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CYCLE XXXII

DIRECTOR Prof. Paolo Pinton

Hyssopus officinalis L. subsp. _aristatus_ (Godr.) Nyman (Italy) and _Ocimum campechianum_ Willd. (Ecuador): botanical identification, chemical and biological characterization

Scientific/Disciplinary Sector (SDS) BIO/15

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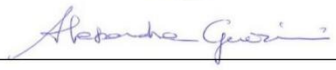
Visto: Il Tutore Si approva Firma del Tutore 

For my daughters Soledad and Doménica, as an example of overcoming and dedication to what I love most.

Paulina

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I want to thank God for the life I have, the blessings received and the opportunity to continue advancing in acquiring knowledge.

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Paulina

Abstract

The main focus of my PhD project is to valorize the biodiversity of both countries, Italy and Ecuador, studying two species belonging to Lamiaceae family: *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman an endemic plant of Italy and *Ocimum campechianum* Willd., a native species from Ecuador. Botanical identification and characterization of the aerial parts of these species through macroscopic and microscopic approaches was done. Screening and structural determination of main phenolics components through TLC and HPLC-DAD was performed, as well as the GC-MS characterization of essential oils (EOs) obtained by hydrodistillation. The main compounds were separated through flash chromatography and structurally identified through NMR. The potential use of the EOs and the alcoholic extracts (AEs) (ethanol 70%, methanol) in health applications was evaluated through *in vitro* antioxidant, antimicrobial, cytotoxic and mutagenic tests. *H. officinalis* subsp. *aristatus* has been harvested in natural habitats in Abruzzo, Civitaretenga (AQ) in 2016, Navelli and on Majella in 2018. The EOs obtained form hydrodistillation were characterized by different main compounds: Civitaretenga EO was characterized by limonen-10-yl-acetate, Navelli EO by *cis*- (43.2%) and *trans*-pinocamphone (11.0%) and Majella EO by methyleugenol (41.5%) and 1,8-cineole (39.7%). These evidences confirm the fundamental importance of the area in which plant grow in guiding the synthesis of secondary metabolites and directing the research towards further investigations into the area of Civitaretenga and other parts of Abruzzo. All AEs showed, in order of relative abundance of the main phenols, chlorogenic, rosmarinic and caftaric acid. The amount of chlorogenic acid for the specimen collected in Civitaretenga was about twice as much as in the other extracts. The AEs highlighted an interesting antioxidant capacity (DPPH test). Regarding the cytotoxicity, no significant results were observed for EOs on A549 cell line, while for the ethanolic extract an encouraging reduction in both the viability and the migration was observed. The cytotoxicity of the main molecules characterizing the extracts did not provide any appreciable results at the tested concentrations. The extracts did not show any cytotoxicity against HaCat cell line, giving indication of safe use, confirmed also by Ames test. The aerial parts of *O. campechianum* were collected from a wild population in the Amazonian region (Pastaza, Ecuador). The EO obtained by hydrodistillation was characterized by eugenol (46.55%), β-caryophyllene (11.94 %), β-elemene (9.06%) and 1,8-cineole (5.35%). The methanolic and ethanolic extracts, chemically characterized for the first time in this research, showed a similar fingerprinting, with the main presence of rosmarinic acid, followed by caftaric, chlorogenic acids as minor components. The ethanolic extract exhibited the greatest abundance of every compound. In particular, the quantities of caftaric and chlorogenic acid were about twice as much as in the other extract, while rosmarinic acid was nearly four times more abundant. A preliminary screening of the extracts against A549 cell line were performed but with negative outcomes. On the other hand, extracts did not show any cytotoxicity against HaCat cell line, giving indication of their safe of use. Regarding the antibacterial activity of essential oil resulted the most active preparation with moderate antimicrobial properties. All extracts exhibited a noteworthy antioxidant capacity (DPPH): in particular, the IC_{50} values of the EO (7.7±0.1 µg/ml) and ethanolic extract (11.1±0.01µg/ml) were close to the one of the positive control (Trolox). Rosmarinic acid and eugenol, respectively tested as main components of AEs and EO showed values close to Trolox. AEs and EO did not reveal mutagenic activity. The results confirmed very promising properties for the formulation of cosmetic and food supplements products.

Abstract

L'obiettivo principale del mio progetto era di valorizzare la biodiversità dei paesi, Italia e Ecuador, studiando due specie appartenenti alla famiglia delle Lamiaceae: *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman una specie endemica italiana e *Ocimum campechianum* Willd. autoctona dell'Ecuador. È stata effettuata l'identificazione botanica e la caratterizzazione delle parti aeree di queste specie attraverso osservazioni al microscopio, lo screening e la determinazione strutturale dei principali componenti fenolici attraverso TLC e HPLC-DAD, nonché la caratterizzazione GC-MS di oli essenziali (EO) ottenuti per idrodistillazione. I principali composti sono stati separati mediante flash cromatografia e identificati strutturalmente tramite NMR. Il potenziale utilizzo degli OE e degli estratti alcolici (EA) (etanolo 70%, metanolo) per applicazioni nell'ambito salutistico è stato valutato mediante test antiossidanti, antimicrobici, citotossici e mutageni *in vitro.* Da *H. officinalis* subsp. a*ristatus*, raccolto in habitat naturali in Abruzzo (Civitaretenga (AQ) nel 2016, Navelli e in Majella nel 2018), sono stati ottenuti OE caratterizzati da diversi composti principali: Civitaretenga OE da limonen-10-il-acetato, Navelli OE da *cis*- (43,2%) e *trans*-pinocanfone (11,0%) e Majella OE da methileugenolo (41,5%) e 1,8-cineolo (39,7%). Queste risultati sottolineano l'importanza fondamentale dell'area in cui le piante crescono nel guidare la sintesi dei metaboliti secondari e nel dirigere la ricerca verso ulteriori indagini nell'area di Civitaretenga e in altre parti dell'Abruzzo. Tutti gli EA hanno mostrato, come principali fenoli, acido clorogenico, rosmarinico e caftarico. La quantità di acido clorogenico per il campione raccolto in Civitaretenga era circa il doppio rispetto agli altri estratti. Gli EA hanno evidenziato un'interessante capacità antiossidante (test DPPH). Per quanto riguarda la citotossicità, non sono stati osservati risultati significativi per gli OE sulla linea cellulare A549, mentre per l'estratto etanolico è stata osservata una incoraggiante riduzione sia della vitalità che della migrazione. Lo studio della citotossicità delle principali molecole che caratterizzano gli estratti non ha fornito risultati apprezzabili alle concentrazioni testate. Gli estratti non hanno mostrato alcuna citotossicità nei confronti della linea cellulare HaCat, fornendo indicazioni per un uso sicuro, confermato anche dal test di Ames. Le parti aeree di *O. campechianum* sono state raccolte allo stato spontaneo nella regione amazzonica di Pastaza (Ecuador). L'OE ottenuto per idrodistillazione era caratterizzato da eugenolo (46,55%), β-cariofillene (11,94%), β-elemene (9,06%) e 1,8-cineolo (5,35%). Gli estratti metanolici ed etanolici, caratterizzati chimicamente per la prima volta in questa ricerca, hanno mostrato come componente principale acido rosmarinico, seguito da acido caftarico e clorogenico. L'estratto etanolico possedeva quantità di acido caftarico e clorogenico circa il doppio rispetto all'altro estratto, mentre l'acido rosmarinico era quasi quattro volte più abbondante. Uno screening preliminare degli estratti contro la linea cellulare A549 ha con esiti negativi. Gli estratti non hanno mostrato alcuna citotossicità nei confronti della linea cellulare HaCat, fornendo indicazioni sulla loro sicurezza d'uso. L'OE è risultato la preparazione più attiva per le proprietà antimicrobiche, comunque moderate. Tutti gli estratti hanno mostrato una notevole capacità antiossidante (DPPH): in particolare, i valori IC_{50} di OE (7,7 ± 0,1 µg/ml) ed estratto etanolico (11,1 ± 0,01µg/ml) erano vicini a quello del controllo positivo (Trolox), come pure quelli dell'acido rosmarinico e dell'eugenolo, rispettivamente componenti principali degli EA e dell'OE. Tutte le preparazioni non hanno rivelato attività mutagena. I risultati hanno confermato proprietà molto promettenti per la formulazione di prodotti cosmetici e integratori alimentari.

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INTRODUCTION

Ever since ancient times, people looked for drugs in nature to identify remedies for their diseases. The beginnings of the medicinal plants' use were instinctive, as is the case with animals. At those times, information either concerning the origins of the illnesses or which plants could be used to cure diseases was not available: all knowledge was empiric. In time, the reasons for the usage of specific medicinal plants for the treatment of certain diseases were being discovered and gradually became founded on scientific bases (Fretes, 2010; Petrovska, 2012). As well known, academic medicine practiced in the 16th and 17th centuries was born in the Mediterranean world, as a result of the crossing between the rich healing traditions of the people living in southern Europe, Northern Africa, and the Middle East. Since the 16th century, with the consolidation of long-distance marine routes, such as those of New World, other cultural hybridizations had a significant impact on medical practice. The assimilation in Europe of American plants was a complex process that led to profound changes in European society and culture. Its introduction and use gave rise to substantial changes in food and medicines used until then by the Europeans, but also in gardens and landscapes, drugs, wood, dyes, and many other things of practical application (López, 2012). In the following centuries, until the advent of iatrochemistry, plants were the source of treatment and prophylaxis. However, in the last few decades, synthetic drugs began to show increasing side effects, and their use began to decrease, accompanied by a newly rediscovered interest in natural drugs in traditional medicine and, in particular, herbal medicines both in developed and developing countries (OMS, 2003).

Plants had always been in active competition for space, light, and soil resources, and such perennial evolutionary pressure has forged a vast array of secondary metabolites (Rolli et al., 2014).

In Central and South America, many aromatic plants are used as flavorings of beverages and soups, as medicine and as herbicides (Charles et al., 1990). Tropical forests contain a significant percentage of the world's plant species, including those with unique and more varied biochemical modes of defense and survival than their temperate counterparts. Consequently, tropical forests can provide natural products with invaluable compounds that are starting points for the development of new drugs. Although relatively few tropical species have been studied for their pharmaceutical potential (generally accepted to be less than 1%), the tropics have yielded numerous invaluable pharmaceutical compounds. Including the anticancer agents: vincristine and vinblastine from *Catharanthus roseus*, the muscle relaxant d-tubocurarine from *Chondodendron* and *Strychnos* species, which were originally used in the Amazon for arrow poisons, and steroids from *Dioscorea* spp. (Andresen et al., 2018; Guerrini et al., 2016; Sacchetti et al., 2004).

In South America, Ecuador is a megadiverse and beautiful country, recognized for its variety and wealth of plants and animals worldwide. Because of its location, the development of the privileged flora and fauna are distributed in the Cordillera de Los Andes, paradisiacal coasts, mysterious Amazon jungles, as well as the natural laboratory of the Galapagos, concentrating 10% of all the plant species in the world. It has more than 17,058 species of vascular plants or flowering plants, as

indicated in the Fourth National Report for the Convention on Biological Diversity (Ministerio del Ambiente del Ecuador, 2010).

The aromatic natural resources of the Amazon are considered an appropriate renewable source for the production of essential oils and flavors, as well as a clear economic alternative to sustainable development, with a real prospect of generating wealth for the region. Numerous studies have been conducted in the Amazonian region of Ecuador, particularly describing the chemodiversity of essential oils as well as other natural bioactive ingredients that are being applied in food, beverages, cosmetics, pharmaceuticals and pesticides (Guerrini et al., 2016; Lubbe & Verpoorte, 2011; Maia et al., 2009; Sacchetti et al. 2006). However, overharvesting of species in the wild for pharmaceutical, herbal, and traditional medicines has often resulted in the depletion of valuable species, and any program that attempts to promote the use of these species must incorporate strategies for the sustainable sourcing of raw materials.

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

To generate scientific development solidly, the Amazon State University (UEA) has encouraged the creation of research groups, with principles of sustainability, transparency and complementarity, that articulate individual efforts and talents, as well as links with other related national and international institutions, through agreements and networks (Dirección de Investigación, UEA, 2018). The mission of the UEA is "To generate science and technology, to train professionals and researchers to meet the needs of the territory, under the principles of integral and balanced sustainable development of the human being of the Amazon Region and Ecuador, conserving their ancestral knowledge and fostering their culture." The mission of the Directorate of research is "to Enhance the technical and operational capacity of the institution through research to promote national development, solve community problems and disseminate the scientific and technological knowledge of Amazonian biodiversity (Dirección de Investigación, UEA, 2018).

Italy is also among the most biodiverse European countries. The variety of biogeographical, geomorphological, and climatic conditions that characterizes its territory makes it an extraordinary area of concentration both of species and of habitat. In Italy, several hot spots have been identified, such as those located in the Tyrrhenian islands, in the Maritime and Ligurian Alps, without counting other areas, such as the Apennine chain, characterized by a high number of endemic species.

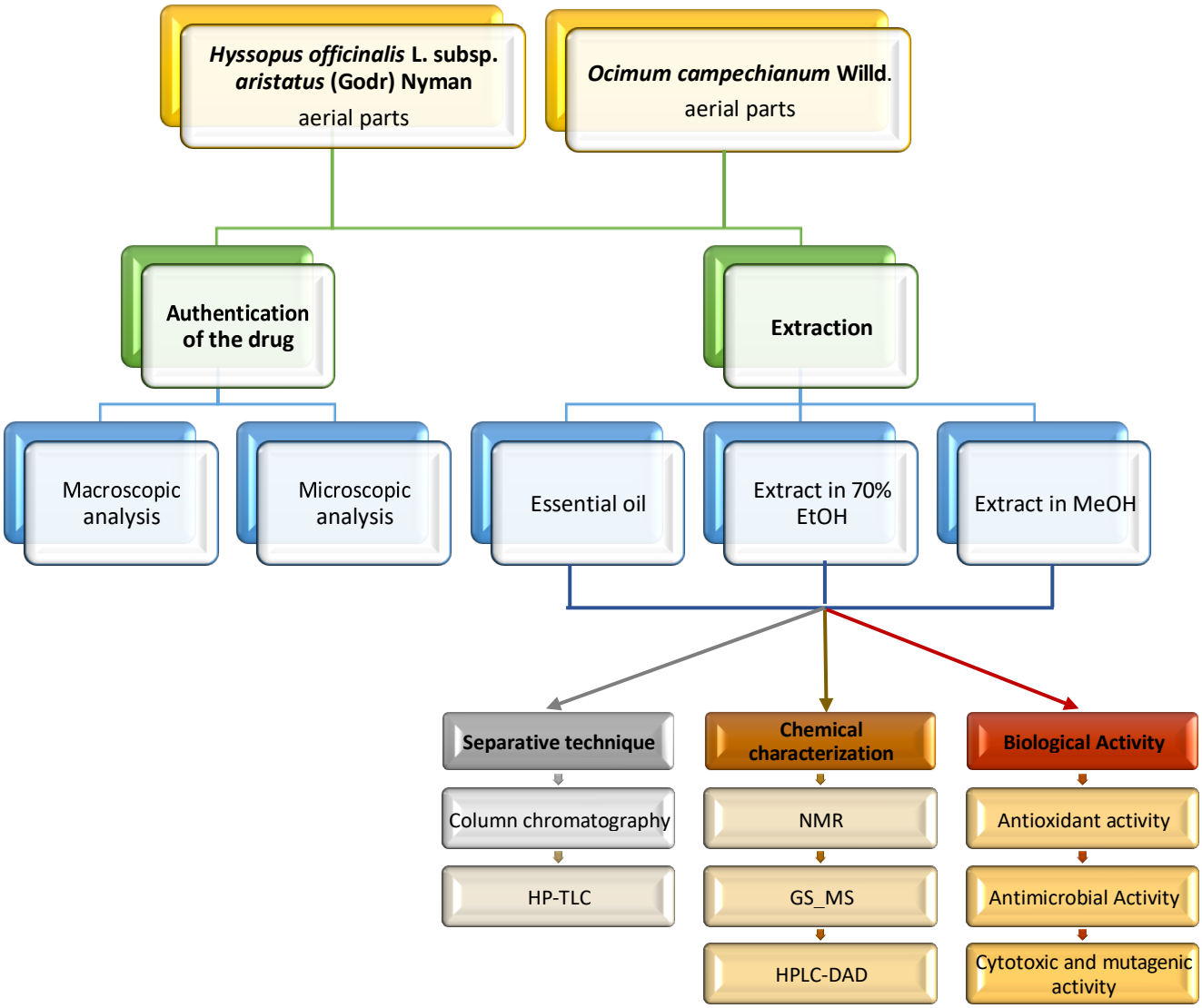
Italy is the European country that has the highest number of species; in particular, it holds about half of the plant species and a third of all animal species currently present in Europe. The Italian vascular flora includes almost 7,000 species, of which 16% are endemic species. At the quantitative level, the highest number of species is found in the regions characterized by more significant environmental variability and by those with larger territories: Piedmont (3,304 species), Tuscany (3,249), Veneto

(3,111), Friuli Venezia Giulia (3,094), Lazio (3,041), Abruzzo (2,989) (ISPRA, 2019). More recently, particular attention has been directed to phytochemical studies on endemic species containing active compounds with an ethnopharmacological history and tradition. For these plants, a wide variety of climates, environmental, geological, geographical, and biological conditions has led to the diversification of species (Bianco et al., 2016).

Goals of the thesis

The main focus of my Ph.D. project is to valorize two species belonging to the Lamiaceae family, *Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman an endemic plant of Italy and *Ocimum campechianum* Willd., a native species from Ecuador, to valorize the biodiversity of both countries within the Framework Agreement of Cooperation between UNIFE and UEA. Botanical identification and characterization of the aerial parts of these species through macroscope and microscope approaches was done. Screening and structural determination of main phenolics components through TLC (Thin-Layer Chromatography) and HPLC-DAD (High-Performance Liquid Chromatography-Diode Array) were performed, as well as the evaluation of the volatile fraction of aerial parts by hydrodistillation and GC-MS characterization (Gas Chromatography-Mass Spectrometry). The main compounds were separated through flash chromatography and structurally identified through nuclear magnetic resonance (NMR). A potential application of essential oils and extracts for health purposes was determined through *in vitro* antioxidant, antimicrobial, cytotoxic, and mutagenic tests.

Exemplification of the experimental work:



1.A Systematic placement of *Hyssopus officinalis* subsp. *aristatus*

Division: Magnoliophyta
Class: Magnoliopsida
Subclass: Asteridae
Order: Lamiales
Family: Lamiaceae
Subfamily: Nepetoideae
Tribe: Mentheae
Genus: *Hyssopus* L.
Species: *Hyssopus officinalis* L.
Subespecies: *H. officinalis* subsp. *aristatus* (Godr.) Nyman

Synonyms of *Hyssopus officinalis*

Hyssopus alopecuroides Fisch. ex Benth.
Hyssopus altissimus Mill.
Hyssopus angustifolius M.Bieb.
Hyssopus beugesiacus Jord. & Fourr.
Hyssopus caucasicus Spreng. ex Steud.
Hyssopus decumbens Jord. & Fourr.
Hyssopus fischeri Steud.
Hyssopus hirsutus Hill
Hyssopus judaeorum Sennen
Hyssopus myrtifolius Desf.
Hyssopus orientalis Adam ex Willd.
Hyssopus passionis Sennen & Elias
Hyssopus polycladus Jord. & Fourr.
Hyssopus pubescens Jord. & Fourr.
Hyssopus recticaulis Jord. & Fourr.
Hyssopus ruber Mill.
Hyssopus schleicheri G.Don ex Loudon
Hyssopus torresii Sennen
Hyssopus vulgaris Bubani
Thymus hyssopus E.H.L.Krause

Synonyms of *Hyssopus officinalis* subsp. *aristatus.*

Hyssopus aristatus Godr.
Hyssopus officinalis subsp. *pilifer* (Pant.) Murb.

Common names:

hyssop, hysope officinale, Ysop, hissopo, hisopo, isop, shen xiang cao, issop obyknovennyj.

Figure 1: Images by Marinella Miglio, Department of Life Sciences, University of Trieste.
http://luirig.altervista.org/flora/taxa/index2.php?scientific-name=hyssopus+officinalis+subsp.+aristatus

1.A.1 General aspects of *H. officinalis*

In the plant kingdom, the Lamiaceae family displays plants and species with polyphenolic contents, and their antioxidant properties are well known (Vlase et al., 2014). *H. officinalis* is a polymorphic species that belongs to the Lamiaceae family (Hajdari et al., 2018). The generic name Hyssopus, comes from the Greek word Hyssopos derived from ezob, which is similar to the hyssop the Jews used to spray; the specific name "officinalis" refers to its medicinal application (Munoz et al., 2001). It is an evergreen shrubby grass (Henry, 1913) that grows in dry, rocky, calcareous (Rosato et al., 2018) sandy soils (Henry, 1904) and in the Mediterranean climatic conditions (Brockman, 2016). It is distributed in Africa, Asia, and Europe. It has been cultivated in Asia-Temperate, China, Europe, and North America (Canada, United States) (U.S. National Plant Germplasm System, 2018).

This plant is cultivated in gardens (Bordeaux, 1959-1961) and known as an ornamental bee's attractant plant (Hajdari et al., 2018). It is a perennial resistant herb with an aromatic (Henry, 1915), pungent (Henry, 1904) odor. It is an erect plant with narrow, bright, and dark green leaves, small blue flowers born in whorled spikes, and sometimes they could be white or pink (Henry, 1915). The parts used are the flowering tops and leaves (Henry, 1907) due to their medicinal properties (Henry, 1914; Henry 1920). The plant contains polyphenolic compounds as apigenin, quercetin, diosmin, and luteolin. The essential oil of the aerial parts contains terpenoids as pinocamphone, isopinocamphone, and β-pinene (Brockman, 2016).

This plant is used to treat rheumatic pains, bruises, wounds, states of anxiety and hysteria, to regulate blood pressure, as well as in physiotherapy over the centuries. However, it is necessary to be alert about dosage due to the presence of epileptic substances such as pinocamphone and

isopinocamphone (Hajdari et al., 2018). The extracts are used as a stimulant, tonic, and in perfumery (Henry, 1931). It acts at the bronchial level, and subsequently, it has beneficial for asthma and colds (Henry, 1904). Other uses of this extract are in the cosmetic industry, and in the food industry as a condiment (Bordeaux 1959-1961; Rosato et al., 2018)

ISO 9841:2013 (confirmed in 2019) specifies certain characteristics of the *H. officinalis* ssp. o*fficinalis* essential oil. The essential oil is obtained through steam distillation of the leaves and presents the following features: it is liquid, transparent with a mobile appearance, the pale color yellow to yellowish-brown, and with a characteristic odor. Moreover, the essential oil has a relative density at 20 ° C (Min 0.920 - Max 0.950), a refractive index at 20 ° C (Min 1.475 - Max 1.486), optical rotation at 20 ° C between (-25 ° and -10 °), acid value of less than or equal to 2.0 and typical chromatographic profile (Table 1).

Table 1 — Chromatographic profile

Components	Minimum %	Maximum %
α-Pinene	0,4	1,5
β-Pinene	7,0	20,0
Sabinene	1,0	3,5
Limonene	0,6	4,0
Myrtenyl methyl ether	0,9	3,0
Pinocamphone	8,0	25
Isopinocamphone	25,0	45,0
β-Bourbonene	0,8	2,6
β-Caryophyllene	1,0	3,0
Alloaromadendrene	1,0	3,0
Germacrene D	1,2	4,5
Elemol	0,2	2,5
Spathulenol	0,1	1,5

Source: ISO 9841 (2013)

1.A.2 Botanical aspects of *H. officinalis.*

H. officinalis is a perennial and semi-perennial herbaceous plant with a pleasant smell. It is a shrub or sub-shrub that grows up to 0.2-0.6m in height. Its stems are straight, woody, and branched at the base. The leaves are lanceolate (2-4 cm long and 2-7 mm wide) opposite on the stem, generally revolute, and it presents covering, and glandular trichomes that produce essential oil: the color is dark green. The flowers can be blue, pink, pale violet, and white or purple. They have an aromatic odor; their exterior is pubescent, with corollas long between 7 to 11 mm. They are hermaphrodite flowers, pollinated by insects mainly by bees and the flowering season is from July to September and even November. The seeds mature from August to October. The ripe fruit is small, oblong, dihedral, dark brown, and up to 25 mm in length. Hyssop reproduction occurs by generative or vegetative propagation. The Hyssop main root is woody and fasciculate. This plant is suitable for a hot climate and grows in well-drained sandy or silty soils. The plants could be cultivated for approximately ten years, and the crops can be harvested several times, in spring, in the flowering stage, and when the

flowers get dry. Hyssop E.O.s (essential oils) is obtained by steam distillation with yields of around 0.2%-1.2% in France, Spain, Italy, Germany, Slovenia, Moldova, Bulgaria, Iran, China, and India (Brockman, 2016; Preedy, 2015; Venditti et al., 2016).

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1.A.3 Traditional uses

It is commonly used as an aromatic herb and medicinal plant, particularly carminative, tonic, antiseptic, expectorant, and antitussive (Brockman, 2016; Rosato et al., 2018). The leaves, tips or young buds, and fresh or dried flowers are consumed as an aromatic condiment for soups and salads. It has a slightly bitter taste due to tannins and an intensive mint aroma: for this reason, its use is moderate. The hyssop is also used to flavored liquors and is part of the official component of Chartreuse (French liquor). An infusion made with leaves is used to control bacterial diseases of plants. The hyssop essential oil obtained from the leaves is antiseptic and is used in perfumery. The average oil yield is 0.5-0.6% (Brockman, 2016; Preedy, 2015). In other studies, the extracts and the essential oil isolated from the hyssop show antiviral and antifungal properties *in vitro* (Vlase et al., 2014).

Hyssop is used to cure respiratory diseases such as tuberculosis, asthma, cough, sore throat, respiratory infection, as well as pain, menstrual cramps. It can help to prevent kidney stone formation, and it promotes the digestion of fatty foods and, finally, shows anthelmintic activity. The decoctions or essential oil is used on the skin to treat burns, frostbite, bruises, skin irritations, and to decrease the effects of skin aging. Hyssop is effective against clinical herpes common virus, HSV-1, and HSV-2 strains. The plant extracts have antibacterial activity against *Propionibacterium acnes*. Essential oil is used externally to treat toothache, earache, and rheumatism; it is also used for massage, promoting positive physiological effects on blood circulation and muscle relaxation. The natural extracts of dried leaves of *H. officinalis* have shown intense anti-HIV activity and are useful to treat AIDS patients (Preedy, 2015)

1.A.4 Phytochemical, pharmacological and toxicological aspects of *H. officinalis* and their subspecies

The chemical variability of the essential oil and other components in plant drugs is due to factors of origin, time of harvest, genetic diversity, and biotic stress.

Preedy (2015) describes in his book, that the hyssop contains polyphenols, as acids (chlorogenic, p-hydroxybenzoic, protocatechuic, ferulic, feruloylquinic, syringic, vanillic, p-coumaric, rosmarinic, caffeic) and flavonoids, polysaccharides, tannins, pigments, and resins. The natural bioactive compounds are hissopin (a glucoside), dosmin and hesperidin (flavonoids), marrubin (a bitter diterpenoid), gentisic, ursolic, and oleanic acids. Its essential oil is a light yellow liquid, has an herbal scent similar to camphor with warm and spicy nuances: the most important components are isopinocamphone (syn. *cis*-3-pinanone), pinocamphone (syn. *trans*-3-pinanone) and their precursor

β-pinene. Others minor components are pinocarvone, sabinene, germacreme D, germacren D-4-ol, α-β-phellandrene, 4-carvomenthol, thymol, carvacrol, ethenol, limonene, linalool, 1,8-cineole, α-terpinen, myrtenyl acetate, methyleugenol and others.

Zheljazkov et al. (2012) conducted field and laboratory experiments to evaluate the productivity and composition of essential oils of hyssop (*H. officinalis*) depending on the year, harvest time and drying; the content of hyssop oil varied from 0.13 to 0.26% (general average of 0.19%). The main components were pinocamphone + isopinocamphone (57-75%) and β-pinene (5-15%). The late hyssop harvest increased the concentrations of β-pinene, myrcene, and limonene + cineole, but reduced the pinocamphone + isopinocamphone.

Figueredo et al. (2012) in their study of the chemical composition of the essential oil obtained by hydrodistillation of the aerial parts of Turkey *Hyssopus officinalis* reported among the main constituents pinocarvone (29.2%), *trans*-pinocamphone (27.2%), β-pinene (17.6%), *cis*-pinocamphone (4.7%) and myrcene (2.92%).

Michalczyket al. (2012) studied with a panel of assessors the effect of adding essential oils of hyssop and coriander at the highest concentration (0.02% v/p) sensorially acceptable on the microbiological and biochemical characteristics of stored ground beef. They reported that the dominant component in the hyssop was the *cis*-pinocamphone.

Capuzzo et al. (2013) extracted hyssop through supercritical fluids technology: they showed that main components were sabinene, isopinocamphone and pinocamphone and their relative concentrations are related to different conditions of pressures, temperatures, extraction times (dynamic and static) and a small quantity of methanol used as cosolvent.

Vlase et al. (2014) when evaluating the HPLC phenolic profile for aerial parts of *Hyssopus officinalis* reported the identification in the ethanolic extract of caftaric, gentisic, caffeic, chlorogenic, *p*-coumaric, rosmarinic and ferulic (the most abundant) acids, three flavonoid glycosides, whose isoquercitrin in the largest amount, rutin and quercitrin and two flavonoid aglycones, quercetin, and luteolin.

The chemotypic profile of essential oils obtained from cultivated populations of *H. officinalis* from different areas (Poland, Spain, Turkey, Italy, Serbia, India) of the Eurasian continent and of *H. officinalis* subsp. *angustifolius* grown in Turkey showed pinocamphone/isopinocamphone monoterpenes as dominant components, but essential oils belonging to plants of *H. officinalis* subsp. *officinalis* grow in Poland, *H. officinalis* var. *decumbens* grown in France and *H. officinalis* subsp. *aristatus* grown in Italy revealed significant differences. Similar degrees of similarity/dissimilarity has also been found for the phenolic profile of *H. officinalis*, *H. officinalis* subsp. *aristatus*, and for *H. officinalis* subsp. *pilifer*, which shares a composition based on chlorogenic acid and its quinic isomeric derivatives, while *H. officinalis* subsp. *angustifolius* grown in Turkey displayed the presence of a composition dominated by p-coumaric acid, benzoic acid, o- coumaric acid, ferulic

acid, and quercetin (Hajdari et al., 2018). *H. officinalis* essential oil exhibited fungistatic and fungicidal effects (Letessier et al., 2001).

