Università degli Studi di Ferrara

Biochimica, Biologia molecolare e Biotecnologie
CICLO XXIII

COORDINATORE Prof. Francesco Bernardi

From ethnomedicine to pharmaceutical biology through Cooperation projects: a cultural and research bridge between Africa and Amazonia

Settore Scientifico Disciplinare BIO/15

Dottorando
Dott. Useli Chiara

_____
(firma)

Tutore
Prof. Sacchetti Gianni

_____
(firma)

Anni 2008/2011

Il tuo indirizzo e-mail
chiara.useli@unife.it
Oggetto:
Dichiarazione di conformità della tesi di Dottorato
Io sottoscritto Dott. (Cognome e Nome)
Useli Chiara
Nato a:
Venezia
Provincia:
Venezia
Il giorno:
04/03/1980
Avendo frequentato il Dottorato di Ricerca in:
Biochimica, Biologia molecolare e Biotecnologie
Ciclo di Dottorato
23
Titolo della tesi (in lingua italiana):
Dall'etnomedicina alla biologia farmaceutica attraverso progetti di Cooperazione: un ponte culturale e di ricerca tra Africa e Amazzonia
Titolo della tesi (in lingua inglese):
From ethnomedicine to pharmaceutical biology through Cooperation projects: a cultural and research bridge between Africa and Amazonia
Tutore: Prof. (Cognome e Nome)
Sacchetti Gianni
Settore Scientifico Disciplinare (S.S.D.)
BIO/15
Parole chiave della tesi (max 10):
ethomedicine phytochemistry bioactivity etnomoedicina fitochimica bioattività
Consapevole, dichiara
CONSAPEVOLE: (1) del fatto che in caso di dichiarazioni mendaci, oltre alle sanzioni previste dal codice penale e dalle Leggi speciali per l'ipotesi di falsità in atti ed uso di atti falsi, decade fin dall'inizio e senza necessità di alcuna formalità dai benefici conseguenti al provvedimento emanato sulla base di tali dichiarazioni; (2) dell'obbligo per l'Università di provvedere al deposito di legge delle tesi di dottorato al fine di assicurarne la conservazione e la consultabilità da parte di terzi; (3) della procedura adottata dall'Università di Ferrara ove si richiede che la tesi sia consegnata dal dottorando in 4 copie di cui una in formato cartaceo e tre in formato pdf, non modificabile su idonei supporti (CD-ROM, DVD) secondo le istruzioni pubblicate sul sito: http://www.unife.it/studenti/dottorato alla voce ESAME FINALE – disposizioni e modulistica; (4) del fatto che l'Università sulla base dei dati forniti, archivierà e renderà consultabile in rete il testo completo della tesi di dottorato di cui alla presente dichiarazione attraverso l'Archivio istituzionale ad accesso aperto "EPRINTS.unife.it" oltre che attraverso i Cataloghi delle Biblioteche Nazionali Centrali di Roma e Firenze; DICHIARO SOTTO LA MIA RESPONSABILITA': (1) che la copia della tesi depositata presso l'Università di Ferrara in formato cartaceo, è del tutto identica a quelle presentate in formato elettronico (CD-ROM, DVD), a quelle da inviare ai Commissari di esame finale e alla copia che produrrò in seduta d'esame finale. Di conseguenza va esclusa qualsiasi responsabilità dell'Ateneo stesso per quanto riguarda eventuali errori, imprecisioni o omissioni nei contenuti della tesi; (2) di prendere atto che la tesi in formato cartaceo è l'unica alla quale farà riferimento l'Università per rilasciare, a mia richiesta, la dichiarazione di conformità di eventuali copie; (3) che il contenuto e l'organizzazione della tesi è opera originale da me realizzata e non compromette in alcun modo i diritti di terzi, ivi compresi quelli relativi alla sicurezza dei dati personali; che pertanto l'Università è in ogni caso esente da responsabilità di qualsivoglia natura civile, amministrativa o penale e sarà da me tenuta indenne da qualsiasi richiesta o rivendicazione da parte di terzi; (4) che la tesi di dottorato non è il risultato di attività rientranti nella normativa sulla proprietà

industriale, non è stata prodotta nell'ambito di progetti finanziati da soggetti pubblici o privati con vincoli alla divulgazione dei risultati, non è oggetto di eventuali registrazioni di tipo brevettale o di tutela. PER ACCETTAZIONE DI QUANTO SOPRA RIPORTATO

