.08±1.60 bc	17.81±0.64 c	17.04±0.68 ab
1	17.98±1.89 c	12.95±2.44 c	16.44±1.70 c	11.64±1.12 b

Oregano germination was 0 in all treatments and there was no seedling length to measure. Values are mean of five replications (mm) ± error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests. *Dose: µL/mL.

T. mastichina essential oil showed no significant differences between control and treated seedlings' length (hypocotyls and/or radical), even without differences between concentrations, against *P. oleracea* (Table 3). However, this essential oil was able to inhibit in a dose-dependent manner both hypocotil and radicle elongation of *L. multiflorum* and *E. crus-galli* with significant inhibitory effect especially between the control and the highest dose (1 μL/mL) (Table 3) assayed.

4. DISCUSSION

Because oregano is the common name of different species used for culinary purposes, such as Greek oregano (*O. vulgare* L. ssp *hirtum*), Turkish oregano (*O. onites* L.), and also Spanish oregano (*Thymus capitatus* (L.) Hoffmanns & Link) and Mexican oregano (*Lippia graveolens* HBK) belonging to other genera, several differences are found in their essential oil composition. In general, phenolic compounds (thymol and carvacrol) and their biogeneticprecursors γ-terpinene and *p*-cymene are the main compounds in oregano essential oils, but with great variability in the percentage depending on the geographical origin. In certain regions of India, *O. vulgare* produces an essential oil rich in *p*-cymene (6.7-9.8%), γ-terpinene (12.4-14.0%), thymol (29.7-35.1%) and carvacrol (12.4-20.9%) (Pande, Tewari, Singh, & Singh, 2012), whereas Turkish oregano essential oil together with the phenolic compounds thymol (15.66%) and carvacrol (24.52%) contain high amounts of linalool (50.53%) (Ozkan & Erdoğan, 2011). Spanish oregano is rich in carvacrol (61.21%), *p*-cymene (15.12%) and γ-terpinene (4.80%) (Viuda-Martos, Ruíz-Navajas, Fernández-López, & Pérez-Álvarez, 2007), and finally, Mexican oregano content also has carvacrol as the main compound (47.41%) followed by *p*-cymene (26.44%) and thymol (3.02%) (Rodríguez-García et al., 2016). Our results are similar

to those reported for *O. vulgare* growing in the Mediterranean area (Viuda-Martos et al., 2007), complying with the values of the monographs, so the commercial oregano analysed here is *O. vulgare* with optimum quality compatible with public health.

Although oxygenated monoterpenes are the main fraction of commercial marjoram essence analysed here and also of marjoram (*O. majorana* L.) harvested in Tunisia at four phenological stages (Sellami et al., 2009), great differences were found between the principal compounds. Terpinen-4-ol (29.13-32.57%), *cis*-sabinene hydrate (19.9-29.27%) and *trans*-sabinene hydrate (3.5-11.61%) were the main components of this fraction in *O. majorana* essential oils harvested in Tunisia, whereas 1,8-cineole (58.59±0.85%), linalool (13.05±0.04%) and α-terpineol (3.33±0.10%) were the main compounds in commercial natural marjoram essence analysed here.

Several studies indicate that terpinen-4-ol is responsible for the antihypertensive (Seong, Hong, Hur, & Lee, 2013) and anticancer (Shapira, Pleban, Kazanov, Tirosh, & Arber, 2016) properties. This compound only reached 0.41% in marjoram essence analysed here. So, an anti-anxiety (Kim, Seo, Min, Park, & Seol, 2014), antimicrobial with application as natural preservative in food packaging (Taqi, Askar, Mutihac, & Stamatin, 2013), anti-inflammatory, antiviral or inhibitory of nuclear factor (NF)-kB effect (Li et al., 2016) could be expected with marjoram essence due to high 1,8-cineol (59.59%) content. Our results are in agreement with the percentages obtained from 20 samples of *T. mastichina* essential oils (1,8-cineol 56.80–69.60%) from Spain (Delgado et al., 2014), and according with ISO quality standards (Table 4) of *T. mastichina* (Mendez-Tovar,

Novak, Sponza, Herrero, & Asensio-S-Manzanera, 2016). So, marjoram essence analysed here could be this last species, known as Spanish marjoram. These results corroborate the need to indicate on the label not only the common name of the species as it appears in marjoram essence purchased, but also the main compounds (terpinen-4-ol or 1,8-cineole), especially when this product is the raw material in perfumery, cosmetic, agricultural and pharmaceutical industries.

Table 4. Quality ranges from the International Standard Organization for *Thymus mastichina* L. (ISO 4728:2003) (Méndez-Tovar et al., 2016), natural marjoram and *T. mastichina* essential oils composition.

Compounds	*T. mastichina* ISO 4728:2003	Marjoram	*T. mastichina*
α-Pinene	1.0-4.5	4.11	4.28
β-Pinene	2.0-5.0	4.35	5.54
Limonene	1.0-6.0	-	-
1,8-Cineole	30.0-68.0	59.59	49.94
Linalool	3.0-48.0	13.50	5.66
Camphor	0.1-2.0	1.04	-
δ-Terpineol	0.2-2.0	0.40	1.86
Borneol	0.1-1.8	1.06	-
Terpinen-4-ol	0.2-1.2	0.41	0.83
Linalyl Acetate	0.2-4.0	2.71	0.04
β-Caryophyllene	0.5-1.5	0.86	0.62

%: Peak area percentage.

In commercial *T. mastichina* essential oil analysed here, 1,8-cineol (49.49±0.37%), linalool (5.66±0.01%) and α-terpineol (5.59±0.01%) were the main compounds. Recent studies between 11 wild populations of *T. mastichina* collected in Spain (Mendez-Tovar et al., 2016) showed that 1,8-cineol (58.52-68.82%) was the main compound in all analysed samples, and linalool (1.16-10.24%) exhibited a large range of variation. Despite this fact it was the least environmentally influenced species between the analysed

ones (Spanish marjoram, spike lavender and Spanish sage). It is an important fact taking into account that 90% of their production in the Iberian Peninsula is harvested from its natural habitat. On the other hand, limonene, camphor and borneol which are included in quality ranges from ISO for *T. mastichina* (Table 4) were not identified in commercial *T. mastichina* essential oil analysed here, despite limonene was described in the label. Although *T. mastichina* is known as Spanish marjoram, qualitative and quantitative differences were found between the two commercial essential oils analysed, which could affect the biological activities, so that we tested also the phytotoxicity of these essential oils against *P. oleracea*, *L. multiflorum* and *E. crus-galli*, important weeds in summer crops of the Mediterranean area.

According to phytotoxicity, 1-O-*cis*-cinnamoyl-β-D-glucopyranose, the most potent allelochemical isolated from *Spiraea thunbergii* Sieb., could be explained by its *cis*-cinnamic configuration which showed a 100 times higher inhibition of lettuce root-growth than *trans*-cinnamic acid, being also able to inhibit the root growth of *Avena sativa*, *Triticum aestivum*, and *Arabidopsis thaliana* (Nishikawa et al., 2013). However, no phenylpropanoids in oregano essential oil that justify the strong inhibitory effect against *P. oleracea*, *L. multiflorum* and *E. crus-galli* seed germination have been detected.

The herbicidal activity of oregano essential oil is not due to the high percentage in oxygenated monoterpenes fraction, because also higher percentages are found in both marjoram (84.49%) and *T. mastichina* (67.71%). The responsible compound was the oxygenated monoterpene carvacrol (60.42% *vs.* 0.02 and 1.63%, respectively) instead of 1,8-cineole

(0.62% *vs.* 59.59 and 49.94%). This is in agreement with previous studies (Angelini et al., 2003) with thymol, carvacrol and 1,8-cineole, in which the two phenolic compounds were more injurious to lettuce and common purslane, than 1,8-cineole at the same concentration. A recent study about the phytotoxic activity of 19 main compounds of essential oils against germination and root length of rigid ryegrass (*Lolium rigidum*), also corroborated that carvacrol, carvone, thymol, *trans*-anethole and linalool were the most phytotoxic components (Vasilakoglou, Dhima, Paschalidis, & Ritzoulis, 2013). However, in a study with 27 monoterpenes, both hydrocarbons and oxygenated ones, against seed germination and primary radicle growth of radish (*Raphanus sativus* L.) and garden cress (*Lepidium sativum* L.), only 1,8-cineol, inhibited their radicle elongation at the lowest concentrations (10^{-5}M, 10^{-6}M) applied (De Martino, Mancini, Almeida, & De Feo, 2010), showing also essential oils with high percentages of 1,8-cineol remarkable interference with germination and seedling growth of certain weeds like silver leaf nightshade (*Solanum elaeagnifolium* Cav.) that was inhibited by *Eucalyptus* spp., such as *Eucalyptus salubris*, *Eucalyptus dundasii* and *Eucalyptus spathulata* oils with 57.6%, 65.5% and 52.9% of 1,8-cineole, respectively (Zhang, An, Wu, Liu, & Stanton, 2012). However, our results showed that marjoram and *T. mastichina* essential oils with high 1,8-cineol content were not able to significantly inhibit *P. oleracea* radicle elongation at all concentrations (0.125, 0.25, 0.50 and 1 μL mL^{-1}) tested (Table 3).

Although it is generally preferable to apply herbicides before crops' emergence, weeds can arise to attack harvests after their germination so a post-control is also required. In this sense, 1,8-cineol and other cineole derivatives have also showed a dose-dependent post-emergence herbicidal

activity against radish and annual ryegrass root and shoot growth (Barton, Clarke, Dell, & Knight, 2014).

On the other hand, linalool, the second main compound in *T. mastichina* and marjoram essential oils with 5.66±0.01% and 13.50±0.04%, respectively, has also been reported as the responsible oxygenated monoterpene of the herbicidal properties of a chemotype (90% linalool) of *Zataria multiflora* essential oil, against spontaneous barley, common rye, common amaranth and bermuda grass (Saharkhiz, Smaeili, & Merikhi, 2010).

Our results corroborate that herbicidal activity of essential oils may be due to both main compounds since the phytotoxic effect of 1,8-cineol against different annual weeds (*Chenopodium album, P. oleracea* and *E. crus-galli*) and crops (*R. sativus, Capsicum annum* and *Lactuca sativa*) was smaller than other aromatic monoterpenes such as thymol and carvacrol (Angelini et al., 2003) and synergistic/antagonistic interaction between their different components, because minor variations in the essential oil constituents affect significantly hypocotyl seedling growth of *P. oleracea* (Table 3). So, for a given weed is possible to develop selective bioherbicides, least injurious to the crops as well as promising alternatives appropriate for uncultivated fields, with more phytotoxic components affecting both crops and weeds.

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CHAPTER 5.

Tee trea and wintergreen essential oils in the management of the invasive species *Cortaderia selloana* and *Nicotiana glauca*

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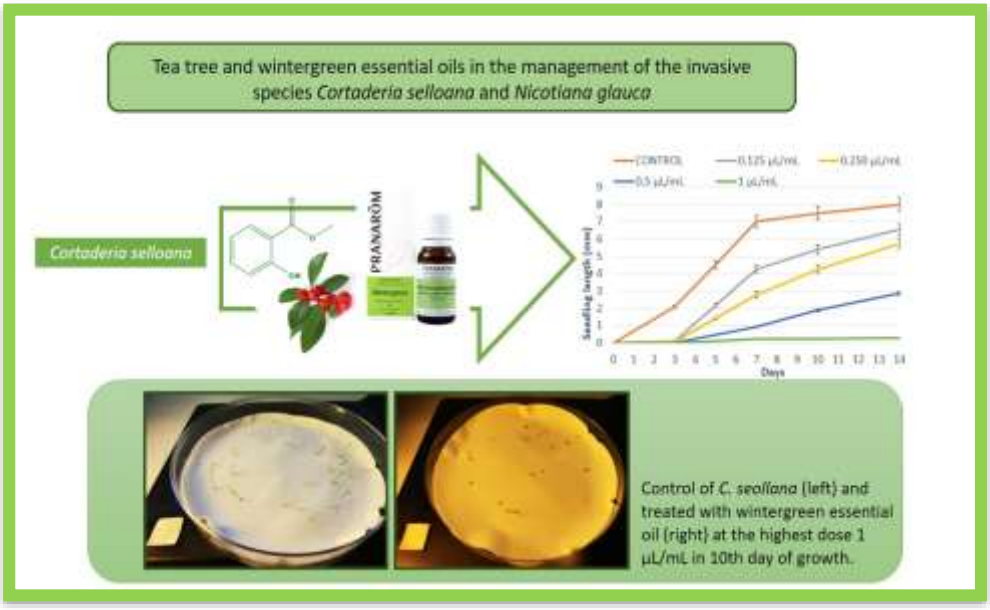

ABSTRACT

Chemical composition of tea tree (*Melaleuca alternifolia*) and wintergreen (*Gaultheria procumbens*) essential oils as well as their phytotoxic effects against two invasive species (*Cortaderia selloana* and *Nicotiana glauca*) were studied. Fifty-eight compounds accounting for between 98.89-99.94% of the total commercial tea tree and wintergreen essential oils were identified by Gas Chromatography-Mass Spectrometry analysis. Tea tree essential oil with terpinen-4-ol (28.37±0.05%) followed by 1,8-cineole (15.81±0.06%), γ-terpinene (15.60±0.03%), α-pinene (10.92±0.08%) and α-terpinene (8.52±0.01%) as the main compounds did not produce significant effect against seed germination and hypocotyl growth of *N. glauca*, but showed significant effects in seed germination inhibition of *C. selloana* (34.69%) as well as in hypocotyl (60.96%) and radicle (62.55%) growth, at the highest dose (1 µL/mL) assayed. High amounts of methyl salicylate (99.63±0.02%) were found in *G. procumbens* essential oil with remarkable phytotoxic effects in *C. seollana*. Methyl salicylate inhibited the seed germination (77.38%) and hypocotyl and radicle growth (96.38% and 96.65%, respectively) at the highest dose (1 µL/mL) assayed. Wintergreen essential oil constitutes an eco-friendly alternative to control the high capacity of invasiveness of *C. selloana.*

Keywords: *Cortaderia selloana*; *Nicotiana glauca;* tea tree; wintergreen; essential oils; phytotoxicity.

1. INTRODUCTION

The naturalization of invasive species brings serious consequences in the ecosystems, from the replacement of the endemic plant species, that could even become extinct, to changes over native fauna as well as alteration of soil chemistry, geomorphological processes, fire regime and hydrology (Cronk and Fuller 2013; Weber 2017). These so hazardous non-native species have been traded outside of their native systems as ornamental plants due to their exotic and attractive appearance (Simberloff *et al.* 2013). In this sense, *Hypericum canariense* L., a native species to the Canary Islands has been introduced and become naturalized in California with detrimental effect on surrounding original plants (Rejmánek 2015). Also, the invasive annual species, *Impatiens glandulifera* Royle native to the western Himalaya and hosted in Europe and North America in the middle of the 19th century, is able to affect soil fungal and bacterial communities and consequently varying soil properties (Gaggini *et al.* 2018). In addition, this species is able to interfere in networks between plant and pollinators achieving an impaired native plant pollination (Vanbergen *et al*. 2018). It is estimated that at least 13,168 vascular plant species have become naturalized in at least one of the 843 regions (including 362 islands) covered by the GloNAF database (van Kleunen *et al.* 2015) with *Sonchus oleraceus* L., *Ricinus communis* L., *Oxalis corniculata* L., *Portulaca oleracea* L., *Eleusine indica* (L.) Gaertn., *Chenopodium album* L. Bosc ex Moq., *Capsella bursa-pastoris* (L.) Medicus, *Stellaria media* (L.) Vill., *Bidens pilosa* L., *Datura stramonium* L. and *Echinochloa crus-galli* (L.) Beauv. as the most widely distributed species (Pyšek *et al.* 2017). Information of aliens species distribution is essential to determine their occurrence, status and impact as well as to prevent new incursions or reduce the impacts of

invasive species (Latombe et al., 2017) Climatic change, among other factors may contribute to the dispersion and tolerance of invasive plant species (Early *et al*. 2016). Regarding this, an important increase in naturalized plant numbers are expected in the next 20 years with more invasions in northern temperate countries and a reduction in tropical and sub-tropical regions (Seebens *et al.* 2015; Dullinger *et al.* 2017). In addition, environmental impacts of alien plant species also result in socioeconomic impacts: out of 128 alien plant species screened in Europe, 96 negative impacts have been recorded on agriculture, animal and forestry production as well as on human infrastructure, health and social life (Rumlerová *et al.* 2016).

In relation to this, an interesting alien invasive species is pampas grass (*Cortaderia selloana* (Schult. & Schult. f.) Asch. & Graebn., Poaceae) original from Brazil, Argentina, Chile and Uruguay which has been traded extensively as an ornamental plant. As a consequence, it has become naturalized in Macaronesia Islands, South Africa, Australia, New Zealand, Hawaiian Islands, the Pacific coast of the USA and Southern Europe (Basnou 2006). Particularly, it is considered invasive in the Mediterranean Basin (Brunel et al. 2010) with a remarkable rapid and deep impact mainly in the north and the eastern coast of the Iberian Peninsula and in the Canary Islands being included in the Spanish Catalogue of Invasive Species (RD 630/2013) (Doménech-Carbó *et al*. 2018). *C. selloana* can tolerate moderate drought, winter frost, intense sunlight and warm summer temperatures as well as a broad variety of physiological soil conditions (Vourlitis and Kroon 2013). This wide tolerance for varying environmental circumstances is presumably the main reason why *C. selloana* is highly invasive together with the fact that it is a gynodioecious species with both

female and hermaphrodite specimens (Doménech-Carbó *et al.* 2018), so it is able to self-fertilize its seeds without a pollinator, typical characteristic of grasses, and regenerate a whole population from a single plant (Kroon 2007). Consequently, *C. selloana* changes soil properties and in this way, the areas affected by this species have lower total soil nitrogen and higher C/N values, with less species, families and life diversity (Domènech *et al.* 2006).