The antibacterial activity of the essential oil is probably due to monoterpenes that interact with the cell membranes against the microorganisms, profoundly damaging the lipid bilayers (Rosato et al., 2018).

Hyssop extracts inhibit oxidation and lipid degradation of the pigments caused by cooking and storage, so it is used to prevent the oxidation and discoloration of lipids in the processed meat (Fernandez-Lopez et al., 2003). At the pharmaceutical level, it is used as antiseptic, against chronic bronchitis and for the treatment of asthma. Also, for applications in treatments of rheumatic pain, bruises, wounds, blood pressure regulation, states of anxiety and hysteria, it has a muscle relaxation activity. Additionally, the new publications on the ethanolic extract of *H. officinalis* it is reported to have gastrointestinal protection properties estimated by the mucus content of gastric adhesion (Džamić et al., 2013).

Preedy (2015) also added that hyssop preparations and essential oils are used in veterinary medicine, in domestic animals as anthelmintic remedies and for the treatment of gastrointestinal disorders. These preparations possess insecticidal properties against flies, flea beetles, green caterpillars, mites, larvae of some worms, cabbage moths, slugs, and others. At the same time, they attract hummingbirds, butterflies, bees, and other animals, who are excellent pollinators for the plants growing in the hyssop's neighborhood.

In general, the extracts and the essential oil isolated from the hyssop showed moderate antioxidant and antimicrobial activity together with antifungal and antifungal properties *in vitro.* Studies with animal models indicated myorelaxant, antiplatelet, and α-glucosidase inhibitory activity for this plant (Fathiazad and Hamedeyazdan, 2011).

The evaluation of possible composition differences between different populations of *H. officinalis* subsp. *aristatus*, which aerial parts were collected in different parts of the Western Balkans (Kosovo and Albania), showed a marked different chemotype established by the profile of essential oil (1,8-cineole vs. isopinocamphone/camphone). The phenolic composition is based on chlorogenic acid and its quinic isomeric derivatives, and it is similar to that of *H. officinalis* subsp. *aristatus* studied by Venditti et al. (2015) and *H. officinalis* subsp. *pilifer* (Hajdari, et al. 2018).

Venditti et al. 2015 investigated the aerial composition parts of the essential oil of *H. officinalis* subsp. *aristatus* (Abruzzo, central Italy) and highlighted a peculiar chemotype, with linalool and methyl eugenol as main constituents. They also explain that *H. officinalis* subsp. *officinalis* is characterized by monoterpene ketones such as pinocamphone and isopinocamphone. And also, by smaller amounts of β-pinene, pinocarvone, limonene, 1,8-cineole, linalool, and camphor. Based on other studies, the chemical variability is due to factors such as origin, harvest time, genetic diversity, and biotic stress. They noted that in the *H. officinalis* subsp. *aristatus* ethanolic extracts were

characterized by chlorogenic acid (3) (3-O-caffeoylquinic acid) was the most abundant, followed by neochlorogenic acid (5-O-caffeoylquinic acid) and minor amounts of criptochlorogenic acid (4-O-caffeoylquinic acid), 4-O-feruloylquinic acid and 5-O-p- hydroxybenzoylquinic acid: rosmarinic and syringic acids were also identified.

Benelli et al. (2017) studied the chemical composition of essential oils obtained from *H. officinalis* subsp. *aristatus* as an alternative to chemical pesticides for vector control actions. The G.C.–M.S. analysis revealed high levels of oxygenated monoterpenes (51.8%), followed by monoterpene hydrocarbons (22.7%), sesquiterpene hydrocarbons (10.4%) and phenylpropanoids (9.9%). The primary compound was the monoterpene alcohol linalool (47.7%). The main representative compounds of the other groups were (Z)-β- ocimene (6.2%) and (E)-β-ocimene (5.4%), germacrene D (5.8%), and methyleugenol (9.9%), respectively. Even if the essential oil contained high levels of the insecticide linalool (47.7%), exhibited low activity (LD_{50} of 99.5 μL L^{-1}) because this monoterpene alcohol is not very effective as larvicidal against *C. quinquefasciatus* (LD_{50} of 247 mg L^{-1}). However, this is a sign that not always the insecticidal activity of essential oil depends on its main molecules, but it reflects the interaction between all essential oil components and insect physiology and behavior.

Džamić et al. (2013), in their study, investigated the chemical composition of *H. officinalis* subsp. *pilifer*. The essential oil showed among the primary compounds 1,8-cineole (36.43%), β-pinene (19.55%), isopinocamphone (15.32%) and *trans*-pinocamphone (6.39%); an aqueous deodorized extract (DAE), analyzed by LC-DAD / ESI-TOF MS, highlighted syringic, caffeoylqunic, feruloylquinic and rosmarinic acid as main compounds. Alinezhad et al. (2013) showed that ethanolic extracts of flowers, leaves, and stems *H. officinalis* var. *angustifolius* have antioxidant activities in the model of linoleic acid-induced by hemoglobin.

1.B.1 Systematic placement of *Ocimum campechianum* Mill.

Phylum: Magnoliophyta
Class: Magnoliopsida
Order: Lamiales
Family: Lamiaceae
Subfamily: Nepetoideae
Tribe: Ocimeae
Genus: *Ocimum*
Species: *Ocimum campechianum* Mill.

Synonyms:
Ocimum guatemalense Gand
Ocimum micranthum Willd.
Ocimum montanum Hook.

Common names:
albahaca del monte, Amazonian basil, wild sweet basil, wild mosquito plant

Figure 2: Images by Paulina Echeverria, Department of Earth Sciences, Amazonian State University. (2018)

1.B.2 General aspects of *Ocimum* genus

Ocimum (Lamiaceae) is a genus of aromatic and tropical plants, with about 65 species, native to tropical and subtropical Central and South America, Africa, and Asia. These species inhabit dry

areas, rocky, and humid soils. The genus has medicinal and economic importance, many species are culinary herbs, and their extracts have anti-inflammatory, analgesic, and antispasmodic properties (O'Leary, 2017). In Ethiopia, the aerial parts of *Ocimum americanum* L. and *Ocimum basillicum var. thyrsiflorum* (L.) Benth. are commonly used to preserve and to flavor butter (Sishu et al., 2010). Their essential oils are used in food, perfumery, and cosmetics industries. In Asian and African countries *O. americanum* is used to treat conjunctivitis, malaria, headache, flavor food; in South Africa, it is often named camphor basil, in Zimbabwe its uses ranges from flavor and fragrance, until the insects repellent and as a preservative for corpses (Matasyoh et al., 2006). The Mediterranean *O. gratissimum* from Africa and *O. sanctum* from India showed strong antibacterial, antifungal, antioxidant, and even adaptogenic activity. A large number of species and varieties is due to cross-pollination, with differences in the composition of the essential oils and morphological characteristics within the genus (Matasyoh et al., 2006). These popular herbs are used both as a fresh and dried food spice and also in traditional medicine (Salles et al., 2006), cosmetics, and toiletries (Sacchetti et al., 2004).

The plants of the *Ocimum* genus are subshrubs or annual or biannual herbs, generally aromatic. The main morphological characters are: stems tetragonal, leaves opposite, simple, generally petiolate, inflorescences composed of several opposite 3–6-flowered cymes (whorls), subtended by persistent or deciduous bracts: the bract scar sometimes developing into a bowl-shaped, gland-like structure which functions as an auxiliary nectary. The calyx is tubular or funnel-shaped, nerved, two-lipped, the superior lip with a round tip, the inferior lip with four lobes with acuminate apices, the two lateral lobes triangular, and the two median lobes subulate, glabrous in the inner surface. The calyx usually enlarges slightly in fruit; corolla is two-lipped, the tube included in the calyx, dorsally gibbous at the midpoint, the superior lip four-lobed, the inferior lobe entire, flat, or slightly concave. The stamens are four, didynamous, exerted, the superior pair attached near corolla base, glabrous or basally pubescent, the inferior pair attached near the corolla mouth, the anthers divergent, sometimes parallel. The style is bifid, the stigmatic branches subequal, subulate, the ovary disk equally four-lobed. The nutlets are four, spherical to ellipsoid or obovoid, the surface reticulate or smooth. (O'Leary 2017).

1.B.2.1 General aspects of *O. campechiamum*

Ocimum campechianum is distributed from Florida and Mexico to northern Argentina in the provinces of Salta, Jujuy, Chaco and Formosa. It is widespread in Central America and northern South America, where it is a common weed in crop fields. It inhabits dry areas, sometimes with rocky soils, exceptionally found in humid soils. *O. campechianum* belongs to the family Lamiaceae, and its common name is basil, maroon basil, chicken basil, and wild basil (O'Leary, 2017).

1.B.3 Botanical aspects

The plant is an annual or biannual herb, 40–60 cm high, with stems reddish or purple, woody at the base, glabrous to slightly puberulous. The leaves are ovate-elliptic, 2–10 cm long and 1–4 cm wide, membranaceous, with 0.2–4 cm long petiole, the base cuneate, the apex acute, the margin with irregular, slightly impressed teeth; the adaxial surface is light green, abaxial surface darker green, glabrous or tuberculous on the veins: both surfaces with glandular dots. The inflorescence is composed of six flowered whorls, spaced 0.8–2 cm apart, grouped in bracteate pseudo-racemes, to 8 cm long in anthesis, to 15 cm long in fruit, with a short peduncle, 1.5–2 cm long in anthesis and fruit. The bracts are ovate or subrhomboidal, persistent, with acute apices; the flower pedicels are hispid, 1–1.5 mm long in flower, becoming reflexed in fruit, 4–7 mm long. The calyx is 2.5–3 mm long in anthesis, to 8 mm long in fruit, margins hispid. The corolla is white, lilac or violet, 3–4 mm long. The stamens are glabrous, 5–6 mm long. The style is 5 mm long. The nutlets are obovoid, 1.5–2 mm long, brown, smooth (O'Leary, 2017).

1.B.4 Traditional uses

The infusion of this plant is used for gastrointestinal diseases such as ulcers, gastritis, intestinal fever, inflammation, dysentery, vomiting, stomach pain, and it has a vermifuge capacity. It is used for catarrh and bronchial infections, in stomatology, for the pain of ears, head, molars, belly, it is a soothing, diuretic and carminative remedy. The essential oil of this plant is a mosquito repellent, active against human pathogens, fungi, insects, larvae, and has antimicrobial, antioxidant, antiprotozoal, contraceptive and anti-inflammatory activity (Jaramillo et al., 2014). *O. campechianum* is known as "field basil" or "wild basil", used by the indigenous population for both culinary and medicinal purposes, excellent antimicrobial properties (Sachetti et al., 2004). The extracts of the plant contain biologically active components having insecticidal, nematocidal, fungistatic, or antimicrobial properties (Simon et al., 1999).

The plant is locally used in the treatment of epilepsy, nervous trouble, and earaches, as a remedy for influenza, colic, and convulsion in children and painful menstruation (Charles et al., 1990).

1.B.5 Phytochemical, pharmacological and toxicologal aspects

The essential leaf oils of *O. campechianum,* have been studied by many authors.

The chemical composition of leaf essential oil of *O. campechianum*, collected in North of Brazil is characterized by eugenol and β-caryophyllene as principal components (de Vasconcelos Silva et al., 1998); the same authors (de Vasconcelos Silva et al., 2004) confirmed eugenol as main component (97.0-84.0%), followed by β-caryophyllene (1.6-7.6%) and bicyclogermacrene (1.4-5.2%) for brazilian *O. campechianum* essential oil.

Vieira R. and Simon (2000) reported that O. *micranthum* from Northeastern Brazil showed high content of eugenol (44.8%) and β-caryophyllene in the leaves, whereas in the inflorescence elemene and eugenol were the major components.

For *O. micranthum* (synonym of *O. campechiamum*) cultivated in the state of Pará (Brazil) leaf essential oil was characterized by high amounts of methyl-(*E*)-cinnamate (36.8%), followed by carvone, limonene, linalool methyl-(*Z*)-cinnamate, β-caryophyllene and (*E*)-β-farnesene (Rosas et al., 2005).

Salles et al. (2006) reported eugenol (64.8%) and β-caryophyllene (11.9%) as the main components of leaf essential oil from Brazil.

The most abundant components identified in the leaf oils from *O. campechianum* collected in distinct areas at Northeastern of Brazil were: eugenol (32.2% - 60.6%), methyleugenol (60.6% - 69.5%), 1,8-cineole (0.9% - 19.7%), and elemicin (0.2% - 65.9%) (Zoghbi et al., 2007).

O. campechiamum leaf essential oil, collected from Bolivar department (Colombia), is reported to show as major compound eugenol (60.37%), followed by 1,8-cineole (12.09%), *cis*-β-terpineol, (4.25%) α-terpineol (4.43%), *cis*-ocimene (1.17%), α-caryophyllene (1.12%), α-selinene (1.15%), α-cadinene (1.27%) (Jaramillo et al., 2014).

O. micranthum leaf essential oil from Ecuador evidenced eugenol (46.5%) as main component, followed by β-caryophyllene (11.9%), β-elemene (9.1%), δ-elemene (4.2%) and a minor amount of linalool (1.5%) (Sacchetti et al., 2004). More recently, the same research groups confirmed eugenol (18.6%) and β-caryophyllene (17.0%) as the main compounds (Scalvenzi et al., 2019). Chemical constituents of essential oils from leaves of *O. campechianum*, collected in Chocó of northwest Colombian, showed as main components methyleugenol (12.0%), germacrene D (10.1%), and eugenol (9.0%) (Benitez et al., 2009).

According to Carovic'-Stanko et al. (2011) analyzed cultivated plant in Croatia and determined the main compounds of *O. campechianum* essential oil: 1,8-cineole (20.31%) and β-caryophyllene (14%).

In conclusion, eugenol, β-caryophyllene and 1,8-cineole are the most abundant compounds revealed in *O. campechianum* leaf essential oil.

Studies on biological activity showed an interesting potential against *Candida* species: antifungal compounds such as eugenol makes it a promising source of new phytotherapeutic agents to treat mycosis (Vieira P. et al., 2014). In the study of composition and functional properties of the essential oil of amazonian basil, *O. campechianum* essential oil revealed a remarkable scavenging effect, in DPPH assay, three times higher than Trolox, taken as positive control (Sachetti et al., 2004) and a promising larvicidal activity against *Aedes aegypti* (Scalvenzi et al., 2019).

Recent research confirmed the traditional use of *O. campechianum* as leaf infusion as a potential source of antihyperglycemic agents. 5-demethyl nobiletin, 5-demethyl sinensetin, together with luteolin, methylrosmarinate, and rosmarinic acid, are the main characteristic compounds. The last two metabolites appear to be responsible for the α-glucosidase inhibitory activity and the antihyperglycemic effect detected in the lyophilized infusion (Ruiz-Vargas et al., 2019).

2.1. Authentication of *H. officinalis* subsp. *aristatus* crude drug

The analysis and the authentication of the dried flowering tops of *H. officinalis* subsp. *aristatus* were carried out at the Pharmaceutical Biology Laboratory of the Department of Life Sciences and Biotechnology of the University of Ferrara, and at the Herbarium of the Botanical Garden of the University of Ferrara. The samples were collected from wild plants in Abruzzo (Civitaretenga 09/2016, Navelli 09/2018, Lama dei Peligni 10/2018) by prof. Luigi Menghini.

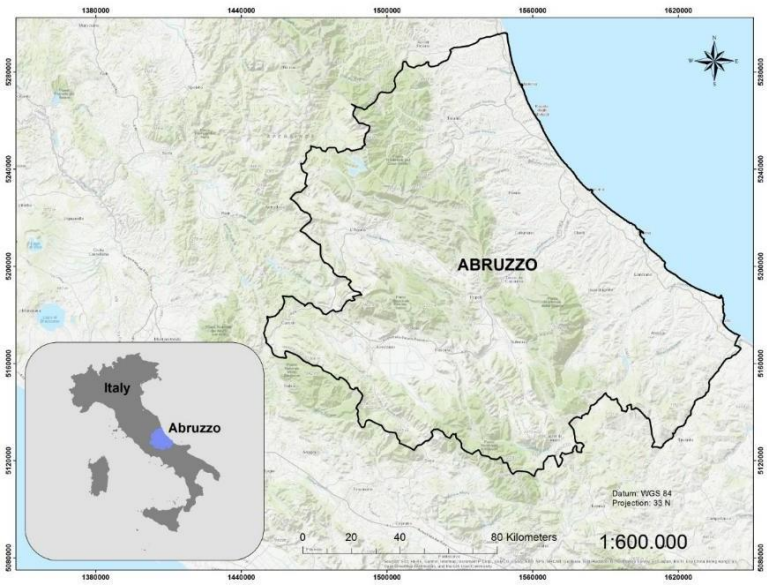


Image 3.- Images of the geographical area of Italy www.conociendoitalia.com/mapa-regiones-de-italia

Verification of authenticity of the samples was made using the stereomicroscope and the optical microscope and comparing them with an authentic sample of *H. officinalis* L. subsp. *aristatus* (Godr.) Nyman. from the Exicata of the Botanical Garden Herbarium at the University of Ferrara. Also, the morphological characteristics of *H. officinalis* and some subspecies and varieties, reported in the scientific literature (Pignatti, 2003) were compared.

2.1.1. Macroscopic analysis

The macroscopic analysis of a species was done through the observation of morphological (aspect and shape) and organoleptic (odor, taste, color) features. This approach can offer a preliminary indication for the identification of the species, also using dichotomous keys (Pignatti, 2003). Regarding the part of the plant defined as a drug, it is also possible to evaluate morphological and organography characters with a stereomicroscope assay; besides, this instrument can detect the foreign matter, as indicated in European Pharmacopeia IX edition (2017). In this research project, a stereomicroscope Nikon SMZ-2B (Japan) was used.

Image 4.- *Hissopus officinalis* subsp. a*ristatus* (Godr.) Nyman

2.1.2. Microscopic analysis

2.1.2.1 Evaluation of morphological features with the optical microscope

For the evaluation of the morphological characters, we used an optical microscope to identify if the parts of the drug correspond to the subspecies and if they have some anomalies. In this analysis, we use chloride hydrate as a means of immersion since it clears cellular structures and eliminates air inclusions. Methylene blue is used to observe the aerial parts of the plant to give a coloration that allows the identification of some cell organelles. For the *H. officinalis* subsp. *aristatus* the Zeiss optical microscope (Axio Lab A1, Germany), was used to recognize the distinctive features of the drug.

2.2 Authentication of *Ocimum campechianum* crude drug

Two samples of the aerial parts of *O. campechianum* Mill. (syn. *Ocimum micranthum* Willd.) were collected in 2017 and 2019 from wild plants in the Ecuadorian Amazon. The authentication was performed with the aid of Dr. David Neill, and a specimen was deposited in the Herbarium ECUAMZ of the State University of the Amazon (UEA) in the CIPCA (Amazon Research, Postgraduate and Conservation Center). The research center is located in the Arosemena Tola canton of the Napo province, at kilometer 44 through Puyo-Tena in Ecuador.

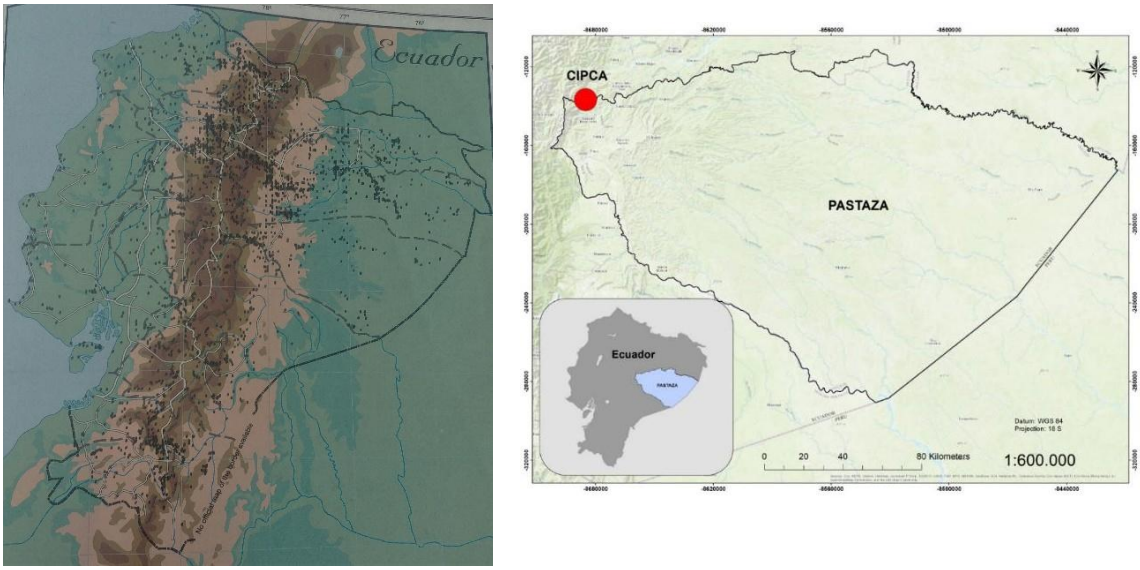


Figure 5.- Image of the geographical area of Ecuador

To compare *O. campechia*mun, with an authentic sample, the Exicata deposited in the Herbarium ECUAMZ of the State University of the Amazon (UEA) in the CIPCA (Amazon Research, Postgraduate and Conservation Center), sample verification of authenticity was made using the stereomicroscope and the optical microscope. Also, the morphological characteristics of *O. campechianum* and other subspecies and varieties reported in the scientific literature (Hamel, et al. 2007) were compared.

2.2.1 Macroscopic analysis

The instrument used for this analysis is the stereomicroscope ELICROM- MOTIC, which allowed to observe the salient distinguishing features of the drug.

Figure 6.- *Ocimum campechianum* Mill

2.2.2 Microscopic exam

2.2.2.2 Evaluation of morphological features with the optical microscope

For the evaluation of the morphological characters of *O. campechianum*, an optical microscope ELICROM- MOTIC was used. In this analysis, we utilized chloride hydrate as a means of immersion since it clears cellular structures and eliminates air inclusions. To observe the aerial parts of the plant, methylene blue was used to give coloration that allowed the identification of some organelles.

2.3 Extractive technique

2.3.2 Steam distillation

Steam distillation is one of the most used methods for the extraction of essential oils, complex mixtures of organic substances of diverse chemical nature, perfumed, with lipophilic characteristics, and highly volatile. The components of essential oils, although volatile, have high boiling temperatures (between 150-300° C), so if subjected to dry distillation, they would alter due to oxidation reactions, polymerization, favored by high temperature. For this reason, the drug is heated in the presence of water, which, together with the oil, forms a system consisting of two immiscible liquids. Dalton's partial pressure law regulates the behavior of the saturated vapors of this system:

$$P_{total} = P_{oil} + P_{water}$$

according to which, in a mixture of two or more gases which do not react with each other, each component exerts a partial pressure equal to that it would exert if it alone occupied the entire volume available. The boiling point of the oil-water mixture corresponds to the temperature at which the sum of the partial pressures (P_{total}) equals the atmospheric one. This phenomenon usually occurs at a lower temperature than that of the less volatile component, which is water. Since the incompatibility between the two liquids leads to higher vapor tensions than those required by Raoult's law, causing a lowering of the point boiling of the mixture compared to that of the individual pure components. In this way, there is also a distillation of the higher boiling components, at a temperature lower than 100 °C, thus avoiding decomposition phenomena of the thermolabile molecules of the oil.

Steam distillation is generally carried out with a Clevenger-type apparatus, following the method prescribed by the European Pharmacopoeia. Two methods can be used for steam distillation, the first one, external steam is used, coming from a steam line, which is passed through the distillation flask; in the second, steam is generated in situ, heating a flask containing both the product to be distilled, and the water. Following the formation of the distillate, the vapors are condensed in a refrigerated tube, and the essential oil, not being miscible with water, is easily separated (Morelli et al., 2005).

2.3.3 Extraction and isolation

Following an extraction process, mixtures are obtained consisting of several compounds that generally belong to chemical classes with similar properties. For the separation of the individual constituents, chromatographic techniques were used.

2.3.3.2 Extraction in hydroalcholic solvent and methanol

To extract phenolic compounds, we used hydroalcoholic solvent and methanol.

The extraction process consists of the following steps: real extraction, partial or total removal of the extraction solvent, and drying.

The solvent, whose choice depends on the solubility of the components to be extracted, can be pure or a mixture of several solvents. The extraction process will also not have to alter the chemical structure of the compounds and may have to extract some of them from others selectively. Furthermore, the solvent must possess a low boiling point, stability, and low viscosity. The end of the process of extraction could be considered when the distribution of the substances to be extracted between the extractive solution and the residual drug reaches an equilibrium of concentration, a constant value. In some processes, it continues the extraction until the drug was exhausted. The parameters that influence the extraction trend and, therefore, the achievement of equilibrium are numerous:

- characteristics of the drug and the solvent
- the proportion of the drug/solvent mixture
- degree of grinding of the drug
- Kinetics of extraction (the rate to reach equilibrium depends on temperature, solvent properties, and agitation)
- temperature
- the pH of the solvent
- interactions between solubilized compounds and plant fibrous material
- degree of lipophilic/hydrophilic (affects the composition of the extract)

2.3.4 Ultrasound extraction

This technique is a particularly rapid type of maceration performed with Vibro-extraction.

The maceration consists of extracting the active ingredients of plant powder, soaking them in a solvent at room temperature. To facilitate a better extraction, the maceration is carried out by ultrasound waves with frequencies higher than 20,000 Hz. Sonication is caused by the use of magnetic or piezoelectric devices. In our laboratories, the container, hermetically sealed, containing the drug and the extraction solvent, is placed by immersion in an ultrasound bath. The main effects are:

- Increase in cell permeability

- Production of cavitations and, therefore, of bubbles in a liquid below its boiling point, producing a superficial local overheating
- Increased mechanical stress.

The effectiveness depends in particular on the duration of the extraction process and the frequency of the device (typically between 25-1000 kHz). The main advantage is reduced times: in 5-10 minutes, it is possible to perform an extraction similar to that of a maceration process, obtained in several hours. However, ultrasounds can cause a degradation of the active ingredients and induce oxidation or radicals reactions (Morelli et al., 2005).

Figure 7. Ultrasonik 104x (Pharmaceutical biology laboratories, Malborguetto 2018)

2.4 Separation technique

In general, it is possible to define the chromatography as a physical method for the separation of the components of a mixture, based on their distribution between two phases, a defined stationary phase consisting of an immobile bed, and the other said mobile phase, which is passed through the stationary phase. The two phases always have the opposite character. The chromatographic separation takes place by the continuous establishment of equilibrium among the constituents of the mixture with the two phases. The different affinity of the single substances for the two phases is the reason for more or less delayed on the stationary phase. Consequently, molecules come out at different times. The different chromatographic techniques can be classified according to various principles. The first classification includes the distinction between planar chromatography (TLC, HPTLC) and column chromatography (gravity chromatography, Flash, GC, VLC, HPLC). Based on the fact that the stationary phase is arranged on a flat surface or can be contained within a cylindrical tube made of glass, steel, fused silica, or plastic. Another classification system takes into consideration the nature of the mobile phase and the stationary phase. The mobile phase can be a gas (GC), a liquid (LC) or a supercritical fluid (SFC), while the stationary phase is a liquid or a solid.

Finally, depending on the type of equilibrium between the two phases during the separation process, four types of chromatography can also be identified:

- adsorption chromatography, if the separation occurs through a continuous sequence of adsorption, or desorption of the various analytes
- partition chromatography if the analytes divide between the two phases either liquid or a gaseous and a liquid.
- ion-exchange chromatography, if the stationary phase has ions bearing a charge opposite to that of the sample and the mobile phase consists of an aqueous buffer.
- size exclusion chromatography, if the stationary phase is a solid consisting of controlled pores, which have the aim to filter the substances on the basis of their size.

The chromatography can finally be classified as a method that takes into account the relative polarity of the two phases: it is called direct chromatography when the stationary phase is strongly polar (as in the case of silica, alumina, etc.), while reverse chromatography if the stationary phase is apolar (e.g., hydrocarbon chains). In the first case, the less polar compounds are eluted first, while in the second case, the predominantly polar compounds are the first to emerge. Generally, it is preferred to work in the reverse phase as it is possible to use water as the solvent for the mobile phase: the advantages are the low cost and the non-toxicity (Morelli et al., 2005; Skoog et al., 2013).

2.4.2 Gravity column chromatography

The gravity column chromatography is an application of adsorption chromatography in which the stationary phase is a solid and mobile phase a liquid. The stationary phase, consisting of silica gel or alumina, is placed inside the glass cylinder, and the mobile phase is introduced from the top of the column. The mixture to be separated is placed in the upper part of the column in a thin layer, and then it penetrates the fixed phase. The eluent passes through the cylinder, and it flows by gravity (in flash chromatography the column is subjected to pressure to favor the process): a dynamic equilibrium is established, so the molecules of the mixture are continuously absorbed and desorbed by the stationary phase and transported from the mobile phase. Based on the affinity of the components for the two phases, separation is performed. The components most related to the mobile phase come out first, while those closest to the stationary phase delay their exit. Operationally, a porous material, such as glass or cotton, or inert material, such as sand or diatomaceous earth, is placed in the lower part of the column to avoid the passage of the stationary phase and to favor the flux of the analytes transporting by the mobile phase.

The stationary phase should be uniform and homogeneous and should not contain air bubbles to avoid troubles as preferred routes and a decrease in the effectiveness of the separation process. Two methods are generally used to prepare a column: the dry method and the wet method. During the dry

method, the column is first filled with dry stationary phase powder, followed by the addition of mobile phase, which is flushed through the column until it is completely wet, and from this point is never allowed to run dry. For the wet method, a slurry is prepared of the eluent with the stationary phase powder and then carefully poured into the column. The top of the silica should be flat, and a layer of sand can protect the top of the silica. Eluent is slowly passed through the column to advance the organic material. The eluent fractions, containing the separated constituents of the mixture, are collected in different fractions collectors. To verify the separation, an TLC control is carried out, and the GC-MS or HPLC-MS can be used to determine the purity of the separated analyte.
Several parameters influence the separation:

- choice of stationary phase
- choice of solvent or eluent mixture
- dimensions of the column, depending on the amount of material to be separated
- elution flow

To optimize the conditions, the components of the mixture are separated, avoiding an overlap of the bands (Morelli et al., 2005; Skoog et al., 2013).