Firma del dottorando

Ferrara, li _____ (data) Firma del Dottorando

_____

Firma del Tutore

Visto: Il Tutore Si approva Firma del Tutore _____

A Mea la mia gatta
e a tutti gli altri

I miei più sentiti ringraziamenti a:

Alessandro Medici, Gianni Sacchetti, Damiano Rossi, Alessandra Guerrini, Immaccolata (Rita) Maresca, Silvia Maietti, Alessandro Grandini, Irene Poppi, Guido Acquaroli, Valentina, Stefania, Claudia, Katia, Gabi, Silvia, Laura e Matteo, Maria Elena e Paco, la Foundación Chankuap e l'Universitad Politécnica Salesiana di Quito, il VIS, gli Amici dei Popoli e i Barabbas' Clown - per gli insegnamenti, i consigli, il conforto e l'ospitalità. Un ricordo particolare va a Mariavittoria Muzzoli, e un grazie speciale a Luca e a tutta la mia famiglia per l'appoggio e la pazienza.

INDEX

ABBREVIATIONS:

(HP)TLC = (High Performance) Thin layer Chromatography

(HP)TLC = (High Performance) Thin Layer Chromatography

ABTS = 2, 2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid

ACO = Avocado Commercial Oil

AHM = Avocado Oil hexane extracted

AMM = Avocado extract emethanol macerated

ASFE = Avocado oil supercritical fluid extracted

ASU = Avocado and Soyabean Unsaponifiale fraction

ATCC = American Type Culture Collection

BHA = Butylated hydroxy anisole

C.a. = *Candida albicans*

CCIE = cyaniding chloride equivalents

CFU = Colony Forming Units

DMSO = dimethyl sulfpxide

DPPH = 1,1-diphenyl-2-picrylhydrazyl

E.c. = *Escherichia coli*

E.f. = *Enterococcus faecalis*

EIA. = hexane/80% ethanolic extract

EMS = Ethylmethansulphonate

EO = Essential Oil

GAE = gallic acid equivalent

GCC = gene conversion

HC = hydrocarbons

HE = hyperoside equivalent

HS = Head Space analysis

HUD = high uneffective dose

IZD = inhibition zone diameter

K.o. = *Klebsiella oxytoca*

L.g.= *Listeria grayi*

M.l. = *Micrococcus luteus*

MC = reverse point mutation

MIC = Minimum Inhibitory Concentration

MUFA = monounsaturated fatty acids

P.a. = *Pseudomonas aeruginosa*

P.v. = *Proteus vulgaris*

PUFA = polyunsaturated fatty acids

S.a.= *Staphilococcus aureus*

S.c. = *Saccharomyces cerevisiae*

SD = Steam distillation

SFE = Supercritical fluid extraction

SFE Supercritical Fluid Extraction

TFA = total fatty acids

TTC = 2,3,5-triphenyltetrazolium chloride

UAE Ultrasound Assisted Extraction

UF = unsaponifiable fraction

UHM = ultrasound assisted n-Hexane maceration

UMM = ultrasound assisted methanol maceratio

INTRODUCTION

1. PhD background and preliminary consideration

The present PhD research has its background in Cooperation and Development projects organized and sponsored by University of Ferrara.

The *Centre for the International Development Cooperation of the University of Ferrara* is linked with non-profit associations and, in particular, Salesians (i.e. Salesian Polytechnic University of Quito), VIS-ONG [Volontariato Internazionale per lo Sviluppo], ADP-ONG [Amici dei Popoli] and Barabba's clown ONLUS, with the aim to promote collaborations based on mutual utilization of intellectual resources and technical strategies, promoting, coordinating and carrying out development co-operation projects, research, didactical, training and updating activities and providing services inherent to topics relevant to developing Countries.

In this context, my work was split into two general research pathways leading to different goals:

- the valorization through chemical and biotechnological lab research strategies of ethnomedical sources (from Rwanda and Amazonia in particular);
- to help and support non-profit Italian associations to practically set up factory production systems which could give social and economic contributes to Natives in valorizing the biodiversity of the Country. This support has been performed as a consequence of technical and researching aspects reached with the previous target (A).

This double approach gave to me the possibility to attend the growing research laboratory, in Amazonian Ecuador and the pharmaceutical biology labs at the University of Ferrara, mainly optimizating extractions, chemical analyses and bioactivity checks of plant sources potentially interesting for the health market.