Another important example is tree tobacco (*Nicotiana glauca* Graham, Solanaceae) which is an invasive species native to South America (Petanidou *et al*. 2012), and introduced in the first half of the 19th century as ornamental plant. It is a naturalized species in Australia, California, Mexico, Hawaii, the north and east Mediterranean region, Canary Islands, and North and Southern Africa (Ollerton *et al.* 2012), and included in the Global Invasive Species Database (https://Global Invasive Species Database 2016) due to its high invasion potential. *N. glauca* disturbs ecosystem structure and reduces native biodiversity growing in a wide variety of open and disturbed habitats including roadsides and lakeshores and becoming a problem in relatively dry areas (Thomas *et al.* 2016; Ayenew *et al.* 2018). Its capacity of water conservation by stomatal closure and osmotic adjustment, as well as the presence of leaf wax that reduces the absorption of excess of radiation provide tree tobacco advantages to invade disturbed areas subject to salinity, drought and high radiations (González *et al.* 2012). These characteristics together its ability to reproduce sexually by pollination mainly through hummingbirds as well as by autogamy when these natural pollinators are missing increases its invasive capacity with high seed production and fast development. In addition it can also arise from root which makes *N. glauca* more difficult to eradicate, improving its

expansion (Álvarez *et al.* 2016). Therefore, *N. glauca* is able to influence the environment, socioeconomic factors as well as livestock and human health impacts ("Invasive Species Compendium" 2018) as a source of the alkaloid anabasine with teratogenic properties (Panter *et al.* 2017).

The management of alien species remains today an aspect of chief importance to guarantee the restoration of affected ecosystems. There are numerous methods including prevention, mechanical, cultural, physical, biological and chemical, to control invasive plants or weeds in natural areas (Tu *et al*. 2001; Melander *et al.* 2005). Between them, herbicides are the most common remedy used to eradicate non-native species. It is estimated that in the USA half a million hectares of public wildlands were sprayed with herbicides in 2010, representing 201 tonnes, in which glyphosate was the most commonly used active ingredient (Wagner *et al.* 2016). Indeed, the application of glyphosate-based formulations has not stopped despite its consequent alteration of soil microorganisms, the notable increase in glyphosate-resistant weeds, the potential direct and indirect health effects as well as the environmental contamination (Anza *et al.* 2016; Duke 2018; Van Bruggen *et al.* 2018). So, it is necessary to find less hazardous natural alternatives to synthetic herbicides to control invasive plant species. Specifically, essential oils due to their short environmental half-life and less toxicity, represent a suitable option to synthetic herbicides. These secondary metabolites from mevalonic and shiquimic pathways are mainly recognised by their aromatic, antioxidant, antibacterial and antifungal properties, for which they have been widely used in several industries (Blázquez 2014). Among these mixtures of natural compounds, tea tree (*Melaleuca alternifolia* Maiden & Betche ex Cheel) essential oil is known by its antimicrobial properties against broad spectrum of microoganisms

(bacteria, fungi, viruses and protozoa) as well as by its analgesic, anti-inflammatory and anticancer activities (Yadav *et al.* 2016). On the other hand, wintergreen (*Gaultheria procumbens* L.) essential oil is well-known, together with its antimicrobial properties, by its good antioxidant and anti-radical profile. The analgesic activity of methyl salicylate can explain its topical use in the treatment of rheumatism (Garg 2005; Nikolić *et al* 2013). So, the aims of this work are firstly to determine the chemical composition of *M. alternifolia* and *G. procumbens* essential oils through Gas Chromatography-Mass Spectrometry (GC-MS) analysis in order to assure their main compounds; and secondly, to test the *in vitro* phytotoxic activity of these essential oils against seed germination and seedling growth of the problematic *C. selloana* and *N. glauca* to evaluate their herbicidal activity and consequently know their potential use as natural alternatives to synthetic herbicides in the management of these invasive species.

2. MATERIALS AND METHODS

2.1. Essential oils

Commercial samples of tea tree (*Melaleuca alternifolia* Maiden & Betche ex Cheel) (Batch 0051451) and wintergreen (*Gaultheria procumbens* L.) (Batch 0F18989) essential oils obtained from their leaves were purchased in Guinama and Pranarôm S.A, respectively. Both were stored at 4 ^{o}C until chemical analysis and phytotoxic assays were carried out.

2.2.Invasive plant species

Mature seeds of the invasive plant species pampas grass (*Cortaderia selloana* (Schult. & Schult. f.) Asch. & Graebn. and tree tobacco (*Nicotiana glauca* Graham) were supplied by the Botanical Garden of Valencia.

2.3.Gas chromatography-Mass spectrometry analysis

GC-MS analysis was carried out with a mass spectrometer 5977A Agilent and a gas chromatograph (Agilent 7890B) apparatus, equipped with a capillary column (95 dimethylpolysiloxane - 5 % diphenyl), Agilent HP-5MSi (30 m long and 0.25 mm i.d. with 0.25 µm film thickness). The column temperature program was 60 ºC during 5 min, with 3 ºC /min increases to 180 ºC, then 20 ºC /min increases to 280 ºC, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the *m/z* 30-500 range with an ionizing voltage of 70 eV. The resulting individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index calculated using co-chromatographed standard hydrocarbons relative to C_8-C_{32} *n*-alkanes, and mass spectra with reference samples or with data already available in the NIST 2005 mass spectral library and in the literature (Adams 2007).

2.4. In vitro assays: C. selloana and N. glauca seed germination and seedling growth with tea tree and wintergreen essential oils

Sets of 20 seeds each with five replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No.1) moistened with 4 mL of distilled water and with 0 (control), 0.125, 0.250, 0.50, and 1 µL/mL of tea tree and wintergreen essential oils. Petri dishes were sealed with parafilm and incubated in a germination chamber Equitec EGCS 301 3SHR model, according to previous assays (Blázquez and Carbó 2015) alternating the humidity with 30.0±0.1 ºC 16 h in light and 20.0±0.1 ºC 8 h in dark. To evaluate the phytotoxic activity of the essential oils, the number of germinated seeds was

counted and compared with those of untreated seedlings. Emergence of the radicle (≥1 mm) was used as an index of germination and seedling length (hypocotyl and/or radicle) data were recorder after 3, 5, 7, 10 and 14 days in each replicate.

2.4.Statistics

Experiments were made with five replicates. Data were subjected to one-way analysis of variance (ANOVA) with SPSS statistics 24 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* one was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Chemical composition of tea tree and wintergreen essential oils

Fifty-eight compounds accounting for between 98.89-99.94% of the total commercial tea tree and wintergreen essential oils were identified by Gas Chromatography-Mass Spectrometry analysis. Components are clustered (Table 1) in homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, aromatic compounds and others, and listed according to Kovat's retention index calculated in GC on apolar HP-5MS column.

Monoterpene compounds (95.13%), both hydrocarbons (45.20±0.07%) and oxygenated (49.94±0.05%) with 16 compounds and 14 identified compounds, respectively were the main qualitative and quantitative fraction found in commercial tea tree essential oil. Monoterpene fraction was also the main phytochemical group in other previously analysed commercial essential oils, such as oregano (*Origanum vulgare* L.), marjoram

(*Origanum majorana* L.) and *Thymus mastichina* L. reaching values of 93.05, 96.93 and 83.36%, respectively. These compounds played an important role in the phytotoxic activity against the seed germination and seedling growth of weeds (*Portulaca oleracea* L., *Lolium multiflorum* L. and *Echinochloa crus-galli* (L.) Beauv.) (Ibáñez and Blázquez 2017).

Terpinen-4-ol (28.37±0.05%) followed by 1,8-cineole (15.81±0.06%) and the monoterpene hydrocarbons γ-terpinene (15.60±0.03%), α-pinene (10.92±0.08%) and α-terpinene (8.52±0.01%) were the main compounds in *M. alternifolia* essential oil. Also, relative large amounts of the oxygenated monoterpene α-terpineol (4.24±0.03%) and the monoterpene hydrocarbons terpinolene (3.16±0.03%), *p*-cymene (2.52±0.01%) and β-pinene (1.87±0.01%) were found. These results accomplish the standard ISO 4730 whose chromatography revealed the same characteristic compounds in tea tree essential oil obtained by steam distillation of the foliage and terminal branches (ISO 2017). Although the chemical composition of tea tree essential oil has been well defined by previous authors (Cox *et al.* 2001; Liao *et al.* 2017), there can be slight variations according to the origin of the plant species. High amounts of terpinolene (45.7%) have been found in samples from China that exceed even the usually major component terpinen-4-ol (44.7%) (de Groot and Schmidt 2016). These quantitative and qualitative variations in chemical composition could be influence the biological activity of tea tree essential oil. According to this, it has been observed that terpinen-4-ol, had more potent acaricidal effect than the following major compounds 1,8-cineole and α-terpineol; showing also terpinen-4-ol a significant synergistic effect with terpinolene, and an antagonistic effect with α-terpineol in killing mites (Tighe *et al.* 2013). In addition, terpinen-4-ol is established to be the responsible compound of the

antimicrobial (Bordini *et al.* 2018), antitumor (Sobral *et al.* 2014) and herbicidal effects (Verdeguer *et al*. 2011). Phytotoxic effects have also been shown with 1,8-cineole, the second main component of tea tree essential oil here analysed. In this sense, *Eucalyptus globulus* Labill. essential oil (1,8-cineole 76.43±0.35%), was able to inhibit the seedling growth of *E. crus-galli* and the radicle development of *L. multiflorum* when 1 µL/mL and doses of 0.125, 0.25, 0.50 and 1µL/mL respectively, of the essential oil were applied (Ibáñez and Blázquez 2018a). Conversely, in wintergreen essential oil only four monoterpene hydrocarbons (0.04±0.00%) α-pinene, β-pinene *p*-cymene and limonene and three oxygenated monoterpenes (0.07±0.01%) 1,8-cineole, linalool and camphor were identified.

Among the sesquiterpene fraction in tea tree essential oil (3.19%), 13 hydrocarbons were identified (3.02±0.03%) from which only aromadendrene (1.44±0.01%) and longifolene (0.90±0.01%) reached percentages close to or greater than 1%, while none of the only five oxygenated sesquiterpenes identified reached 0.1%. Aromadendrene like compounds can be detected in higher amounts in essential oils coming from *Melaleuca* species, like *M. styphelioides* in which isoaromadendrene epoxide (7.45%) and *allo*-aromadendrene have been found (1.18%) (Albouchi *et al.* 2017), as well as in other species, such as in the essential oil of *E. globulus* fruits, where aromadendrene (31.17%) the main compound, followed by 1,8-cineole (14.55%) showed antimicrobial and synergic properties against antibiotic-susceptible and antibiotic-resistant pathogens (Mulyaningsih *et al.* 2010). Regarding to longifolene (22.0%), this sesquitepene hydrocarbon was also together the oxygenated monoterpene 1,8-cineole (22.9%) the main compounds of the essential oil of *E. oleosa* F. Muell leaves from Tunisia with antimicrobial properties, too

(Hassine *et al.* 2012). From the sesquiterpene fraction, β-caryophyllene (0.01±0.00%) was the only sesquiterpene hydrocarbon detected in a slight percentage in wintergreen essential oil here analysed.

On the other hand, aromatic compounds both C_6-C_3 and C_6-C_1 from shiquimic pathway was the principal fraction of *G. procumbens* essential oil. Methyl chavicol (0.58±0.01%) not detected in wintergreen, was the only aromatic compound found in tea tree essential oil. This fraction (99.82±0.01%) with six compounds identified represented the main qualitative and quantitative group of wintergreen essential oil here analysed. Methyl salicylate (99.63±0.02%) was by far the principal compound of *G. procumbens* essential and of the fraction with benzaldehyde, ethyl benzoate ethyl salicylate, methyl *o*-anisate and eugenol, that did not reach 0.20% but also. These results are according to previous works that confirm methyl salicylate as the main compound in the essential oil wintergreen leaves (Kujur et al. 2017; Singh and Ali 2017). Also, methyl salicylate is popularly known by its pharmacological properties as analgesic, astringent, carminative, diuretic, stimulant, antispasmodic and antiseptic (Cock 2015). Furthermore, it has been included in a pesticidal composition together with rosemary essential oil showing at doses with pesticidal effects not phytotoxicity or dermal sensitivity (Bessette and Lindsay 2016). However, methyl salicylate has been observed to exert certain phytotoxicity against *P. oleracea* inhibiting its seed germination between 31.40-44.19% as well as the radicle and hypocotyl elongation between 37.78-47.75% and 52.63-52.34, respectively at different doses (0.125, 0.25, 0.50 and 1µL/mL) (Ibáñez and Blázquez 2018b).

Table 1. Chemical composition of commercial tea tree from *M. alternifolia* and wintergreen from *G. procumbens* essential oils.

RI	RI_{Ref}	Compound	Tea tree	Wintergreen
Monoterpene hydrocarbons			45.20±0.07	0.04±0.00
926	926	Tricyclene	0.04±0.00	-
932	930	α-Thujene	0.02±0.00	-
940	939	α-Pinene	10.92±0.08	0.02±0.00
953	954	Camphene	0.20±0.00	-
980	979	β-Pinene	1.87±0.01	0.01±0.00
987	987	3-*p*-Menthene	0.09±0.00	-
993	990	Myrcene	0.68±0.01	-
1006	1002	α-Phellandrene	0.32±0.00	-
1012	1011	δ-3-Carene	0.47±0.01	-
1019	1017	α-Terpinene	8.52±0.01	-
1022	1024	*p*-Cymene	2.52±0.01	t
1025	1026	1-*p*-Menthene	0.54±0.00	-
1027	1029	Limonene	-	0.01±0.00
1043	1037	*cis*-β-Ocimene	0.07±0.01	-
1054	1050	*trans*-β-Ocimene	0.18±0.01	-
1060	1059	γ-Terpinene	15.60±0.03	-
1087	1088	Terpinolene	3.16±0.03	-
Oxygenated monoterpenes			49.93±0.05	0.07±0.01
1031	1031	1,8-Cineole	15.81±0.06	0.03±0.01
1071	1070	*cis*-Sabinene Hydrate	0.03±0.02	-
1076	1072	*cis*-Linalool Oxide	0.29±0.01	-
1098	1099	α-Pinene Oxide	0.02±0.00	-
1095	1096	Linalool	0.63±0.01	0.03±0.00
1137	1137	*cis*-*p*-Mentha-2,8-dien-1-ol	0.04±0.00	-
1138	1139	*trans*-Pinocarveol	0.19±0.01	-
1145	1146	Camphor	-	0.01±0.00
1159	1160	Isoborneol	0.11±0.01	-
1170	1169	Borneol	0.06±0.01	-
1180	1177	Terpinen-4-ol	28.37±0.05	-
1186	1186	Dill Ether	0.08±0.02	-
1190	1188	α-Terpineol	4.24±0.03	-
1222	1220	α-Fenchyl Acetate	0.05±0.01	-
1270	1269	*trans*-Ascaridol Glycol	0.02±0.00	-
Sesquiterpene hydrocarbons			3.02±0.03	0.01±0.00
1351	1352	α-Longipinene	0.10±0.01	-

1370	1371	Cyclosativene	0.02±0.00	-
1372	1374	Longicyclene	0.06±0.00	-
1389	1391	Sativene	0.04±0.01	-
1403	1407	Longifolene	0.90±0.01	-
1419	1419	β-Caryophyllene	0.02±0.00	0.01±0.00
1432	1433	β-Gurjunene	0.03±0.00	-
1440	1441	Aromadendrene	1.44±0.01	-
1454	1454	α-Humulene	0.02±0.00	-
1461	1460	*allo*-Aromadendrene	0.19±0.01	-
1488	1490	β-Selinene	0.02±0.00	-
1495	1496	Viridiflorene	0.16±0.00	-
1500	1500	α-Muurolene	0.02±0.00	-
Oxygenated sesquiterpenes			0.17±0.01	-
1577	1578	Spathulenol	0.02±0.00	-
1591	1590	Globulol	0.06±0.00	-
1593	1595	Cubenan-11-ol	0.03±0.00	-
1601	1600	Rosifoliol	0.04±0.00	-
1632	1632	γ-Eudesmol	0.02±0.00	-
Aromatic compounds			0.58±0.01	99.83±0.01
959	960	Benzaldehyde	-	t
1173	1173	Ethyl Benzoate	-	t
1195	1191	Methyl Salicylate	-	99.63±0.02
1199	1196	Methyl Chavicol	0.58±0.01	-
1269	1269	Ethyl Salicylate	-	0.18±0.00
1333	1337	Methyl *o*-Anisate	-	t
1358	1359	Eugenol	-	0.01±0.00
Others			-	t
1002	1002	3-Hexenyl Acetate	-	t
TOTAL			98.89±0.01	99.94±0.01

[a]RI, retention index relative to C_8-C_{32} *n*-alkane on HP-5MSi column; RI Kovats index in Adams 2007; values are mean relative area (%) ± standard deviation of three samples; t trace amount less than 0.01.