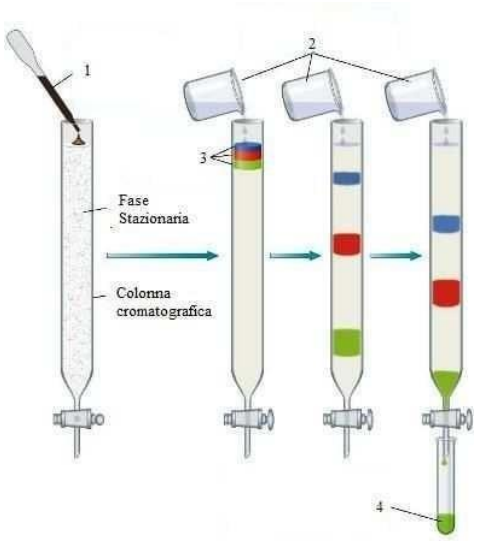


Figure 8. Gravity column chromatography

2.5 Characterization techniques

2.5.2 GC-MS

Gas chromatography (GC) is a chromatographic analysis technique exploited mainly for the analysis of mixtures of volatile compounds. In the field of natural substances, it is, in fact, very used for the characterization of essential oils. There are many advantages: high resolution, reliability, and repeatability of results, relatively short analysis times. Schematically, a gas chromatograph consists of:

- a source of gas as a mobile phase
- a sample injection system
- a column in which the separation occurs, contained in a thermostat oven
- a detector to highlight the output of the various molecules separated
- a system to register data and to transform them in the form of a chromatogram.

The principle on which gas chromatography is based is the same as the one described for column chromatography. The stationary phase is contained within the column and can be solid or liquid with adsorbent properties, supported on inert material. The mobile phase is instead a gas, also called carrier gas. Generally, chemically inert gases with low viscosity and high purity (99.999%), such as nitrogen, helium, argon, hydrogen, or carbon dioxide, are selected. The chromatographic column is inside a thermostat oven at a specific temperature, fixed or programmed over time. The speed at which the compounds move within the column depends on the affinity they have for the stationary phase. The limitations of gas chromatography are represented by the fact that the sample must be volatile in a temperature range between the room temperature and 300-330 °C, a value beyond which the stationary phase would degrade, and the thermolability of sample's components would emerge. The derivatization of some functional groups of the chemical compounds can increase the volatility and facilitate the analysis.

The characterization of the compounds is made by their retention time, i.e., the time between samples' injection time in the gas chromatograph and the moment of maximum response of the detector (apex of the peak on the chromatogram). To improve the characterization of the components in a mixture, the so-called retention indices can be used, among which the most widely used are the Kovàts retention indices, such as arithmetic indices. This index provides a normalized retention time, using a linear equation in which the retention time of each peak is compared to that of a mixture of n-alkanes, injected with the same analytical conditions of the unknown mixture.

For the qualitative analysis of substance mixtures, gas chromatography can be coupled with spectroscopic or spectrometric techniques. The gas chromatograph present in our laboratories is, in fact, interfaced with a mass spectrometer, which provides the mass spectrum in addition to the retention time, giving information on the structure of the individual constituents, through the characteristic fragmentation of each molecule. The mass spectrometry experiment consists of the following operations:

- ionization: the molecule is transformed into a positively (or negatively) charged ions or radicals
- acceleration: the ions are accelerated to provide the same kinetic energy to everyone

- deflection: the ions are deflected by a magnetic field based on their mass; the deviation also depends on the number of ion charges
- detection: the mass spectrometer detector electronically detects the ion beam.

The instrument used in this project is equipped with an electronic impact ionization source (EI), which supplies enough energy to remove an electron from the sample molecule and to generate the formation of a positive ionic radical, called a molecular ion. In this type of ionization, the sample must be a gas or a natural volatile substance whose molecules will be hit by an electron beam generated by a heated metal filament. The generated molecular ion can, in turn, be unstable and break into smaller fragments, either charged or not, according to precise rules that depend on the structure of the molecule. Only the charged species will be affected by the accelerating electromagnetic field, and therefore only the ions will be accelerated deflected and revealed.

The analyzer of our instrument is an ion trap, which selects and separates ions based on their mass/charge ratio (m / z). This system employs three electrodes to trap and accumulate ions in a narrow volume cavity, the so-called ion trap, to achieve high sensitivity. The two side electrodes have a small hole in the center through which the ions pass. The mass spectrum is generated by varying the electrical potential to sequentially eject ions from the trap to the detector, according to an increase m/z value. The mass spectrum correlates the relative intensity of an ionic species (directly proportional to concentration), concerning the m/z ratio. In the mass spectrum, the heaviest ion (the one with the highest m/z value) is likely to be the molecular ion. Since the masses of an electron is practically negligible, the molecular ion will have a mass corresponding to that of the unknown sample. All other signals to the left of the molecular ion will derive from its own.

Finally, the most intense peak is called the base peak, and it is the more stable ionic species or the one that is more easily formed and reaches the detector in higher concentration; we attribute to it an intensity equal to one hundred and the intensity of all the other peaks is expressed as a percentage of this. A further comparison with a library of spectra, commercial or internal to the instrument in use allows a more straightforward interpretation of the results obtained (Scalvenzi, 2019; Skoog et al, 2013).

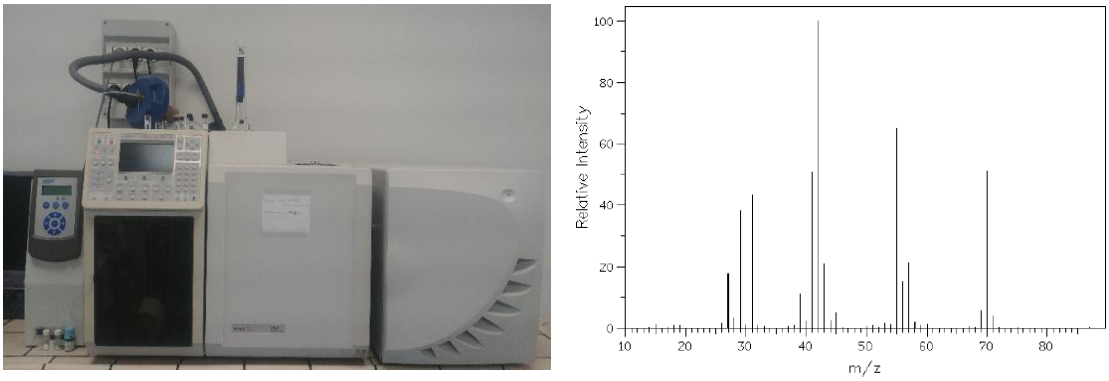


Figure 9. GC-MS (Pharmaceutical biology laboratories, Malborghetto 2018**).** Example of fragmentation of a molecule

2.5.3 TLC and HPTLC

Thin-layer chromatography (TLC) is a chromatographic technique in which the stationary phase, consisting of granular homogeneous material (silica gel, active alumina, etc.), made to adhere to planar support consisting of a glass or metal plate. A small aliquot of the sample is deposited to be separated on the seeding line, close to the base of the plate. The lower end of the plate (Figure10) is brought into contact with an appropriate mobile phase that goes up along the thin layer by capillarity. Depending on the affinity for stationary and mobile phases, the components of the mixture (extracts obtained from the crude drug) could be separated.

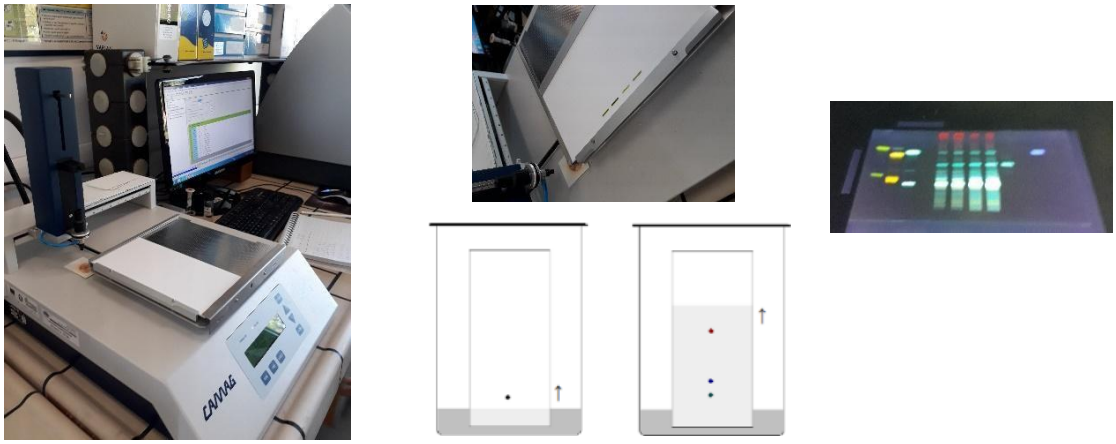


Figure 10. Chromatographic separation in TLC (Pharmaceutical biology laboratories, Malborghetto 2018**)**

The deposition of the mixture on the plate (standard height of 10 cm) is made with an automatic depositor (CAMAG-Linomat5) where the sample quantities to be deposited (in µL), the distance of the deposition from the base of the plate (in general are set 1cm), the distance between the bands (in mm) and the deposition rate (sec/µL) are defined through a specific software, taking into consideration the solvent in which the mixture of the compounds is dissolved. When the plate is developed, the solvent is evaporated under a chemical hood; some compounds, which can be characterized as pigments, are visible to the white or UV light (more frequently 254 and 366 nm), while for other chemical classes it is necessary to proceed with a derivatization with specific chromogenic reagents to make visible bands. The silica gel is often mixed with an inorganic fluorescent indicator F254, that show a green fluorescence when illuminated with UV light al 254nm. HPTLC (High-Performance Thin Layer Chromatography) is an analytical technique similar to TLC, but allows a higher resolution of the compounds since the stationary phase has much smaller particle size (10 µm). The identification of the different components is possible through the Rf (delay factor) of the individual bands and comparing it with the pure molecule or with literature data (Morelli et al., 2005; Skoog et al., 2013).

2.5.4 HPLC-DAD

HPLC (High-Performance Liquid Chromatography), born around the '60s, is nothing more than the instrumental evolution of classical column chromatography. This chromatographic technique is based on the use of a liquid mobile phase, pressurized in the system, and of a solid stationary phase packed in a chromatographic column. Reverse-phase chromatography (RP-HPLC) is so called since the stationary phase has an apolar character (opposite to the traditional chromatographic method), while the eluent mixture is predominantly polar. An advantage compared to column chromatography is that this method uses pumps able to exert high pressures (in the order of 300 kg/cm^2), which allow to maintain an adequate flow of mobile phase and to separate very complex mixtures in a short time. The elution can also be carried out in two different ways: in isocratic mode, maintaining the composition of the eluent mixture constant throughout the whole analysis, or in gradient mode, if two or more different solvents are mixed in variable proportions or the flow changes during the chromatographic run. The sample to be separated is introduced into the system by a microsyringe and a loop valve, mixed to the mobile phase, and then transported under pressure into the column in contact with the stationary phase.

Typically the analytical columns for HPLC have a length of 15-25 cm, an internal diameter of 3-5 cm, and the two ends are closed by a porous stainless-steel disk, with support functions for the stationary phase and to filter the eluent. The separation of molecules depending on their chemical structure. The compounds have a different affinity for the two phases and, for this reason, have a different time to exit from the column, defined retention time. The separated molecules enter then in a detection apparatus, which allows revealing specific signals, for each component of the mixture, that can be amplified and sent to a recorder, which highlights them in the form of peaks and provides the chromatogram.

From the retention time, it is possible to deduce the identity of the eluted compounds, and from the peak areas the content in the mixture. The detector used in this project is a diode array detector (DAD, which provides for each peak, the retention time and the UV-visible spectrum of the single separate molecule or of the molecules that coelute (Morelli et al., 2005; Skoog et al., 2013).

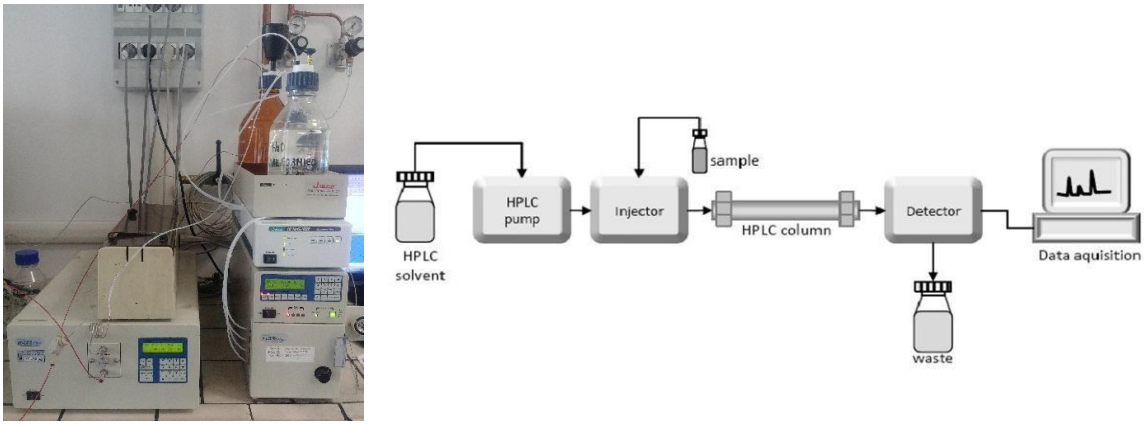


Figure 11. HPLC scheme (Pharmaceutical biology laboratories, Malborghetto 2018**)**

2.5.5 NMR

Nuclear magnetic resonance (NMR) is one of the most widely used spectroscopic tools for the structural elucidation of a molecule based on the magnetic properties of the nuclei of individual atoms and isotopes. Many nuclei can be studied at NMR, but the most investigated are the isotope 1 of hydrogen (^{1}H) and the isotope 13 of carbon (^{13}C). The NMR offers a lot of information related to the molecular structure, thahow the atoms are bonded together in the molecule. For example, by evaluating the NMR spectrum of the ^{1}H isotope, it is possible to deduce:

- how many types of H are present in the molecule
- the number of H present for each type
- some information about the other types of atoms bonded to H present in the molecule and the stereochemistry of a particular type of H
- the enantiomeric excess of a mixture of enantiomers

Combining the information deduced from ^{1}H and ^{13}C and bidimensional experiments it is possible to determine the skeleton of the new molecules.

NMR experiments are based on the fact that some nuclei have magnetic properties since they are able to rotate around their own axis (spin movement). The circular movement of the nucleus gives to the particles the capacity to be moving charges that generate a magnetic field. The spin of the nucleus is indicated as the quantum number of spin (I), and only the nuclei with I different from zero can be studied at the NMR. In the absence of an external magnetic field (B_0), all the nuclei of a population of ^{1}H have the same energy and are randomly oriented. In the presence of B_0 instead, the nuclei with I different from zero arrange their rotation axis according to a well-defined number of orientations. The ^{1}H, having a spin quantic number equal to 1/2, has only two possible orientations available: one in a low energy level (α level), where the nuclei generating a magnetic field favorable to B_0 are arranged, and one in a higher energy level (β level), where the nuclei generating a magnetic field oriented against B_0 are placed. Also, the ^{13}C nuclei behave in two energy levels, since they have the same value as I.

Nuclear magnetic resonance occurs when the nuclei absorb sufficient energy to change their spin movement and, therefore, their magnetic moment (μ) with respect to the applied magnetic field. Since the energy absorption is quantized, the exact amount of energy equal to the ΔE existing between the energy levels α and β must be absorbed. If the sample to be analyzed is irradiated with a frequency equal to ΔE, the magnetically active nuclei can enter into resonance, absorb energy passing from α to β or vice versa, yielding energy passing from β to α. The magnitude of the energy gap is directly proportional to the applied field, and follows this expression:

$$\Delta E = h \gamma B0 / 2\pi$$

Where gamma is the gyromagnetic ratio of the nucleus, h is the Planck constant, and B_0 is the intensity of the applied magnetic field. If electromagnetic waves are used as an energy vector, the frequency of absorption will be:

$\sqrt{} = \gamma / 2\pi B0$

Energy absorption can only occur if there is an excess of nuclei in the α level compared to that β. When the number of protons in α becomes equal to the number of protons in β, the net energy absorption of the system ends, and therefore the absorption NMR signal disappears. The signal will be re-observable only if the nuclei have time to "relax," i.e., to restore the excess of nuclei in α concerning β. The energy emitted by the nuclei that descend from β to α during the relaxation phase is mainly dispersed in the form of radiation. But a part is re-emitted as radiofrequency: this last has the same energy as the absorption one and is detected for the recording of the NMR spectrum. Taking into account the isotope ^1H, the NMR experiment makes sense because not all protons of a molecule resonate at the same frequency since the chemical and electronic environment of a molecule are often different from each other. It has been verified that the electrons moving around the nucleus generate a local magnetic field (CM_{local}) whose lines of force are opposed to B_0. Each proton will, therefore, be subject to a total magnetic field, equal to the difference between B_0 and CM_{local}, whose intensity influences its ΔE and, therefore, the frequency that must absorb to enter into resonance. The local magnetic field is decreased by electronegative atoms near the proton; this effect, called the deschermating effect, causes that the total magnetic field of the proton becomes higher, and the absorption occurs at higher frequencies.

In general, the resonance frequency absorbed by each different proton present in the molecule will be shifted by the reference frequency (generally the absorption frequency of tetramethylsilane is used) of a few parts per million and therefore of a few Hz; this change of position is called "chemical shift." This parameter can define how many types of different protons contains a molecule since the number of signals present in the spectrum is related to different protons. Instead, to determine the number of protons present for each type, it is considered the area subtended by each signal that is directly proportional to the number of equivalent protons (Silverstein et al., 2014).

NMR spectrometers are divided into two classes:

Continuous-wave spectrometers, in which the sample is immersed in B_0 and then irradiated by performing a slow scan in the appropriate frequency range. The recording of energy absorption as a function of frequency generates the spectrum. This method has a long time and can only be suitable for naturally abundant isotopic nuclei.

Spectrometers that work with the pulse method, in which the sample is immersed in B_0 and irradiated with a high-power radio frequency energy pulse. This pulse simultaneously excites all the nuclei of the sample; the excited nuclei immediately begin to decay in the fundamental state and radiate the absorbed energy in part as radiofrequency. A detector collects this emission of

frequencies producing an FID (Free Induction Decay), which is the sum of all the radiations emitted by the nuclei over time. The information contained in the FID is then converted by Fourier Transform into a well-defined spectrum, related to absorption frequency.

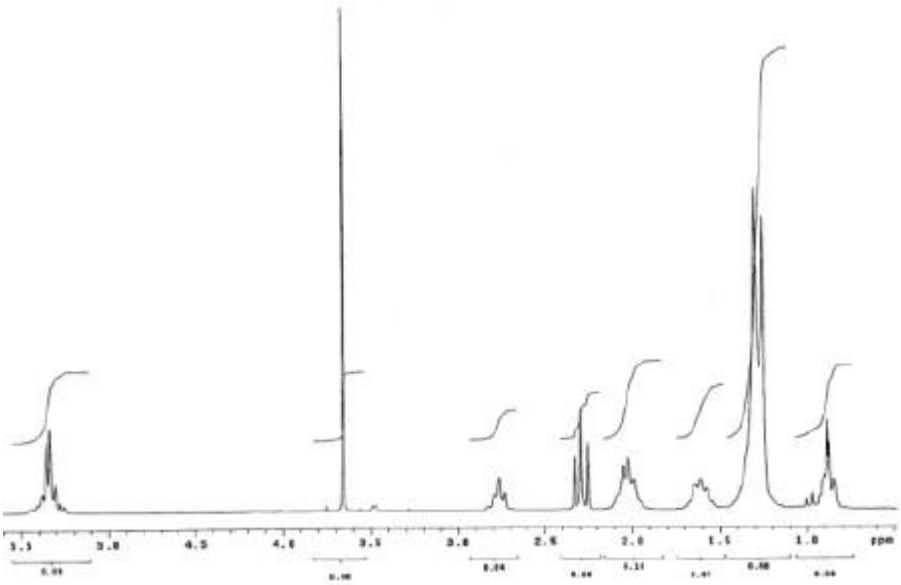


Figure 12. Example of ^{1}H-NMR spectrum.

2.6 Biological activity

2.6.2 Antioxidant activity

Antioxidants are compounds capable of counteracting the oxidation process that originates from free radicals, species capable of independent existence (hence the adjective "free") containing one or more electrons not paired. The free radicals carry out their destructive action mainly towards the cells, in particular in the fats that form the membranes (lipoperoxidation), in the sugars, in the proteins, in the enzymes, and in the DNA where they alter the genetic information. To counteract the excessive production of radical species, the human organism has developed sophisticated mechanisms to maintain redox homeostasis, increasing its elimination, or blocking its formation. They include endogenous antioxidant defenses with enzymes capable of catalyzing reactions of free radical elimination and some proteins capable of binding to the ions responsible for oxidation (Fe^{2+} and Cu^{+}), which are flanked by exogenous defenses, represented in part by the antioxidants taken with the diet such as ascorbic acid, vitamin C, α-tocopherol, vitamin E, carotenoids, polyphenols and other compounds with low molecular weight.

Antioxidants can be classified according to their mechanism of action in:

* "Preventive" antioxidants, capable of inhibiting or counteracting the formation of radicals by preventing the oxidation phenomenon in the "initiation" phase. In this class, we can

insert all the enzymes that are capable of decomposing peroxides and hydroperoxides, for example, the enzyme superoxide dismutase (SOD) and the catalase enzyme (CAT);

* Antioxidants that "break chains," capable of preventing the propagation in one or more levels, trapping the free radicals that are formed during the oxidation reaction. They have the advantage of acting faster than the radical chain propagators with respect to the reaction speed of the latter with the substrate; in addition, they give rise to stable radicals, which, in turn, do not cause the origin of the oxidative chains.

The determination of the antioxidant activity of a Phyto-complex or an active ingredient is a complex operation at the operational and interpretive level. There are several *in vitro* and *in vivo* tests that can be used to determine this activity, each of which can reveal a particular uniqueness of a biological expression that often varies in its biochemical mechanisms. One of the most used methods is the "DPPH test", for determining the antiradical activity on the radical 1,1-diphenyl-2- picryl-hydrazyl (DPPH). The assay is based on the detection of the ability to neutralize the DPPH radical by possible antioxidant compounds. This method is fast, simple, economical, and widely used to measure the ability of the compounds to act as free radicals or hydrogen donors, and it applies to both hydrophilic and lipophilic substances.

In this reserach, the antioxidant activity of the essential oil, MeOH, and 70% EtOH extracts were analyzed by a qualitative and quantitative DPPH test, which uses a stable radical 2,2- diphenyl-1-picrylhydrazil (DPPH). When the powder was solubilized in ethanol acquires a purple color while, in the presence of antioxidant compounds, for the neutralization of the free radical, the color changes to yellow.

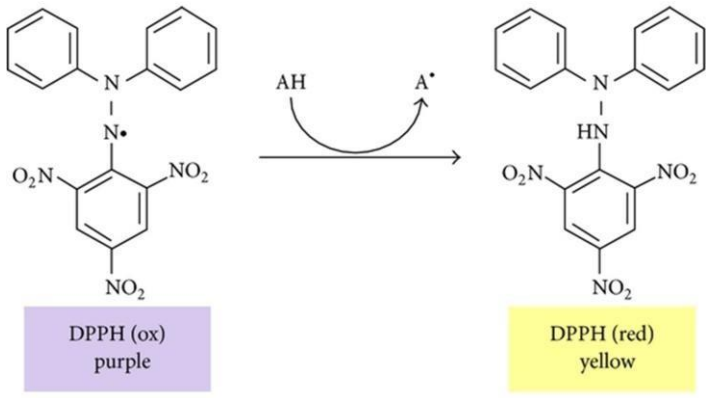


Figure 13. DPPH

2.6.3 Cytotoxic activity

2.6.3.2 Cancerogenesis

Traditionally the carcinogenesis process is divided into three phases: induction, promotion, and progression.

Induction is the first phase, characterized by epigenetic and genetic alterations and supported by functional and metabolic changes; it can occur spontaneously following organic processes associated with senility, hormonal conditioning, as a result of complex alterations of the microenvironment or exposure to carcinogens. Genetic alterations can lead to the loss of regulation of biochemical control pathways associated with proliferation, survival, and cell differentiation, which in turn influence relevant factors, including the type of metabolism and DNA repair function. At this stage, the chemopreventive action should be carried out for the morbid processes that precede the direct manifestation of induction, such as inflammatory processes or the metabolic neutralization of environmental mutagens (Siddiqui et al., 2005). The promotion phase is considered a relatively long and reversible process in which pre-neoplastic proliferating cells accumulate progressively. By this time, the process can be modified by chemopreventive agents that can influence growth rates and correct the metabolic setting of neoplastic cells or condition the histological and hormonal environment that supports and promotes the evolution towards the next phase.

Progression is the phase in which the lesion evolves towards the development of invasive cancer, and is the final stage of neoplastic transformation. In this phase, there is a rapid increase in the size of the tumor. The cells undergo further mutations with the enhancement of invasive capacity even at a distance (metastasis). The chemopreventive agents at this stage should be more correctly qualified as adjuvant agents of chemotherapy treatment were no longer directed towards prevention but at the eradication of the morbidly consolidated disease (Marín-Aguilar et al., 2017)

Among the most effective chemotherapy drugs for cancer therapy are many molecules of vegetable origin and their derivatives. It is sufficient to cite as an example the taxol obtained from Taxus brevifolia, the podophyllotoxin derivatives from *Podophyllum haxandrum* and *P. peltatum*, vincristine and vinblastine from *Catharanthus roseus*, camptothecin, topotecan and irinotecan from *Camptotheca acuminata*. These molecules act through various mechanisms such as cytostatics. In consideration of their efficacy and toxicity, they are used in the well-established and advanced phases of the pathology with heavy side effects. They are, therefore, not attributable to the category of chemopreventive agents (Meybodi et al., 2017).

The chemopreventive agents are the object of intense research activity, widely represented by thousands of publications in specialist journals. The prevention of cancer pathologies and the adjuvant action against pharmacological chemoresistance and the non-specific toxicity effects of chemotherapeutic drugs constitutes a priority research orientation in the context of the study of cancer therapy. The need to evaluate thousands of extracts and herbal preparations of various origins (phytochemicals) has led to the development of many *in vitro* experimental procedures that allow cost-effective screening and allow the dissection of the molecular mechanisms used in the interaction between phytochemicals and cells. The research proceeds in the primary evaluation of potential cytotoxic effects against in vitro cell lines, to identify possible cytostatics and quantify the range of concentrations to be used in the subsequent tests aimed at identifying the mechanisms

of interference with the genetic and epigenetic metabolic functionality. If the toxicity appears to be manifested at very low dose levels (less than 10 micromolar) and with a significant specificity towards neoplastic cells, it would be advisable to direct the research towards the identification of specific molecules that can be used as chemotherapeutic agents. This approach has been successfully followed in the isolation of the chemotherapeutic drugs listed above as examples following the wide-ranging screening, sometimes on thousands of plants (Iqbal et al., 2017).

Cytotoxicity is not necessarily a prerequisite for the identification of chemopreventive molecules. In this case, it is necessary to evaluate much more articulated effects often with actions of interference between various molecules and in the context of networks of biological interactions of high complexity. In other words, the study of chemopreventive agents in the comparison of tumors requires mechanistic insights into both the chemical characterization and the functional biological response. This justifies the slowness of the results aimed at the clinical application.

2.6.3.3 *In vitro* citotoxic activity

The use of cell cultures *in vitro* constitutes an inescapable prerequisite for any subsequent experimental approach in the context of the study of the interaction between complex chemical systems or single molecules with living systems. In this context, it is possible to use primary cell cultures that are deriving from explants directed from tissues coming from anatomical districts, or secondary cultures in which the cells descend from primary cultures transformed by chemical, physical or biological agents. Among the latter, immortalized crops can set up a stabilized cell line with continuous growth and virtually unlimited lifespan. The transformation into a continuous cell line is carried out: the following selection of spontaneous mutations, treating with carcinogens, following exposure to viruses, or by selection from primary explants from neoplastic tissue. The immortalized cells are not necessarily referable to the neoplastic tissue, even if they have acquired one of the most relevant peculiarities. They are widely used *in vitro* systems due to the advantages they offer compared to primary lines, such as cryoscopic preservation, continuous and unlimited growth, and reduced use of serum. They can, following unique treatments, differentiate into structures that effectively simulate physiological tissues. This last aspect (the possibility of differentiating into other cell types), can, however, constitute a severe drawback, wanting to maintain the reproducibility of the outcomes in differentiated conditioning contexts (Atanasov et al., 2015).

Due to their manageability, the stabilized tumor cell lines are often used in the search for new molecules that can exert an effective toxic and cytostatic action against neoplastic tissues. This preliminary approach is widely followed in the initial screening of complex molecular systems, such as phytocomplexes, and allows the first selection for subsequent research activities that are much more expensive and complicated.

Overall, the *in vitro* cell lines are advantageous as they are simplified and highly reproducible systems, they allow the analysis of cellular and molecular mechanisms, the control of the growth environment, they are cheap and rapid.

Instead, they are disadvantageous in that they are simplified compared to the integrated organism they are exposed to substances other than those *in vivo*, and the substances administered can interact with the culture medium.

To evaluate the differential toxicity of plant extracts on neoplastic and non-neoplastic cells, a tumor cell line identified with the abbreviation A549 and an immortalized line identified with the acronym HaCaT was used in this project.

Characteristics of the cell lines used:

A549

They are epithelial, basal, and alveolar cells of human adenocarcinoma obtained from pulmonary tumor removal in 1972 (Foster et al., 1972). They adhere to the culture flask as a single layer but can also be used in suspension, and if kept in culture for a long time, they tend to differentiate. They synthesize lecithin and high levels of unsaturated fatty acids. They are also used for the study of metabolism, expressing various cytochrome P450 isoenzymes: among these, CYP 1B1 is peculiar to neoplastic cells. In these cells, the genetic family p53 is not mutated.

HaCaT

Immortalized aneuploid keratinocytes are spontaneously transformed from human skin explants (Schoop et al., 1999). They are not neoplastic cells and are used for the simulation of healthy epithelial tissue, and as in the present project, as a healthy control regarding neoplastic cells. They adhere to the culture flask in a single layer.

2.6.3.4 MTT test

The MTT test is a colorimetric assay widely used to evaluate cytotoxicity in the in vitro studies of the identification of toxic molecules (Mosmann, 1983); it takes its name from the acronym derived from the compound used (3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide).

The MTT test was the first homogeneous cell viability test developed for the screening of a large number of compounds and preparations. The MTT substrate is prepared in a physiologically balanced solution, added to the cells in culture, usually at the final concentration of 0.2 - 0.5 mg/ml and incubated for 1 or 4 hours. The amount of formazan (admitted proportional to the number of viable cells) is measured spectrophotometrically at 570 nm.