At the beginning of my PhD, I had been working for three months in an Amazonian cultural association supported by Salesians, Foundación Chankuap (Macas - Ecuador), which markets Amazonian Essential Oils and raw material through "Fair Trade", with the aim to support European exportation of that products and to learn technical know-how. In parallel,

thanks to the activities of the *Centre for the International Development Cooperation of the University of Ferrara,* I started a relationship with two Rwandese centre: Hameau de Jenes (Musha - Rwanda) and Salesian School of Gatenga (Kigali - Rwanda), with the aim to explore the exploitation of cultivated plants and derivates and the possibility to improve their markets, related to Essential, fixed oils and blended essential oils. So, after the PhD starting phase in Ecuador, I planned a research linked to Cooperation necessities and PhD research, regarding Rwandese Avocado fixed oil and Amazonian Curcuma, Yerba luisa, Basil and Ginger essential oils with the target to explore their chemical and biological properties, and the possibility to employ all those derivates together as formulations.

In Rwanda Avocado oil could be used both as alternative cosmetic ingredient for simple formulations for local market (i.e. skin-care formulation), and as a cooking oil with interesting nutraceutical properties. The first part of my studies was focused on preliminary extraction procedures of Avocado oils with two purposes: 1) to trace the phytochemical fingerprint of Rwandese avocado oil in comparison with more studied and marketed qualities (i.e. Mexican's), regarding biodiversity and its potential prospective towards health; 2) to perform an appropriate oil extraction process for developing local weaving factories.

Then, I focused my attention on setting up bioactivity and safety tests of Amazonian essential oils related with their chemical and biological fingerprints, starting from studies performed in pharmaceutical biology laboratories of University of Ferrara (Sacchetti et al., 2004; Sacchetti et al., 2005; Guerrini et al., 2011; Rossi et al., 2011; Maietti et al., in press).

In the last part of my PhD, it has been explored the possibility of applicative uses of fixed oil and essential oils for "fair trade" markets, such as healthy formulations.

However, before the official start of the PhD, and during PhD research path, I contribute to chemically and biologically characterize other plant species, always interesting as natural resources for health purposes, following typical research pattern of the pharmaceutical biology labs. In synthesis, some of these studied-plants are object of original papers to which I contributed for their realization, reported as conclusive paragraph of the thesis (Guerrini et al., 2011; and Maietti et al., in press).

As a conclusion of this background, it is my opinion that scientific publications derived from this kind of research profile – from ethnomedicine to laboratory led by international Cooperation projects– could help scientists to find new integrated opportunities for human beings progress (i.e. new chemicals for new drugs to treat old and new diseases) with ethical respect to different cultures from different Countries.

My personal experience as PhD student linked to a Cooperation project and to similar previous PhDs, would try to give another little contribute to this approach: M. Gabriela Moreno Rueda, *Biotrasformazioni di terpeni e oli essenziali con batteri e funghi isolati da frutti del genere Citrus della foresta amazzonica (Ecuador)*, (2009); Laura Scalvenzi, *Amazonian plants from ethnomedicine through pharmaceutical biology approaches: a PhD experience in connecting forest with laboratory*, (2010); Matteo Radice, *Studi di attività biologica cosmeceutica di derivati di specie della zona sud orientale amazzonica ecuadoriana* (2010).

2. "The Developing Countries"

Developing countries: Countries in the process of change directed toward economic growth, that is, an increase in production, *per capita* consumption, and income. The process of economic growth involves better utilization of natural and human resources, which results in a change in the social, political, and economic structures.

Synonym: under-developed Country, third-world Country, less-developed nation, less-developed Country, developing nation, under-developed nation, third-world nation (http://medconditions.net).

Personal note: in these years of global economic crisis, the geography of developing countries – in light of the above noted definitions – is dramatically changing, leaving wider opportunities to the valorizations of local resources, such biodiversity is.

Definition and criteria: Human Development Index (HDI)

Economic criteria have tended to dominate discussions. One criterion is Gross Domestic Product (GDP) that refers to the market value of all final goods and services produced within a country in a given period. Another measure is the Human Development Index (HDI), which combines an economic measure, national income, with other measures like indices for life expectancy and education. The Human Development Index (HDI) is a comparative measure of life expectancy, literacy, education and standards of living for Countries worldwide. It is a standard means of measuring well-being, especially child welfare. It is used to distinguish whether the Country is a developed, a developing or an under-developed Country, and also to measure the impact of economic policies on quality of life. There are also HDI for states, cities, villages, etc. by local organizations or companies.