3.2. In vitro phytotoxic activity of tea tree and wintergreen essential oils against seed germination and seedling growth of C. selloana and N. glauca

Several studies have tested the phytotoxicity of the essential oils among the most widely naturalized species, showing a relationship between composition, dosage, soil and weeds. Although monoterpene hydrocarbons

may be show great herbicidal effect against certain weeds (Blázquez 2014), in general the results showed that oxygenated monoterpenes as well as aromatic compounds are the responsible of the main phytotoxic effects. So, the phytotoxic effect of tea tree and wintergreen essential oils with 49.93% and 99.83% of oxygenated monoterpenes and aromatic compounds respectively has been tested against the seed germination and seedling growth (Tables 2 and 3, Figures 1 and 2) of *C. selloana* and *N. glauca*.

The invasive *C. selloana* species could be controlled by both *M. alternifolia* and *G. procumbens* essential oils. The highest dose (1 µL/mL) of tea tree essential oil assayed, significantly inhibit their seed germination in 34.69% respect to control (Table 2). The high percentage of the aromatic compound methyl salicylate in wintergreen essential oil (Table 1) was able to inhibit in a dose dependent manner the seed germination of *C. selloana* with significant effect regarding control (reduction of 33.34% and 77.38%) at 0.50 and 1 µL/mL (Table 2). *N. glauca* was a more resistant species to theses essential oils. Seed germination of this invasive species was no affected at neither doses (0.125, 0.25, 0.50 and 1 µL/mL) tested of tea tree essential oil, being also lower the phytotoxic effect shown by wintergreen essential oil. Only an inhibition percentage of 15.96% was reached with the highest doses (1 µL/mL) of *G. procumbens* essential oil applied.

Table 2. *In vitro* effects of tea tree from *M. alternifolia* and wintergreen from *G. procumbens* essential oil against *Cortaderia selloana* (CS) and *Nicotiana glauca* (NG) seed germination.

		Seed germination (%± e.d.)				
Dose		Control	0.125 µL/mL	0.25 µL/mL	0.5 µL/mL	1 µL/mL
Tt	CS	98.00±2.00 a	92.00±2.55 a	92.00±2.00 a	86.00±4.30 a	64.00±7.81 b
	NG	91.00±3.32 a	60.00±7.58 a	65.00±11.29 a	72.00±11.02 a	65.00±7.91 a
W	CS	84.00±2.45 a	83.00±2.00 a	77.00±6.04 a	56.00±3.67 b	19.00±6.21 c
	NG	94.00±4.00 a	90.00±3.54 a,b	87.00±2.55 a,b	91.00±1.87 a,b	79.00±3.32 b

Values are mean percentage of five replications ± error deviation after 14 days of incubation. Means followed by different letters in the same line indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests. Tt: Tea tree essential oil; W: Wintergreen essential oil.

As occur with the application of synthetic herbicides, in some cases do not reach the expected results. Glyphosate and imazapic only achieved to reduce belowground biomass of the invasive species *Liriope spicata* Lour. by 43 and 45%, respectively, at 180 days after treatment (Enloe *et al*. 2015), and as preliminary studies showed that herbicides mixes could improve the control of certain weeds (Rolando et al. 2011), it is quite interesting to obtain significant results at very low doses of essential oils and it would not be recommended to test higher doses of these essential oils but to apply mixtures of them. Previous studies with winter savory, oregano or peppermint essential oils with other oxygenated monoterpenes different from their main compounds were able to produce at these doses a complete inhibition of the seed germination of other problematic weeds (Ibáñez and Blázquez 2017; Ibáñez and Blázquez 2018c).

The parallel study of the seedling growth corroborated that *C. selloana* is the most susceptible invasive species to tea tree and wintergreen essential oils (Figure 1) and methyl salicylate, the main principle of wintergreen essential oil, the most phytotoxic compound in reduction both hypocotyl and radicle growth of invasive species tested (Table 3).

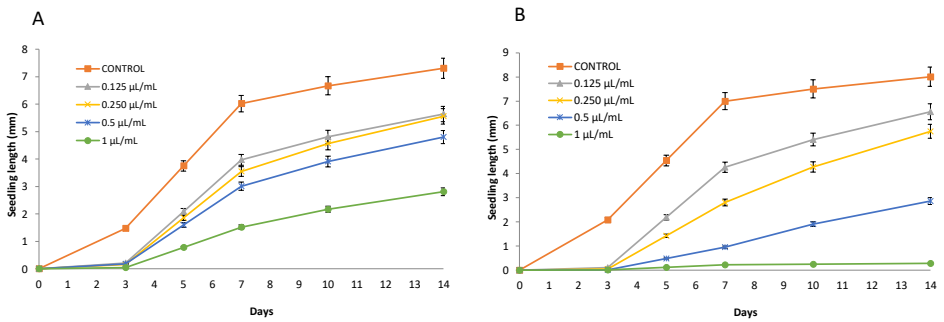

Figure 1. Values of seedling length (mm) (mean ± s.e.) of C. selloana control and treated with tea tree (A) and wintergreen (B) essential oils at 0.125, 0.25, 0.50 and 1 µL/mL.

Table 3. *In vitro* effects of tea tree from *M. alternifolia* and wintergreen from *G. procumbens* essential oil against *Cortaderia selloana* and *Nicotiana glauca* seedling growth.

*Doses		*C. selloana*		*N. glauca*	
		Hypocotyl (mm)	Radicle (mm)	Hypocotyl (mm)	Radicle (mm)
Tt	0	4.56±0.17 a	2.75±0.20 a	2.53±0.24 a	3.14±0.27 a
	0.125	3.79±0.45 a,b	1.85±0.18 b	1.46±1.19 a	2.18±0.15 a,b
	0.25	3.70±0.08 a,b	1.85±0.16 b	1.71±0.45 a	2.17±0.47 a,b
	0.5	3.28±0.25 b	1.51±0.23 b,c	1.41±0.49 a	2.25±0.37 a,b
	1	1.78±0.32 c	1.03±0.14 c	1.24±0.33 a	1.73±0.24 b
W	0	4.14±0.56 a	3.88±0.36 a	4.72±0.30 a	3.87±0.23 a
	0.125	4.14±0.13 a	2.42±0.27 b	2.95±0.35 b	3.00±0.36 a,b
	0.25	3.86±0.43 a	1.89±0.44 b	2.94±0.14 b	3.00±0.03 a,b
	0.50	1.55±0.41 b	1.31±0.33 b	2.79±0.31 b	2.76±1.16 b
	1	0.15±0.07 c	0.13±0.08 c	2.78±0.18 b	2.74±0.26 b

Values are mean of five replications ± error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey B tests. * Doses: µL/mL; Tt: Tea tree essential oil; W: Wintergreen essential oil.

It was especially noteworthy the phytotoxic effect of wintergreen essential oil on *C. selloana* because very high values up to 96.38 and 96.65% of reduction of both hypocotyl and radicle growth, respectively, were recorded at the highest dose (1 µL/mL) assayed according to control (Table 3, Figure 1b). Moreover, considerable decrease in hypocotyl and radicle development of *C. selloana* was observed after the application of a lower dose (0.50 µL/mL) of wintergreen essential oil reaching 62.56 and 66.24%, respectively (Table 3). Comparable percentages of reduction were obtained with the highest dose (1 µL/mL) of tea tree essential oil in *C. selloana* reducing the hypocotyl and radicle length in 60.96 and 62.55, respectively (Table 3, Figure 1a).

On the other hand, *N. glauca* was more resistant species to treatment with tea tree and wintergreen essential oils (Table 3, Figure 2). Tea tree essential oil did not affect the hypocotyl growth of *N. glauca* at neither dose (0.125, 0.25, 0.50 and 1 µL/mL) tested (Table 3) and only slight differences between 30.57-44.9% of reduction were observed in the radicle development of *N. glauca* with tea tree essential oil (Table 3). Wintergreen essential oil was able to inhibit significantly both hypocotyl and radicle enlargement (Figure 2b) with percentages of inhibition compared with the control of 37.50 to 41.02% for the hypocotyl and from 22.48 to 29.20% for the radicle elongation at all doses applied.

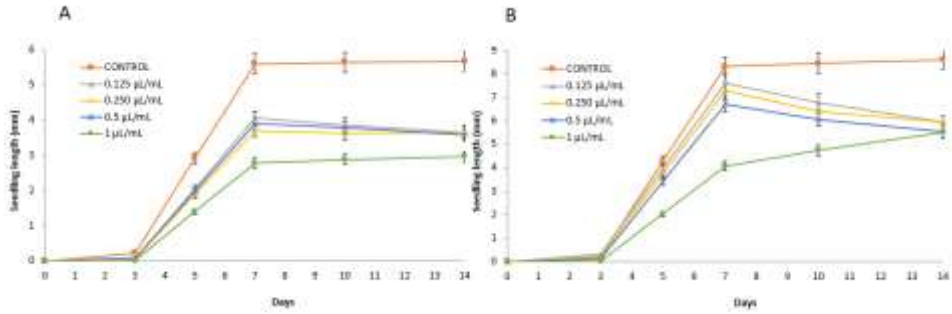

Figure 2. Values of seedling length (mm) (mean ± s.e.) of N. glauca control and treated with tea tree (A) and wintergreen (B) essential oils at 0.125, 0.25, 0.50 and 1 µL/mL.

In relation to these results, tea tree essential oil could be used as a post-emergent bioherbicide to control *C. seollana*, whereas wintergreen essential oil showed a powerful pre and post-emergent herbicide effects against this invasive species. Due to, bioherbicides represent less than 10% of all biopesticides (fungicides, bactericides, insecticides and nematicides) being most of them constituted by fungus and bacteria. Methyl salicylate like pelargonic acid (Beloukha®, Katoun®), bioherbicide obtained from rapseed oil (Cordeau *et al.* 2016), provide interesting alternative in the formulation of new herbicides derived from plant products. In this sense, it is interesting to study the effect of methyl salicylate over food crops to ensure its beneficial effects and harmlessness. Regarding this, previous works have studied the influence of methyl salicylate on rice seeds obtaining an increase of rice seedling emergence, development and disease resistance of rice (Kalaivani et al. 2016). In fact, methyl salicylate is involved in plant defence reaction, and can act as a key airborne signal, which activates disease resistance and the expression of defence-related genes in healthy neighbouring plants (Shulaev et al. 1997).

In summary, tea tree and wintergreen essential oils with terpinen-4-ol (28.37±0.05%) and methyl salicylate (99.63±0.02%) as the main compounds, respectively, could be used in the control of invasive plant species. *N. glauca* was more resistant to tea tree and wintergreen essential oils than *C. selloana* regarding seed germination and seedling growth. Wintergreen essential oil showed more phytotoxic effect in both non-native species. Methyl salicylate effectively controlled the seed germination and seedling growth of *C. seollana* in a dose-dependent manner. Together its pharmacological properties, this aromatic compound from wintergreen essential oil represent a potential source of bioherbicides.

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CHAPTER 6.

Phytotoxicity of essential oils from culinary herbs against seed germination and seedling growth of selected weeds

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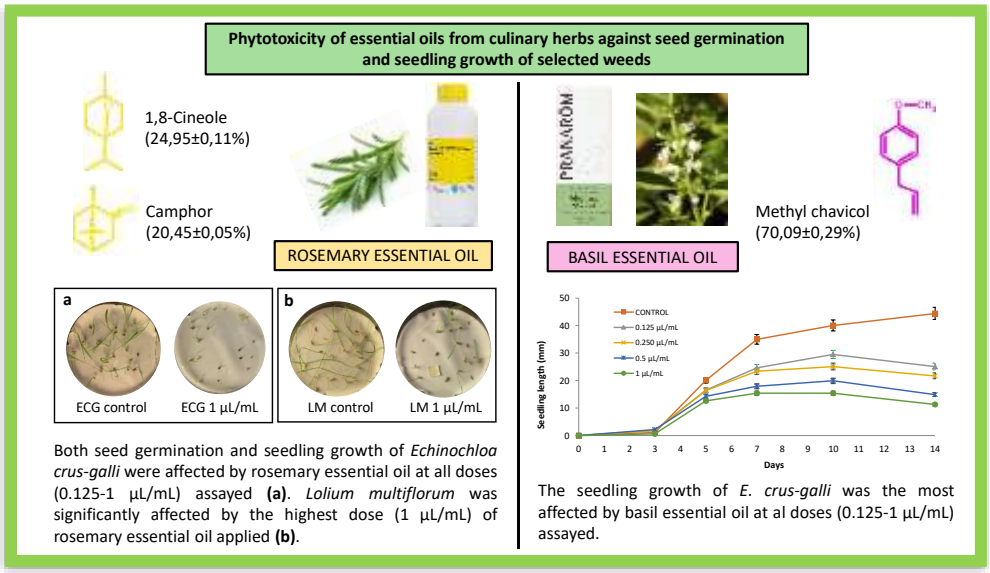

ABSTRACT

Chemical composition of *Rosmarinus officinalis* L. and *Ocimum basilicum* L. ssp. *basilicum* essential oils as well as their phytotoxic effects against seed germination and seedling growth of *Portulaca oleracea*, *Lolium multiflorum* and *Echinochloa crus-galli* has been investigated. Seventy-eight compounds accounting between 98.10-99.15% of the total commercial oils were identified by GC/MS analysis. The oxygenated monoterpenes 1,8-cineole (24.95±0.11%) and camphor (20.45±0.05%) were the main compounds of rosemary essential oil, whereas large amounts of the aromatic compound methyl chavicol (79.09±0.29%) was found in basil essential oil. Rosemary essential oil significantly inhibited the seed germination of *L. multiflorum* and *E. crus-galli* and the seedling growth of the three weed, whereas basil essential oil only showed significant effects in hypocotyl and/or radicle length depending of the weed and dose.

Keywords: rosemary; basil; essential oils; GC-MS; weed control.

1. INTRODUCTION

In agriculture, the visible consequences of climate change such as the Earth's rising temperature, extreme weather events, shifting seasons as well as scarcity in precipitations [1] have led to the proliferation and propagation of weeds, pests and diseases that lessen crop yields and therefore increase production, collection and industrial processing costs [2]. Food and Agriculture Organization (FAO) especially warns about the increasing presence of invasive weeds, which interfere and compete against cultivated plants for light, water and nutrients [3,4]. Simultaneously, they are indirect transmitters of pests as they are hosts of viruses and insect vectors that help them propagating. According to FAO member expert, 'weeds are the principal enemy of farmers' due to the fact that they cause a higher number of losses in crop production than any other disaster. These damages reach numbers of 95000 million dollars in global food production, representing 380 million tons of wheat [5]. Despite the most affected countries are the developing ones, weeds also disturb developed countries invading pathways, gardens, historical monuments as well as causing allergies, fires, etc. So, one of the most important challenges nowadays is to fight against invasive exotic species, which constitute a growing threat for native plants [6].

In this sense, *Echinochloa* genus, especially barnyard grass (*Echinochloa crus-galli* (L.) Beauv) mimics rice plants, producing inevitable crop yield losses once *E. crus-galli* can be easily recognized [7]. Another weed species to take into consideration is the well-known common purslane (*Portulaca oleracea* L.), annual weed which although is edible and consumed in several countries, it is also considered a spontaneous weed of orchards,

pathways and gardens in many others [8]. Purslane infests a wide variety of crops, such as sweet maize, tomato, sunflower, rice and cotton among others due to its opportunistic properties [3,9]. Manual weeding as innocuous and unique method to eliminate them results non-viable, leading to the increased use of synthetic herbicides with problems of toxicity as well as the appearance of long-term resistance.

Regarding resistance, Italian ryegrass (*Lolium multiflorum* Lam.) has shown 10-fold levels of resistance to glyphosate, the world's most widely used herbicide since 1974 [10,11], in comparison to a susceptible population due to its constant application after many years [12,13]. *L. multiflorum* resistance to glyphosate has been recorded in orchards spread through the American continent, Spain and more recently in Japan [10,14]. Although the mechanism of developing resistance is not completely known, it has been demonstrated that a proline 106 to serine amino acid substitution of EPSP synthase decreases glyphosate binding and confers moderate levels of glyphosate resistance [13]. Furthermore, additional concerns related to synthetic agro-chemicals are the potential biodiversity damages [15], especially against human health: it causes skin and mucosa irritation, head and stomach ache, vomiting, unconsciousness, etc., through the intoxication, ingestion of contaminated food, inhalation and direct contact [16]. World Health Organization (WHO) warns about certain synthetic herbicides because can cause human cancer. In this sense, glyphosate produces cancer in lab animals as well as chromosome and human DNA damage, being consequently classified as 'probable carcinogenic' (Group 2A) substance [17].

Therefore, it is necessary to reduce the dependence on synthetic weed killers and give priority to other natural compounds that neither damage both environment and living organisms beings nor promote resistance appearance [18]. Between these natural alternatives, essential oils are being employed to control crop pests [19,20] apart from their very well-known anti-inflammatory, anticancer, antiviral, repellent, antibacterial, antifungal or antioxidant activities and wide employment in perfumery, cosmetics, pharmaceutical and food industry [21-23].

Among Lamiaceae family [24-26], rosemary (*Rosmarinus officinalis* L.) is a quite famous aromatic plant due to its numerous health effects such as digestive, anti- inflammatory, anti-nociceptive, diuretic, antihepatotoxic, antispasmodic or neuroprotective against Alzheimer and Parkinson diseases [27]. Its essential oil has been tested against *Sporothrix brasiliensis* and S. schenckii isolated from humans, cats, dogs and environmental soils, being a promising product for treatment of sporotrichosis in refractory cases to itroconazole [28]. In addition, it has been combined with thyme essential oil for the control of *Listeria monocytogenes* in mortadella packaging [29] as well as part of the active packaging of refrigerated beet meat for its preservation prolonging the shelf-life until day 15 [30], being also used as potential bio-fumigant in control of *Callosobruchus maculatus* (F.) in chickpea seeds without affecting the food product [31]. According to its phytotoxic potential, rosemary essential oil supressed germination rate of *Avena sterilis* and *Sinapis arvenis*, weed species commonly found in wheat growing areas, affecting in a lower extent wheat cultivars [32].