The vital cells with active metabolism convert the yellow MTT, in a violet-colored product, the formazan, which is the molecule that absorbs at 570 nm. When cells die, they lose the ability to convert MTT to formazan, so color formation is an indicator of the number of viable cells. Formazan accumulates as an insoluble precipitate inside the cells, and it is deposited near the cell surface and in the culture medium. Formazan must, therefore, be solubilized before spectrophotometric determination. The exact cellular mechanism of the reduction of MTT in

formazan is not well understood, but probably involves the reaction with NADH or similar reducing molecules that transfer electrons to the MTT. Some evidence has oriented the hypothesis that MTT is stored in highly hydrophobic structures, such as lipid droplets (Stockert et al., 2012). As a rule, the conversion to formazan of MTT can be traced to the activity of the enzyme succinic dehydrogenase, which is active in viable cells. Therefore, in the final analysis, the MTT test is an enzyme activity test and, as such susceptible to all the confounding factors typical of this type of test. This is why it is often complemented with alternative assessments that determine other metabolic activities of the cell, including the DNA synthesis test with Bromodeoxyuridine, the Trypan Blue test, which identifies dead cells instead of vital ones, the evaluation of Lacticodehydrogenase, the activity of specific cell death proteases or the measurement of NADH concentration (Riss et al., 2004).

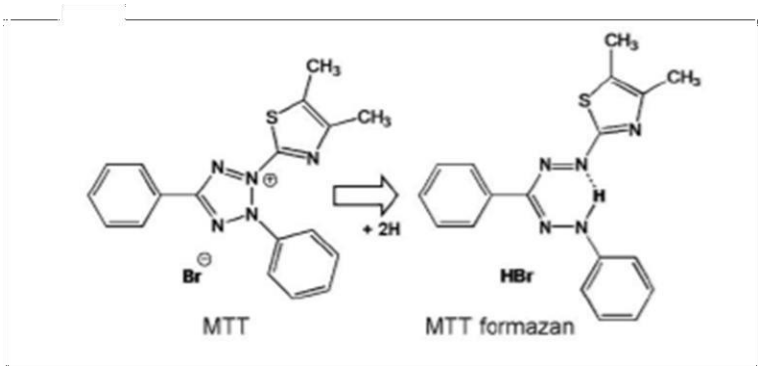


Figure 14. Reduction reaction of MTT (Stockert et al., 2012)

2.6.3.5 Cell migration

Migration by cells into normal physiological processes in adjacent histological districts is due to various situations in the ontogenetic context, from the first embryonic processes, such as gastrulation and development of the nervous system, to vascular germination (even in fully developed organisms), wound healing, colonization with immune system cells (Kalluri, 2009).

In the pathological field, cell migration can be qualified as a cellular invasion, that is, as the overcoming of frequently impassable histological barriers (such as the basement membrane for epithelial cells). The fibrosis, resulting from necrotizing inflammation and colonization by neoplastic cells, represent the most recurrent emblematic examples of this pathological process (Kalluri, 2009). In particular, the cells that generate metastases represent the main problem in the treatment of cancer due to the often poor prognosis. The *in vitro* study of neoplastic cell invasion processes constitutes a relevant approach to this topic.

In *vitro* tests that exploit the cellular response to a chemotactic stimulus, if configured in a system with two compartments separated by a porous membrane, can be used for the study of migration and cellular invasion. The cells are induced to migrate from an upper to a lower compartment following the application of a chemotactic gradient. The porosity of the membrane that separates the two compartments must be chosen according to the size of the cells.

2.6.3.5.1 Transwell migration test (Boyden chamber assay)

Among the systems of this type, the transwell assay was initially introduced by Boyden (and is therefore often called the Boyden chamber assay) to analyze the chemotactic responses of leukocytes (Boyden, 1962). It is essential to choose a pore diameter, which allows active transmigration, which is slightly smaller than the cell diameter to avoid non-specific mechanical passages. Commercially available membranes have pore diameters between 3 and 12 micrometers. There are two possibilities for the detection and quantification of migrated cells. The cells that passed the membrane can be fixed on it, stained with cytological dyes, such as hematoxylin, toluidine, crystal violet, or Giemsa, and counted under the microscope. It is crucial to determine the right incubation time, which corresponds to the time when the cells appear on the underlying side of the filter. This parameter can vary significantly between different cell types and affects the time when the cells left on the upper side of the membrane are removed with a small cotton swab.

A second method consists of removing the migrated cells after staining with a fluorescent agent, with a dissociating agent such as trypsin, and then proceeding with quantification with a fluorescence reader. This second technique avoids the critical phase of mechanical removal of the cells left on the upper side of the membrane. Active removal from the membrane can also be used to recover cells capable of migrating, which may constitute a more specific sub-population susceptible to further investigation.

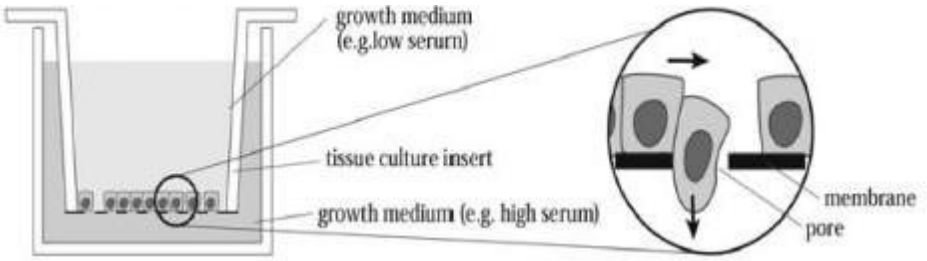


Figure 15. Boyden chamber (Kramer et al., 2013)

2.6.3.5.2 Invasion test

The use of a reconstituted basal membrane in vitro (Matrigel®), applied over the porous filters used in the chemotaxis test, allows a rapid and quantitative assessment of the invasive potential of tumor cells. Matrigel® occludes pores, blocking the passage of non-invasive cells. In contrast, invasive cells can degrade the matrix and migrate through the gel layer and reach the lower chamber. For the rest, we proceed correctly as for the migration test.

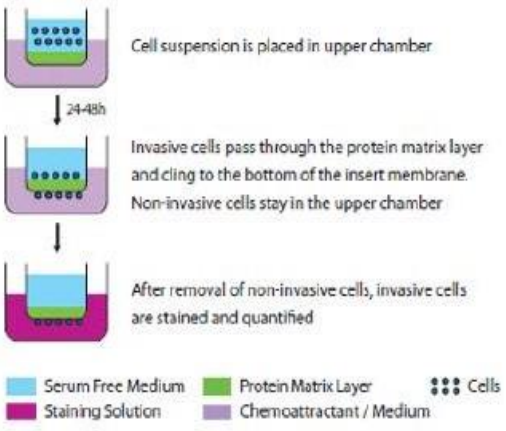


Figure 16. Invasion test (https://www.biocat.com/cell-biology/cell-invasion)

2.6.3.5.3 *In vitro* wound-healing assay (scratch test)

This widespread, technically undemanding and economical test can be used to study two-dimensional cell migration (Rodriguez et al., 2005).

The test is carried out by delicately engraving an area of confluent cells and generating a "wound." The continuity solution thus obtained is monitored under a microscope, following the movement of the cells from the areas that converge towards the scratch area. The cell movement can be calculated by measuring the decrease of the discovered region, at different times, until the partial resolution of the continuity solution. The simplest, but also least precise, way of generating the "wound" involves the use of a plastic tip, for example, the tip of a Gilson pipette. However, it should be considered that the speed of cell migration just before wound closure is often higher than in later times. Therefore, the variation of the gap width before the beginning of cell migration is a critical factor, which can compromise the reproducibility of the outcomes.

Furthermore, some cells remain adhered to the edge of the scratch and can re-adhere to the plate moving into the wound area later. These drawbacks can be avoided by trying to obtain a solution of continuity by not practicing "cutting," but by hindering cell growth: this can be achieved using septate silicone inserts that cover a part of the cell growth area keeping it free from cells. The insert is then removed, allowing the cells to move towards the free area. This second method overcomes the described drawbacks and allows a better reproducibility; it can, however, be more expensive, implying the purchase of silicone inserts.

The movement of the cells is quantified by measuring the decrease of the discovered region at successive times up to the resolution of the continuity solution.

By coating the plates with materials of biological origin such as collagen, fibronectin, or Matrigel®, before cellular seeding, it is possible to simulate different tissue contexts and evaluate the relevance of the stromal context.

If the test is carried out over extended periods (more than 24 hours), the outcomes are affected by the confounding factor due to cell proliferation, which becomes comparable if not majority compared to cell motility. It is possible to overcome this limit by resorting to inhibitors of

cytodieresis, such as cytochalasin, or to culture media with a low content of growth factors (fetal bovine serum in a percentage of 1-2%, against the percentage of growth of the culture between 10 and 20%), which make cell replication negligible concerning migration.

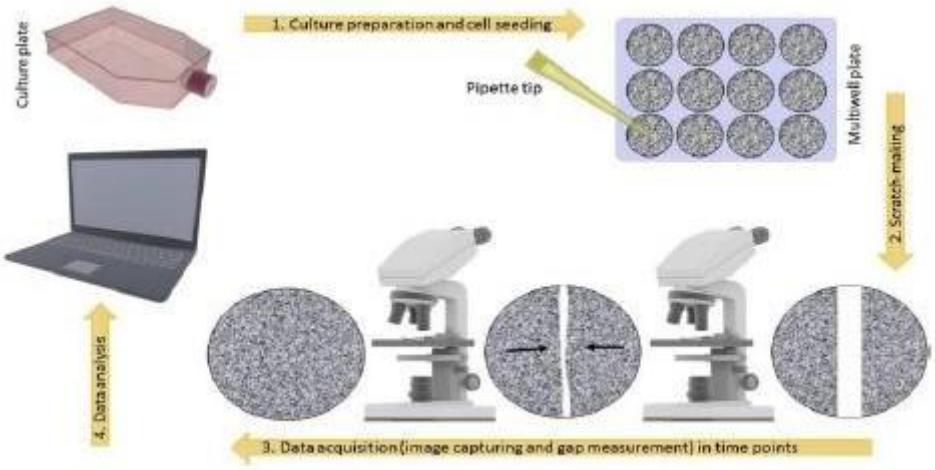


Figure 17. Scratch test (Grada et al., 2017)

2.6.4 Mutagenic test

2.6.4.2 Ames test

The Ames Salmonella/microsome mutagenicity assay (Salmonella test; Ames test) is a short-term bacterial reverse mutation assay specifically designed to detect a wide range of chemical substances that can produce genetic damage that leads to gene mutations. The test is used to evaluate the mutagenic properties of molecules and extracts. The Ames test uses amino acid-dependentt strains of *Salmonella typhimurium* and *Escherichia coli,* each carrying different mutations in various genes in the histidine operon. These mutations act as hot spots for mutagens that cause DNA damage via different mechanisms. In the absence of an external histidine source, cells cannot grow and form colonies. Only those bacteria that revert to histidine independence (his+) are able to form colonies. The number of spontaneously induced revertant colonies per plate is relatively constant. However, when a mutagen is added to the plate, the number of revertant colonies per plate is increased, usually in a dose-related manner. The Ames test is used worldwide as an initial screen to determine the mutagenic potential of new chemicals and drugs (Tejs, 2008).

MATERIALS AND METHODS

3.1 Extractive techniques

3.1.1 Ultrasound extraction

9 g of the dry aerial part of both drugs are ground to obtained 7.9 g of powder sample. 1 g of the powdered drug was added to 10 ml of MeOH (drug/solvent ratio of 1:10) and 1 g to 7 ml of EtOH and 3 ml of distilled water to obtain 70% EtOH. The extraction was performed by an ultrasound device (Ultrasonik 104X, Ney Dental International, MEDWOW, Cyprus, the total volume 10.4 L, internal dimensions: 146 x 292 x 241 mm, frequency: 48 kHz) for 30 minutes at room temperature. After centrifugation of the MeOH extract, the supernatant was transferred to a test tube and dried with a rotavapor, while in the 70% EtOH extract, it was reduced in volume by a rotavapor, then freeze-dried to remove water.

Figure 18.- Extraction in MeOH and EtOH 70% *H. officinalis*. subsp. *aristatus*

3.1.2 Steam destillation

The technique used to produce the essential oil of *H. officinalis* subsp. *aristatus* and *Ocimum campechiamun*, was the hydrodistillation. 25 g of plant material was weighed in a 1000 ml flask: 300 ml of distilled water was added together with some glass beads to prevent violent boiling. The flask was inserted in a heating mantle, which allows steam formation in situ, and subsequently connected to the distillation column and condenser (Clevenger type equipment). The optimized distillation time is 3 hours.

The samples were stored in the dark at − 18 °C in a freezer until further analysis. The yield of essential oil is expressed as the volume percentage of essential oil on air-dried plant material.

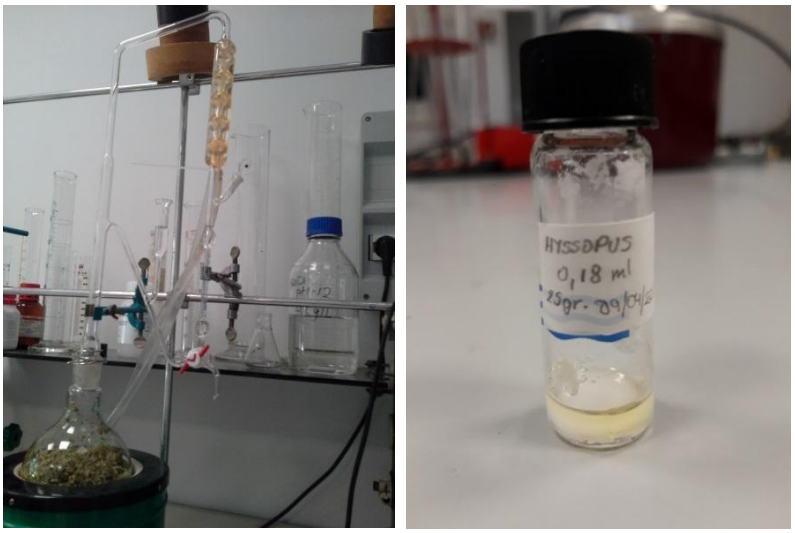


Figure 19.- Steam destillation *H. officinalis* subsp. *aristatus* essential oil

3.2. Characterization of the extracts

3.2.1 GC-MS and GC-FID analysis

The GC-MS was used to analyze the essential oils of *H. officinalis* subsp qualitatively. *aristatus* and *O. campechiamun*, the GC-FID, to obtain quantitive data.

The GC-MS analysis was performed with a Varian 3800 chromatograph equipped with a Varian Factor Four VF-5ms column (5%-phenyl-95%-dimethylpolysiloxane, internal diameter: 0.25 mm, length: 30 m) interconnected with a Varian mass spectrometer SATURN MS-4000, with electronic impact ionization, ion trap analyzer and software provided with the NIST database for the identification of components. The experimental conditions used were the following: helium carrier gas (1ml/min), ionization energy (EI) 70 eV, emission current of 10μA, the scan rate of 1 scan/sec, mass range 40-400 Da, 150 °C temperature of the ion trap, transfer line temperature 300 °C.

Operating conditions for GC were the following: injector temperature 280°C, carrier (Helium) flow rate of 1 mL/min, and split ratio 1/50. The initial oven temperature was 55°C and then increased to 100°C at a rate of 1 °C/min, successively to 250°C at a rate of 5°C/min and then constant at 250°C for 15 min.

The analysis was carried out by introducing 1μL of a solution consisting of 10 μL of pure essential oil dissolved in 1mL of methylene chloride in the gas chromatograph injector. The acquisition time was 90 minutes.

The FID analysis was performed in the same conditions above described, and FID temperature was 250°C.

The spectrum and arithmetic indices of the detected molecules were compared with the data present in the NIST library and with the literature (Adams, 2007).

3.2.2 HPLC analysis

HPLC analyses of ethanolic and methanolic extracts of *H. officinalis* subsp. *aristatus* and *O. campechiamun* were performed.

The analysis was performed using a JASCO modular HPLC instrument (Tokyo, Japan), PU-2089Plus, with MD-2010Plus DAD photodiode detector and a 20 µl loop. For the analyses, a Kinetex-C18 column (150x4.6 mm, 100 Å) was used.

The extracts in 70% EtOH (concentration of 0.69 mg/ml) and in MeOH (concentration of 0.60 mg/ml) were analyzed. The mobile phase used was composed of two solvents:

A) Water (1% formic acid)

B) MeOH

The method provides a gradient elution, as shown in the table:

Time (min)	Solvent A	Solvent B
0.00	95	5
50.00	70	30
65.00	70	30
70.00	95	5
85.00	95	5

Table 2. Gradient of the mobile phase for HPLC-DAD analysis

The column is maintained at a temperature of 30°C, and the eluent flow is adjusted to 0.7 ml/min. For the calibration curve, five solutions containing the three components identified in the hydroalcoholic extracts, a different concentration, were prepared: for caftaric acid, the concentration range was 2.5-50 µg/mL, chlorogenic acid 10-250 µg/mL, for rosmarinic acid 10-50 µg/mL. All standards were purchased by Extrasynthese (Cedex, France).

The substances were taken from pure samples at 1 mg/ml and then diluted with MeOH. For each solution, three chromatographic runs were performed.

3.2.3 HPTLC analysis

This technique has been applied to methanol and ethanol extracts and to the essential oils. The method used for the separation of flavonoids consists of the following mobile phases:

- first stage: ethyl acetate - formic acid - acetic acid - distilled water (100: 11: 11: 20) with elution up to 4 cm.
- second phase: toluene - ethyl acetate - acetic acid (100: 90: 10) with elution up to 8 cm.

To characterize the terpenes, present in the essential oil, however, the following eluent was used: Toluene - ethyl acetate (93: 7)

Once the elution was carried out, the chromatographic plates were visualized at wavelengths of 366 nm and 254 nm with the help of CAMAG Visualizer.

The plates were then derivatized with:

1. Reagent with sulfuric acid/vanillin
1) 1% solution of vanillin in ethanol;
2) 10% sulfuric acid in ethanol

   the plate containing the essential oils is sprayed with solution 1, followed immediately by solution 2; then, it was heated at 100°C for 5-10 min. After the treatment, monoterpene alcohols and their esters show a blue or violet-blue color.

2. Reagent with NP/PEG (1% diphenylboryloxyethylamine in MeOH, 5% polyethylene glycol-4000 in EtOH): the plate is sprayed with NP and then with PEG. This reagent is used for the detection of flavonoids and aloins. After the treatment, an intense fluorescence, reinforced by PEG, be developed at visible and UV-366 nm light.

3.2.4 Analysis by ^{1}H-NMR and ^{13}C-NMR

15 mg of essential oil and the pure separated compound was dissolved in 1 ml of $CDCl_3$ and analyzed at ^{1}H-NMR and ^{13} C-NMR at 400 MHz. To confirm the structural identity of the main components, the spectrum of essential oil was compared with that of the molecules isolated from chromatographic column.

3.2.5 Gravity colum chromatography

For the isolation of the unknown compound of the essential oil, a gravity column chromatography was performed.

250 µl of essential oil was dissolved in a few drops of the mobile phase, choose through preliminary TLC performed to show the better composition of the eluent. The mobile phase identified for the separation was: hexane: ethyl acetate (98: 2)

The chromatographic column (height 15 cm, diameter 2.5 cm) is prepared with the wet method using silica gel (silica gel 60 mesh, particle size: 0.035-0.070 mm) as the stationary phase. The sample is placed at the head of the column. Then the mobile phase is introduced and eluted through the stationary phase by gravity.

The fractions were analyzed by TLC, and the plate was sprayed with the phosphomolybdic acid solution (20% phosphomolybdic acid in EtOH) (Wagner et al., 2009). When sprayed, the plate is heated to 120 °C- The components of the essential oil will show an intense blue color on a yellow background. The tubes containing our pure analyte were collected in a flask, and the eluent was evaporated in the rotary evaporator. Subsequently, the sample was dried with nitrogen.

An aliquot of analyte was taken to perform a purity test on the gas chromatograph.

3.3 Antioxidant activity

3.3.1 HPTLC derivatization with DPPH

The chromatographic plates deposited in twice were visualized at the wavelength of 366nm and 254nm by the CAMAG Visualizer and subsequently derivatized in first part with NP/PEG reagent (1% diphenylborilosisethylamine (NP) in MeOH; 5% Polyethylene glycol (PEG) in EtOH) and finally derivatization with DPPH (200 mg of DPPH in 100 ml of 70% EtOH).

3.3.2 DPPH microplate reader asssay

Being a colorimetric analysis, it is necessary to identify a range within which it is possible to evaluate the antioxidant power better. The evaluations of the antioxidant activity were carried out in the in MeOH and the hydroalcoholic EtOH extract of *H. officinalis* subsp. *aristatus*, making them react with a stable radical, 2,2-diphenyl-1-picrylhydrazide (DPPH, Sigma-Aldrich). The experiment was carried out in 96-well plates and with a microplate reader for data acquisition (Microplate Reader 680 XR, Biorad): the wavelength was fixed to 515 nm, the closest to the absorption peak of the radical form.

The hydroalcoholic extracts were serially diluted (with a factor of 1: 1) to obtain 7 different concentrations in a range of 0.51-172 µg/ml for MeOH and hydroalcoholic extract, 0.8-40µg/ml for rosmarinic acid and 0.6µg/ml for chlorogenic acid. Likewise, Trolox (6-hydroxy-2,5,7,8-tetramethyl-chromium-2-carboxylic acid) was prepared, used as a positive control (200µL/ well, concentration of 0.04 mg/ml in 50% ethanol), in a dilution range of 0.04-20 µg/mL. Finally, 100µl of a DPPH solution (final 0.208mM) in 50% ethanol was added to each well. As a negative control, only DPPH was used, and the 50% ethanol solution was white instrumental. The plates were incubated at room temperature with shaking and in the dark for 40 minutes, then analyzed in the microplate reader at a wavelength of 515 nm. With the values obtained, we calculate IC_{50} (extract concentration capable of eliminating 50% of the DPPH radical) of the hydroalcoholic extract in MeOH, EtOH, rosmarinic acid, chlorogenic acid, and Trolox, according to the following

$$\%\ \text{DPPH removed} = \left(1 - \frac{\text{AC} - \text{Awhite}}{Acontrol - Awhite}\right) * 100$$

Where AS is the absorbance of the well with the sample and the DPPH, *Acontrol* is the absorbance of the wells with the DPPH alone, and *Awhite* is the absorbance of the wells with 50% ethanol. Lower IC_{50} value corresponds to the higher antioxidant capacity (Guerrini et al., 2016).

3.4 Antimicrobial activity

The qualitative and quantitative antimicrobial activity of the methanol and ethanol 70% extracts and of the essential oil of *H. officinalis* subsp. *aristatus* was carried out on Gram-positive bacteria *Staphylococcus aureus* (DSM 799).

To carry out this analysis, the cell culture sample must be prepared at least 48 hours in advance, causing the cells to proliferate in a controlled environment with appropriate nutrients.
The strain, contained in a cryovials with nitrogen and glycerine, is inoculated in a liquid medium consisting of 0.6 g of Tryptone-Soya Broth in 20 mL of distilled water, previously sterilized for 15 minutes at 121°C. The culture is incubated for 24 hours at 37°C under mechanical agitation. Subsequently, a dilution is made by taking 100 mL of broth containing the bacterial sample and introducing it into a second sterilized liquid medium. The culture is again incubated at 37°C for 24 hours in constant agitation.
The medium containing the bacterial colony will be used to investigate the antibacterial properties of extracts of *H. officinalis* subsp. *aristatus*

3.4.1 HPTLC bioautography assay

This technique evaluates the active ingredient of the extract that turns out to be the antimicrobial agent. A solid culture medium consisting of 8.0 g of tryptone-soybean agar is prepared in 200 ml of distilled water, sterilized for 15 minutes at 121°C. 5ml of liquid medium containing the bacterial cell culture and 0.625 µl of vitalizing dye (20mg/ml of dye in 1 ml of sterile distilled water) were inoculated. The dye serves to highlight the presence of bacterial cells. 2,3,5-Triphenyltetrazolium chloride containing a tetrazolium salt that colors the tissues of the bacterial cells was used. The microorganisms store the dye through an active transport mechanism and, through its dehydrogenase enzymes, divide the N-N double bonds of the molecule to form the red TPF (1,3,5-triphenylformazan) of red color.
Subsequently, chromatographic plates are prepared, using the appropriate eluents:

- one plate (4.5x10 cm) for the extract in MeOH and EtOH 70%
- one plate (9x10 cm) for the essential oil containing essential oil and thymol (standard).

Once the plates have been developed, they are inserted into Petri dishes, and the solid culture medium is poured on them; it is left in incubation for 24 hours at 37°C. In the presence of bacterial colonies, we would have a red color, in correspondence with the bands where the antimicrobial active ingredients are present, we would have a yellow coloration (Guerrini et al., 2016).

3.4.2 Determination of the MIC value

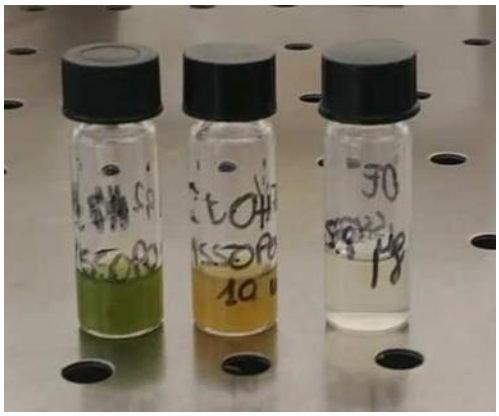
MIC is the lowest inhibitory concentration or the lowest concentration of the antibacterial substance that inhibits the reproduction of the microorganism. This value is calculated using a method that involves a series of successive dilutions of our bacterial cell culture.
Starting from the mother cell culture, eight successive dilutions are made (dilution factor 1:10) with sterile distilled water. A known volume of some diluted cell cultures (having dilution factors 10^6, 10^7, 10^8, 10^9) is sown in Petri dishes on a solid medium (Tryptone-Soya-Agar) and placed in an incubator at 37°C. After 24 hours the colony count is performed to establish, based on the dilution

factor, the quantity of bacteria presents in the stock solution. The mother cell culture is stored at 4°C to inhibit bacterial activity and alter the cellular microbial amount within the suspension.

For each extract, a solution containing the analyte, the DMSO, and the culture broth (Mueller Hinton Broth, MHB) is prepared.

The experiment is conducted in 96-well plates (microplates) with a capacity of 200µL. An antibacterial drug, chloramphenicol (concentration range 20 - 0.32 µg/mL) was used as a positive control. As a negative control, 2µL of dimethylsulfoxide (DMSO), as white and to check for possible contamination, the only inoculation medium was used.

Following the guidelines of the "Clinical and Laboratory Standards Institute" (CLSI) seven serial dilutions of the three analytes with factor 1:2 were made starting from 50µg/mL in MHB medium for methanol and ethanol extracts and 20 µg/mL for the essential oil, with a volume of 100µL per well. To these were added 100µL of bacterial inoculum at a concentration of $5x10^5$ CFU/mL. The microplates were incubated with agitation (110 rpm) (Clinical and Laboratory Standards Institute, 2012).



Fugure 20.- Stock solutions for the determination of MIC, 70% EtOH extract, MeOH extract, the essential oil.

After 9-12 hours of incubation at 37° C, in each well, a vital dye is inoculated, the yellow 3- (4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) which it turns towards blue in the presence of bacterial cells. This happens thanks to the intervention of bacterial dehydrogenases that reduce MTT in formazan. The dye had a concentration of 0.62 mg/mL, and in each well 40µL of the solution was inserted: the microplate was incubated at 37°C for 30 minutes under stirring. In the wells where antimicrobial substances are present, the coloring will remain yellow. Based on the dilution factor, knowing the inoculated bacterial concentration, the MIC will be determined (Guerrini et al., 2016).

3.5 Cytotoxic activity

3.5.1 Prepation of cell cultures

A549 and HaCaT cells were propagated in 75cm^2 flasks, in Ham's F12 medium, instead of the HaCaT in DMEM medium: both were added with a 2 mM solution of L-glutamine, 100 U/ml of penicillin and 100 mg/ml of streptomycin. The operations described below must be performed in conditions of maintenance of sterility and using a laminar flow hood (SAFE FATE ELITE FASTER / NICOTRA). After an initial sterilization phase, the incubator operating parameters (Memmert INC153 CO2 Incubator) is set at 37 °C in an atmosphere saturated with humidity and 5% conditioned in carbon dioxide. Every treatment of cell culture must be carried out at 37 °C in order to avoid thermal shock.

Starting and maintainance of cultures

40 ml of 20% medium are prepared in FBS (Fetal Bovine Serum) and are brought to 37 °C in a heating bath. The vial containing the cells (volume 1 ml) was frozen, and the content was transferred in the falcon, already containing the medium (16 ml) at temperature, as quickly as possible. The content of the falcon is transferred to the plate, and the culture is placed in the incubator.

After 4-5 hours, the adherence of the cells is verified, the supernatant is eliminated, and 20 ml of fresh medium is added to 20% of FBS. Then the culture is transferred to the incubator overnight.

The following day the medium is replaced at 20% in FBS with a 15% FBS medium, proceeding as follows: the previous culture medium is discarded, washed three times with 10 ml aliquots of PBS, in order to eliminate culture residues that would cause trypsin inactivation. 1 ml of trypsin solution is added, the culture is kept in the incubator for 6 minutes until the cells are detached. Then 9 ml of culture medium are added to the flask, and the dispersion is mobilized. Finally, an aliquot of the old crop is transferred to a new flask with a new medium.

In the subsequent culture propagation, it is defined how many fractions of the previous culture must be propagated on the basis of cell crowding and the number of cells needed. The percentage of FBS can vary from 20 to 10%, depending on the maintenance times of the culture and the biomass required.

Preparation and cryopreservation of cell lines

In order to maintain a constant cell line aliquot it is necessary, at the end of the incubator maintenance, to prepare a vial containing the cellular dispersion that can be preserved at extended times in liquid nitrogen. The list of operations are:

- filter the FBS with a 0.2-micron filter;
- trypsinize the cellular dispersion;
- centrifuge for 5 minutes at 1000 rpm in Falcon, discard the supernatant;
- resuspend the pellet in an Eppendorf with 500 µL of the medium at 37 ° C at 20% FBS;
- add 450 µL of filtered FBS and 50 µL of DMSO;
- transfer the contents of the Eppendorf into the freezing vial;
- freeze for 30 minutes at -20 ° C;

- keep overnight at -80 ° C;
- transfer the vial into liquid nitrogen.