Kofi Annan, former Secretary General of the United Nations, defined a developed Country as follows: "*A developed Country is one that allows all its citizens to enjoy a free and healthy life in a safe environment.*" (Secretary-General Kofi Annan, Bangkok (United Nations Information Services), Tenth United Nations Conference on Trade and Development ("UNCTAD X"), Bangkok, 12 February 2000).

The UN also notes: “In common practice, Japan in Asia, Canada and the United States in North America, Australia and New Zealand in Oceania, and most European Countries are considered "developed" regions or areas. In international trade statistics, the Southern African Customs Union is also treated as a developed region and Israel as a developed Country; Countries emerging from the former Yugoslavia are treated as developing Countries; and Countries of eastern Europe and of the Commonwealth of Independent States (code 172) in Europe are not included under either developed or developing regions.” (http://unstats.un.org).

The diagram below (fig. 1) shows a World map indicating the Human Development Index category by Country (2010).

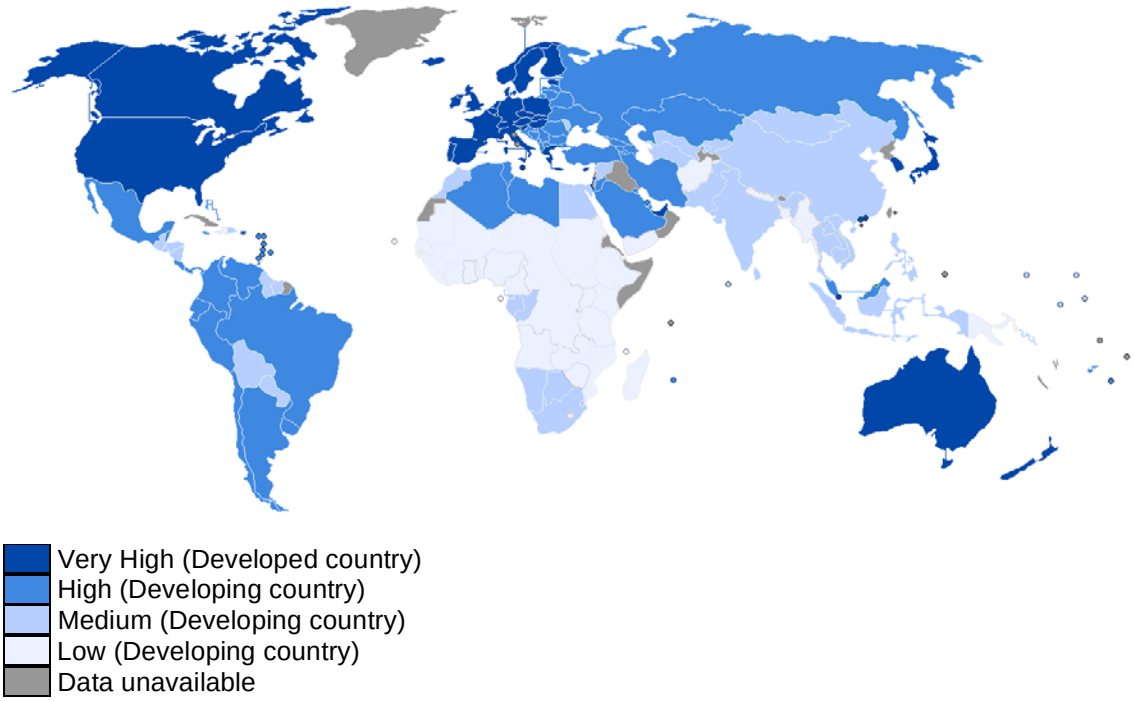


Fig. 1 Thematic World map indicating the HDI (Human Development Index) category by Country; data collected on 2010. Both Ecuador and Rwanda has Emerging and Developing economies (from International Monetary Found data www.imf.org).

Both Ecuador and Rwanda are considered developing country, with the Ecuadorian HDI higher than the Rwandese one.