Another interesting culinary spice belonging also to the Lamiaceae family is basil (*Ocimum* spp.), popularly used as food additive to prevent microbial

arise [33]. *Ocimum tenuiflorum* extracts produced a reduction in seedling growth of Italian ryegrass (*L. multiflorum*), barnyard grass (*E. crus-galli*) between other weeds [34]; similarly, *Ocimum basilicum* L. ssp. *basilicum* essential oil is highly phytotoxic against ferns, gingers and delicate flowers when used as insecticide against *Planococcus ficus* (Signoret) (Hemiptera: Pseudococcidae) causing leaves losses of more than 50% [35].

So, the aims of this work was analyze by Gas Chromatography-Mass Spectrometry the chemical composition of rosemary (*Rosmarinus officinalis* L.) and basil (*Ocimum basilicum* L. ssp. *basilicum*) essential oils, two widely culinary spices in order to determine through the seed germination and seedling growth of *P. oleracea*, *L. multiflorum* and *E. crus-galli* the potential bioherbicide effects of its species mainly used in several dishes of the Mediterranean diet.

2. MATERIAL AND METHODS

2.1. Plant material

Commercial samples of rosemary (*Rosmarinus officinalis* L.) (Batch 0037337) essential oil purchased from Guinama (Valencia, Spain), and *Ocimum basilicum* L. ssp. *basilicum* essential oil (Batch 0F22144) supplied by Pranaròm International, were stored at 4 ºC until chemical analysis and phytotoxic studies.

2.2. Weeds

Mature seeds of annual weeds of *Portulaca oleracea* L., *Lolium multiflorum* Lam. and *Echinochloa crus-galli* (L.) Beauv., were purchased from Herbiseed (website: www.herbiseed.com).

2.3. Gas chromatography-Mass spectrometry (GC-MS)

GC-MS analysis was carried out with a 5973N Agilent apparatus, equipped with a capillary column (95 dimethylpolysiloxane - 5 % diphenyl), Agilent HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 µm film thickness). The column temperature program was 60 ºC during 5 min, with 3 ºC/min increases to 180 ºC, then 20 ºC/min increases to 280 ºC, which was maintained for 10 min. The carrier gas was Helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the *m/z* 30-500 range with an ionizing voltage of 70 eV.

2.4. Identification

The individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index calculated using standard hydrocarbons relative to C_8-C_{32} *n*-alkanes, and mass spectra with reference samples or with data already available in the NIST 2005 mass spectral library and in the literature [36].

2.5. Herbicidal activity

Sets of 20 seeds each with five replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No.1) moistened with 4 mL of distilled water and with 0 (control), 0.125, 0.250, 0.5, and 1µl/ml of rosemary and basil essential oils. Petri dishes were sealed with parafilm and incubated in a germination chamber Equitec EGCS 301 3SHR model, according to previous assays [37] alternatig 30.0±0.1 ºC 16h in light and 20.0±0.1 ºC 8h in dark and with (*E. crus-galli*) and without (*P. oleracea, L. multiflorum*) humidity.

To evaluate the herbicidal activity of the essential oils, the number of germinated seeds was counted and compared with those of untreated

seedlings. Emergence of the radicle (≥1 mm) was used as an index of germination and seedling length (hypocotyl and/or radicle) data were recorder after 3, 5, 7, 10 and 14 days in each replicate.

2.6. Statistical analysis

Experiments were made with five replicates. Resulting data were subjected to one-way analysis of variance with SPSS statistics 22 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* one was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq 0.05$.

3. RESULTS

3.1. Essential oil composition

Seventy-eight compounds accounting between 98.10- 99.15% of the total commercial rosemary and basil essential oils were identified by GC/MS analysis. Components are clustered (Table 1) in homologous series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, oxygenated sesquiterpenes, aromatic compounds and others and listed according to Kovat's retention index calculated in GC on apolar HP- 5MS column.

Table 1: Chemical composition of rosemary and basil essential oils.

RI	Compound	Rosemary	Basil
Monoterpene hydrocarbons		38.27±0.13	0.25±0.02
926	Tricyclene	0.31±0.00	-
939	α-Pinene	16.70±0.12	0.04±0.01
944	β-Fenchene	0.13±0.01	-
955	Camphene	9.94±0.04	-
976	Sabinene	-	0.01±0.00
979	β-Pinene	6.14±0.01	0.03±0.00
992	Myrcene	1.27±0.01	0.03±0.01
1005	α-Phellandrene	0.11±0.00	-
1012	δ-3-Carene	0.05±0.00	-
1020	α-Terpinene	0.12±0.00	-
1027	*p*-Cymene	3.12±0.03	0.01±0.00
1031	Limonene	-	0.05±0.01
1043	*cis*-Ocimene	0.03±0.01	-
1052	*trans*-Ocimene	-	0.08±0.01
1063	γ-Terpinene	0.19±0.00	-
1090	Terpinolene	0.17±0.01	-
Oxygenated monoterpenes		57.70±0.09	16.20±0.36
1033	1,8-Cineole	24.95±0.11	0.17±0.01
1070	*cis*-Sabinene hydrate	0.03±0.00	-
1075	*cis*-Linalool oxide	-	0.15±0.01
1089	*trans*-Linalool oxide	-	0.13±0.01
1098	*trans*-Sabinene hydrate	0.06±0.01	-
1101	Linalool	1.13±0.02	14.58±0.26
1116	α-Fenchol	0.03±0.01	-
1123	β-Fenchol	0.02±0.01	-
1142	1-Terpineol	0.07±0.02	-
1149	Camphor	20.45±0.05	-
1155	Menthone	-	0.05±0.01
1160	Isoborneol	1.33±0.01	-
1164	*iso*-Menthone	-	0.01±0.00
1165	*neo*-Menthol	-	0.01±0.00
1169	Borneol	3.02±0.04	-
1175	Menthol	-	0.37±0.02
1179	Terpinen-4-ol	0.20±0.15	-
1183	*p*-Cymen-8-ol	0.02±0.01	-
1190	α-Terpineol	2.50±0.18	-
1197	γ-Terpineol	0.33±0.01	-

1222	α-Fenchyl acetate	0.02±0.01	-
1244	Neral	-	0.20±0.14
1258	Geraniol	-	0.04±0.01
1260	Linalool acetate	0.32±0.00	-
1273	Geranial	-	0.46±0.02
1288	Bornyl acetate	3.23±0.03	-
1294	Menthyl acetate	-	0.02±0.01
1382	Geranyl acetate	-	0.01±0.00
Sesquiterpene hydrocarbons		3.08±0.03	1.62±0.17
1351	α-Cubebene	0.04±0.00	-
1376	α-Ylangene	0.02±0.00	-
1376	α-Copaene	0.10±0.01	0.03±0.01
1388	β-Bourbonene	-	0.01±0.00
1403	Longifoleno	0.07±0.00	-
1415	*cis*-α-Bergamotene	-	0.01±0.00
1419	β-Caryophyllene	2.31±0.02	0.34±0.04
1430	β-Copaene	0.02±0.00	-
1437	*trans*-α-Bergamotene	-	0.56±0.06
1439	Aromadendrene	0.01±0.00	-
1444	*cis*-β-Farnesene	-	0.05±0.01
1454	α-Humulene	0.27±0.00	0.15±0.02
1459	*trans*-β-Farnesene	-	0.19±0.02
1481	γ-Muurolene	0.05±0.01	0.17±0.02
1500	α-Muurolene	0.02±0.00	-
1508	β-Bisabolene	0.01±0.01	0.07±0.01
1514	γ-Cadinene	0.03±0.00	-
1524	δ-Cadinene	0.12±0.00	0.03±0.01
Oxigenated sesquiterpenes		0.06±0.00	0.20±0.02
1577	Spathulenol	-	0.04±0.00
1582	Caryophyllene oxide	0.06±0.00	0.10±0.01
1607	Humulene epoxide	-	0.03±0.01
1684	α-Bisabolol	-	0.02±0.00
Aromatic compounds		0.03±0.01	79.69±0.22
1209	Methyl Chavicol	-	79.07±0.29
1255	*p*-Anis aldehyde	-	0.03±0.01
1256	Chavicol	-	0.02±0.01
1286	*trans*-Anethole	-	0.01±0.00
1359	Eugenol	0.01±0.01	0.03±0.01
1405	Methyl Eugenol	0.02±0.00	0.02±0.01

1567	*p*-Methoxy Cinnamaldehyde	-	0.51±0.06
Others		-	0.14±0.02
853	3-Hexen-1-ol	-	0.01±0.01
971	3,7-Dimethyl-2-octene	-	0.05±0.01
986	6-Methyl-5-hepten-2-one	-	0.07±0.00
1003	Octanal	-	0.03±0.01
1009	Hexenyl acetate	-	0.01±0.00
	Total identified	99.15±0.01	98.10±0.27

RI, retention index relative to C_8-C_{32} *n*-alkane on HP-5MS column; values are means ± standard deviation of three samples.

Seventeen oxygenated monoterpenes (57.70±0.09%), 13 monoterpene hydrocarbons (38.27±0.13%) 13 sesquiterpene hydrocarbons (3.08±0.03%), one oxygenated sesquiterpene (0.06%) and two aromatic components (0.03±0.01%) were the compounds identified in rosemary essential oil. Monoterpene compounds with the oxygenated monoterpenes 1,8-cineole (24.95±0.11%) and camphor (20.45±0.05%) followed by the monoterpene hydrocarbons α-pinene (16.70±0.12%), camphene (9.94±0.04%) and β-pinene (6.14±0.01%) were the principal components. Between the sesquiterpene fraction β-caryophyllene with 2.31±0.02% was the main compound, being caryophyllene oxide (0.06%) the only oxygenated sesquiterpene identified. The phenylpropanoids, eugenol and methyl eugenol, were detected in low amount among the compounds biosynthesized by the shikimic biogenetic pathway.

Aversely, aromatic fraction (79.69±022%) with seven identified compounds, was the main phytochemical group of basil essential oil, followed by the oxygenated monoterpenes (16.20±0.36%) and

sesquiterpene hydrocarbons (1.62±0.0.17%) with 13 and 11 compounds, respectively. The phenylpropanoid methyl chavicol was the most abundant compound in basil essential oil (79.07±0.29%), followed by the oxygenated monoterpene linalool (14.58±0.26%). No higher percentages than 0.1% were found between the seven monoterpene hydrocarbons and only β-caryophyllene (0.34%), α-*trans*-bergamotene (0.56%), α-humulene (0,15%), β-*trans*-farnesene (0.19%), γ-muurolene (0.17%) and *p*-methoxy-cinnamaldehyde (0.51%) reached percentages higher than 0.1% in the sesquiterpene hydrocarbons and aromatic fractions respectively.

3.2. *Seed germination and seedling growth inhibition against P. oleracea, L. multiflorum and E. crus-galli*

The effect of rosemary and basil essential oils against seed germination and seedling growth of *P. oleracea*, *L. multiflorum* and *E. crus-galli* is shown in Tables 2-3 and Figures 1, 2 and 3, respectively. Despite *R. officinalis* essential oil did not exert significant weed killer capacity against *P. oleracea* germination at none of the tested doses, there was a significant difference between those dishes containing the highest dose (1 µL/mL) of rosemary essential oil and control ones with *L. multiflorum* (Table 2); and remarkable significant differences between the seed germination of *E. crus-galli* control plates and all doses assayed, meaning rosemary essential oil was obtained (Table 2).

On the other hand, no significant effect in the seed germination of the three weeds resulted after basil essential oil exposure at all the doses applied (Table 2).

Table 2: *In vitro* effects of rosemary and basil essential oils against *P. oleracea*, *L. multiflorum* and *E. crus galli* seed germination.

Concentration (µL/mL)	*P. oleracea*	
	Rosemary	Basil
Control	83.00±5.83 a	85.00±5.24 a
0.125	78.00±5.83 a	77.00±2.55 a
0.25	76.00±4.30 a	81.00±3.67 a
0.5	74.00±4.58 a	86.00±4.85 a
1	73.00±2.00 a	77.00±4.36 a
Concentration (µL/mL)	*L. multiflorum*	
	Rosemary	Basil
Control	73.00±3.39 a	73.00±3.39 a
0.125	68.00±5.83 a,b	73.00±3.39 a
0.25	69.00±3.32 a,b	71.00±3.32 a
0.5	64.00±4.30 a,b	63.00±4.90 a
1	53.00±3.39 b	62.00±5.61 a
Concentration (µL/mL)	*E. crus-galli*	
	Rosemary	Basil
Control	86.00±6.00 a	86.00±6.00 a
0.125	22.00±7.35 b	83.00±2.56 a
0.25	18.00±9.17 b	83.00±3.39 a
0.5	13.00±8.31 b	83.00±2.55 a
1	1.00±1.00 b	78.00±1.23 a

Values are mean of five replications ± standard error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests.

Regarding seedling growth, rosemary essential oil showed at all doses assayed significant inhibitory effect with respect to control in both hypocotyl and radicle of *P. oleracea* and at the doses of 0.25-1 µl/ml and all dose of hypocotyl and radicle respectively of *E. crus-galli* (Table 3, Figures 1a and 2a), showing also significant differences in a dose-dependent manner in hypocotyl and radicle of *L. multiflorum* (Table 3, Figure 3a).

Table 3: Effects of rosemary and basil essential oils on seedling length (hypocotyl and radicle) of *P. oleracea*, *L. multiflorum* and *E. crus-galli*.

Concentration (µL/mL)	Rosemary		Basil	
	P. oleracea			
	Hypocotyl	Radicle	Hypocotyl	Radicle
Control	9.60±1.03 a	11.60±1.69 a	11.60±1.69 a	11.60±1.69 a
0.125	6.60±0.68 b	6.60±0.68 b	7.60±0.60 b	14.40±1.97 a
0.25	6.60±0.25 b	6.60±0.25 b	7.80±0.86 b	12.40±0.68 a
0.5	6.20±0.66 b	4.80±0.37 b	7.40±0.40 b	12.60±1.36 a
1	4.80±0.80 b	4.20±0.20 b	6.00±0.32 b	11.20±0.86 a
Concentration (µL/mL)	*L. multiflorum*			
Control	48.50±3.35 a	39.20±2.14 a	48.50±3.35 a	39.20±2.14 a
0.125	34.99±2.18 b	30.04±1.75 a,b	40.90±1.10 a,b	31.36±0.78 b
0.25	29.14±1.43 b,c	31.95±2.44 a,b	35.36±1.42 b	35.37±1.88 a,b
0.5	23.19±0.54 c,d	27.01±4.33 b	35.21±1.80 b	24.98±1.82 c
1	19.90±1.61 d	22.18±0.94 b	25.01±2.04 c	20.85±1.57 c
Concentration (µL/mL)	*E. crus-galli*			
Control	23.66±3.80 a	20.78±1.46 a	23.66±3.80 a	20.78±1.46 a
0.125	18.60±3.53 a	4.00±1.64 b	12.32±0.67 b	12.78±0.31 b
0.25	7.00±1.14 b	4.00±2.45 b	8.72±0.31 b	12.92±0.38 b
0.5	6.40±0.40 b	2.00±2.00 b	6.32±0.86 b	8.58±0.28 c
1	2.00±0.00 b	0.00±0.00 b	5.68±0.45 b	5.60±0.41 d

Values are mean of five replications ± error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests.

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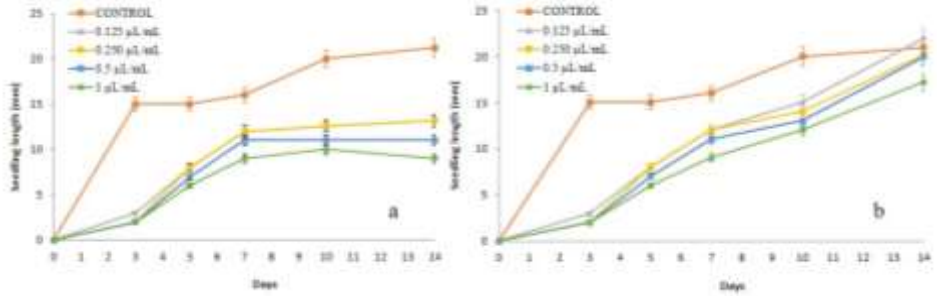

Figure 1: Values of seedling length (mm) (mean ± s.e.) of P. oleracea control and treated with rosemary (a) and basil (b) essential oils at 0.125, 0.25, 0.5 and 1 µl/ml measured over 14 days.

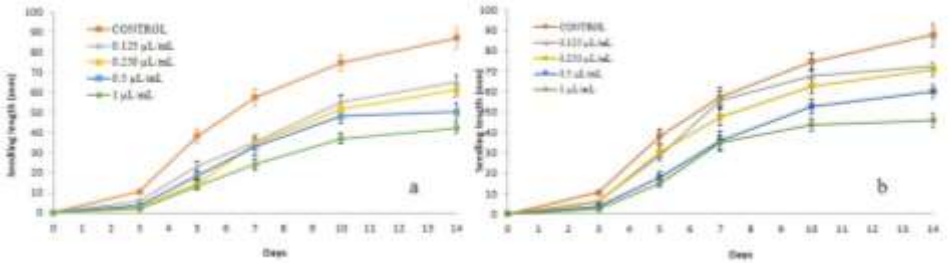

Figure 2: Values of seedling length (mm) (mean ± s.e.) of E. crus-galli control and treated with rosemary (a) and basil (b) essential oils at 0.125, 0.25, 0.5 and 1 µL/mL measured over 14 days.