Quantification of cell number with Burker-Turk chamber

To standardize the quantifications, it is necessary to trace the cellular numerosity to defined values by the various protocols. To this aim, the counting is carried out with the optical microscope using the Burker-Turk chamber. The list of operations are the following:

- take 10 µl of the Trypan Blue dye and place them in 1.5 ml Eppendorf (the Trypan Blue is photosensitive, then proceed under the hood with the light off by wrapping the container with aluminum foil to avoid light irradiation);
- add 10 µl of cell suspension and mix;
- insert 10 µl of this solution in both chambers of the Burker-Turk chamber and proceed with the microscope (Primo Vert inverted microscope, Zeiss) to quantify the cellular size.

Following this procedure, the cellular dispersion was diluted in the ratio one to two. It can easily be traced to the number of first cells. If the number of cells is so high as to make counting difficult, further dilution must be carried out before adding the dye solution.

Preparation of solutions

In the preparation of the essential oil samples, we start from a 200 mg /ml stock solution obtained by solubilizing the sample in pure DMSO. This is then diluted with 2% FBS up to a titer of 200 µg/ml of sample and 0.1% in DMSO. The solutions having a different titer of the sample under examination are obtained by dilution with medium containing FBS at 2% and DMSO 0.1%.

For the ethanolic extract, a certain amount of lyophilisate is weighed, and the medium with FBS at 2% is added to obtain a stock solution of 200 µg/ml; the successive dilutions are always made with medium with only 2% FBS.

The solutions of pure compounds are obtained by weighing the pure standard and solubilizing it in pure DMSO, to reach a concentration of 100 mM. This is diluted with the required amount of medium at 2% FBS up to a titer equal to 100 µM, and the subsequent solutions are obtained by adding medium with FBS 2% and DMSO 0.1%.

3.5.2 Cell vitality test - MTT test

The test is conducted over several days according to the following procedure.

First day

Starting from a suspension containing $1x10^5$ cells/ml at 10% in FBS, 200 µl of suspension are sown In each well in 96-well microplate. The plate is then incubated at 37 °C in an oven for 5 hours. After verifying the state of the culture, the medium at 10% in FBS is replaced with a 2% FBS medium. This FBS title, while allowing the maintenance of the crop, prevents its propagation and keeps the cellular number constant.

Second day

In each well of the plate, after washing with PBS, the culture medium is replaced with the solutions to be tested and with the positive and negative controls. As negative controls, we used medium with only FBS 2% and medium with FBS 2% and DMSO 0.1%, used in the dilutions of the different solutions. Instead, as a positive control, we used doxorubicin.
Therefore, a further incubation period of 72 hours is carried
out. Third day
- Prepare the working solution in MTT (0.5 mg/ml) under the hood in the dark: 1.2 ml of MTT stock solution in 10.8 ml of PBS are added, and it is kept in the dark;
- Remove the medium and wash with 200 µl of PBS per well (37 °C);
- add 100 µl of MTT solution to each well (always in the dark);
- incubate the culture for 4 hours under standard conditions;
- Remove the supernatant and add 150 µl of DMSO per well trying to suspend the formazan salts;
- Proceed to the spectrophotometric reading in a microplate reader at 570 nm (Microplate Reader 680 XR).

3.5.3 Migration test

This test allows studying cell migration *in vitro* in response to a chemotactic stimulus. In particular, the Boyden chambers are used that, thanks to a porous membrane that separates two environments, allow to simulate what happens *in vivo*. Cells must be done grown in culture flasks in a medium containing 10% FBS. Once they reach the confluence, they are trypsinized and counted with Burker's chamber. In our experiments we used a final solution of $1 * 10^5$ cells per ml, in an FBS-free medium. Therefore the cells were centrifuged (5 minutes at 1000 rpm), and once the supernatant was eliminated, the necessary amount of medium was added; all must be stored in the 37 °C bath to maintain the ideal temperature for the cells.
The cell migration experiment was performed using the following protocol:
- In a microplate of 24 wells, in sterile conditions, the number of desired Boyden chambers is inserted, equivalent to the number of samples to be tested, possibly in the same row;
- 100 µl of medium without FBS is added in each chamber (area above the membrane);
- 100 µl of the solution containing $1 * 10^5$ cells per ml are deposited with the medium
- in the area outside the Boyden chamber, 750 µl of medium containing 2% FBS is added;
- the whole must be incubated overnight at 37 ° C in a controlled atmosphere (5% CO_2 and 95% humidity);
- after incubation, remove what is in the upper part of the Boyden chamber, and perform two washes with PBS;
- using a 37% formaldehyde solution in PBS, left in contact with the cells for about 2 minutes, the cells are fixed;

- after removal of the fixing solution and subsequent washing with PBS, 100% methanol is added and left to act for 20 minutes;
- then the methanol is removed and washed twice with PBS. It is left to dry, and it is then stained with a cellular solution of Giemsa dye at 5% in PBS for 15 minutes, avoiding exposure to light;
- after this period, the dye is eliminated, it is washed twice with PBS, the excess cells are removed with a cotton bud from the upper chamber, and the observation is carried out under a microscope.

3.5.4 Invasion test

We proceed precisely as for the migration test, with a small modification. It is necessary to place inside the inserts a layer of Matrigel® that simulates the basal membrane. The day before sowing the cells in the chambers, 100 µl of a solution of Matrigel® in a medium without FBS, at a concentration of 200 µg/ml, must be placed in the upper part of the Boyden chamber, and the microplate must be incubated at 37 °C overnight. This gel solidifies at temperatures between 20 and 40 °C, so it is necessary to work with previously cooled equipment. The next day it will not be necessary to deposit 100 µl of medium without FBS.

3.5.5 Wound healing test

From the primary trypsinized culture, a pellet is separated by centrifugation and dispersed again in the culture medium. Then the cells are inoculated into a 24-well plate to have 100% confluence in 24 hours. The timing and number of cells needed to achieve this condition can be established with some preliminary tests. We have verified that about one million cells can be used for both types of cells.

In a sterile environment, using a 200 µl Gilson pipette tip creates a vertical wound through the cell monolayer taking care not to cut the surface of the well.

The culture medium is carefully aspirated and with it, the cellular debris. Then the medium is added with the molecules to be tested to the desired concentration, against the walls of the well to cover the bottom of the well itself, avoiding to detach other cells. A microscopic image must then be taken as the starting point, and the culture is left in an incubator set according to the parameters defined for growth.

At different times the plate is removed from the incubator and positioned under the microscope to resume a snapshot, and wound closure is checked. At the end of the experiment, we proceed by measuring the distance of the two edges of the wound at different times.

To maintain the cells in a state of quiescence, while retaining their motility, various modalities can be followed. In this project, we have maintained the FBS titer at 2%, and this has allowed us to avoid necrosis or apoptosis, without excluding the necessary metabolic processes.

3.6 Ames Test

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

The plates were incubated for three days at 37°C and the colonies were counted manually (Colony Counter 560 Suntex, Antibioticos, Italy). The results were considered positive when the number of reverting colonies was at least twice that of the negative control (Rossi et al., 2013).

RESULTS AND DISCUSSION

4.1.A Authentication of *H. officinalis* subsp. *aristatus* drug

4.1.A.1 Macroscopic analysis

Observation of the macroscopic features of the dried sample of *H. officinalis* subsp. *aristatus* flowering tops have allowed the definition of:

• **Drug:** stems, leaves, calyces, flowers, fruits.

• **Color:** blue-violet flowers, brownish-green the other parts

• **Odor**: aromatic, typical of Lamiaceae family

• **Morphological traits**: subshrub with the stem with a woody base, lanceolate leaves with revolute margins, floral leaves with an arista on the apex. Hermaphroditic flowers with the pentamerous sympetalous corolla, synsepalous conical calyx; the androcean consisting of 4 protruding stamens, gynaeceum with superior ovary which could contain the fruit, a tetrachenium

4.1.A.2 Stereomicroscope and microscopic assay

The observation of the morphological characters of the *H. officinalis* subsp. *aristatus* flowering tops highlighted:

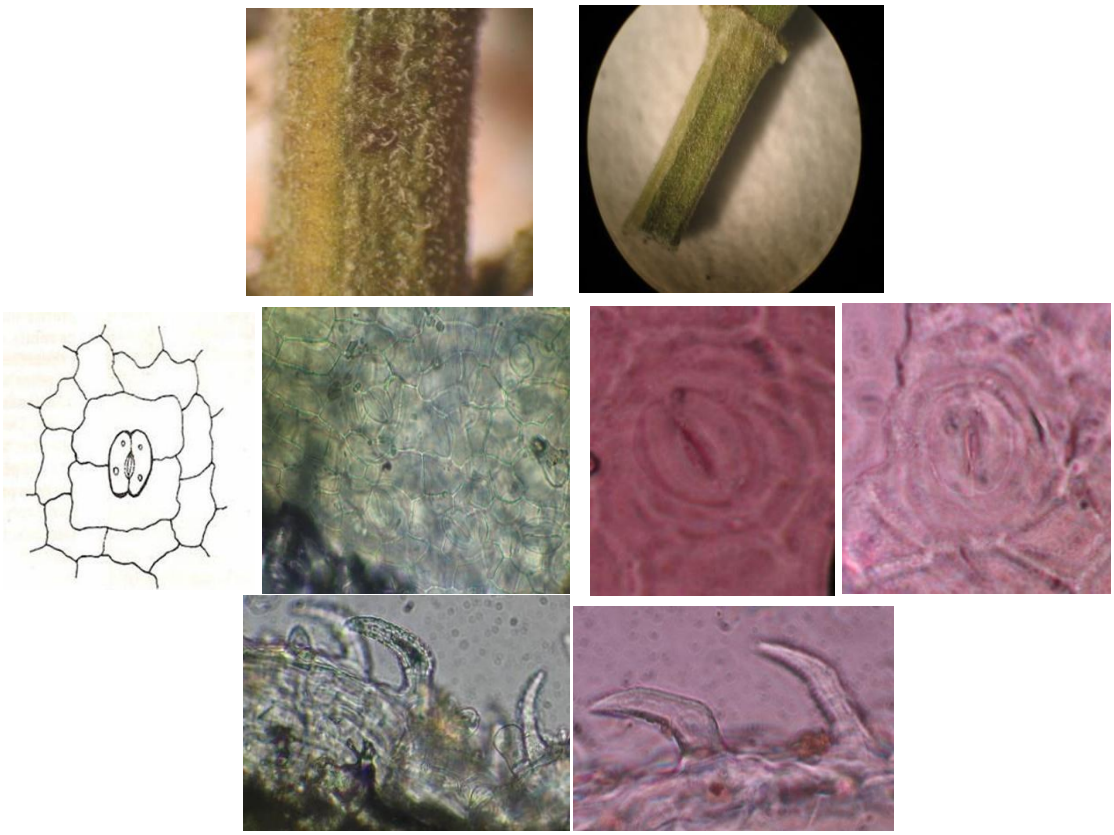
Stems: Woody stems with tiny trichomes (0.1 mm), diacytic stomata, and hooked trichomes. Quadrangular stem typical of Lamiaceae family **a)**

Leaves: 20-30 mm long leaves, with generally revolute at the margins; floral leaves have an aristate tip, characteristic of this species. **b)**

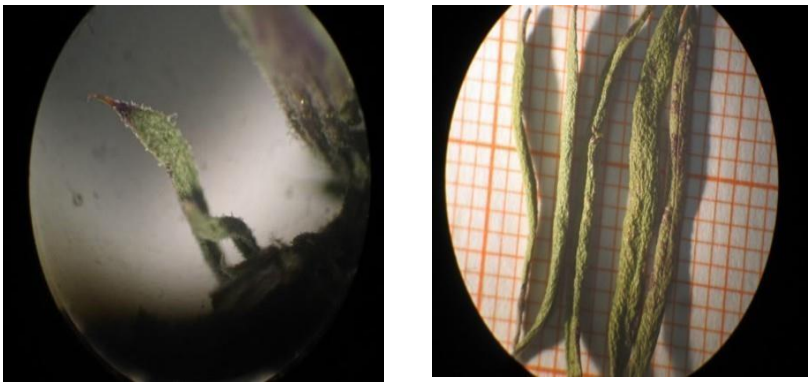
Calyx: synsepalous and conical calyx with aristate teeth; epidermis with numerous sinuous cells **c)**

Flowers: Conical calyx describes above; blue-violet sympetalous corolla with 4 long protruding stamens. On the surface, octo and bicellular glandular trichomes present. **d)**

Fruits: in the form of tetrachenium. **e).**

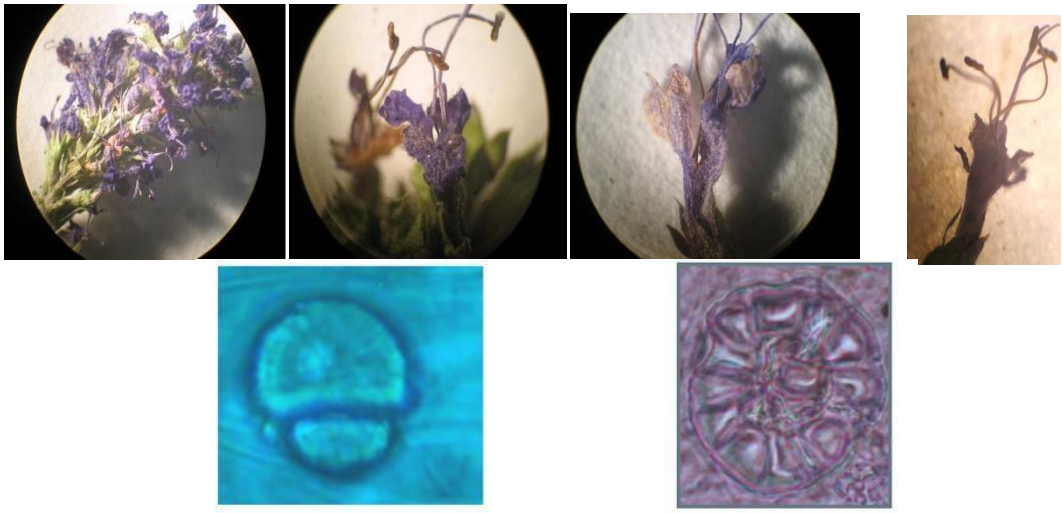


a) Stem (30x and 20x), diacytic stomata on epidermis (400x and 1000x), hooked trichomes (400x)

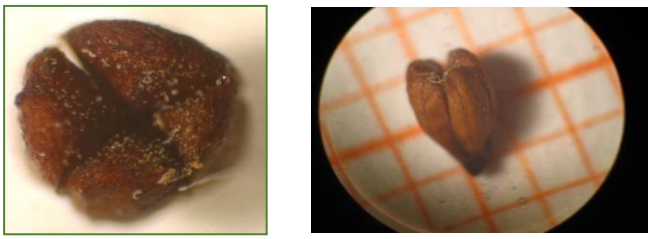


b) Leaves (20x and 8x)

c) Calyx (20x and 50x) and sinuous cells of epidermis (400x)



d) Flowers with conical calyx and an aristate teeth (20x); blue-violet corolla with 4 long protruding stamens (20x); bi and octocellular glandular trichomes (1000x).



e) Fruits (50x and 30x): tetrachenium

Figure 21. Morphological characters of *H. officinalis* subsp. a*ristatus*

4.1.B Authentication of *O. campechianum* drug

4.1.B.1 Macroscopic analysis

Observation of the macroscopic features of the dried sample of *O. campechianum* flowering tops has allowed the definition of:

• **Drug:** stems, leaves, calyces, flowers, fruits.
• **Color:** white-purple flowers; brownish-green the other parts
• **Odor**: aromatic, typical of Lamiaceae family;
• **Morphological traits:** Annual aromatic herb or with the wood base. Quadrangular stem, elliptic, or ovate leaves with the petiole. Racemiformes inflorescence: the flower has the glabrous or pubescent calyx, internally bilabiate, white-purple bilabiate corolla; 4 stamens (Hamel et al., 2007).

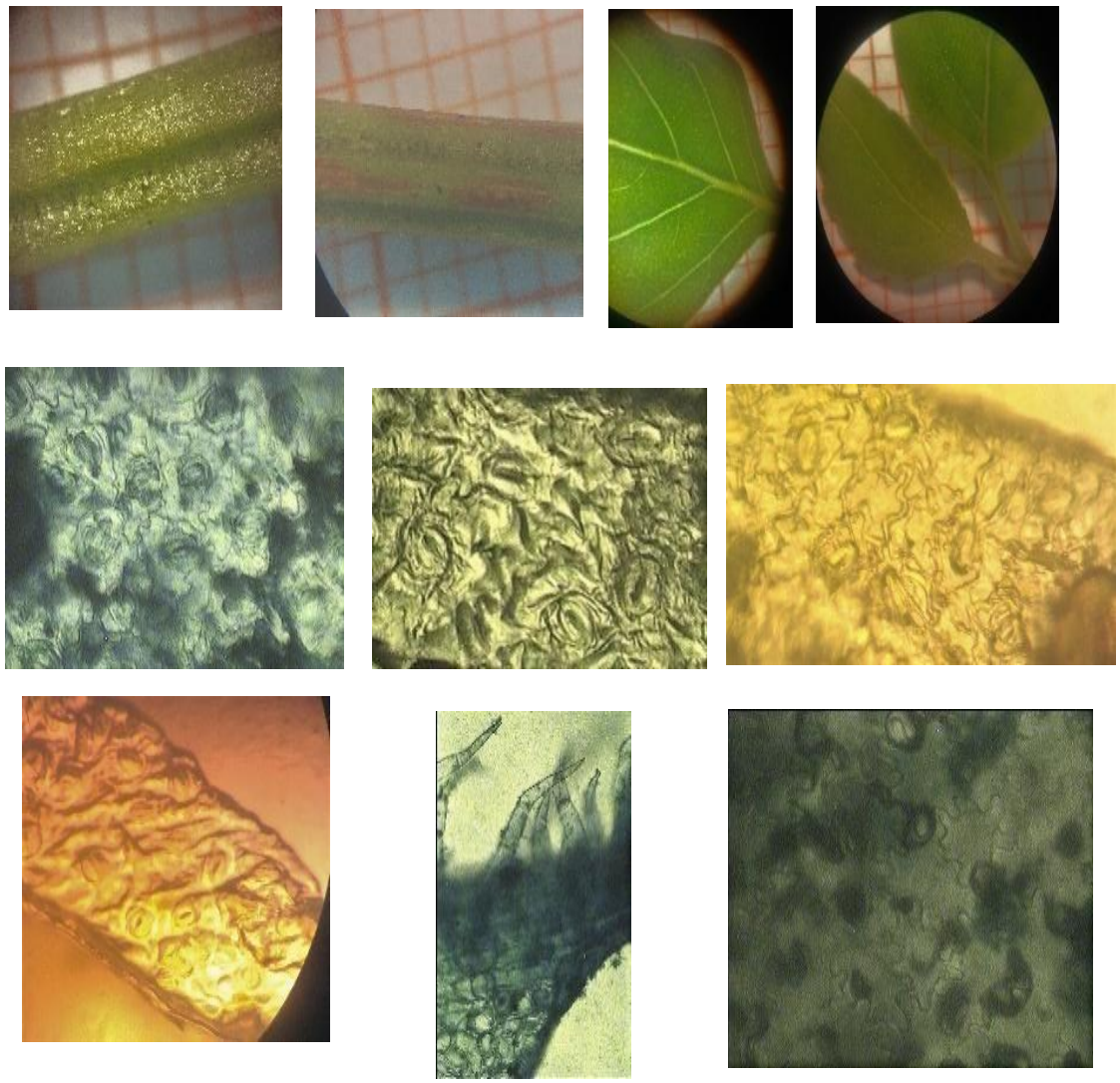
4.1.B.2 Stereomicroscope and microscopic assay

The observation of the morphological characters of the *O. campechianum* flowering tops highlighted

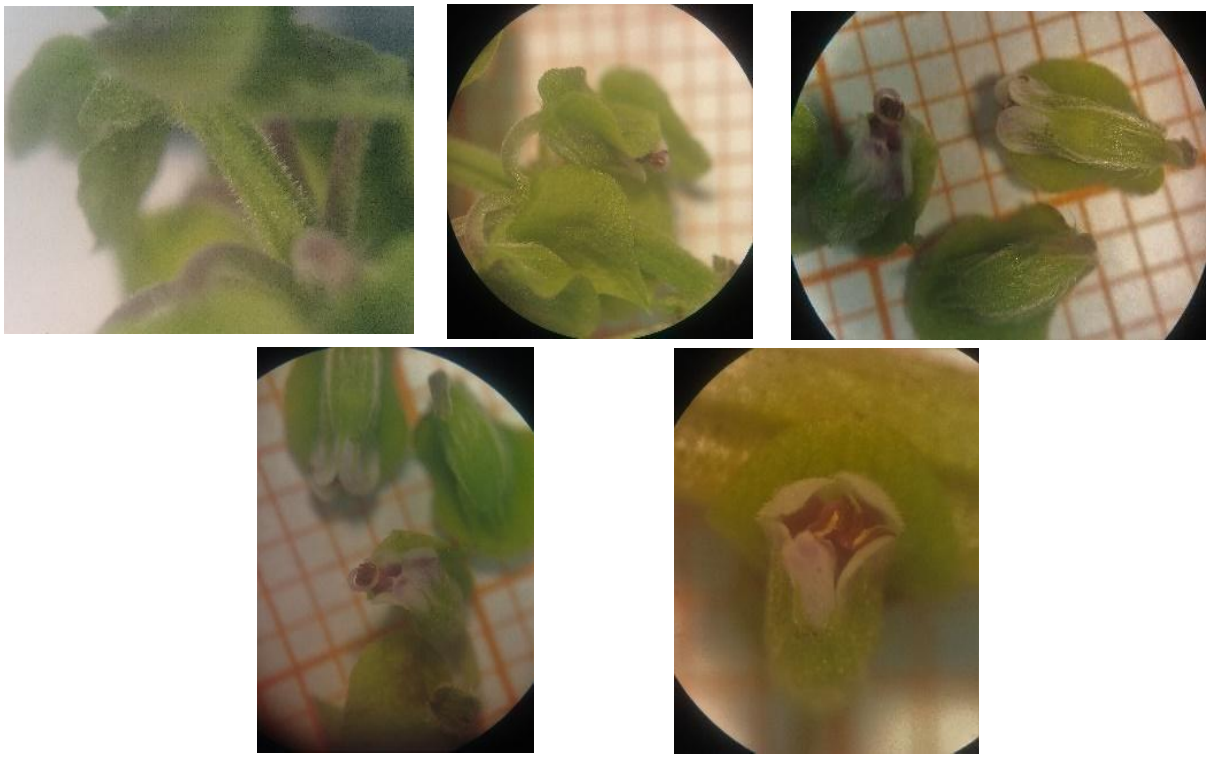
Stems: erect glabrous or pubescent stem of a quadrangular section, typical of Lamiaceae; epidermis with sinuous cells and anomocytic stomata

Leaves: elliptic, ovate lamina with an attenuated base with petiole; epidermis with sinuous cells nad anomocytic stomata **a)**

Calyx: 4-dentate bilabiate calyx, pubescent. Corolla: white-purple bilabiate corolla **b)** Fruits: tetraquenium. **c**



a) Stem, leaves (20-50x), stomata and trichomes (400x)



b) Calyx and flowers (30-50x)

c) Fruits (30-50x)

Figure 22. Morphological characters of *O. campechianum*

4.2 Extraction and chemical characterization of *H. officinalis* subsp. *aristatus*

4.2.1 Extraction and characterization of *H. officinalis* subsp. *aristatus* essential oils

The essential oils extracted by hydrodistillation of the aerial parts of *H. officinalis* subsp. *aristatus* was light yellow and showed a yield from 0.28 to 0.70% (table 3), similar to that reported by other authors (Džamić et al., 2013; Hajdari et al., 2018; Venditti et al., 2015) (Table 4).

Compound	*H. officinalis* subsp. *aristatus* 09/2016 (Civitarenga) Yield: 0.70% % Area	*H. officinalis* subsp. *aristatus* 09/2018 (Navelli AQ) Yield: 0.28% % Area	*H. officinalis* subsp. *aristatus* 10/2018 (Majella) Yield: 0.40% % Area	exp.AI[a]
alfa-pinene	0.3	0.2	0.3	928
Sabinene		0.3		965
beta-pinene	1.7	3.1	4.3	972
delta-2-carene		0.1		1012
para-cymene		1,35		1019
(S)(-)-limonene[b]	5.8	0.2	**7.6**	1024
1,8-cineole	15.5	**4.4**	**39.7**	1027
cis-beta-ocimene	0.7	0.3		1033
cis-sabinene hydrate		0.2		1064
para-2,4(8)mentha-diene		0.1		1079
trans-sabinene hydrate		0.2		1099
cis-thujone		0.3		1103
trans-thujone		0.1		1114
dehydro-sabina ketone		0.1		1115
cis-para-menth-2-en-1-ol		0.1		1120
Nopinone		0.5		1133
trans-sabinol		1.9		1133
trans-pinocarveol			0.4	1133
camphor		0.2		1140
***trans*-pinocamphone**		**11.0**		1153
pinocarvone		1.1		1155
***cis*-pinocamphone (o iso-pinocamphone)**	-	**43.2**		1168
terpinen-4-ol		1.0		1174
para-cymen-8-ol		0.2		1185
thuj-3-en-10-al		1.6		1189
myrtenol		1.3		1191
trans-carveol		0.1		1217
(S)(-)-limonen-10-ol methyl ether[b,c]	1.9			1235
carvotanacetone		0.2		1243
carvone	0.3	0.2		1244
(S)(-)-limonen-10-ol	2.8			1291
perilla alcohol		0.2		1296
beta-bourbonene	0.4	0.9	1.0	1381
4,7,10,13,16,19-Docosahexaenoic acid, methylester			0.3	1387
methyleugenol		**15.8**	**41.5**	1402
(S)(-)-limonen-10-yl-acetate[b,c]	**67.9**			1410
germacrene D	1.2			1476
bicyclogermacrene	0.4			1489
elemol			0.4	1547
ledol			1.0	1566
spathulenol	0.4	1.9	0.9	1577
caryophyllene oxide		0.7	1.5	1578
TOTAL IDENTIFIED	99.3	93.9	98.9	

[a]Experimental arithmetic index; [b]configuration determine d with optical rotation comparing data with literature; [c]structure identification through ^{1}H and ^{13}C NMR

Table 3. Results of GC-MS (identification of compounds) and GC-FID (%Area)

Figure 23. Extraction of *H. officinalis* subsp. a*ristatus* essential oil

Regarding the two samples collected in 2018, the chemical characterization highlighted the two chemotypes already known in the literature (Piccaglia et al., 1999; Hajdari et al., 2018), characterized by the prevalence of methyleugenol (41.5%), 1,8-cineole (39.7%) and (S)(-)- limonene (7.6%) for that from Majella area and *cis*-pinocamphone (43.2%), methyleugenol (15.8%), *trans*-pinocamphone (11.0%) and 1,8-cineole (4.4%) for the other from Navelli area.

Considering also other data in literature data (Džamić et al. ,2013; Rosato et al., 2018; Venditti et al., 2015) methyleugenol, 1,8-cineole, *cis*-pinocamphone, *trans*-pinocamphone, and β-pinene are the main compounds of *H. officinalis* subsp. *Aristatus*essental oil.

There was instead a substantial difference in the composition of the essential oil obtained from the flowering tops of 2016 annually (Civitaretenga area) if compared to the previous chemotypes. It showed a fingerprinting with the predominance of (S)(-)-limonen-10-yl-acetate (67.9%), which is not detectable in the previous literature data, followed by 1,8-cineole (15.5%), (S)(-)-limonene (5.8%), limonen-10-ol (2.79%), limonen-10-ol methylether (1.86%), β-pinene (1.74%) and germacrene D (1.15%). It is therefore composed to a greater extent of monoterpenes, deriving from limonene and only from a 2% of sesquiterpenes. When comparing our data with those in the literature, we noted a certain discrepancy. Piccaglia et al. (1999), Hajdari et al. (2018), Dzamic et al. (2013), Venditti et al. (2015), and Rosato et al. (2018) not detected limonen-10-yl acetate.

This molecule is found to be an FDA flavoring substance, identified in mandarins, lemons, peppermint, and grapefruit. In the European Union, however, it was removed from the list of flavorings by EFSA (Commission Regulation (EU) No 246/2014 of 13 March 2014 amending Annex I to Regulation (EC) No 1334/2008 of the European Parliament and of the Council as regards removal from the Union list of certain flavoring substances), since suspected of genotoxicity.

This possible new chemotype could be explained with peculiar biodiversity of the specific area, but we will perform further analyses on plants collected in the same area in different years to support a possible hypothesis.

Plant	Subspecies	Location	Months	Plant Material	Method	% yield	Bibliography
Hyssopus officinalis L.	ssp. *aristatus*		July-September - 2014		Hydrodistillation	0.24 – 2.0% ranged	(Hajdari et al., 2018)
		Albania		dry aerial part		1.7 - 2.0%	
		Kosovo		dry aerial part		0.24 - 0.56%	
Hyssopus officinalis L.	ssp. *aristatus*	(Abruzzo-central Italy)	September 2014	Flowering aerial parts	Hydrodistillation	1.1%	
		India	September– October , 2015	dry plant samples	(MAE) microwave assisted extraction	23.4 ± 0.36 (g/100 g of DW)	(Nile et al., 2017)
					(EAU) ultrasound-assisted extraction	20.6 ± 0.48 (g/100 g of DW)	
					(SE) Soxhlet extraction	15.1 ± 0.25 (g/100 g of DW)	
					(MC) maceration	12.7 ± 0.14. (g/100 g of DW)	
Hyssopus officinalis L.	----------	Mediterranean			hydrodistillation	0.6%	(Brockman, 2016)
Hyssopus officinalis L.	----------	Poland		dry plant	Steam Distillation	0.8 to 1.3 %. (literature)	(Zawislak, 2016)
			July 2006	beginning of plant flowering		0.23 %	
			August 2007	plant regrowth-end		0.19 %	
Hyssopus officinalis L.	ssp. *aristatus*	(Abruzzo-central Italy)	September 2014	fresh samples	hydrodistillation	1.1%	(Venditti et al., 2015)
			September 2014	dried samples		0.6%	
			August 2013	dried samples		0.7%	
Hyssopus officinalis L.	----------	Lublin	June July August 2007-2008	Herb dry vegetative stage beginning of flowering full blooming	Steam distillation	0.6% 1.2% 1.7%	(Zawiślak, 2013)
Hyssopus officinalis L.	subsp. *pilifer*	Sicevo East Serbia	July 2008	dry aerial part	hydrodistillation	0.6%	(Džamić et al., 2013)
Hyssopus officinalis L.	----------	Turkey	August 2009	aerial dried parts	hydrodistillation	2.29%, 0.7% to 1.13% ranged	(Figueredo et al., 2012)
Hyssopus officinalis L.	----------	Iran	June	flowering stage	hydrodistillation	1.3%,	(Fathiazad and Hamedeyazdan, 2011)
Hyssopus officinalis L.	ssp. *officinalis*	Urbino- Italy	September	flowering stage fresh plants grown at 100 m and 1000 m	hydrodistillation	2.30 and 3.1 ml / kg	(Fraternale et al., 2004)

Table 4. Yield of *H. officinalis* extracts and essential oils

The essential oil analyzed in GC-MS allowed to separate the components of the essential oil.