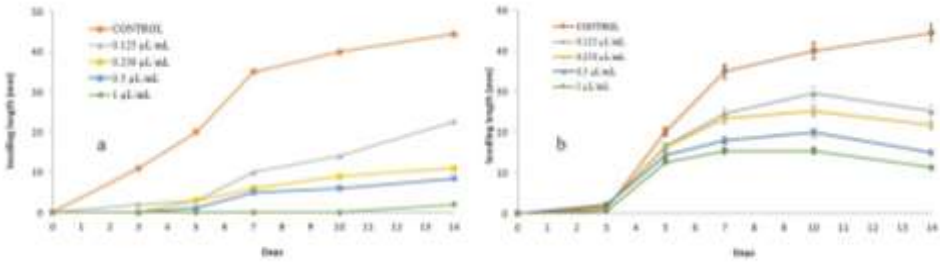

Figure 3: Values of seedling length (mm) (mean ± s.e.) of L. multiflorum control and treated with rosemary (a) and basil (b) essential oils at 0.125, 0.25, 0.5 and 1 µL/mL*measured over 14 days.*

Basil essential oil showed only significant differences between control and treated hypocotyl length, showing no significant effects, even a slight stimulating effect on radicle elongation against *P. oleracea* (Table 3, Figure 1b). Significant differences between hypocotyl and radicle control and all doses applied were found against *L. multiflorum* (Table 3, Figure 3b), being this essential oil able to inhibit significantly at all doses assayed both hypocotyl and radicle elongation of *E. crus-galli* (Table 3, Figure 2b).

4. DISCUSSION

The chemical composition of commercial rosemary and basil essential oil has been analyzed in order to determine their phytotoxic effect against food weeds.

In relation to rosemary essential oil, the commercial sample here analysed is comparable to rosemary growing in Tunisia [38], rich in the oxygenated monoterpenes 1,8- cineole (24.95±0.11%) and camphor (20.45±0.05%), but like in other aromatic plants, the geographic location affects significantly their chemical composition; for instance, α-pinene (16.70±0.12%), which is the third major compound in our commercial sample (Table 1), is the main component with 40.55-45.10% of Brazilian rosemary essential oil [39], affecting the pharmacological activity of the essential oil employed: rosemary essential oil rich in 1,8- cineole has shown promising antibacterial properties against *Staphylococcus aureus* [40] as well as against other multi-drug-resistant microorganisms [41], while α-pinene, common in pines and cedar, has been recently studied for its pharmacological effects on central nervous system activity [42] as well as for its human physiological relaxation [43]. More specific researchers have reported numerous activities of rosemary essential oil against many

agricultural pest of worldwide importance, like the acaricidal effect against twospotted spider mite on greenhouse tomato [44] at not phytotoxic concentrations to the host plant. However, rosemary essential oil is also able to decrease the germination percentage, shoot lengths of prickly lettuce and radish [45]. Our results demonstrated that rosemary essential oil has a selective herbicidal effect because seed germination of *P. oleracea* was not inhibited, whereas *L. multiflorum* seed germination only was significantly reduced at the higher dose applied (1 µL/mL) and *E. crus-galli* was the most sensible weed to rosemary essential oil with significant inhibitory effects at all doses (0.125, 0.25, 0.50 and 1 µL/mL) tested (Table 2).

The second selected essential oil to test food weed control was basil essential oil (*Ocimum basilicum* L. ssp. *basilicum*) with high content in the phenylpropanoid methyl chavicol with 79.07±0.29%, followed by linalool (14.58±0.26%). According to the main components in *Ocimum basilicum* essential oil, there exist several chemotypes in which methyl chavicol-rich and linalool- rich are included [46]. Depending on the cultivar of *O. basilicum*, methyl chavicol and linalool appear in higher or lower amounts, and in this sense, in *O. basilicum* var. *purpureum*, methyl chavicol represents 57.3% and linalool, 18.0%; whereas in *O. basilicum* var. *thyrsiflora* methyl chavicol achieves 20.0% and linalool, 68.0% [47]. In previous studies, *O. basilicum* essential oil has been able to display herbicidal effect against *Solanum lycopersicum* root and hypocoyl length, with 85% and 78.8% inhibition, respectively, so it could be used as post-emergence treatment [48]. In our study no significant seed germination inhibition has been found, showing basil essential oil significant inhibitory effect at all the doses assayed on *P. oleracea* hypocotyl, and in both hypocotyl and radicle elongation of *L. multiflorum* and *E. crus-galli*,, with

a percentage of inhibition of 48.4% and 46.8% for *L. multiflorum* (hypocotyl and radicle, respectively) and 76.0% and 73.1%, against *E. crus-galli*, that corroborate, a post-emergence treatment.

5. CONCLUSION

The phytotoxic activity of rosemary was correlated with the high content of oxygenated monoterpenes. Rosemary essential oil showed a selective effect against *E. crus-galli* seed germination. Significant inhibitory effects on seedling length obtained with both rosemary and basil essential oils against the tree food weeds could be employed as a post-emergence treatment. Further studies *in vivo* conditions are needed to determine no phytotoxic effects on crops.

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CHAPTER 7.

Post-emergent herbicidal activity of *Eucalyptus globulus* Labill. essential oil

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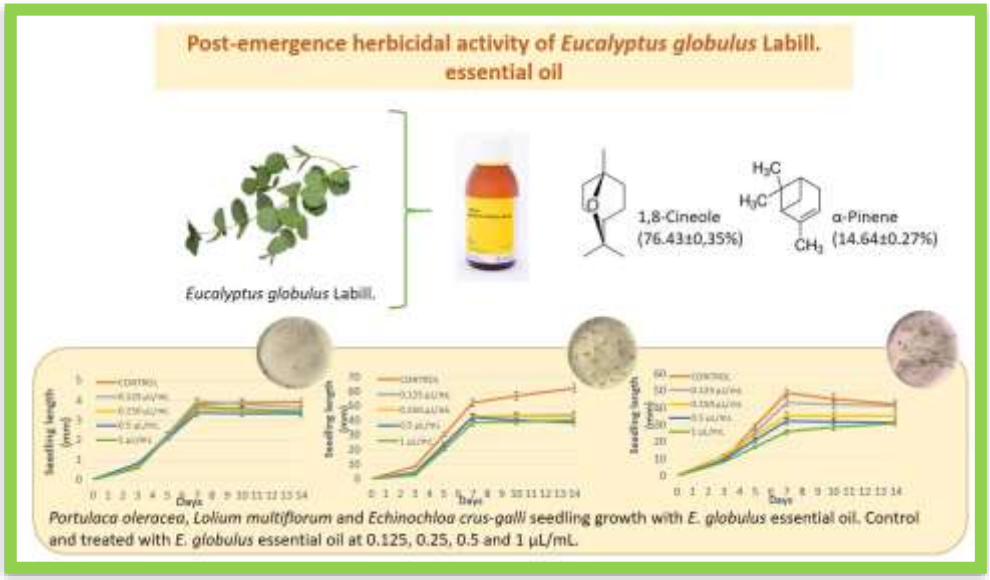

ABSTRACT

Weed resistances to synthetic herbicides, as well as consequent health and environmental problems, are important items to find more eco-friendly natural alternatives to weed control. *Eucalyptus globulus* Labill. essential oil has been traditionally used against respiratory troubles as well as insect repellent due to 1,8-cineole content. Chemical composition of commercial *E. globulus* essential oil and its phytotoxic activity against three common annual weeds (*Portulaca oleracea* L., *Echinochloa crus-galli* (L) Beauv. and *Lolium multiflorum* Lam.) has been studied. Twenty-eight compounds reaching 99.83% of the total essential oil were identified by Gas Chromatography-Mass Spectrometry analysis. The oxygenated monoterpene 1,8-cineole (76.43±0.35%), followed by the monoterpene hydrocarbon α-pinene (14.64±0.27%) were the main compounds. *E. globulus* essential oil lacks of phytotoxicity against the seed germination of the tested weed, showing significant effect on hypocotyl and radicle elongation of *E. crus-galli* at the highest dose (1 µL/mL) assayed and radicle inhibitory effects at all concentrations applied (0.125, 0.25, 0.50 and 1 µL/mL) against *L. multiflorum*. *E. globulus* essential oil could be used in the management of *E. crus-galli* due to its post-emergence herbicidal activity.

KEYWORDS: *Eucalyptus globulus;* essential oil; GC-MS; phytotoxic activity.

1. INTRODUCTION

Eucalyptus *(Eucalyptus globulus* Labill.) is a tree belonging to Myrtaceae family whose leaves are traditionally used in cough and flu disorders by its anticatarrhal, expectorant and antipneumonic properties [1,2]. Its essential oil has been included in a mixture with other volatile oils to relieve muscular aches, arthritis and respiratory troubles [3], being recently corroborated the mucolytic effect of the fluid extract obtained from the leaves of *E. globulus* together *Borago officinalis* L. and *Sambucus nigra* L. [4]. In addition, their essential oil is also a recognized insecticidal agent commonly used not only as an alternative pediculicide with a 100% of effectiveness in humans [5] but also as insect repellent against harmful creatures in agriculture, such as housefly (*Musca domestica*) and *Acanthoscelides obtectus* [6-8]. In this sense, *E. globulus* essential oil is being studied for pest control in food production, due to its wide-spectrum antimicrobial activity against storage foodstuff pathogens, like certain bacteria including *Escherichia coli* and *Pseudomonas aeruginosa* [9], fungal strains with a dose-dependent fungicidal effect against *Aspergillus flavus* and *A. parasiticus* and their aflatoxin production [10] and also against the normal development of *Fusarium verticillioides* by delaying spore germination causing a reduction in fumonisin production, too [11], as well as against other food spoilage microorganisms, such as yeasts strains (*Candida albicans* and *Sacchromyces cerevisiae*) [9]. Regarding this, there is an increased interest in the research of the industrial application of these properties, for instance *E. globulus* essential oil is incorporated as a natural antimicrobial ingredient in edible films exhibiting its antimicrobial and antioxidant properties and consequently enhancing microbial safety and shelf-life of food [12].

It is interesting to note the pesticide activity of natural compounds and their potential applications, particularly for a sustainable agriculture [13,14] due to the rising agrochemical problematic: the World Health Organization (WHO) warns about synthetic pesticides that have been seen to cause serious public health effects along years as consequence of the presence of considerable levels of pesticide residues in ground and surface water, as well as in food purchased in supermarkets, with their subsequently cause of human acute poisonings and even more cancer and other chronic illnesses [15,16]. Furthermore, it is of indispensable consideration the constant emergence of resistances by practically every type of organisms after the extensive use of pesticides making this fact one of the top four environmental problems in the world [15].

According to this, it is popularly known the case of glyphosate, the world's best known herbicide, whose resulting resistances have been described in many worldwide species, like common ragweed (*Ambrosia artemisiifolia* L.) in several row crops of the south-eastern USA following other still unknown mechanisms of action [17], annual ryegrass (*Lolium rigidum* L.) in Australia [18] or barnyardgrass (*Echinochloa crus-galli* (L.) Beauv.) in cotton fields of the midsouthern United States [19].

Regarding this, the phytotoxic effect of several essential oils continues to be studied against seed germination and seedling growth of some weeds [20]. For instance, *Citrus aurantiifolia* essential oil has demonstrated herbicidal effect against three agricultural weeds, *Avena fatua, Echinochloa crus-galli* and *Phalaris minor*, reducing their germination at ≥0.25-0.50 mg/mL as well as the coleoptile and root growth at ≥0.10-0.50 mg/mL [21]. Thyme (*Thymus vulgaris*), summer savory (*Satureja hortensis*), clove

(*Syzgium aromaticum*) and cinnamon (*Cinnamomum zeylanicym*) essential oils have provided phytotoxic results causing electrolyte leakage and cell death of dandelion leaf (*Taraxacum officinale* Weber in Wiggers) [22], showing also *S. hortensis* essential oil nanoemulsion changes on germination, growth and morphophysiological features of *Amaranthus retroflexus* L. and *Chenopodium album* L. [23].

Regarding *E. globulus* essential oil, it is able to exert strong deleterious effects on the germination of *Amaranthus retroflexus* and *Portulaca oleracea* L. [24], seed germination and seedling growth of *Parthenium hysterophorus* L. [25] as well as on germination percentage and germination rate, radicle length, plumule length, primary root and pedicle length, and seedling height of *A. blitoides* and *C. dactylon*, at increasing concentrations [26].

Together the phytotoxic effects it is important to find selective herbicides that only disturb the seed germination and seedling development of weeds, without toxic effects on food crops.

So, the aims of this work are firstly to standardize through gas chromatography-mass spectrometry analysis the chemical composition of the commercial *E. globulus* essential oil in order to assure its main compounds and secondly, to determine their *in vitro* phytotoxic activity against seed germination and seedling growth of *P. oleracea*, a cosmopolitan annual weed of tropical and subtropical climates, *L. multiflorum*, a grass distributed along temperate climates affecting mostly cereals and *E. crus-galli*, an annual plant seriously influencing irrigation crops, especially rice, in order to obtain eco-friendly herbicides.

2. MATERIALS AND METHODS

2.1. Essential oil

Commercial sample of eucalyptus (*E. globulus* Labill.) (Batch 0065901) essential oil purchased from Guinama Lab. (Valencia, Spain), was stored at 4 ºC until chemical analysis and phytotoxic studies were carried out.

2.2. Seeds

Mature seeds of annual weeds of common purslane (*Portulaca oleracea* L.), Italian ryegrass (*Lolium multiflorum* Lam.) and barnyard grass (*Echinochloa crus-galli* (L.) Beauv.), were purchased from Herbiseed, UK (website: www.herbiseed.com).

2.3. Gas Chromatography-Mass Spectrometry analysis

GC-MS analysis was carried out with a 5973N Agilent apparatus, equipped with a capillary column (95 dimethylpolysiloxane - 5 % diphenyl), HP-5MS UI (30 m long and 0.25 mm i.d. with 0.25 µm film thickness). The column temperature program was 60 ºC during 5 min, with 3 ºC/min increases to 180 ºC, then 20 ºC/min increases to 280 ºC, which was maintained for 10 min. The carrier gas was helium at a flow-rate of 1 mL/min. Split mode injection (ratio 1:30) was employed. Mass spectra were taken over the *m/z* 30-500 range with an ionizing voltage of 70 eV.

2.4. Identification

The individual compounds were identified by MS and their identity was confirmed by comparison of their Kovat's retention index calculated using standard hydrocarbons relative to C_8-C_{32} *n*-alkanes, and mass spectra with reference samples or with data already available in the NIST 2005 Mass Spectral library and in the literature [27].

2.5.Herbicidal activity

Sets of 20 seeds each with five replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No.1) moistened with 4 mL of distilled water and with 0 (control), 0.125, 0.250, 0.5, and 1µL/mL of *E. globulus* essential oil. Petri dishes were sealed with parafilm and incubated in a germination chamber Equitec EGCS 301 3SHR model, according to previous assays [28] alternating 30.0±0.1 ºC 16 h in light and 20.0±0.1 ºC 8 h in dark and with (*E. crus-galli*) and without (*P. oleracea*, *L. multiflorum*) humidity. To evaluate the herbicidal activity of the essential oil, the number of germinated seeds was counted and compared with those of untreated seedlings. Emergence of the radicle (≥1 mm) was used as an index of germination and seedling length (hypocotyl and/or radicle) data was recorder after 3, 5, 7, 10 and 14 days in each replicate.

2.6. Statistical analysis

Experiments were made with five replicates. Resulting data were subjected to one-way analysis of variance with IBM SPSS statistics 22 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* one was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1. Chemical composition of E. globulus essential oil

Twenty-eight compounds reaching 99.83% of the total commercial *E. globulus* essential oil were identified by Gas Chromatography-Mass Spectrometry analysis. Compounds are clustered (Table 1) in homologous

series of monoterpene hydrocarbons, oxygenated monoterpenes, sesquiterpene hydrocarbons, and oxygenated sesquiterpenes and listed according to Kovat's retention index calculated in GC on apolar HP-5MS column.

Highest quantities of monoterpene compounds (98.78%) were found in *E. globulus* essential oil. Twelve monoterpene hydrocarbons (21.93±0.08%) and seven oxygenated monoterpenes (76.85±0.13%) constituted the monoterpene fraction. The oxygenated monoterpene 1,8-cineole (76.43±0.35%) followed by the monoterpene hydrocarbon α-pinene (14.64±0.27%) were the main compounds. Among the monoterpene hydrocarbons, also large quantities of β-pinene (3.26±0.03%) and γ-terpinene (2.00±0.02%) were found.

β-Caryophyllene and longifolene with 0.51±0.01 and 0.30±0.00%, respectively, were the principal components among the sesquiterpene hydrocarbons. No higher percentages than 0.1% were found among the others sesquiterpene hydrocarbons identified.

Finally, caryophyllene oxide (0.04±0.00%) was the only oxygenated sesquiterpene found in *E. globulus* essential oil here analyzed.

The results obtained were similar to recent research [29] with 1,8-cineole (76.6%) and α-pinene (12.9%) as the main compounds of *E. globulus* essential oil and different from those obtained from samples of Pakistan [30] in which 1,8-cineole is the main compounds (56.5%) followed by limonene (28%) and α-pinene (4.2%).

Eucalyptus spp. are characterized by a great variability of the main compounds in their essential oils. Thus, in samples collected in Pakistan,

large amount of citronellal (22.3%) and citronellol (20.0%) have been found in *E. citriodora* essential oil. Limonene (14.3%) and terpinen-4-ol (10.2%) were the main compounds in *E. crebra* essential oil; 1,8-cineole (15.2%) and α-pinene (12.1%) in *E. tereticornis* essential oil, while linalool (17.0%) followed by 1,8-cineole (16.1%) were the principal components in *E. camaldulensis* [31]. However *E. camaldulensis* is also characterized by a high content of spathulenol (41.46%) and *p*-cymene (21.92%) in samples collected in Valencia (Spain) [32]. The different chemical composition may be attributed to geographical, environmental and climatic differences [31].

1,8-Cineole, the main constituent of the essential oil from *E. globulus* and other species, is a well-known wide-spectrum antibacterial agent [7]. Synergic effects against antibiotic-resistant pathogens have been observed with its combination with the sesquiterpene hydrocarbon aromadendrene, the main compound of essential oil from the fruits of *E. globulus* [33], and also with chlorhexidine digluconate, against methicillin-resistant *Staphyloccocus aureus* in planktonic and biofilm cultures [34,35].

On the other hand, its combination with α-pinene, the second main component of *E. globulus* essential oil here analysed provide beneficial effects against cellular oxidative stress, preventing reactive oxygen species-induced damage involved in the pathogenesis of several neurodegenerative diseases such as Alzheimer's disease [36].

Table 1. Chemical composition of commercial *E. globulus* essential oil.

RT	RI	Compound	*E. globulus* Peak Area (%)
Monoterpene hydrocarbons			21.93±0.08
6.58	925	α-Thujene	0.01±0.00
6.88	933	α-Pinene	14.64±0.27
7.36	936	Camphene	0.18±0.00
7.58	952	Thuja-2,4(10)-diene	0.01±0.00
8.48	973	β-Pinene	3.26±0.03
9.11	987	Myrcene	0.77±0.01
9.65	998	α-Phellandrene	0.58±0.01
10.20	1012	α-Terpinene	0.14±0.01
11.25	1037	(*Z*)-β-Ocimene	0.11±0.01
11.68	1047	(*E*)- β-Ocimene	0.03±0.00
12.13	1056	γ-Terpinene	2.00±0.02
13.46	1083	Terpinolene	0.22±0.01
Oxygenated monoterpenes			76.85±0.13
11.10	1033	1,8-Cineole	76.43±0.35
13.87	1091	Linalool	0.02±0.01
14.04	1094	α-Pinene oxide	0.01±0.00
17.59	1171	Terpinen-4-ol	0.04±0.01
18.01	1479	*p*-Cymen-8-ol	0.01±0.01
18.24	1184	α-Terpineol	0.31±0.02
22.62	1278	Bornyl acetate	0.03±0.00
Sesquiterpene hydrocarbons			1.01±0.00
25.38	1341	α-Cubebene	0.05±0.00
26.21	1360	Longicyclene	0.01±0.00
27.71	1393	Longifolene	0.30±0.00
28.34	1408	β-Caryophyllene	0.51±0.01
29.74	1443	α-Humulene	0.06±0.00
30.72	1467	γ-Muurolene	0.01±0.01
31.68	1490	α-Muurolene	0.01±0.00
32.60	1513	δ-Cadinene	0.06±0.00
Oxygenated sesquiterpenes			0.04±0.00
34.85	1571	Caryophyllene oxide	0.04±0.00
	TOTAL		99.83±0.21

RI, retention index relative to C_8-C_{32} *n*-alkane on HP-5MS column; values are mean ± standard deviation of three samples.

3.2. Seed germination and seedling growth inhibition of P. oleracea, L. multiflorum and E. crus-galli, by E. globulus essential oil

The phytotoxic effect of *E. globulus* essential oil against seed germination and seedling growth of three well-known weeds, *P. oleracea*, *E. crus-galli* and *L. multiflorum*, is shown in Table 2 and Figures 1-3.

E. globulus essential oil had no effect against *P. oleracea*, *E. crus-galli* and *L. multiflorum* seed germination. No significant differences were found between control and all dose (0.125, 0.25, 0.5 and 1 µL/mL) assayed (Table 2).

According to seedling growth, no significant differences in the seedling development (hypocotyl and radicle) of *P. oleracea* were observed after the application of *E. globulus* essential oil at all the concentrations assayed in comparison to control (Table 2, Figure 1).

However, both hypocotyl and radicle of *E. crus-galli* were significantly inhibited at the highest dose tried (1 µL/mL) reaching 31.17 and 18.71% of growth reduction with respect to control (Table 2, Figure 2). Regarding *L. multiflorum* evolution, although no significant differences were observed in its hypocotyl growth (Table 2), the radicle development was considerably reduced between 41.22-52.95% without differences between all concentrations applied (0.125, 0.25, 0.50 and 1 µL/mL) (Table 2, Figure 3).

Regarding *L. multiflorum* evolution, although no significant differences were observed in its hypocotyl growth (Table 2), the radicle development was considerably reduced between 41.22-52.95% without differences between all concentrations applied (0.125, 0.25, 0.50 and 1 µL/mL) (Table 2, Figure 3).

Table 2. *In vitro* effects of *E. globulus* essential oil against *P. oleracea, L. multiflorum* and *E. crus-galli* seed germination and seedling growth.

Dose*	*P. oleracea*		
	Germination	Hypocotyl growth	Radicle growth
Control	67.00±4.06 a	2.40±0.34 a	1.50±0.28 a
0.125	70.00±6.12 a	2.33±0.43 a	1.29±0.16 a
0.25	64.00±2.45 a	2.08±0.13 a	1.32±0.19 a
0.5	66.00±4.85 a	1.83±0.35 a	1.50±0.33 a
1	70.00±1.58 a	1.83±0.19 a	1.81±0.22 a
Dose	*E. crus-galli*		
	Germination	Hypocotyl growth	Radicle growth
Control	74.00±3.32 a	22.97±1.75 a	18.92±1.26 a
0.125	87.00±2.55 a	22.95±1.20 a	18.34±1.09 a,b
0.25	73.00±2.55 a	17.76±1.09 a,b	17.70±0.73 a,b
0.5	74.00±3.32 a	17.92±1.83 a,b	13.45±0.82 a,b
1	78.00±5.39 a	15.81±2.14 b	15.38±1.78 b
Dose	*L. multiflorum*		
	Germination	Hypocotyl growth	Radicle growth
Control	67.00±2.00 a	29.18±1.26 a	32.92±2.63 a
0.125	55.00±2.74 a	24.46±0.55 a	19.35±1.22 b
0.25	63.00±3.39 a	25.08±1.55 a	18.01±0.78 b
0.5	63.00±5.39 a	24.77±1.97 a	13.95±1.68 b
1	63.00±3.00 a	24.60±0.61 a	15.49±1.19 b

[a] Values are mean of five replications ± standard error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at p>0.05 according to T3 Dunnet and Tukey tests. *Dose: µL/mL.

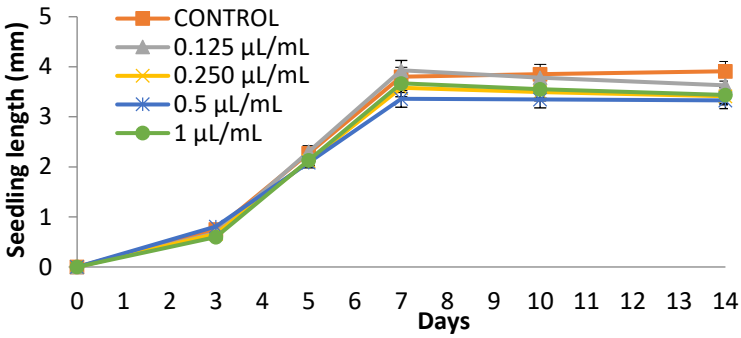

Figure 1. P. oleracea seedling growth with E. globulus essential oil. Control and treated with E. globulus essential oil at 0.125, 0.25, 0.5 and 1 µL/mL.

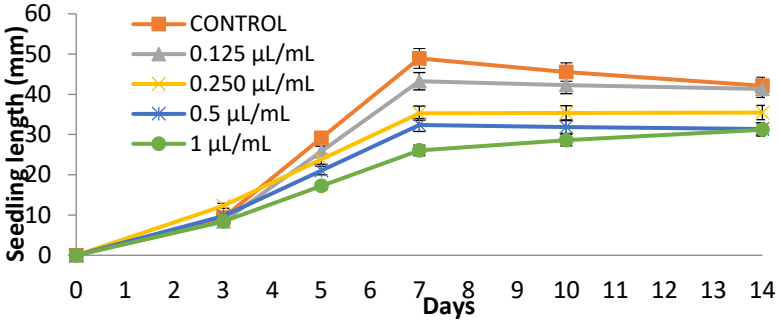

Figure 2. E. crus-galli growth with E. globulus essential oil. Control and treated with E. globulus essential oil at 0.125, 0.25, 0.5 and 1 µL/mL.

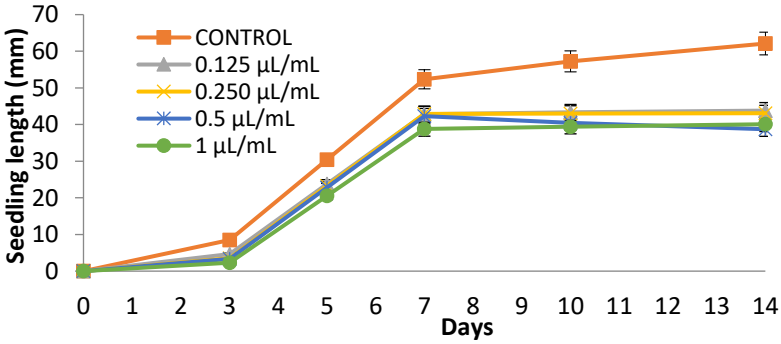

Figure 3. L. multiflorum seedling growth with E. globulus essential oil. Control and treated with E. globulus essential oil at 0.125, 0.25, 0.5 and 1 µL/mL.

The herbicidal potential is closely related with the essential oil composition, weeds and doses applied. In this ways, at the same doses and weed, *E. globulus* essential oil with 1,8-cineole (76.43%) has no phytotoxic effect against *P. oleracea* seed germination and seedling growth whereas *E. camaldulensis* essential oil with spathulenol (41.46%) as main compound was able to completely inhibit the seed germination of this cosmopolitan weed [32].

Regarding the weed, *E. globulus* essential oil showed at the doses employed significant effect on *E. crus-galli* seedling growth without phytotoxic effects against *E. crus-galli* seed germination while a higher doses (100 and 250 µg/mL*) E. tereticornis* essential oil with α-pinene, 1,8-cineole and β-pinene as the main compounds significantly affected both seed germination and seedling growth of *E. crus-galli* [37]. Finally lower doses of *E. globulus* essential oil showed significant effects on seed germination and seedling development of others weed such as *Amaranthus blitoides* and *C. dactylon* [26].

4. CONCLUSION

E. globulus essential oil with 1,8-cineole (76.43%) and α-pinene (14.64%) as the main compounds represents a potentially effective bioherbicide in the management of *E. crus-galli*. Further studies are needed with higher doses of *E. globulus* essential oil in order to corroborate a potential use also as a pre-emergent herbicide as well as its phytotoxic effects against food crops mainly rice and other cereals.

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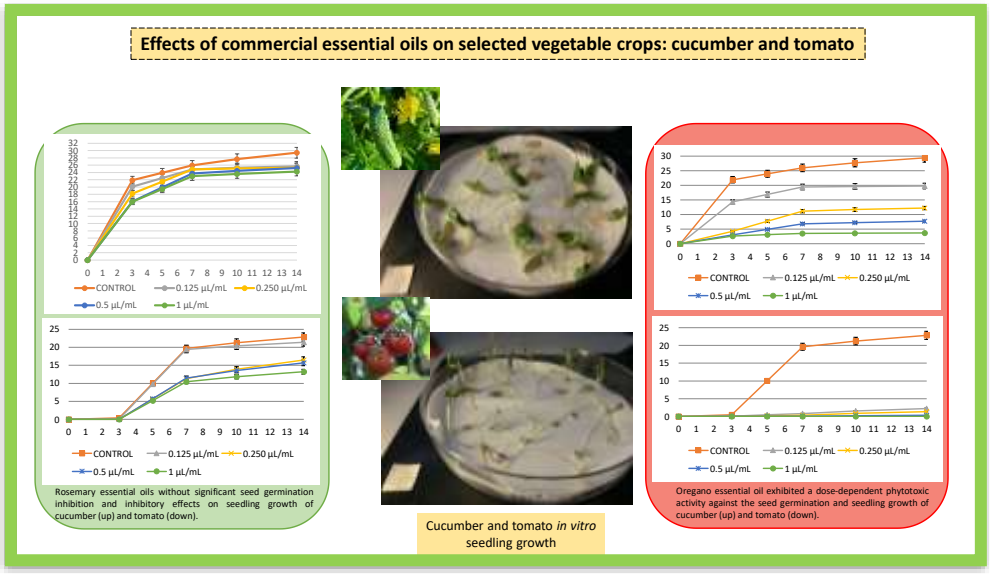
Further publications

CHAPTER 8.

Effects of commercial essential oils on selected vegetable crops: cucumber and tomato

María Dolores Ibáñez and María Amparo Blázquez

In process



ABSTRACT

Essential oils of oregano (*Origanum vulgare* L.), rosemary (*Rosmarinus officinalis* L.), mastic thyme (*Thymus mastichina* L.), basil (*Ocimum basilicum* L.), tea tree (*Melaleuca alternifolia* Maiden & Betche ex Cheel), eucalyptus (*Eucalyptus globulus* Labill.), wintergreen (*Gaultheria procumbens* L.) and marjoram (*Origanum majorana* L.), with demonstrated herbicidal effects against several weeds, were tested at 0.125, 0.250, 0.50 and 1 µL/mL over the seed germination and seedling growth of cucumber (*Cucumis sativus* L.) and tomato (*Solanum lycopersicum* L.), in order to ensure their harmlessness against these Mediterranean food crops. Oregano (carvacrol 60.42%) was the most damaging essential oil, exhibiting a dose-dependent phytotoxic activity against the seed germination and seedling growth of cucumber and tomato, whereas rosemary (1,8-cineole 24.95%, camphor 20.45% and α-pinene 16.70%) essential oil was the least injurious in cucumber and tomato seed germination. The cultivated vegetable crop cucumber was more resistant to tomato at all tested essential oils. Seedling growth studies showed that the radicle was more sensitive than the hypocotyl to the essential oils. In cucumber, only *M. alternifolia* essential oil significantly affected the hypocotyl growth of cucumber (34.61%) at the highest dose (1 µL/mL) assayed. Rosemary essential oil could be used as pre-emergent bioherbicide in the control of weeds affecting cucumber and tomato crops.

Keywords: essential oils, phytotoxicity, cucumber, tomato, food crops.

1. INTRODUCTION

Essential oils represent 'green' alternatives to agrochemicals as they exert low mammalian toxicity, and provide eco-chemical approach in the management of crop pests of a cost effective manner (Koul *et al*. 2008; Srivastava *et al.* 2015). Essential oils and also their active components have shown insecticidal efficacy (Isman *et al.* 2011), nematicidal, ovicidal, fungicidal and bactericidal effects as well as the capacity to inhibit growth, food intake, and oviposition of pathogens and pests that are major threats for agricultural yield (Isman *et al.* 2011; Pavela and Benelli 2016; Martínez *et al*. 2017).

Likewise, weeds represent one of the most severe problems in agricultural manufacture today. Losses due to weeds are one of the major limiting factors in basic crops worldwide. Economic loss of about USD 11 billion was estimated due to weeds in 10 major crops of India viz. groundut (35.8%), soybean (31.4%), greengram (30.8), perlmillet (27.6%), maize (25.3%), sorghum (25.1%), sesame (23.7%), mustard (31.4%), direct-selected rice (21.4%), wheat (18.6%) and transplanted rice (13.8%) (Gharde *et al*. 2018). According to the Food and Agriculture Organization (FAO), considerable reduction percentages due to weeds have also been recorded in vegetables (28%), fruit species and vineyards (29%), and in tobacco (37%) (Petrova *et al*. 2015).

The herbicidal potential of essential oils as well as the synergistic effects among their components have been by far studied (Amri *et al.* 2013) demonstrating the phytotoxicity of certain essential oils against weed seed germination and consequently its viability in weed control technology (Tworkoski 2002). Among the plant families with essential oils belonging

promising herbicidal capacity are Lamiaceae, Myrtaeae, Asteraceae and Anacardiaceae families, containing components with high activity present in these mixtures such as α-pinene, limonene, 1,8-cineole, camphor, carvacrol and thymol (Amri *et al.* 2013). Registered products for weed control based on essential oils and their individual compounds like Barrier H®, Avenger Organic Weed Killer® and Organic Interceptor® including citronella oil, d-limonene and pine oil, respectively are marketed in several countries (Giepen *et al.* 2014). Specifically, from the listed compounds above, thymol and carvacrol have shown strong inhibition of plant germination (Synowiec *et al.* 2017). These monoterpenes are mainly detected in essential oils of some species belonging to Lamiaceae, Verbenaceae and Ranunculaceae families, such as oregano, thyme and savory (Ibáñez and Blázquez 2018a, 2017; Naghdi Badi *et al.* 2017) being well known by their antimicrobial, antiviral, antifungal and antioxidant properties as well as for control of plants diseases and extension of the shelf-life of fruits and vegetables (Edris 2007; Solgi and Ghorbanpour 2014).