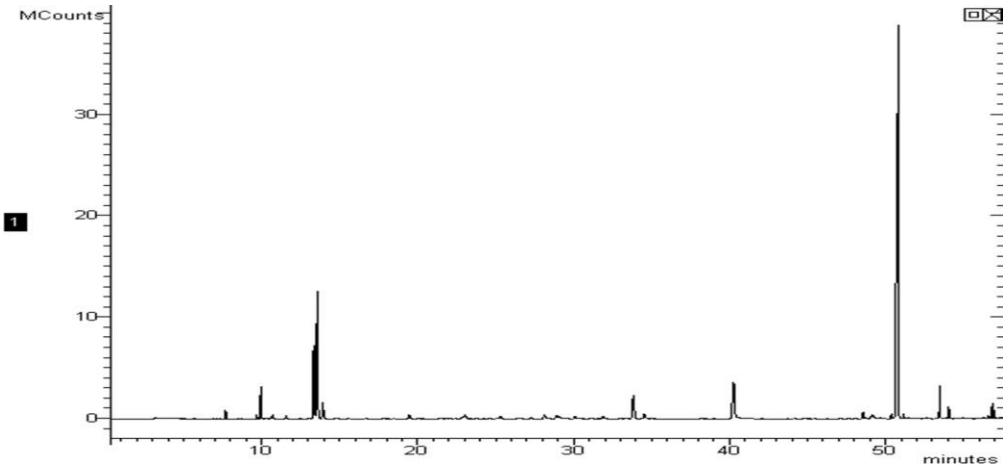


Figure 24.- Chromatogram of the essential oil from Civitaretenga analyzed by GC-MS

The structure of an unknown molecule, limonen-10-ol methyl ether, was deduced comparing the fragmentation pattern to limonen-10-ol and observing the Arithmetic index and fragmentation pattern of a couple of compounds: thymol and thymol methyl ether. The behavior of limonen-10-ol and limonen-10-ol methyl ether is like the couple thymol and thymol methyl ether.

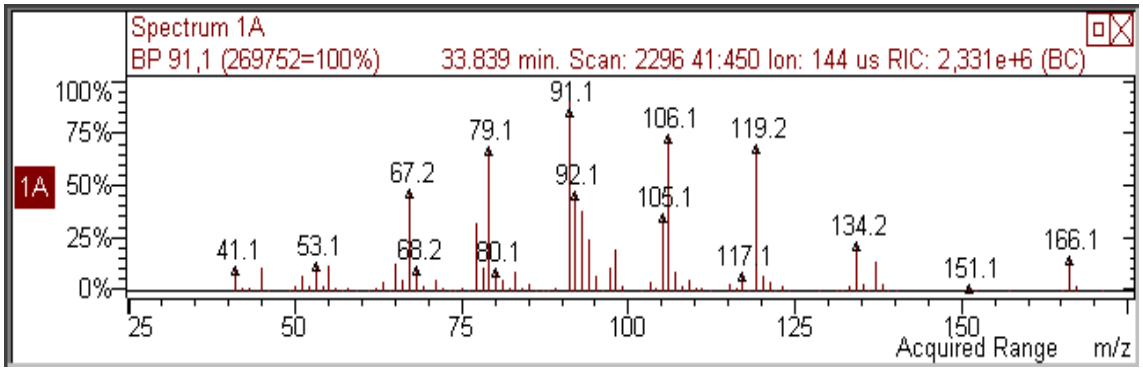


Figure 25.- Mass spectrum related to limonen-10-ol methyl ether

The structure of the main component (67.85%) of the essential oil was not elucidated with the mass spectrum only. The unknown molecule had spectrum like limonen-10 ol, but different Arithmetic index: this behavior with electron impact ionization is typical of molecule esters, whose molecular ion is not visible. Isolation by gravity column chromatography and characterization with nuclear magnetic resonance spectroscopy was necessary.

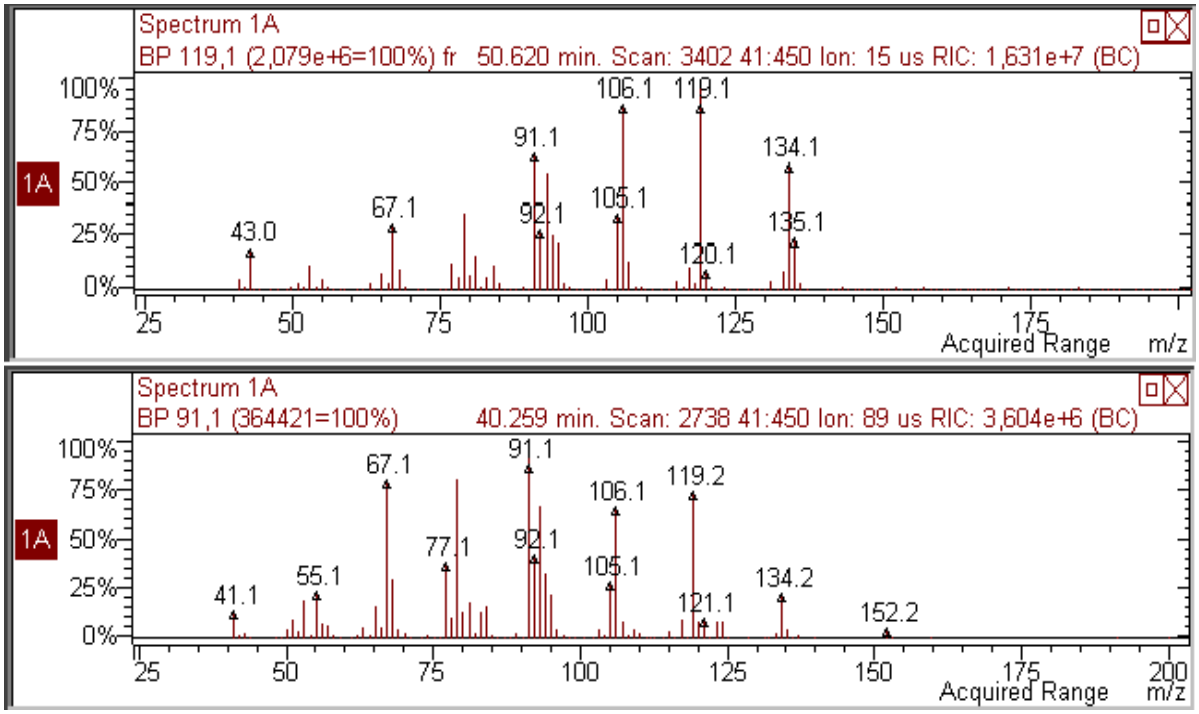


Figure 26. Mass spectrum related to the unknown substance and with limonen-10-ol

For the isolation of the main component, we chromatographed 250 µL of essential oil.

Two pure main fractions were separated. Preliminarily, purity was evaluated by HPTLC (hexane: ethyl acetate / 98: 2). The chromatographic plate was visualized by the CAMAG display at 254 and 366 nm. Then it was derivatized with a phosphomolybdic acid solution since terpenes do not absorb at these wavelengths.

The fractions with the same R_f were joined, and, with the use of a rotary evaporator, most of the eluent was removed, then the residue was dried under nitrogen flow. 58 mg of the unknown analyte was obtained. To assess the purity of the collected fractions, an aliquot was taken and analyzed in the GC-MS:

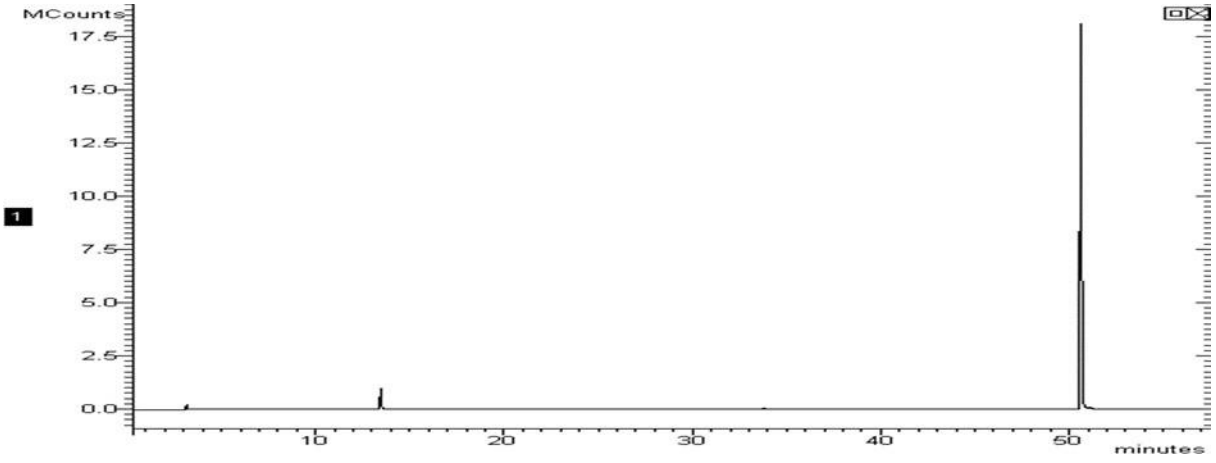


Figure 27.- Chromatogram of the separated unknown compound, analyzed in the GC-MS.

. The sample purity in GC-MS was 94%.

^{1}H-NMR and ^{13}C-NMR analyses were performed on the unknown isolated compound. We are comparing the spectrum of the essential oil of *H. officinalis* subsp. *aristatus* with those related to the isolated molecule and limonene we could deduced the chemical structure.

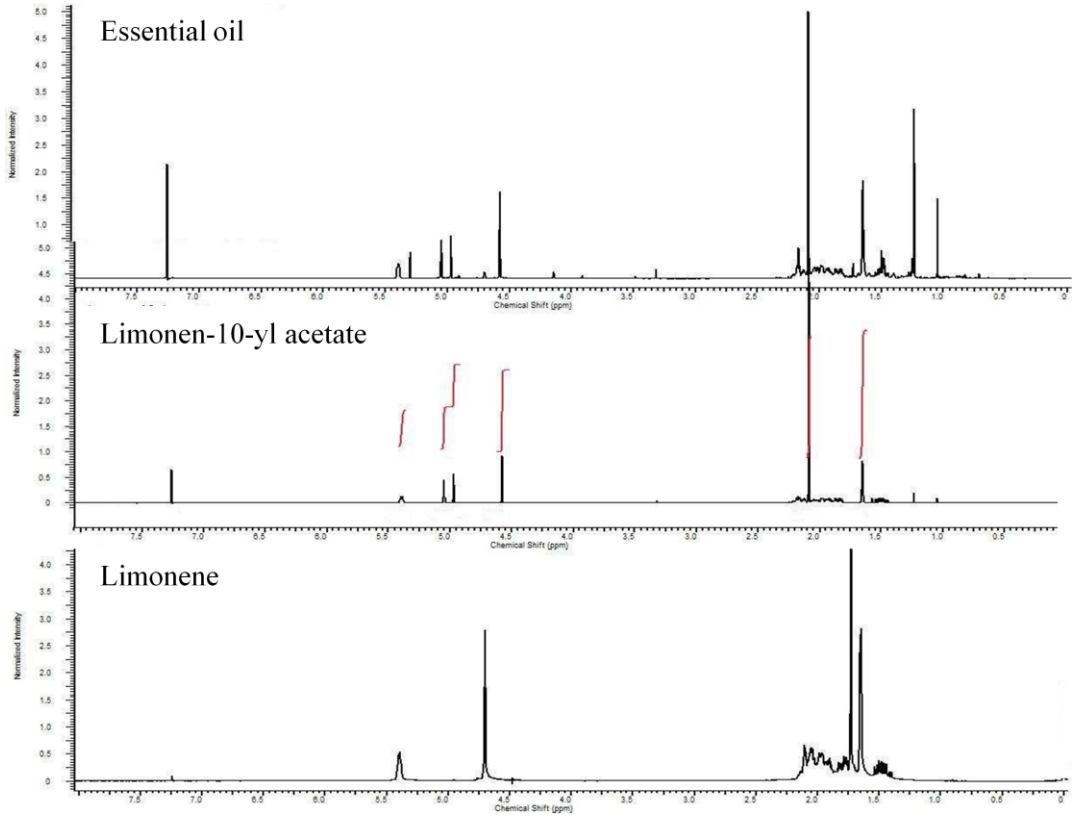


Figure 28.- ^{1}H-NMR spectra of the essential oil compared to that of limonen-10-yl-acetate and that of limonene.

The analysis of the ^{1}H-NMR spectrum showed that in the unknown molecule:

- the 1.8 ppm methyl group of isopropenyl limonene disappeared when it becomes a methylene group with diastereotopic protons that had a chemical shift of 4.95 and 5.05 ppm.
- a methyl group appeared with a typical acetate chemical shift at 2.05 ppm.
- the chemical shift of the olefinic protons of the limonene isopropenyl group at 4.70 ppm moved to 4.55 ppm, due to the presence of the acetate group.

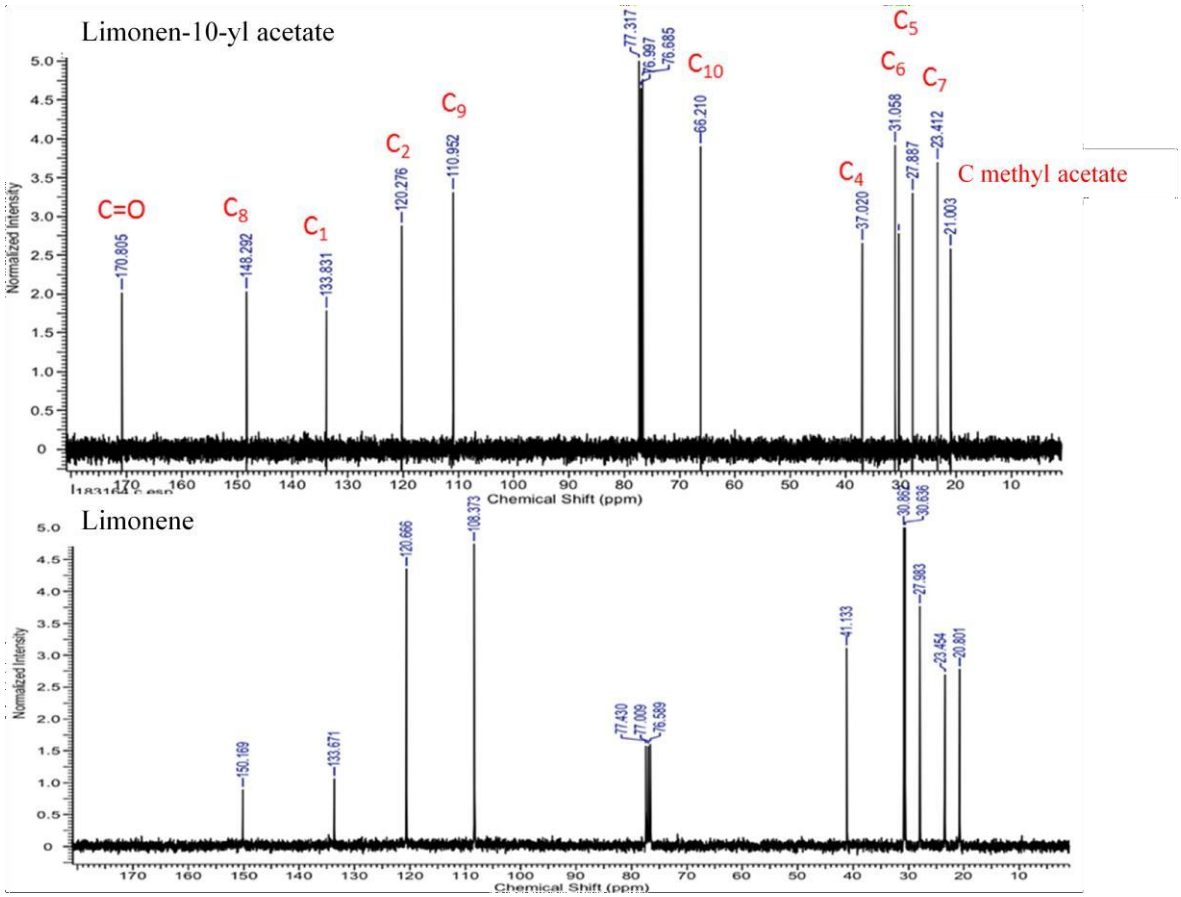


Figure 29.- ^{13}C-NMR spectrum related to the limonen-10-yl- acetate compared to that of the limonene.

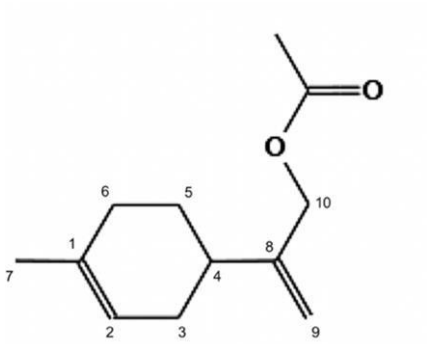


Figure 30. Limonen-10-yl acetate

The analysis of the ^{13}C-NMR spectrum justified the molecular skeleton of the isolated substance, hypothesized with proton nuclear magnetic resonance, and confirmed its identity as limonen-10-yl acetate.

4.2.2 Extraction and characterization of *H. officinalis* subsp. *aristatus* methanolic and ethanolic extract

The yield of methanolic extract was 8.13 ± 2.98% for the sample of Civitaretenga, calculated as the average of two extracts.

The 70% ethanolic extracts were performed twice for all samples, and the yields were reported in table 5.

The extract in 70%EtOH for Civitaretenga drug it had higher yield (12.99± 0.83%) than the extract in MeOH and the qualitative chemical profile is very similar when analyzed by HPLC DAD: for this reason, the quantification of molecules was performed in 70%EtOH extract taking into consideration that this solvent is preferable for human health use.

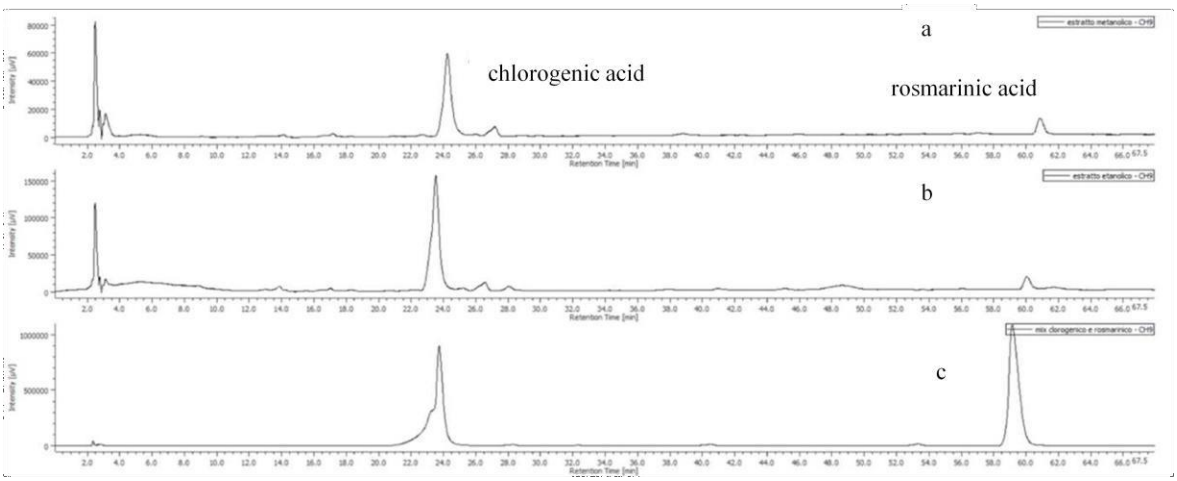


Figure 31 - Comparison between the chromatograms obtained by HPLC-DAD: a) methanol extract, b) ethanol extract, c) chlorogenic acid and rosmarinic acid

The identification of the molecules in these extracts was based on enrichment test and the comparison of their retention times, mass, and UV spectra, with those of the reference standards and literature data.

All phytocomplexes showed qualitatively similar profiles in which caftaric acid (UV: λmax = 327nm; $[M-H]^-$: m/ z 311), chlorogenic acid (UV: λmax = 327nm; [$[M-H]^-$: m/z 353) and rosmarinic acid (UV: λmax = 327nm; $[M-H]^-$: m/z 359)

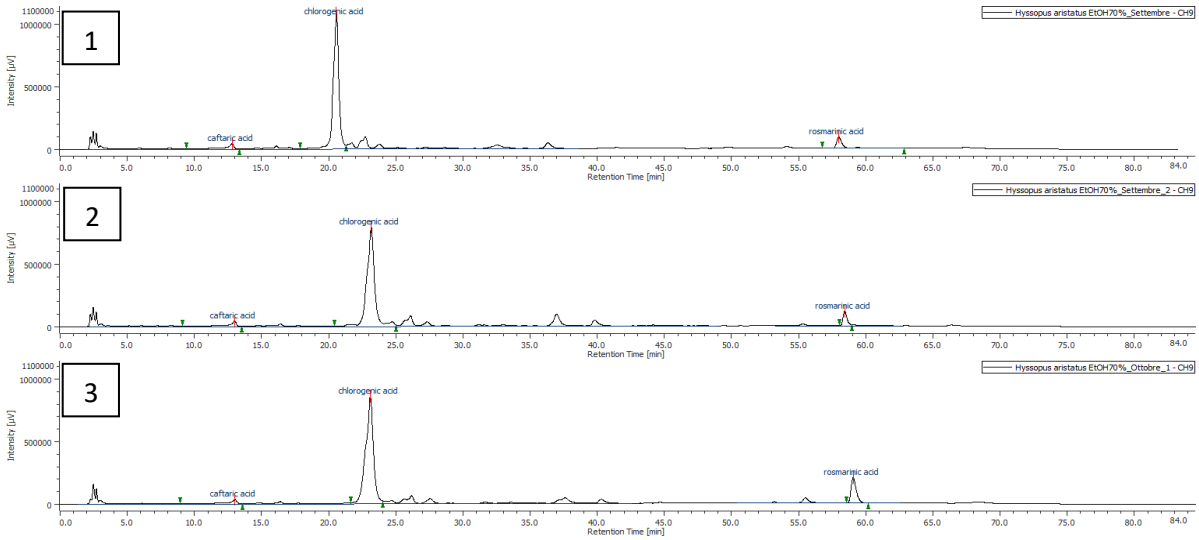


Figure 32- Comparison of the chromatograms (RP-HPLC-DAD) for the extracts obtained in 70%ethanol from the aerial parts of *H. officinalis* subsp. *aristatus* collected in the two years and in the three areas: 1- September 2016 - Civitaretenga; 2- September 2018 - Navelli; 3- October 2018 - Majella.

The chromatograms also showed the presence of other molecules, not identified, at retention times subsequent to those of chlorogenic acid, which show a very similar UV profile. These compounds could be isomers of chlorogenic acid (5-O-caffeoylquinic acid and 4-O-caffeoylquinic acid) as already highlighted in the literature (Hajdari et al., 2018)

The method of quantification was validated, and parameters are reported in table 5.

Compound	RT	Calibration range (µg/mL)	Correlation coefficient (r^2)	LOD (µg/mL)	LOQ (µg/mL)
caftaric acid	13.227	50-2.5	0.997784	0.403	1.221
chlorogenic acid	25.213	250-10	0.996071	0.561	1.874
Rosmarinic acid	64.013	50-10	0.996804	0.483	1.609

Table 5. concentrations of chlorogenic acid, rosmarinic acid and caftaric acid, contained in the five solutions for the construction of the calibration curve

From the point of view of the quantification, the RP-HPLC-DAD analyzes showed a clear prevalence of chlorogenic acid compared to the other molecules identified, in all the extracts of the two years and of the three areas. Furthermore, the 2016 extract showed a greater abundance of all the molecules concerning those of 2018, even with a higher yield (Table 6).

70% ethanol extract	yield	Caftaric acid	Clhorogenic acid	Rosmarinic acid
	%	mg dofstandard / g dried drug ± dev. St.		
H. officinalis subsp. *aristatus* - Settembre 2016 – Civitaretenga	12.99	307.53 ± 46.24	7397.38 ± 231.99	759.50 ± 26.14
H. officinalis subsp. *aristatus* - Settembre 2018 – Navelli	9.33	285.79 ± 6.29	3684.78 ± 9.04	278.01 ± 8.45
H. officinalis subsp. *aristatus* - Ottobre 2018 – Majella	7.39	212.59 ± 0.39	3300.20 ± 42.22	523.96 ± 2.50

Table 6 - Quantification of the molecules identified in the extracts obtained in 70% ethanol extract *H. officinalis* subsp. *aristatus* drugs collected in the two years and in three areas: September 2016 - Civitaretenga; September 2018 - Navelli; October 2018 - Majella.

4.3 Antioxidant activity of *H. officinalis* subsp. *aristatus*

4.3.1 *H. officinalis* subsp. *aristatus* DPPH bioautographic assay

To highlight the possible antioxidant properties of the extracts, a preliminary analysis was carried out through a thin layer chromatographic. Considering Civitaretenga drug had higher amounts of phenolic acids, we evaluated its extracts.

Band	Deposited quantity	Substance
1 (upper Rf)	3.0 µL	**Rosmarinic acid**
1 (lower Rf)	2.0 µL	**Apigenin 7-glucoside**
2 (upper Rf)	1.0 µL	**Caffeic acid**
2 (lower Rf)	3.0 µL	**Chlorogenic acid**
3	10.0 µL	**MeOH Extract**
4	10.0 µL	**70% EtOH extract**

Table 7.- Deposition sequence of substances on HPTLC plate

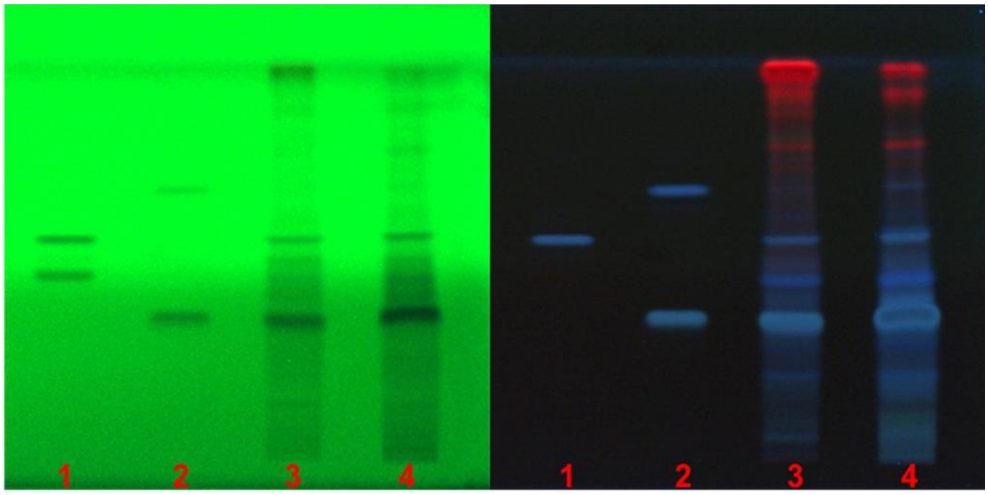


Figure 33.- Non derivatized HPTLC, observed at wavelengths of 354 nm (left) and 366 nm (right) with the substances shown in the Table 7.

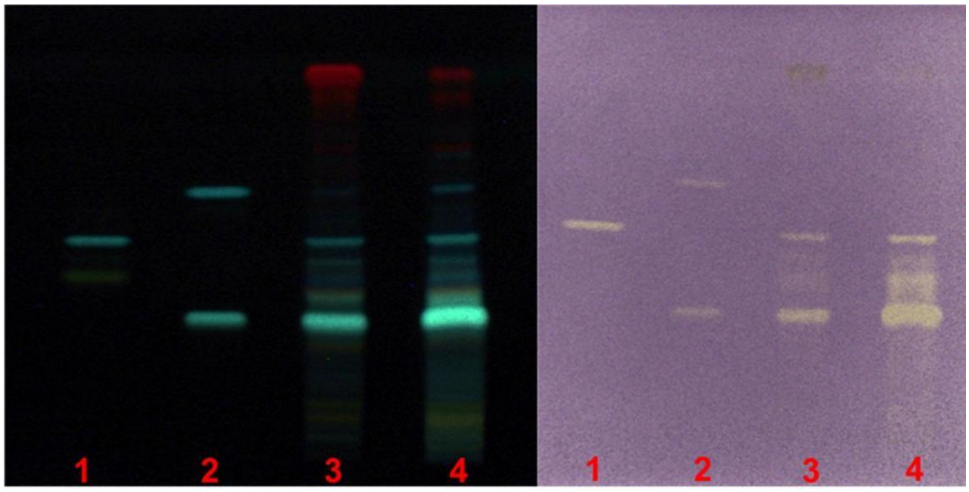


Figure 34.- HPTLC derivatized with NP/PEG (left) and dwith DPPH (right) containing the substances shown in the Table 3.

From the HPTLC carried out, we could view that after the derivatization with NP/PEG, some compounds belonging to the class of polyphenols are more evident. From qualitative analysis, we noted that chlorogenic acid is extracted in a higher way in the 70% EtOH extract than in the MeOH one. Caffeic acid activity was also evaluated. This compound, from the non-derivatized TLC observed at 366 nm, appears to be present in the ethanolic extract, however, once the plate has been reacted with DPPH, the standard has shown activity, but the corresponding band has not been highlighted in the extract. Caffeic acid could be present in low quantities or not be present in the extract, and therefore the isomers of chlorogenic acid could be found at the same Rf. Considering this, we did not proceed with further investigation into the biological activity of caffeic acid. As for the antioxidant activity of the extracts, after derivatization with DPPH we noted that rosmarinic acid and chlorogenic acid are among the main responsible for this biological activity.