Regarding this, the phytotoxic activity of essential oils should be selective, being convenient do not harm adjacent food crops. So, the aim of this work is to study the effects of the essential oils of oregano (*Origanum vulgare* L.), rosemary (*Rosmarinus officinalis* L.), mastic thyme (*Thymus mastichina* L.), basil (*Ocimum basilicum* L.), tea tree (*Melaleuca alternifolia* Maiden & Betche ex Cheel), eucalyptus (*Eucalyptus globulus* Labill.), wintergreen (*Gaultheria procumbens* L.) and marjoram (*Origanum majorana* L.), on the seed germination and seedling growth of cucumber (*Cucumis sativus* L.) and tomato (*Solanum lycopersicum* L.) in order to know their harmlessness in these food crops.

2. MATERIALS AND METHODS

2.1. Essential oils

Commercial samples of oregano (*Origanum vulgare* L.) (Batch 0042451), rosemary (*Rosmarinus officinalis* L.) (Batch 0037337), tea tree (*Melaleuca alternifolia* Maiden & Betche ex Cheel) (Batch 0051451), eucalypt (*Eucalyptus globulus* Labill.) (Batch 0065901) and marjoram (*Origanum majorana* L.) (Batch 0042773) essential oils purchased by Guinama (Valencia, Spain). On the other hand, basil (*Ocimum basilicum* L.) (Batch 0F22144) and wintergreen (*Gaultheria procumbens* L.) (Batch 0F18989) were supplied by Pranarôm International S.A. and mastic thyme (*Thymus mastichina* L.) (Batch TM010711) essential oil was obtained from Planalto Dourado TM (Freixedas, Portugal).

All essential oils were stored at 4 ^{o}C until phytotoxic assays were carried out.

2.2. Food crop seeds

Mature seeds of common Mediterranean crops cucumber (*Cucumis sativus* L.) and 'Muchamiel' tomato (*Solanum lycopersicum* L.) were purchased from Intersemillas S.A. (Valencia, Spain).

2.3. In vitro assays: cucumber and tomato seed germination and seedling growth with essential oils

Sets of 20 seeds each with five replicates per treatment were homogenously distributed in Petri dishes (9 cm diameter) between two layers of filter paper (Whatman No.1) moistened with 4 mL of distilled water and with 0 (control), 0.125, 0.250, 0.50, and 1 µL/mL of oregano rosemary, mastic thyme, basil, tea tree, eucalyptus, wintergreen and marjoram essential oils. Petri dishes were sealed with parafilm and incubated in a germination

chamber Equitec EGCS 301 3SHR model, according to previous assays (Blázquez and Carbó 2015) alternating 30.0±0.1ºC 16 h in light and 20.0±0.1ºC 8 h in dark and with 70% of humidity (cucumber) and no humidity (tomato). To evaluate the phytotoxic activity of the essential oils, the number of germinated seeds was counted and compared with those of untreated seedlings. Emergence of the radicle (≥1 mm) was used as an index of germination and seedling length (hypocotyl and/or radicle) data were recorder after 3, 5, 7, 10 and 14 days in each replicate.

2.4. Statistical analysis

Experiments were made with five replicates. Data were subjected to one-way analysis of variance (ANOVA) with SPSS statistics 24 software. Tukey's *post hoc* test was used when variances remained homogeneous (Levene's test) and T3 Dunnett's *post hoc* one was employed if not, assuming equal variances. Differences were considered to be significant at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

In order to know the potential lack of selectivity of essential oils over food crops when used as weed-killer agents, the potential phytotoxic effect of oregano, rosemary, mastic thyme, basil, tea tree, eucalyptus, wintergreen and marjoram essential oils was evaluated *in vitro* against the seed germination and seedling growth of the well-known Mediterranean vegetables cucumber and tomato at 0.125, 0.25, 0.50 and 1 µL/mL (Table 1, Table 2).

3.1. Effects of essential oil in the seed germination of cucumber and tomato

Oregano essential oil exhibited a dose-dependent phytotoxic activity against the seed germination of cucumber and tomato, especially affecting this last crop with a percentage of inhibition of 51.43% at the lowest dose (0.125 1 µL/mL) tested and total inhibition at the highest dose (1 µL/mL) applied (Table 1).

Table 1. *In vitro* effects of oregano, rosemary, mastic thyme, basil, tea tree, eucalyptus, wintergreen and marjoram essential oils on cucumber and tomato seed germination.

Essential oil	Concentration (µL/mL)	Seed germination (% ± s.e.)	
		Cucumber	Tomato
Oregano	Control	100.00±0.00 a	70.00±5.48 a
	0.125	99.00±1.00 a	34.00±5.79 b
	0.25	93.00±2.55 a,b	29.00±3.32 b
	0.5	88.00±2.00 b	9.00±5.57 c
	1	91.00±1.87 b	0.00±0.00 c
Rosemary	Control	100.00±0.00 a	70.00±5.48 a
	0.125	99.00±1.00 a	77.00±4.06 a
	0.25	95.00±3.16 a	75.00±4.18 a
	0.5	99.00±1.00 a	74.00±5.34 a
	1	95.00±2.24 a	73.00±2.55 a
Mastic thyme	Control	98.00±1.23 a	80.00±7.75 a
	0.125	97.00±2.00 a	59.00±9.93 a
	0.25	94.00±2.92 a	56.00±11.23 a
	0.5	96.00±1.87 a	53.00±10.32 a
	1	92.00±3.39 a	42.00±13.29 a
Basil	Control	99.00±1.00 a	68.00±3.39 a
	0.125	95.00±1.58 a	54.00±14.78 a
	0.25	95.00±1.58 a	54.00±6.60 a
	0.5	98.00±1.23 a	64.00±3.32 a
	1	98.00±2.00 a	45.00±8.22 a
Tea tree	Control	99.00±1.00 a	71.00±2.45 a
	0.125	96.00±2.45 a	62.00±5.61 a,b
	0.25	96.00±1.87 a	55.00±3.54 a,b
	0.5	97.00±1.23 a	53.00±7.35 a,b

	1	96.00±1.87 a	51.00±1.87 b
Eucalyptus	Control	98.00±1.23 a	68.00±3.39 a
	0.125	94.00±2.92 a	66.00±3.67 a
	0.25	99.00±1.00 a	72.00±5.61 a
	0.5	93.00±3.39 a	70.00±6.71 a
	1	99.00±1.00 a	43.00±13.00 a
Wintergreen	Control	98.00±1.23 a	75.00±7.07 a
	0.125	94.00±2.45 a	74.00±3.67 a
	0.25	96.00±1.87 a	71.00±2.92 a
	0.5	93.00±2.55 a	71.00±1.87 a
	1	93.00±2.00 a	68.00±2.56 a
Marjoram	Control	100.00±0.00 a	70.00±5.48 a
	0.125	95.00±1.58 a	67.00±4.64 a
	0.25	96.00±1.00 a	68.00±5.61a
	0.5	94.00±2.45 a	55.00±5.70 a
	1	94.00±2.45 a	46.00±7.14 a

Values are mean percentage of five replications ± error deviation after 14 days of incubation. Means followed by different letters in the same column indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests.

Oregano essential oil has already demonstrated in previous works its broad-spectrum phytotoxicity against the seed germination not only of weeds but also of food crops. At the same doses, oregano essential oil with carvacrol (60.42±0.07) and *p*-cymene (15.52±0.02) as the main compound (Figure 1), showed a total *in vitro* inhibitory effect against the seed germination of the weeds common purslane (*Portulaca oleracea* L.), Italian ryegrass (*Lolium multiflorum* Lam.) and barnyard grass (*Echinochloa crus-galli* (L.) Beauv.) (Ibáñez and Blázquez 2017).

Furthermore, oregano essential oil significantly inhibited the seed germination of the food crops *Lepidium sativum* L, *Lactuca sativa* L. and *Rhaphanus sativus* L, at 1.25-2.5 µg/mL; 0.25-2.5 µg/mL and 0.125-2.5 µg/mL, respectively (De Almeida *et al.* 2010).

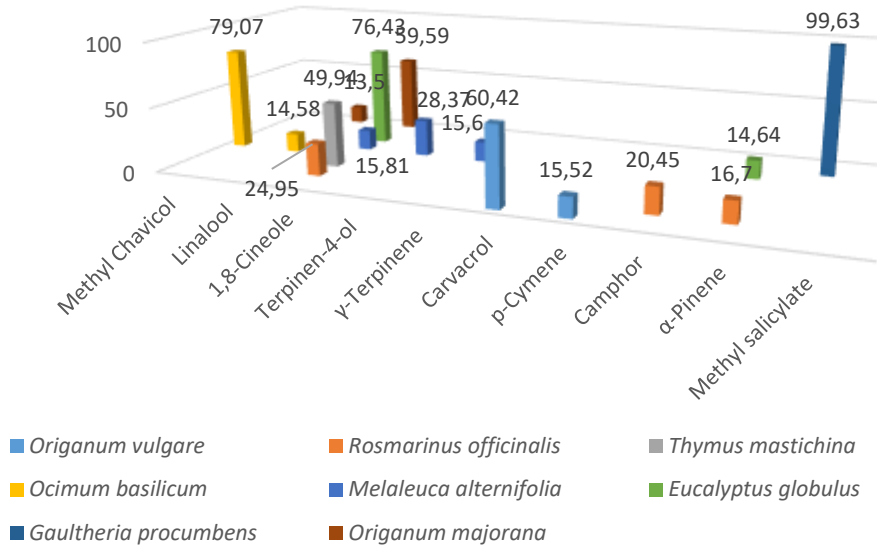


Figure 1. Main compounds of the tested essential oils (Ibáñez and Blázquez 2018a, 2018b, 2018c, 2018d, 2017).

Other essential oils, like *Satureja montana*, with high content in carvacrol (43.34±0.09), thymol (23.20±0.06) and *p*-cymene (11.41±0.01) also showed total seed germination inhibition of *P. oleracea*, *L. multiflorum* and *E. crus-galli* and significant phytotoxic effect in seed germination of maize, rice and tomato seeds (Ibáñez and Blázquez 2018a).

Tea tree essential oil with terpinen-4-ol (28.37±0.05), followed by 1,8-cineole (15.81±0.06) and γ-terpinene (15.60±0,03) (Figure 1), significantly decreased the seed germination of tomato (28.17%) at the highest dose (1 µL/mL) tested with respect to control. However, this essential oil did not cause any inhibitory effect in the seed germination of cucumber at any dose (0.125, 0.25, 0.50 and 1 µL/mL) assayed (Table 1). In fact, tea tree essential oil has been included in non-phytotoxic biocide composition because it has been observed that the antiseptic activity of its component terpinen-4-ol is harmless for plants (Pipko *et al*. 2005). Furthermore, other *Melaleuca* spp.

with different chemical composition, such as *M. armillaris* (*cis*-calamenene 9.0% and torreyol 15.1%), *M. styphelioides* (methyl eugenol 91.1%) and *M. acuminate* (*trans*-pinocarveol 25.1%, dihydrocarveol 23.6%, myrtenol 12.3% and 1,8-cineole 11.7%) essential oils were also ineffective against germination of both crops and weed species: *Rhaphanus sativus* L,, *Lepidium sativum* L., *Triticum durum* L., *Sinapis arvensis* L., and *Phalaris caraniernsis* L. (Amri *et al.* 2012).

On the other hand, rosemary, mastic thyme, basil, eucalyptus, wintergreen and marjoram essential oils showed no significant inhibition of the seed germination of both cucumber and tomato at all doses (0.125, 0.25, 0.50 and 1 µL/mL) assayed (Table 1). But different chemical composition or assays in other food crops or weeds have confirmed its influence in seed germination. For instance, rosemary essential oil collected in Iran with α-pinene (25.8-27.7%), camphor (8.60-9.0%), camphene (6.5-7.7%) and 1,8-cineole (9.4-9.6%) as the main components significantly inhibited the seed germination of the weed plant prickly lettuce (*Lactuca serriola* L.) and the crop *Raphanus sativus* L. at all doses (300, 600, 900, 1200, 1500 and 1800 µL/L) applied and in all phenological stages of the essential oil (Alipour and Saharkhiz 2016). Rosemary essential oil (α-pinene 25.85%, 1,8-cineole 9.67%, camphor 9%, camphene 7.79%, verbenone 6.8% and borneol 6.38%) was able to decrease significantly in dose dependent manner (100-1200 µL/L) the seed germination and germination rate of *Amaranthus retroflexus* L., (Hazrati *et al.* 2018), common weed infesting cucumber (Bakhshayeshan-Agdam et al. 2015; Ngouajio and Mennan 2005) and tomato (Qasem 1992). A total seed germination inhibition of *A. retroflexus* was obtained at 800 µL/L, whereas tomato showed great resistance to

rosemary essential oil with a reduced germination percentage of 53.4% at 800 µL/L (Hazrati et al. 2018).

Regarding mastic thyme also know Spanish marjoram and marjoram essential oils containing 1,8-cineole (49.49±0.37%, 59.59±0.85%) and linalool (5.66±0.01%, 13.05±0.04%), respectively, in addition to cucumber and tomato, also showed not phytotoxic effects in the seed germination of *P. oleracea, L. multiflorum* and *E. crus-galli* at the doses (0.125, 0.25, 0.50 and 1 µL/mL) applied, even with significant stimulatory effect in *P. oleracea* at the highest dose (1 µL/mL) tested of mastic thyme essential oil (Ibáñez and Blázquez 2017). Equally, basil essential oil, with methyl chavicol (79.07±0.29%) and linalool (14.58±0.26%) was not weed-killer against *P. oleracea, L. multiflorum* and *E. crus-galli* and basil essential oil from Greece containing linalool (50.2%) eugenol (15.5%) and *trans*-methyl cinnamate (13.6%) neither influenced the plant number of other weeds, including common purslane (*P. oleracea* L.), black nightshade (*Solanum nigrum* L.) and common lambsquarters (*Chenopodium album* L.), even increased the number of germinated seeds in this last one; moreover, although it caused a decrease in the germination of *E. crus-galli* under laboratory conditions, this result was not confirmed *in vivo* (Dhima *et al*. 2010). However, recently powerful bioherbicidal effect of basil essential oil from Belgrade with linalool (48.6%) and 1,8-cineole (9.7%) against the germination of velvetleaf (*Abutilon theophrasti* Medik.) has been reported (Sarić-Krsmanović *et al.* 2019).

Similarly, *Eucalyptus globulus* essential oil with eucalyptol as major component also affected the germination percentage as well as the germination rate of weeds, including *Amaranthus blitoides* and *Cynodon*

dactylon (L.) Pers (Rassaeifar *et al.* 2013), without significant phytotoxic effects against other weed like *P. oleracea*, *E. crus-galli* and *L. multiflorum* (Ibáñez and Blázquez 2018d) and finally wintergreen essential oil principally constituted by methyl salicylate (99.63±0.02%) (Figure 1) showed a significant drop in the seed germination of *P. oleracea* (Ibáñez and Blázquez 2018c).

The results showed that rosemary, mastic thyme, eucalyptus, wintergreen and marjoram essential oils could be used in the treatment of the weeds affecting cucumber and tomato crops, instead other essential oils like *Cryptocarya massoy* (Oken) Kosterm. essential oil that achieved a reduction of 67.2% in the seed germination of cucumber in respect with the negative control (Rolli *et al*. 2016), peppermint (*Mentha piperita* L.) essential oil with an increasing inhibition dependent on dose of the seed germination of tomato (Ibáñez and Blázquez 2018a) or clove (*Syzygium aromaticum* (L.) Merr. & L.M. Perry) essential oil that affected the seedlings of both cucumber and tomato (Meyer *et al.* 2008).

3.2. Seedling growth inhibition of cucumber and tomato with essential oils

3.2.1. Essential oils phytotoxicity against cucumber

Oregano essential oil (carvacrol 60.42%) showed a strong dose-dependent phytotoxic activity against the hypocotyl and radicle growth of cucumber (Table 2, Figure 2), being especially remarkable its inhibitory effect at the higher doses (0.5 and 1 µL/mL) reducing the development of the hypocotyl and the radicle in 66.15-69.35% and 77.36-94.98%, respectively, according to control (Table 2).

Table 2. *In vitro* effects of oregano (OV), rosemary (RO), mastic thyme (TM), basil (OB), tea tree (MA), eucalyptus (EG), wintergreen (GP) and marjoram (OM) essential oils (EOs) against cucumber and tomato seedling growth.