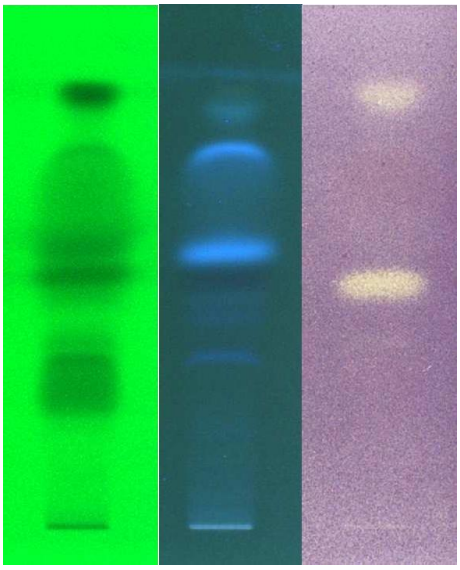


Figure 35.- HPTLC with 40 µL of essential oil (50 mg / ml CH_2Cl_2) deposited using: toluene: ethyl acetate (93: 7) as a mobile phase. From left to right: non-derivatized plate at 254 nm, non-derivatized plate at 366nm, derivatized plate with DPPH.

The essential oil, deposited in high concentration, did not show high antiradical power when derivatized with DPPH. Before evaluating the opportunity to identify the molecules having this activity, the IC_{50} of the essential oil was determined.

4.3.2 Determination of the IC_{50}

The IC_{50} (µg/mL) was determined using the microplate reading method. In addition to the extracts, a synthetic antioxidant (Trolox) used as a positive control together with thymol, as an example of active terpene, some standard molecules of the phytocomplex, potentially responsible for the antioxidant activity such as chlorogenic acid and rosmarinic acid, were tested.

The results are shown in Table 8:

Substance	IC_{50} (µg/mL)	DS	Concentration range(µg/mL)
MeOH extract	57.03	1.97	1.99÷172.00
70%EtOH cxtract	38.79	2.12	1.99÷172.00
Rosmarinic acid	4.31	0.54	0.63÷40.00
Chlorogenic acid	7.29	0.17	0.47÷30.00
Trolox	4.28	0.48	0.31÷20.00

Table 8.- IC_{50} for each analyzed compound, with its relative standard deviation and concentration range used.

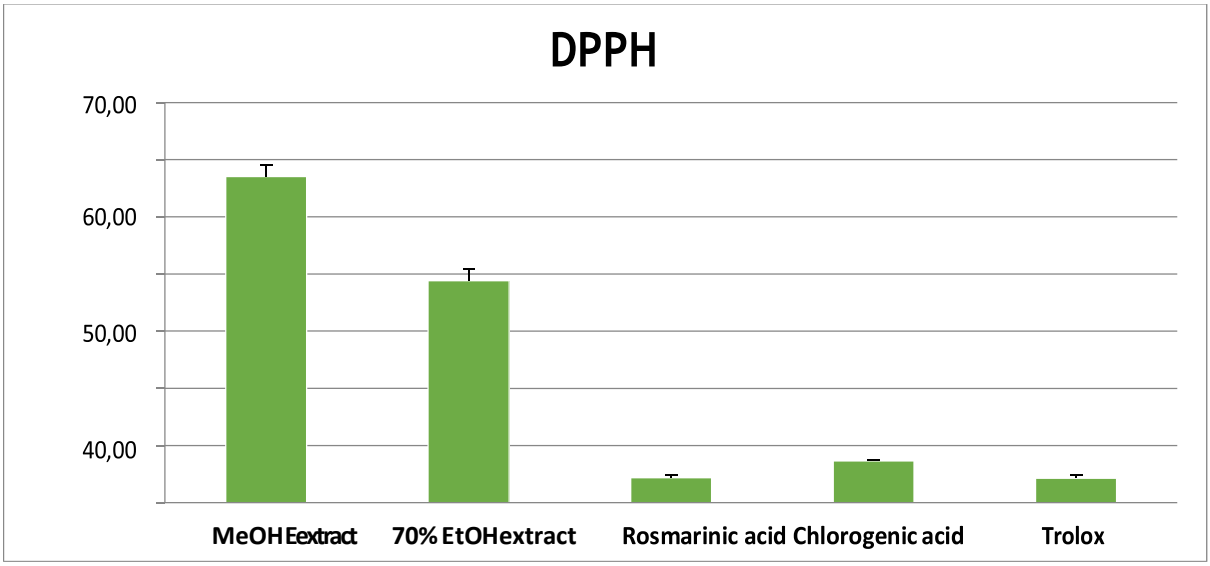


Figure 36.- DPPH: histogram of IC_{50}

The results for essential oil are shown in table 9:

Substance	IC_{50} (µg/mL)	SD	Concentration range (µg/mL)
Essential oil	20047.16	222.36	312.50÷20000
Thymol	357.56	34.70	23.20÷1485
Trolox	4.44	0.37	0.31÷20.00

Table 9.- IC_{50} for each analyzed compound, with its relative standard deviation and concentration range used.

The essential oil did not possess antioxidant activity. The weak alone of inhibition in bioautographic HPTLC could be due to minor components.

The MeOH and EtOH extracts showed antioxidant activity of 57.03 µg/mL and 38.79 µg/mL, respectively. In line with the results, a methanol extract tested by Džamić et al. (2013) highlighted antioxidant capacity.

4.4 Antimicrobial activity of *H. officinalis* subsp. *aristatus*

The antimicrobial activity of *H. officinalis* subsp. *aristatus* was tested using two methods.

4.4.1 HPTLC bioautographic assay

Th This method highlights which active ingredient has antimicrobial activity. In particular, it was considered *S. aureus* as a model among the most sensitive strains (Guerrini et al., 2016). HTPLCs of the three extracts were prepared. MeOH and EtOH extracts were tested as following indicated:

Band	Deposited quantity	Substance
1 (upper Rf)	3.0 µL	**Rosmarinic acid**
1 (lower Rf)	2.0 µL	**Apigenin 7-glucoside**
2 (upper Rf)	1.0 µL	**Caffeic acid**
2 (lower Rf)	3.0 µL	**Chlorogenic acid**
3	10.0 µL	**MeOH extract**
4	10.0 µL	**70%EtOH extract**

Table 10.- Deposition sequence of substances on HPTLC plate

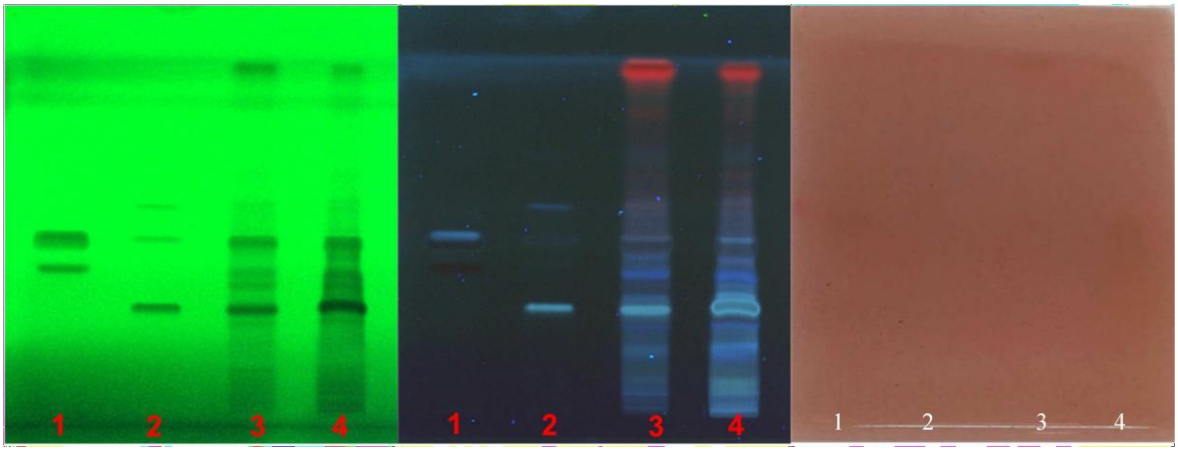


Figure 37.- HPTLC bioautographic assay. From left to right: 254 nm non-derivatized plate, 366nm non-derivatized plate, *S.aureus* incubated on the plate.

After incubation of *S.aureus* on HPTLC, the entire surface of the plate did not change color: the two extracts have no components with antimicrobial activity.

The essential oil was tested as following indicated:

Band	Quantity deposited	Substance
1	20.0 µL	**Essential oil (50mg/ml)**
2	6 µL	**Thymol (3 mg/ml)**

Table 11.- Deposition sequence of substances on HPTLC plate

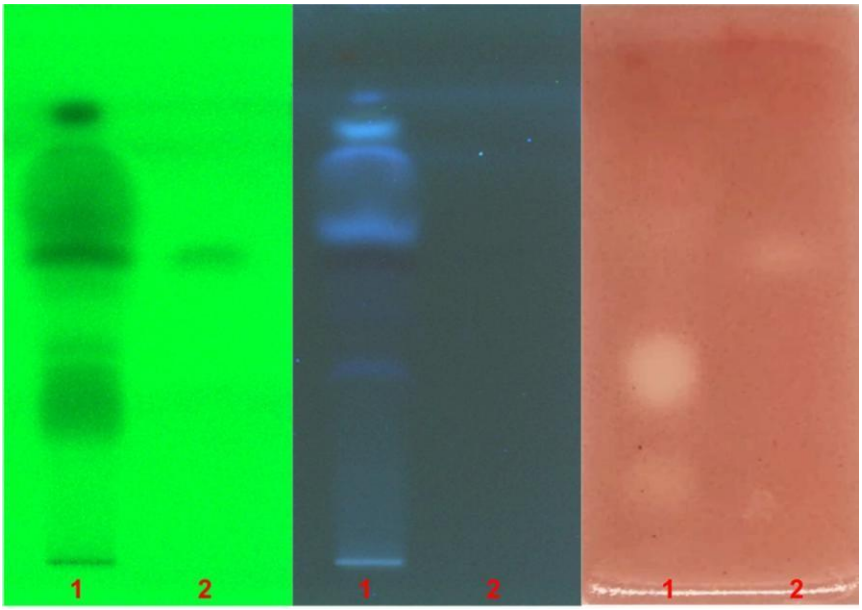


Figure 38.- Biouatographic HPTLC of essential oil. From left to right: non-derivatized plate at 254 nm, non-derivatized plate at 366nm, *S.aureus* incubated on the plate.

Bioautographic HPTLC of the essential oil from Civitarenga drug evidenced decoloration bands at R_f about 0.5. Before evaluating the opportunity to identify the molecules having this activity, the MIC of the essential oil was determined.

4.4.2 MIC determination

The assay was performed on a 96-well plate using a microplate reader. Chloramphenicol was used as a positive control.

The results are shown in Table 12:

Substance	Concentration range µg/mL	MIC (µg/mL)	MIC (mg/mL)
Essential oil	31.25 ÷ 2000	> 2000	**> 2**
MeOH extract	78,.125÷ 2000	>2000	**>2**
70% EtOH extract	78.125÷ 2000	>2000	**>2**
Chloramphenicol	1.5625÷100	12,5	**0.0125**
Rosmarinic acid	31.25 ÷ 2000	2000	**2**
Chlorogenic acid	31.25 ÷ 2000	> 2000	**> 2**
Limonen 10-yl-acetate	31.25 ÷ 2000	> 2000	**> 2**
Thymol	**31.25 ÷ 2000**	**250**	**0,5**

Table 12.- MIC values of tested substances against *S. aureus* strain

All tested substances do not have interesting antibacterial activity. Rosato et al., assaying the essential oil of *H. officinalis* subsp. *aristatus* against *S. aureus* found a moderate MIC value of 3.7 mg /mL, probably comparable with our results.

In our practice of laboratories we assumed that moderate values above 2 mg /ml, in agreement with the literature, are not worthy of note for further study of the essential oil activity.

4.5 Cytotoxic activity of *H. officinalis* subsp. *aristatus*

The essential oils from plants collected in 2018 showed a weak toxic activity on A549 at concentrations above 100 µg / ml. At these levels of concentration, it is not possible to attribute a significant efficacy in consideration of the specificity of the effect and the concentration levels much higher than those accredited in the context of international evaluations (International Cancer Institute, 30 µg/ml). The 2016 chemotype, on the other hand, did not show a significant toxic action against the cell lines taken into consideration (Figure 39).

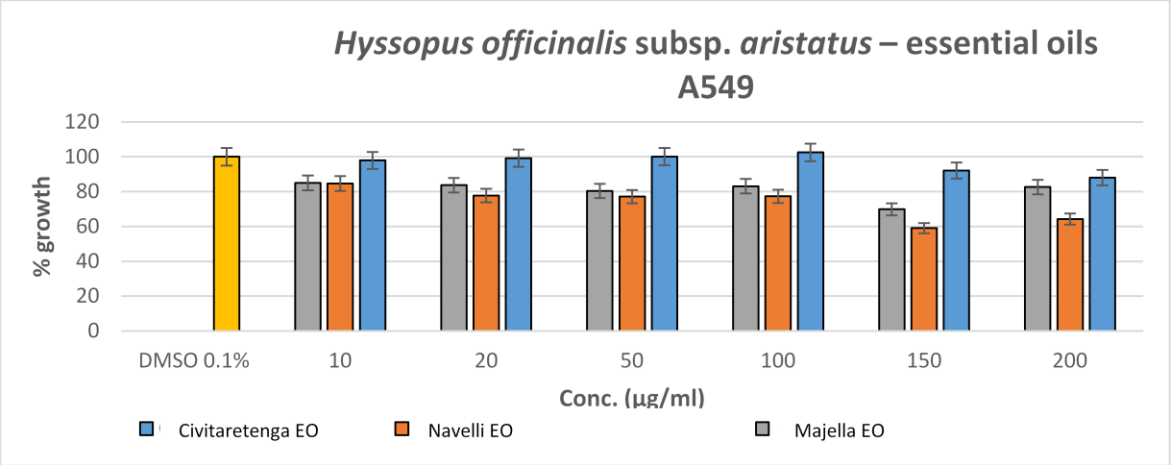


Figure 39. –Cytotoxic activity of essential oils against A549 cell line

Regarding the invasion test on the A549 cell line, unreliable results have been obtained: further tests to a more accurate refinement of the technique will be necessary.

The ethanolic extracts showed a significant dose-dependent toxic effect on A549 since the concentrations above 50 µg /ml with a prevalence of toxicity on the extracts of 2018 (Majella sample), which are gradually differentiating themselves from the others at higher concentrations (Figure 40).

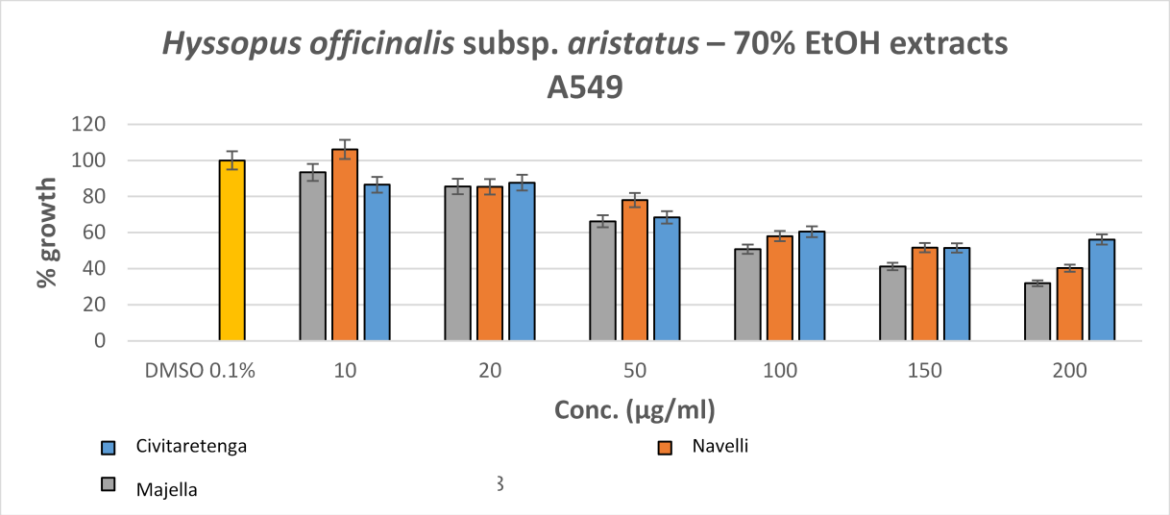


Figure 40. –Cytotoxic activity of ethanolic extracts against A549 cell line

The evaluation of cytotoxicity on the same cell lines by the main compounds of the phytocomplexes (Figures 41 and 42) did not show significant effects. These evidences do not appear to be in agreement with what reported in the literature (Burgos-Moròn et al., 2012) regarding chlorogenic acid which showed a weak but significant cytotoxic effect against the A549 cell line. The discrepancy of these outcomes compared to our experiments is justified by the different level of concentration used by the authors which is about 10 times higher than that used by us. Rosmarinic acid also did not show cytotoxicity at the tested concentrations. In literature we have not found evidence of its activity on these cancer cells (Figure 42).

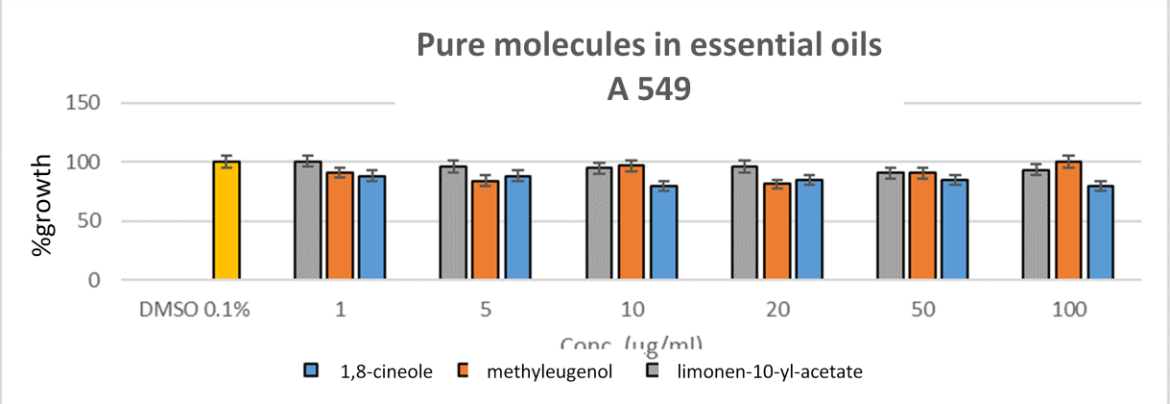


Figure 41. –Cytotoxic activity of main compounds of essential oils against A549 cell line

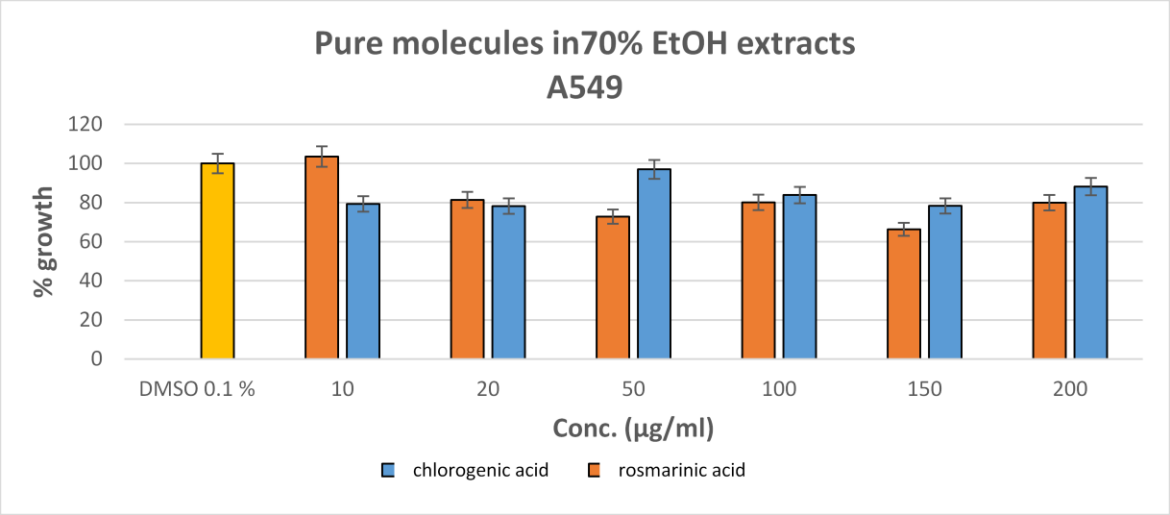


Figure 42. –Cytotoxic activity of main compounds of ethanolic extracts against A549 cell line

To verify the selectivity of the obtained results on the cancer cell line, the tests were extended with a similar method to the HaCaT cell line. These cells are easily propagated as they are immortalized, but retain the characteristics of normal keratinocytes.

The essential oils did not show any cytotoxic activity (Figure 43), while a toxic action of ethanolic extracts is shown, which did not evidenced a selective effect towards cancer cell line (Figure 44).

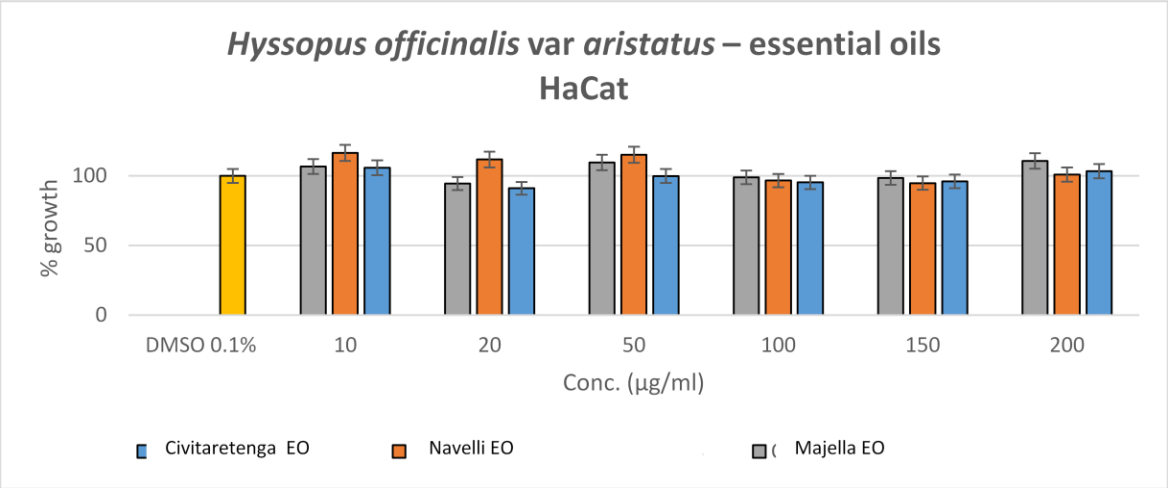


Figure 43. –Cytotoxic activity of essential oils against HaCat cell line

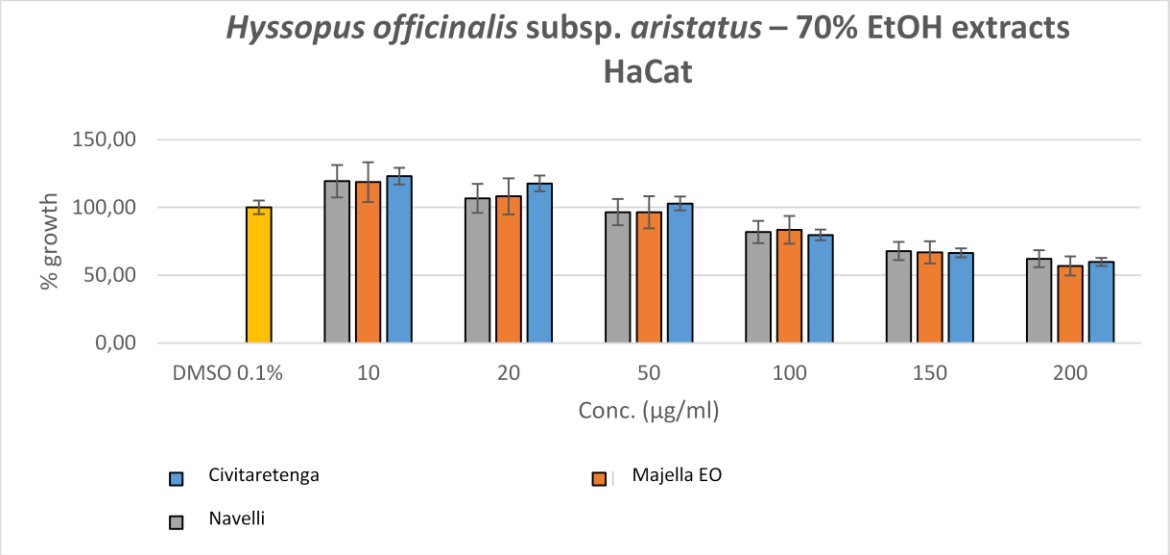


Figure 44. –Cytotoxic activity of ethanolic extratcs against HaCat cell line

The Wound Healing test is used for the assessment of two-dimensional motility, while the test that uses transwells more properly evaluates three-dimensional mobility. This last test is carried out to support the invasion test in which a layer of Matrigel® is placed at the base of the transwell to bring the motion of the cells back to the crossing of the basal membrane and the diffusion in the stroma tissue.

These methods were applied only on the hydroalcoholic extracts at sub-toxic concentrations in consideration of the encouraging results related to the cytotoxic action towards A549 cells. In the wound healing test there is a slight reduction in motility compared to untreated cells (negative control) but significantly lower than the results obtained with myricetin, taken as a positive control in the literature (Shih et al., 2009) (Figure 45).

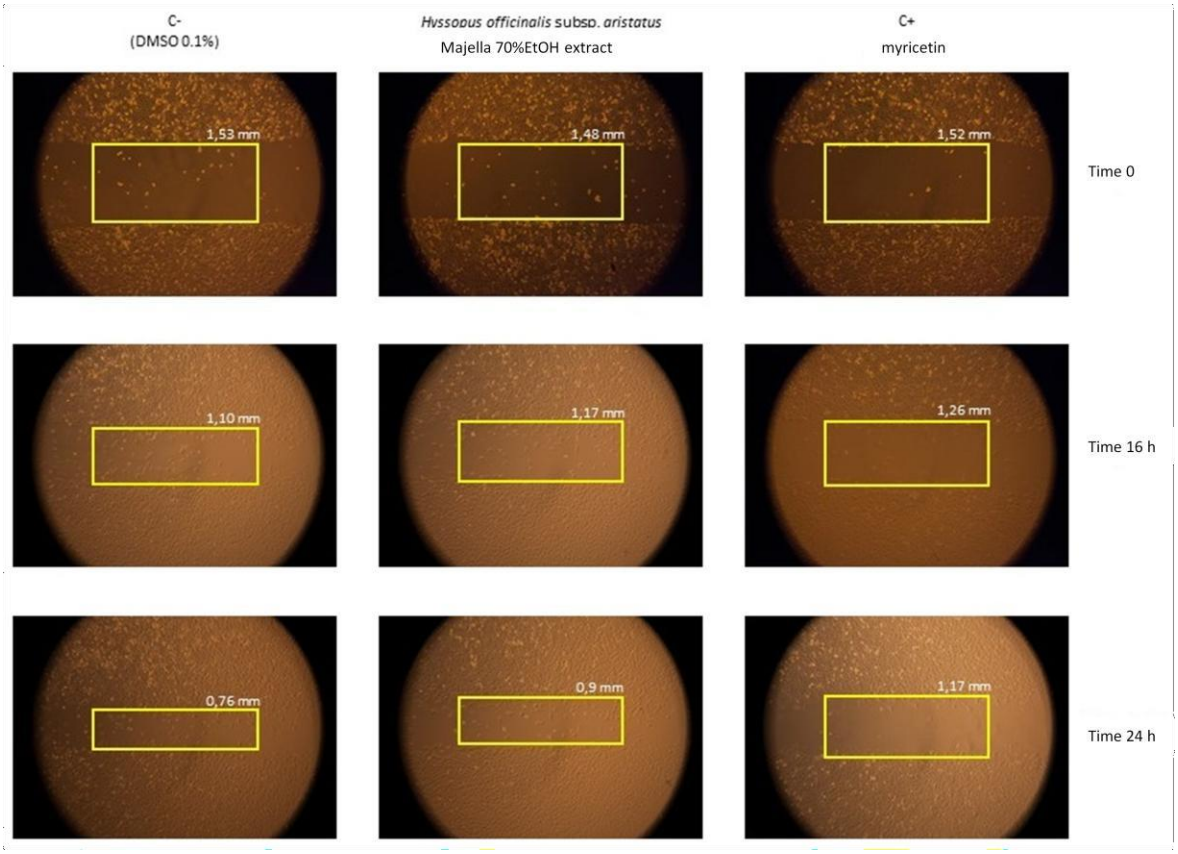


Figure 45. *Wound Healing* at successive times carried out to ethanolic extract of Majella *H. officinalis* subsp. *aristatus*, on A549 cells and negative (cells treated with DMSO 0.1%) and positive (myricetin) controls.

The migration test (in the absence of Matrigel®) showed a weak activity compared to the negative control (Figure 46), while no appreciable results were obtained regarding the invasion test (in the presence of Matrigel®) (Figure 47). This modest anti-invasive activity could also be attributed to the presence of rosmarinic acid, since this molecule is already known in the literature for this biological effect even if on a different cell line (colon carcinoma, Ls174-T) from the one we used (Xu et al., 2010). For a more accurate evaluation, experiments will also be performed at increasing concentrations of rosmarinic acid alone.

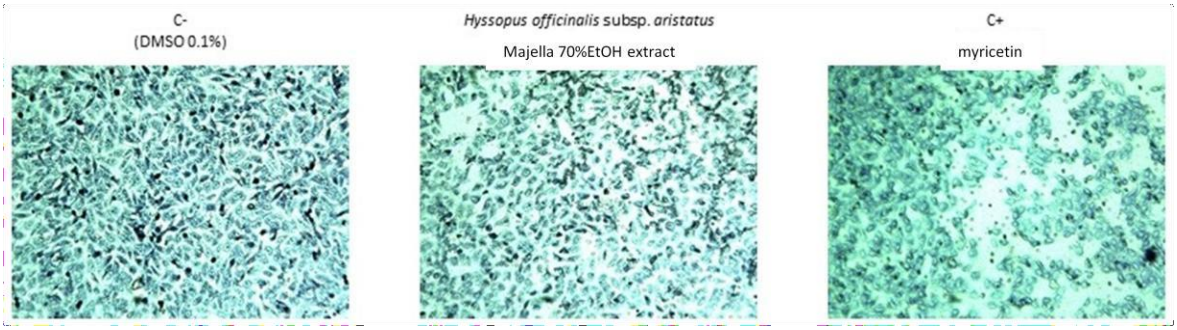


Figure 46. *migration test* at successive times carried out to ethanolic extract of Majella *H. officinalis* subsp. *aristatus*, on A549 cells and negative (cells treated with DMSO 0.1%) and positive (myricetin) controls.

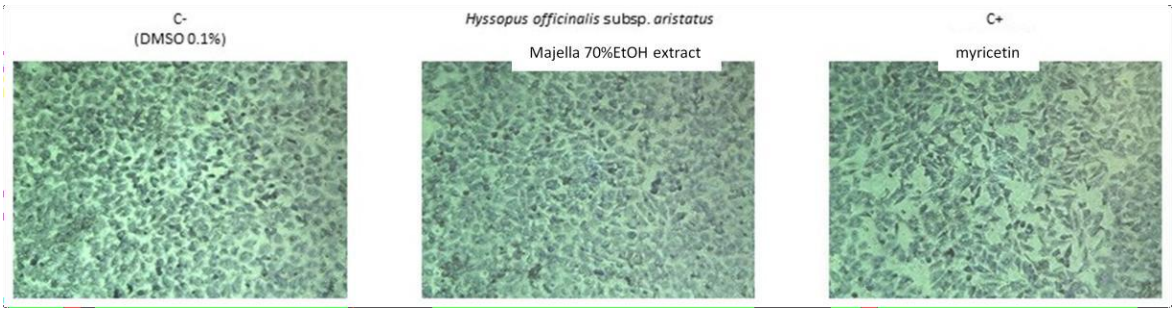


Figure 47. *Invasion test* at successive times carried out to ethanolic extract of Majella *H. officinalis* subsp. *aristatus*, on A549 cells and negative (cells treated with DMSO 0.1%) and positive (myricetin) controls.

4.6 Ames test of *H. officinalis* subsp. *aristatus*

The mutagenic activity was tested using the widespread and consolidated Ames test to verify the genotoxic safety of *H. officinalis* subsp. *aristatus* extracts and essential oils. We tested all the extracts and the essential oil of the Civitaretenga sample, in addition to the essential oil from the Navelli sample: for the Majella sample we have not enough material to perform the test. It envisages the use of engineered *Salmonella typhimurium* strains, auxotrophic yields for histidine and biotin and highly susceptible to potential mutagenic substances. The interpretation of results is rather simple and is based on the fact that for each strain a mutation reversion occurs, making it auxotrophic for histidine and biotin by restoring the wild-type status at a characteristic frequency. By placing these bacteria in contact with the substance to be tested and growing on a non-histidine-free soil, biotin will assist in the development of colonies derived exclusively from reverting bacteria. The substance is considered safe if the number of revertent counts remains within a range that does not exceed at least twice that of the revertents found in the negative control (in which only the solvent used to dissolve the substances to be tested is added); the substance is considered a potential mutagen if the number of revertents is twice that of the negative control (Maron and Ames, 1983). It is also important to observe a dose-dependent pattern to exclude false positives, such as isolated reversing peaks, called by Ames "spikes". There are numerous strains of salmonella produced for mutagenic activity tests, each possessing a different mechanism and sensitivity. We have decided to test extracts and essential oils on two different salmonella strains that can detect almost all potential mutagens; among the most stable strains and most commonly used in this type of test these are TA98, TA100. The TA98 strain is useful for detecting mutations that cause frameshifts, deletions or genomic insertions, it only changes the mutation site, which makes them complementary and most sensitive to some substances rather than others. TA100 mainly detects substances that cause base-pair replacement and have a different genome repair system. Table 13 shows the obtained results.

The results showed that none of the tested essential oils and extracts were potentially mutagenic. Only for Navelli essential oil results were not reliable, because we had not enough amount of substance to test all concentrations.

	Hyssop EO Civitaretenga				Hyssop EO Navelli			
	TA98 (rev/C-)		TA 100(rev/C-)		TA98(rev/C-)		TA 100(rev/C-)	
conc. %	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9
5	0.9	0.7	0.8	0.7	-	-	-	-
10	0.9	1.3	0.7	0.7	-	-	-	-
20	0.5	1.0	0.9	0.7	0.0	0.0	0.5	0.7
50	0.3	1.3	0.1	0.1	-	-	-	-
100	0.5	0.9	0.1	0.0	0.0	-	0.0	0.5
C+ (NF)	3.8	4.3	5.6	4.6	3.8	4.3	5.6	4.6

	Hyssop EtOH extract Civ.				Hyssop MeOH extract Civ.			
	TA98(rev/C-)		TA 100(rev/C-)		TA98(rev/C-)		TA 100(rev/C-)	
conc. %	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9
5	0.0	0.0	0.0	0.0	1.1	1.6	1.0	0.9
10	0.0	0.0	0.0	0.0	0.8	1.7	0.8	0.9
20	0.0	0.0	0.0	0.0	0.5	1.8	1.3	0.7
50	0.0	0.0	0.0	0.0	0.8	1.7	1.3	0.9
100	0.0	0.0	0.0	0.0	0.7	1.3	0.0	0.8
C+ (NF)	3.8	4.3	5.6	4.6	3.8	4.3	5.6	4.6

Table 13. Results of Ames test

4.7 Extraction and chemical characterization of *O. campechianum*

4.7.1 Extraction and chemical characterization of *O. campechianum* essential oil

For the essential oil thirty-one compounds were identified, corresponding to 97.11% of the total. Monoterpenes oxygenated represent 54.37%, (alcohols 48.96%, phenolics 46.55%), monoterpene hydrocarbons 6.78%, sesquiterpenes hydrocarbons 32.85% and sesquiterpenes oxygenated 2.7% of all the compounds detected and identified, which is consistent with others research (Rolli et al., 2014). The compounds of *O. campechianum* essential oil are reported in Table 14; distillation yields were of 0.68%. Among the monoterpene, the most abundant was found in eugenol (46.55%) and 1,8-cineole (5.35%), among the sesquiterpenes β-caryophyllene, was most abundant

(11.94%), following by β-elemene (9.06%), δ-elemene (4.17%) and bicyclogermacrene (2.9%), which is consistent with results reported by others authors (de Vasconcelos et al., 2004; Jaramillo, et al., 2014; Salles, et al. 2006;Sacchetti et al., 2004).

Compound	**Exp. AI**	***O. campechianum* %Area**
α-Pinene	939	0.24
Camphene	954	0.07
β-Pinene	979	0.75
Myrcene	991	0.26
1,8-Cineole	1031	5.35
Cis-Ocimene	1037	2.69
Trans-Ocimene	1050	0.35
Linalool	1097	1.49
Allo-Ocimene	1132	2.42
Borneol	1169	0.14
Mentha-1,5 dien-8 ol	1170	0.33
α-Terpineol	1189	0.45
Neral	1238	0.06
Elemene isomer	1321	0.63
δ-Elemene	1338	4.17
Eugenol	1359	46.55
Elemene isomer	1379	0.63
β-Elemene	1391	9.06
Tetradecane	1400	0.05
β-Caryophyllene	1419	11.94
α, *cis*-Bergamotene	1435	0.13
α-Humelene	1455	2.4
Germacrene D	1485	0.13
β-Selinene	1490	0.86
Bicyclogermacrene	1500	2.9
Spathulenol	1578	1.15
Caryophyllene oxyde	1583	1.23
Hexadecane	1600	0.15
β-Eudesmol	1651	0.17
Selin-11-en-4-α-ol	1660	0.15
Heptadecane	1700	0.21
Total identified		97.11

Table 14.- Chemical characterization of essential oil from aerial parts of *O. campechianum* in GC-MS and GC-FID

4.7.2 Extraction and characterization of *O. campechianum* methanolic and ethanolic extracts

The quantification of phenolic acids revealed that rosmarinic acids is the most abundant in the two extracts (table 15). This is the first contribution of phenolics quantification for *O. Campechianum.*

O. campechianum	70%EtOH (mg/g extract)	MeOH (mg/g extract)	70%EtOH (mg/g drug)	MeOH (mg/g drug)
Catfaric acid	0.35±0.01	0.57±0.01	0.068±0.001	0.031±0.001
Chlorogenic acid	0.27±0.01	0.046±0.019	0.053±0.001	0.0025±0.0010
Rosmarinic acid	22.3±0.1	21.8±0.1	4.33±0.01	1.17±0.01

Table 15.- HPLC-DAD quantification of main phenolic acids

The culinary and medicinal herbs of the Lamiaceae family are known to be rich sources of polyphenolic compounds, particularly phenolic acids. Basil contains high levels of phenolic acids that contribute to its strong antioxidant capacity. The high concentration of rosmarinic acid, in particular, has been associated with medicinal herbs with rosmarinic acid as a quality marker, for example, common rosemary (*Rosmarinus officinalis*).

Rosmarinic acid has been reported to have antiviral, antibacterial, anti-hepatitis, antimutagenic, anti-allergic, anticarcinogenic and anti-inflammatory properties also inhibit HIV-1 and blood clotting. Moreover, it has therapeutic potential in the treatment or prevention of bronchial asthma, spasmogenic disorders, peptic ulcer, hepatoxicity, atherosclerosis, ischemic heart disease, cataract, cancer, and poor sperm counts. In addition, rosmarinic acid inhibits seasonal allergic rhinoconjunctivitis (SAR) by two independent mechanisms: inhibition of the inflammatory response and scavenging of reactive oxygen species (ROS) (Muñoz et al., 2011).

Rosmarinic acid levels in the study of variations in phenolic composition and antioxidant properties of 15 samples of *Ocimum basilicum* L. from the USA. varied from 0.06 mg/g to 6.09 mg/g on the dried matter. Caftaric acid levels ranged from 0.09 mg/g to 0.49 mg/g for *Ocimum basilicum* L., for "Genovese Italian" basil have also determined concentrations of caftaric acid ranging from 0.002 to 0.02 mg/g in commercially purchased dried basil samples (Kwee and Niemeyer, 2011).

The methanolic and hydroalcoholic extracts of leaves of *O. campechianum* showed a relevant concentration of rosmarinic acid. The ethanolic extract exhibited the greatest abundance of every compound. In particular, the quantities of caftaric and chlorogenic acid were about twice as much as in the other extract, while rosmarinic acid was nearly four times more abundant.

4.8 Antioxidant activity of *O. campechianum*

4.8.1 *O. campechianum* DPPH bioautographic assay

A preliminar analysis of the extracts was carried out using bioautographic DPPH assay.

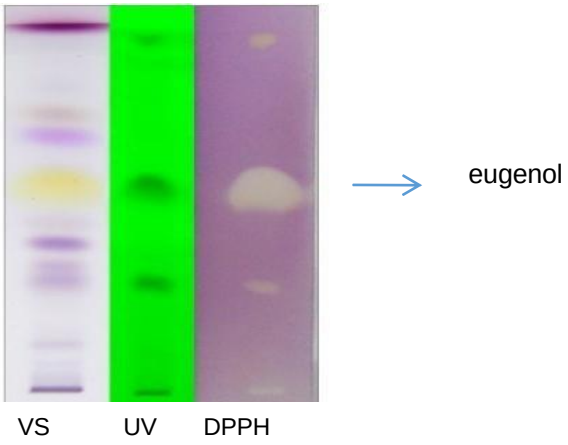


Figure 48.- HPTLC of *O. campechianum* essential oil. VS = vanillic sulphuric acid reagent; UV = ultraviolet detection (254 nm); DPPH

Eugenol was the main component, as determined by GC-FID, and the most active compound. Eugenol which is an aromatic agent in the food industry and a fragrance agent in the cosmetic industry, it is also used in endodontics as a sedative in dental surgery. Eugenol induces effects on the central nervous system in mammals (hypothermia, decreased motor activity, anticonvulsants, and general anesthesia), present antibacterial, antiviral, antifungal, antioxidant and prooxidant activity. It is important to note that both eugenol and its methyl eugenol analog have potent central nervous system antidepressant activity (Benitez et al., 2009).

The hydroalcoholic extracts with the presence of phenolics acids, as the view for *H. officinalis* subsp. *aristatus* could be an interesting DPPH activity.

4.8.2 Determination of the IC_{50}

In addition to extracts and essential oil, trolox was also tested, as a positive control together eugenol as active monoterpene.

The results obtained are shown in table16 :

Antioxidant activity *O. campechianum*			
Substance	**IC_{50} (µg/mL)**	**SD**	**Range conc. µg/mL**
70% EtOH extract	11.10	1.13	5.023÷32.5
MeOH extract	52.15	2.72	4.484÷287.0
Trolox	3.66	0.29	0.31÷20
Essential oil	7.77	0.07	2.44÷156.25
Eugenol	5.64	0.27	3.91÷250.0

Table 16.- IC_{50} values forr each substance analyzed, with its relative standard deviation and concentration range used.

All extracts exhibited an interesting antioxidant capacity (DPPH). The hydroalcoholic extract showed activity close to the one of essential oil (11.1±0.01µg/ml), while the methanolic displayed a weaker activity when compared to the previous extracts (52.3±2.7 µg/ml), rosmarinic acid (previously tested with hyssop) and eugenol values close to Trolox. The results confirmed very promising properties for all the extracts, for exploitations in cosmetic and food supplements products.

4.9 Antimicrobial activity of *O. campechianum*

The antimicrobial activity of *O. campechianuim* was tested using two methods.

4.9.1 HPTLC bioautographic assay on *S. aureus.*

This method highlights which active ingredient has antimicrobial activity.
HPTLCs of the three extracts were prepared.

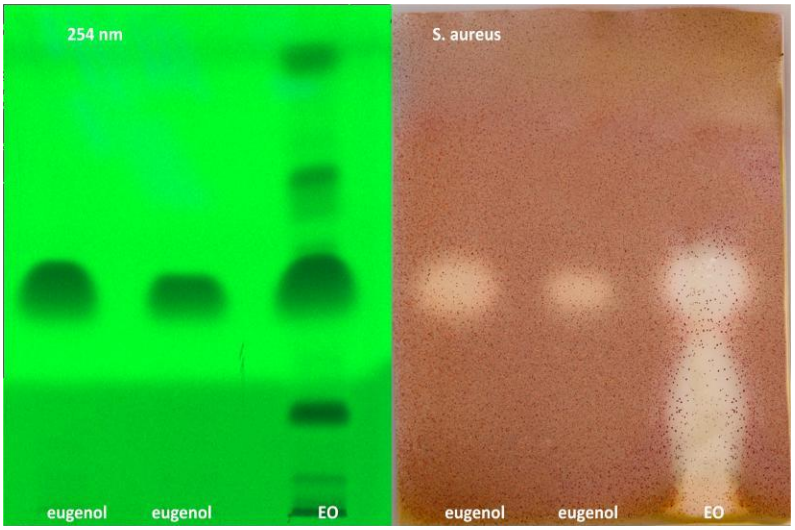


Figure 49.- HPTLC of essential oil (2 µL) and eugenol, the last in two different concentrations(1 and 0.5 µL)

Essential oil evidenced the high antibacterial activity of eugenol.

band	Quantity deposited	Substance
1 (superior)	2.0 µL	Rosmarinic acid
1´(lower Rf)	4.0 µL	Rutin
2 (lower Rf)	2.0 µL	Chlorogenic acid
2´(lower Rf)	2.0 µL	Catfaric acid
2´´(upper Rf)	3.0 µL	Caffeic acid
3	30.0 µL	MeOH Extract
4	20.0 µL	70%EtOH Extract

Table 17.- Disposition of substances on HPTLC

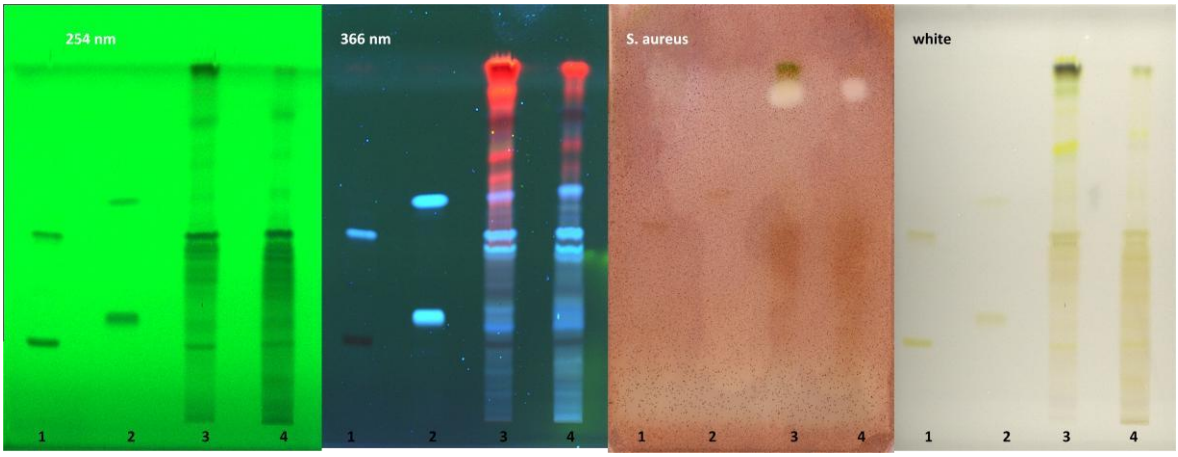


Figure 50.- HPTLC of *O. campechianum* extracts

Probably only minor components, not identified, are responsible of the decoloration of HPTLC in bioautographic assay.

4.9.2 MIC determination

The assay was performed on a 96-well plate in microplate reader.

Substance	MIC (µg/mL)	Concentratio range (µg/mL)
Essential oil	5000	39.05 - 5000
EtOH Extract	> 2000	15.63 - 2000
MeOH Extract	> 2000	15.63 - 2000
Eugenol	1000	7.81 - 1000
Rosmarinic acid	5000	39.05 - 5000
Thymol	250	3.91 - 500
Cloramphenicol	10	0.16 - 20

Table 18.- MIC values evaluated on *S. aureus* for *O.campechianum*

The eugenol have moderate acitivity and also the essential oil on this strain of *S. aureus*. The data is in part in good accord with literature.

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

. **Table 19.** MIC by microdilution method of EOs and their main components *(Baser and Buchbauer, 2015)*

In literature, the essential oils of *O. basilicum* exhibited strong antimicrobial activity against a lot of microorganisms. Results obtained from the disc diffusion method, followed by measurement of minimum inhibitory concentration (MIC), indicated that *S. aureus* and *B. subtilis* were the most

sensitive microorganisms showing largest inhibition zones (22.2-24.4 mm), (20.4-26.1 mm), and lowest MIC values (0.9 mg/mL), (0.8 mg/mL), respectively (Hussain, et al. 2008).

It was possible that the strain tested in our laboratories is not too sensible to eugenol.

The moderate activity was obtained also against *Pseudomonas syringae* pv. *syringae*: the essential oil resulted in the most active preparation with a MIC of 2.5 mg/ml. The essential oil could be interesting for application as a biopesticide.

4.10 Cytotoxic activity of *O.campechianum*

The cytotoxic activity was evaluated in A549 cells (human lung carcinoma) for the essential oil, methanol, and ethanolic extract and doxorubicin was used as positive control (concentration range 0.1-20 µg/mL, IC50 = 2.92 µg/mL) and the data were compared with the values returned from the medium with DMSO 0.1% (negative control).

A preliminary screening of the extracts against A549 cell line were performed but with negative outcomes. On the other hand, extracts did not show any cytotoxicity against HaCat cell line, giving indication of safety of use of the extracts.

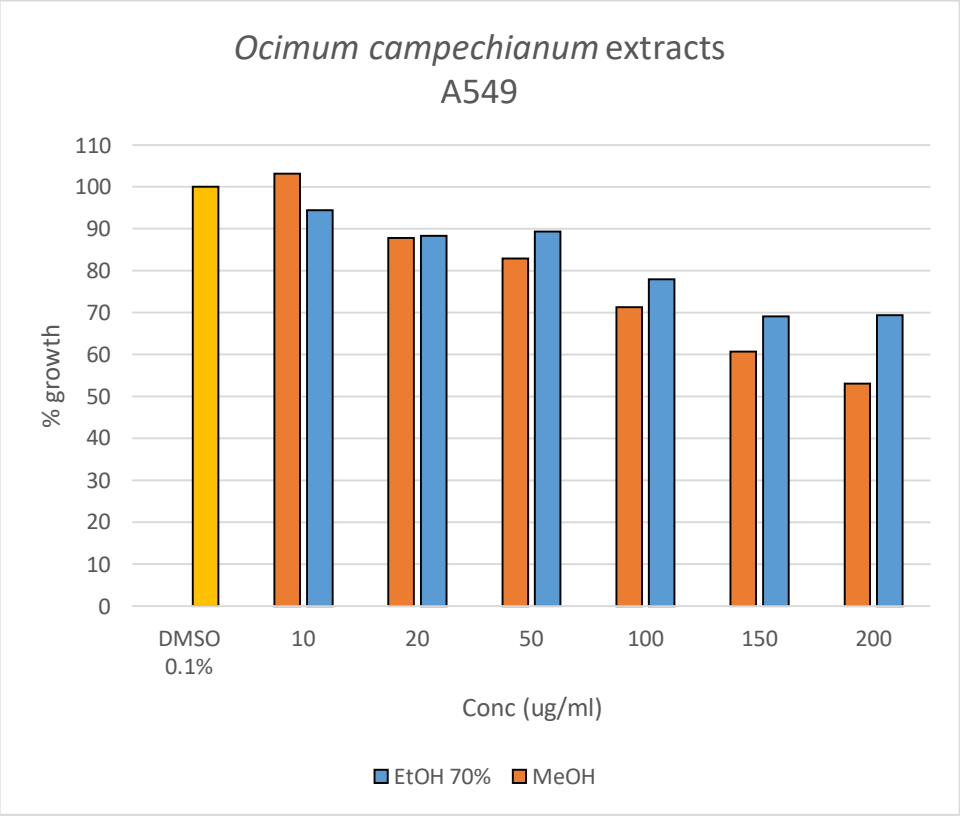


Figure 51. MTT test on A549 cell line for extracts

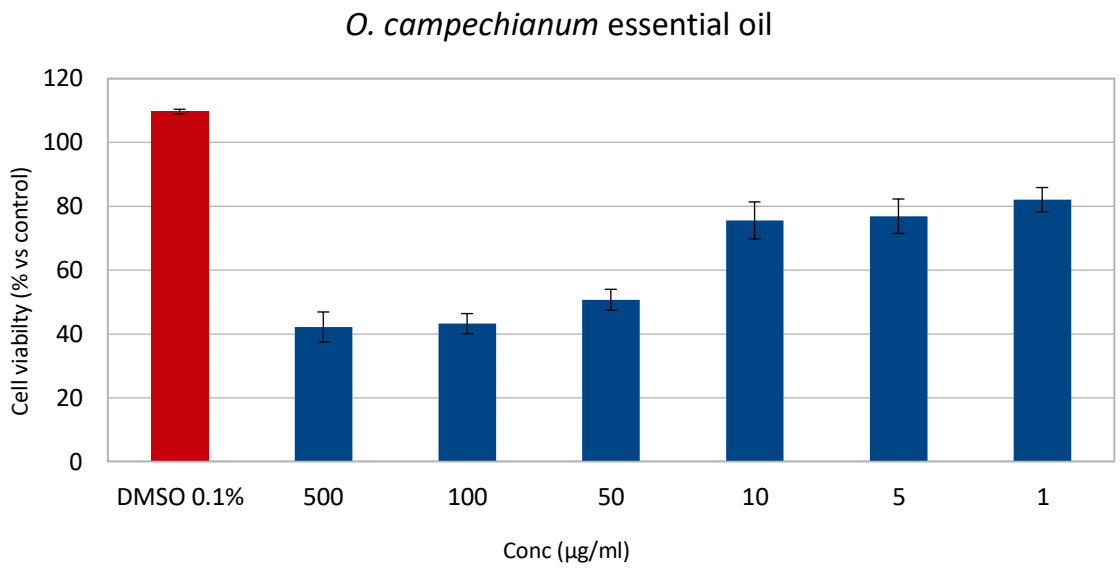


Figure 52. MTT test on A549 cell line for essential oil

4.11 Ames test of *O. campechianum*

The results showed that essential oil and extracts were not potentially mutagenic.

	O.campechianum EO				EtOH extract				MeOH extract			
	TA98		TA 100		TA98		TA 100		TA98		TA 100	
conc. %	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9	- S9	+ S9
5	0.9	0.7	1.0	0.8	0.7	1.0	0.9	0.8	0.4	0.9	1.2	0.9
10	0.9	0.5	1.0	0.8	0.7	1.2	0.9	1.0	0.7	0.9	1.1	0.9
20	0.9	0.6	0.9	0.7	0.7	1.0	0.9	0.9	0.5	0.7	0.9	0.9
50	1.1	0.3	0.5	0.2	1.0	0.5	1.1	1.0	0.3	0.8	1.0	0.9
100	0.3	0.0	0.0	0.2	0.7	0.7	1.0	0.7	0.6	0.5	1.1	1.0
C+ (NF)	4.4	4.3	5.4	4.6	4.4	4.3	5.4	4.6	3.8	4.3	5.6	4.6

Table 20. Results of Ames test

CONCLUSIONS

H. officinalis subsp. *aristatus* has been harvested in natural habitats in Abruzzo, Civitaretenga (AQ) in 2016, Navelli, and Majella in 2018. The abundant presence of limonen-10-yl-acetate characterizes the essential oil of the specimen harvested at Civitaretenga (AQ). This molecule is absent in the essential oils obtained from the aerial part of plant species collected in 2018 that showed composition already reported in the literature. In particular, the essential oil obtained from the specimen harvesting in Navelli (AQ) showed a fingerprinting characterized by the quantitative amount of *cis*- (43.2%) and *trans*-pinocamphone (11.0%), while that obtained from the harvesting in Majella (Abruzzo), is particularly rich in methyleugenol (41.5%) and 1,8- cineole (39.7%). As for the hydroalcoholic extracts, all the phytocomplexes showed qualitatively similar profiles characterized mainly, in order of relative abundance, by chlorogenic acid, rosmarinic acid, and caftaric acid. The most prosperous sample was found to be the one harvested in 2016 at Civitaretenga. This evidence confirms the fundamental importance of the area in which plant growing, therefore also in the production of secondary metabolites, and direct the research towards further investigations into the area of Civitaretenga and other parts of Abruzzo.

The hydroalcoholic extracts showed a similar fingerprinting for all specimens, characterized by the leading presence of chlorogenic, rosmarinic, and caftaric acid (RP-HPLC-DAD), and they also exhibited a great antioxidant capacity (DPPH). The plant collected in Civitaretenga in 2016 showed the highest abundance of every compound. In particular, the quantity of chlorogenic acid was about twice as much as in the other specimens. Regarding the cytotoxicity, no significant results were observed for essential oils, while a slight but significant reduction in both the viability and migration in the ethanol extract was observed. The cytotoxic evaluation of the quantitatively relevant molecules of the ethanolic extract did not yield any significant results at the tested concentrations, which are 10 times lower than those evaluated in the literature (resulting weakly active). In migration tests, on the other hand, an encouraging effect of slowing down motility was observed for the ethanolic extract, which, however, is not confirmed in the invasion test. Extracts did not show any cytotoxicity against HaCat cell line, giving an indication of the safe use of the extracts, also confirmed by the Ames test. These results point towards methodological insights and further refinement of the experimental parameters in biological tests.

O. campechianum is a plant species of the Lamiaceae family, widespread across Central and South America, and it was chosen because of its broad spectrum of biological activities. It is traditionally used as an ingredient for infusions and beverages or as a flavoring agent for foods, against cough, bronchitis, and general infections or as anti-inflammatory, antipyretic, to treat conjunctivitis and even as diuretic and emmenagogue. Literature also reported an excellent antioxidant capacity and, recently, a larvicidal activity *in vitro* for its essential oil.

The crude plant drug was characterized based on the essential oil, methanolic, and hydroalcoholic (ethanol 70%) extracts composition. The chemical characterization of essential oils, performed with GC-MS analyses, revealed a chemical profile characterized mainly by eugenol (46.55%), β-caryophyllene (11.94 %), β-elemene (9.06%) and 1,8-cineole (5.35%).

The methanolic and hydroalcoholic extracts, chemically characterized for the first time in this research, showed a similar fingerprinting, with the central presence of rosmarinic acid, followed by caftaric, chlorogenic acids as minor components (RP-HPLC-DAD, NMR). The ethanolic extract exhibited the highest abundance of every compound. In particular, the quantities of caftaric and chlorogenic acid were about twice as much as in the other extract, while rosmarinic acid was nearly four times more abundant. A preliminary screening of the extracts against A549 cell line was performed but with negative outcomes. On the other hand, extracts did not show any cytotoxicity against HaCat cell line, giving an indication of the safe use of the extracts. The antibacterial activity against S. aureus and P. syringae pv. syringae was also tested, and essential oil resulted in the most active preparation with moderate antimicrobial properties. All extracts exhibited a remarkable antioxidant capacity (DPPH). The highest activity was shown, once again, by the essential oil with an IC50 of 7.7±0.1 µg/ml, close to the value of the positive control (Trolox). The hydroalcoholic extract exhibited bioactivity close to the one of essential oil (11.1±0.01µg/ml), while the methanolic displayed a weaker activity when compared to the previous extracts (52.3±2.7 µg/ml). Rosmarinic acid and eugenol, tested as central components of extracts and essential oil, respectively, showed values close to Trolox. Extracts and essential oil did not reveal mutagenic activity. The results confirmed very promising properties for the formulation of cosmetic and food supplements products.

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Congress Poster

Alessandra Guerrini, Immacolata Maresca, Massimo Tacchini, Gianni Sacchetti, Ilaria Burlini, Mónica Paulina Echeverría Guevara, Giustino Orlando, Claudio Ferrante, Luigi Menghini *"Hyssopus officinalis* subsp. *aristatus* (Godr.) Nyman from Abruzzo region: morphological features, chemical and biological characterization of essential oil and hydroalcoholic extract" Poster in 113° Congress of Società Botanica Italiana -*V International Plant Science Conference (IPSC)*, Fisciano, 12-15 september 2018.

Massimo Tacchini, Laura Scalvenzi, Matteo Radice, Ilaria Burlini, Immacolata Maresca, Gianni Sacchetti, Alessandro Grandini, Alessandra Guerrini, M nica Paulina Echeverría Guevara "*Ocimum campechianum* Mill. leaves: an Amazonian promising drug". Poster Presentation: 114° Congress of Società Botanica Italiana-VI International Plant Science Conference (IPSC), Padova, 4-7 September 2019.

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