Seedling growth of cucumber (mm ± s.e.)						
EOs	Dose*	Control	0.125	0.25	0.5	1
OV	Hyp	8.45±0.85 a	5.58±0.74 a,b	3.81±0.46 b,c	2.86±0.32 c	2.59±0.15 c
	Rad	21.16±1.02 a	14.16±1.30 b	8.38±1.76 b,c	4.79±1.97 c,d	1.06±0.21 d,e
RO	Hyp	8.45±0.85 a	8.17±0.29 a	8.28±0.35 a	8.09±0.51 a	8.52±0.81 a
	Rad	21.16±1.02 a	17.52±0.78 a,b	17.03±1.32 a,b	17.12±0.92 a,b	15.74±1.58 b
TM	Hyp	10.34±0.33 a	10.30±0.53 a	10.00±0.54 a	9.63±0.15 a	9.68±0.10 a
	Rad	18.61±0.29 a	18.60±0.33 a	16.81±0.75 a,b	16.96±0.40 a,b	14.98±0.43 b
OB	Hyp	8.35±0.10 a	8.33±0.17 a	8.75±0.40 a	8.82±0.49 a	8.15±0.85 a
	Rad	23.13±1.54 a	14.80±0.77 b	13.69±0.57 b	13.86±0.29 b	13.45±0.49 b
MA	Hyp	8.35±0.10 a	8.08±0.28 a	8.06±0.25 a	7.96±0.42 a	5.46±0.84 b
	Rad	23.13±1.54 a	17.77±0.73 b	17.22±0.40 b	16.32±0.77 b,c	13.00±0.91 c
EG	Hyp	10.34±0.33 a	11.10±0.65 a	11.30±0.77 a	11.13±0.42 a	10.08±0.90 a
	Rad	18.61±0.29 a	17.23±1.88 a,b	17.07±0.97 a,b	17.38±0.78 a,b	13.83±0.62 b
GP	Hyp	10.34±0.33 a	9.49±2.14 a	8.18±0.33 a	8.05±0.24 a	8.39±0.98 a
	Rad	18.61±0.29 a	16.27±0.87 a,b	16.25±0.94 a,b	16.25±0.85 a,b	13.32±1.07 b
OM	Hyp	8.45±0.85 a	8.49±0.16 a	8.45±0.76 a	8.86±1.04 a	8.78±0.95 a
	Rad	21.16±1.02 a	18.72±0.52 a,b	16.45±0.48 b,c	14.80±0.89 c	14.56±0.84 c
Seedling growth of tomato (mm ± s.e.)						
OV	Hyp	8.28±0.86 a	0.97±0.36 b	0.76±0.18 b	0.21±0.15 b	0.00±0.00 b
	Rad	14.54±1.94 a	1.26±0.34 b	0.45±0.18 b	0.09±0.06 b	0.00±0.00 b
RO	Hyp	8.28±0.86 a	7.17±0.81 a,b	6.11±0.52 a,b	6.09±0.83 a,b	4.51±0.48 b

TM	Rad	14.54±1.94 a	14.22±0.87 a	10.40±1.48 a,b	9.60±0.32 a,b	8.72±0.56 b
	Hyp	8.05±1.40 a	2.87±0.70 a,b	2.86±1.95 a,b	2.66±0.71 b	2.18±1.05 b
OB	Rad	14.78±1.16 a	11.75±2.25 a,b	5.25±2.71 b,c	5.17±0.88 b,c	3.16±1.29 c
	Hyp	10.09±1.56 a	4.72±2.00 a,b	4.36±0.54 a,b	4.73±1.72 a,b	1.82±0.65 b
MA	Rad	14.41±1.34 a	9.75±2.49 a,b	9.27±1.16 a,b	6.46±0.58 b	6.97±1.85 b
	Hyp	7.25±1.35 a	5.09±0.92 a,b	3.10±0.50 b	3.10±0.73 b	2.39±0.32 b
EG	Rad	16.71±1.47 a	12.31±1.57 a,b	11.82±1.72 a,b	11.43±2.02 a,b	8.51±0.59 b
	Hyp	7.94±0.81 a	5.11±1.28 a	6.16±0.71 a	5.95±2.56 a	2.20±1.10 a
GP	Rad	18.87±1.14 a	14.05±1.64 a	13.14±0.57 a	14.31±1.12 a	4.90±2.03 b
	Hyp	16.96±1.22 a	11.35±1.42 b	11.19±1.01 b	10.37±0.58 b	10.29±0.86 b
OM	Rad	13.24±0.92 a	9.80±0.97 b	9.62±0.60 b	8.27±0.50 b	7.36±0.82 b
	Hyp	8.28±0.86 a	7.19±0.15 a	7.01±0.77 a	6.77±2.11 a	3.97±1.02 a
	Rad	14.54±1.94 a	10.66±1.08 a,b	9.92±1.07 a,b	7.81±0.67 b	7.66±1.45 b

Values are mean percentage of five replications ± standard error after 14 days of incubation. Means followed by different letters in the same row indicate that are significantly different at $p < 0.05$ according to T3 Dunnet and Tukey tests. *Dose: µL/mL; Hyp: Hypocotyl; Rad: Radicle.

Oregano essential oil (carvacrol 44.0%) has been previously reported to also affect the seedling growth of other food crops, particularly the radicle elongation of *L. sativum*, *L. sativa* and *R. sativus* at 1.25 µg/mL and 2.5 µg/mL (Arminante et al. 2015; De Almeida et al. 2010). However, *in vitro* and greenhouse antifungal studies with *Origanum onites* L. essential oil (carvacrol 68.23%) against root and stem rot of cucumber by *Fusarium oxysporum* f. sp. *radicis-cucumerinum* (Forc) showed *in vivo* no phytotoxicity on cucumber at the highest dose 900 mg/mL assayed (Soylu and Incekara 2017).

Previous studies have demonstrated that the development of cucumber is also sensitive to other essential oil like *Schinus terebinthifolius* Raddi essential oil obtained from fresh leaves, unripe and ripe fruits that caused significant inhibition of the radicle growth of cucumber (50.5-84.5%) at 10,000 µg/mL (Cláudio *et al.* 2007). Several mechanisms have been proposed to explain the phytotoxicity of the essential oils in cucumber. In this sense, peppermint (*Mentha* x *piperita* L.) essential oil was able to interfere with respiratory functions, significantly inhibited the root respiration of cucumber at concentrations higher than 60 mg/L with a IC_{50} of 324 mg/L, lower than that necessary (IC_{50} of 593 mg/L) to produce the mitochondrial respiration inhibition (Mucciarelli *et al*. 2001) or clove essential oil that was able to influence the development of cucumber seedlings through the loss of water content, lipid peroxidation and antioxidative enzyme activity causing the destruction of cell membrane components by mechanisms different from those of paraquat (Park *et al.* 2011).

In our study, rosemary, mastic thyme, basil, eucalyptus, wintergreen and marjoram essential oils did not inhibit significantly the elongation of cucumber hypocotyl at neither dose (0.125, 0.25, 0.50 and 1 µL/mL) assayed, inhibiting significantly the radicle elongation by 25.61%, 19.51%, 41.85%, 25.69%, 28.43% and 31.20% respectively with respect to control at the highest dose (1 µL/mL) applied (Table 2, Figure 2). Tea tree essential oil at the highest dose assayed showed significant inhibitory effect in both hypocotyl (34.61%) and root (43.80%) development (Table 2, Figure 2). Previous studies showed than rosemary, mastic thyme, eucalyptus, wintergreen and marjoram essential oils were able to inhibit both hypocotyl and radicle elongation of weeds infesting cucumber and other crops, such as *L. multiflorum*, *E. crus-galli* and *P. oleracea* (Ibáñez and Blázquez 2018d, 2018b, 2018c, 2017). However, eucalyptus essential oil reduced the seedling height of other weeds like *Amaranthus blitoides* in 61.65-37.92 and 34.67-22.17 mm under laboratory and greenhouse conditions, respectively (Rassaeifar *et al.* 2013) as well as the growth reduction of lettuce when eucalyptus leaves was incorporated into the soil as green manure during 30-day of treatment (Puig *et al.* 2018).

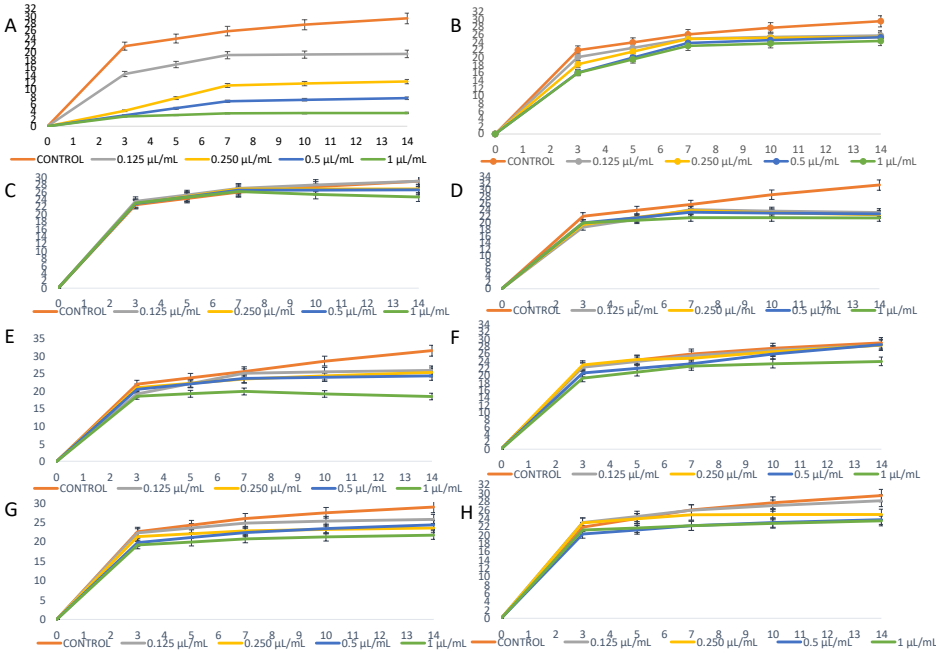


Figure 2. Seedling growth of cucumber (mm) (mean ± s.e.) with oregano (A), rosemary (B), T. mastichina (C), basil (D), tea tree (E), eucalyptus (F), wintergreen (G) and marjoram (H) essential oils. Control and treated with 0.125, 0.25, 0.50 and 1 µL/mL.

In general, it was observed that the radicle of cucumber was more sensitive than the hypocotyl to the tested essential oils. Previous studies have also reported the extra retarded root elongation of cucumber after the application of *Cryptocarya massoy* essential oil reaching 90% reduction percentage at only 100 µL/L (Rolli *et al.* 2016).

3.2.2. *Essential oils phytotoxicity against tomato*

The seedling development of tomato was more sensitive than cucumber to the tested essential oils (Figure 2 *vs* Figure 3). All essential oil here studied exerted certain phytotoxicity over its hypocotyl and radicle (Table 2, Figure 3).

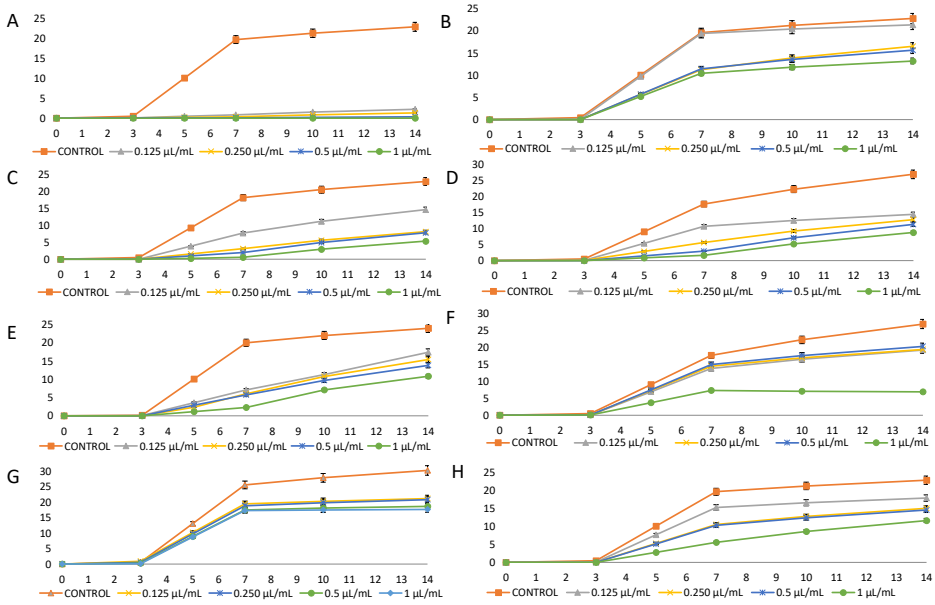


Figure 3. Seedling growth of tomato (mm) (mean ± s.e.) with oregano (A), rosemary (B), T. mastichina (C), basil (D), tea tree (E), eucalyptus (F), wintergreen (G) and marjoram (H) essential oils. Control and treated with 0.125, 0.25, 0.50 and 1 µL/mL.

Oregano essential oil was the most harmful essential oil for tomato as although it significantly inhibited the seedling growth starting from 0.125 µL/mL without remarkable differences between all doses (0.125, 0.25, 0.50 and 1 µL/mL) assayed. The reduction percentages observed were quite considerable reaching 88.29-100% and 91.33-100% in both hypocotyl and radicle, respectively (Table 2). This potent herbicidal activity is consequence of carvacrol (60.42%) contain (Figure 1) which is the main component in oregano essential oil here used and other *Origanum* spp. like *O. acutidens* (carvacrol 87.0%) (Kordali et al. 2008). In fact, due to their strong phytotoxicity, carvacrol and their isomer thymol have been added together with clove oil in a herbicidal mixture (Rohlfsen 2008).

Previous studies have also reported that 1,8-cineole, the main component in eucalyptus (76.43%) marjoram (59.59%) and mastic thyme (49.94%)

essential oils here employed (Figure 1), has dose-dependent herbicidal activity against weeds and food crops, such as annual ryegrass (*Lolium rigidum*) and *R. sativus*, being this activity further improved by its derivatives (Barton *et al.* 2010). In this sense mastic thyme essential oil produced reduction percentages of both hypocotyl (72.92%) and radicle (78.62%) growth of tomato in a dose-dependent manner (Table 2).

Although rosemary (1,8-cineol 24.95%, camphor 20.45% and α-pinene 16.7%) and wintergreen (methyl salicylate 99.63%) essential oils were the less phytotoxic essential oils, also significantly affected the seedling length of tomato with values of reduction of hypocotyl (45.53-39.33%) and radicle elongation (40.03-44.41%), respectively at the highest dose (1 µL/mL) assayed (Table 2). Rosemary essential oil showed phytotoxic activity against weeds and food crops in general as it also reduced the seedling growth of *B. tectorum* and tomato by 56.70 and 26.70%, respectively, compared with control (Hazrati et al. 2018)

These results are according to other studies in which rosemary essential oil has minimal allelopathic effect than *Thymus vulgaris* and *Pimpinella anisum* on tomato (Shokouhian et al. 2016).

4. CONCLUSIONS

The essential oils of *O. vulgare, R. officinalis, T. mastichina, O. basilicum, M. alternifolia, E. globulus, G. procumbens* and *O. majorana* rich in both oxygenated (carvacrol, 1,8-cineol, camphor and terpinen-4-ol) and hydrocarbonated (α-pinene, *p*-cymene and γ-terpinene) monoterpenes as well as aromatic compounds (methyl chavicol and methyl salicylate) able to inhibit seed germination of several weeds showed less phytotoxic effects on seed germination of cucumber and tomato. In general, these crops were

more tolerant than weed to the majority of these essential oils. So, the tested essential oils could be a source of bioherbicides for selective weed control.

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Annexes

ANNEX I. EXAMPLES OF ESSENTIAL OILS: CHEMICAL COMPOSITION AND MAIN BIOLOGICAL ACTIVITIES.

Plant name	Biological activities	References
Anise (*Pimpinella anisum* L.)	-Carminative. -Antimicrobial, insecticidal, antiviral, muscle relaxant of tracheal chain, anticonvulsant, analgesic, antioxidant.	(1,2)
Basil (*Ocimum basilicum* L. ssp. *basilicum*)	-Alleviation of mental fatigue, colds, spasms, rhinitis and pain derived from cysts in ovary. First aid treatment for wasp stings and snakebites. -Antimicrobial, insecticidal, antioxidant and enzyme inhibitory effects: management of type II diabetes and hypertension.	(3–6)
Eucalyptus (*Eucalyptus globulus* Labill.)	-Antiseptic (wounds, cuts, scratches and punctures), fever, anti-inflammatory (gingivitis, rheumatism).	(7)
Ginger (*Zingiber officinale* Rosc.)	-Antioxidant, pain relief, anti-inflammatory, antidiabetic, lipid-lowering, anti-obesity (nutraceutical).	(8,9)
Lavender (*Lavandula angustifolia* Mill.)	-Antimicrobial. -Analgesic (rheumatism, headache), anti-inflammatory (bruises), prevention of hair loss, athlete's foot.	(10,11)
Lemon eucalyptus (*Eucalyptus citriodora* Hook)	-Antioxidant, antimicrobial, insect repellent.	(12–14)
Marjoram (*Origanum majorana* L.)	-Treatment of insomnia, nervousness and stress, rheumatism and muscle aches.	(15–17)

	-Antioxidant, antimicrobial, anti-inflammatory, cardio and hepatoprotective.	
Oregano (*Origanum vulgare* L.)	-Wound disinfection and pain relief. -Antioxidant, anti-inflammatory antidiabetic, antimicrobial, antiviral and antifungal. -Toxic against houseflies.	(18–20)
Peppermint (*Mentha piperita* L.)	-Antimicrobial, antioxidant. -Anti-inflammatory, antiseptic, bactericidal, antibronchitic, expectorant, antispasmodic and carminative.	(21,22)
Rosemary (*Rosmarinus officinalis* L.)	-Analgesic, antioxidant.	(23)
Scots pine (*Pinus sylvestris* L.)	-Antimicrobial, larvicidal.	(24,25)
Spanish marjoram (*Thymus mastichina* L.)	-Antioxidant.	(26)
Tea tree (*Melaleuca alternifolia* Maiden & Betche ex Cheel)	-Antimicrobial, analgesic, anti-inflammatory. -Anti-acne, antiseptic, treatment of mosquito bites and athlete's foot, immunology enhancer, deodorant.	(27,28)
Turmeric (*Curcuma longa* L.)	-Anti-inflammatory, antidiabetic, antineurodegenerative.	(29)
Wintergreen (*Gaultheria procumbens* L.)	-Antioxidant, antimicrobial, analgesic: treatment of rheumatism.	(30,31)

Winter savory (*Satureja montana* L.)	-Stimulator of the digestive system (stomachic and eupeptic). -Expectorant. -Antimicrobial, antioxidant and inhibitor of human serum cholinesterase. Treatment of foodborne and neurological diseases, wounds and other infections. -Acute toxicity against mosquito larvae.	(32–34)

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