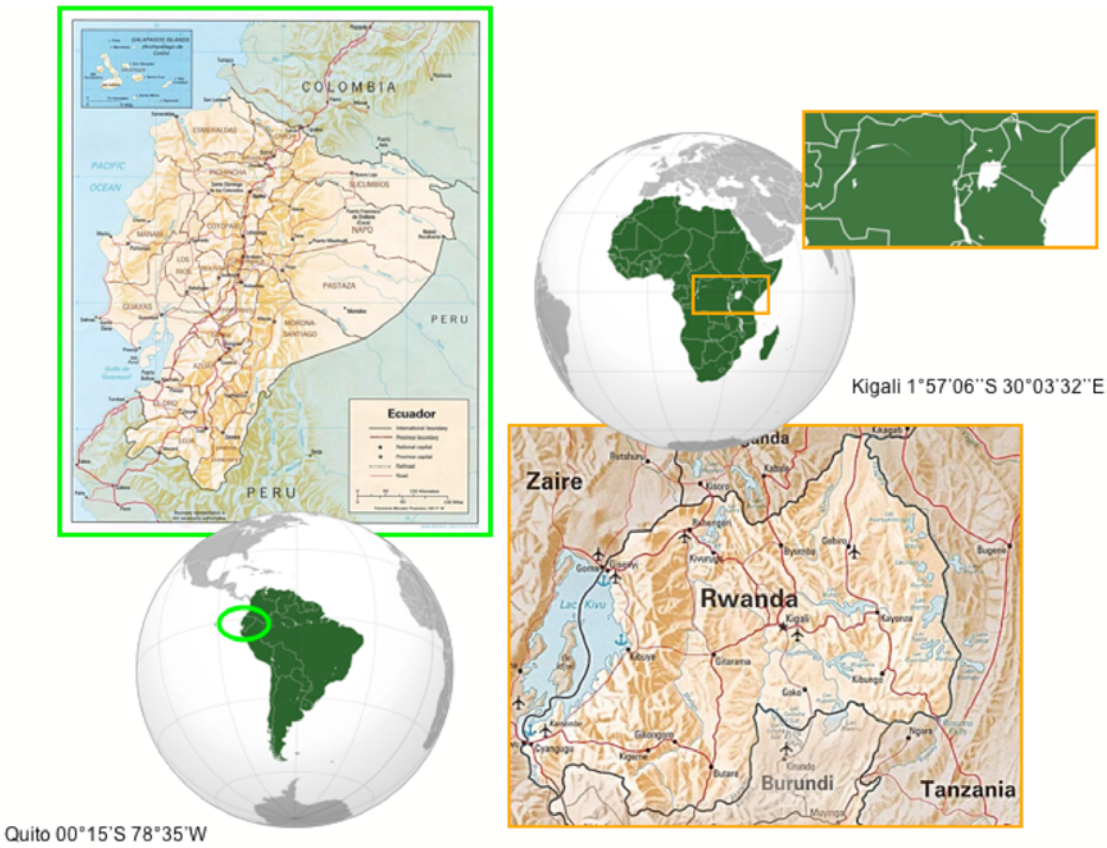


Fig. 2 Location of Ecuador and Rwanda on the planisphere and relatives maps. Both Countries straddle Equator; Rwanda and Amazonian Ecuador has temperate climate, with two rainy seasons and two dry seasons.

Notes on Ecuador and Rwanda

The **Republic of Ecuador** is a representative democratic republic in South America, bordered by Colombia on the north, Peru on the east and south, and by the Pacific Ocean to the west. Its capital city is Quito, which was declared a World Heritage Site by UNESCO in the 1970s for having the best-preserved and least altered historic centre in Latin America. The Country's largest city is Guayaquil. The Ecuadorian biodiversity makes this Country one of the 17 megadiverse Countries in the world. The new constitution of 2008 is the first in the world to recognize legally enforceable Rights of Nature, or ecosystem rights. The economy is heavily based on cultivation and exporting plant-derived products typical of Ecuadorian biodiversity (coffee, cacao, guayusa, bananas etc.). Petroleum, fishing and

agronomic resources contributes to Ecuadorian economy definition ("Background Note: Ecuador. US Department of State" 2010 http://www.state.gov).

The **Republic of Rwanda**, indeed, is a Country in central and eastern Africa with a population of approximately 11.4 million (2011). The population is young and predominantly rural, with a density among the highest in Africa. Rwanda's economy suffered heavily during the 1994 Genocide, but has since strengthened. The economy is based mostly on subsistence agriculture; coffee and tea are the major cash crops for export (World Trade Organization (WTO) (2004-09-30). "Continued reforms and technical assistance should help Rwanda in its efforts to achieve a dynamic economy" (*Trade policy review: Rwanda*. Retrieved 2009-02-04).

STRATEGY AND GOALS

1. PhD Strategy and goals

This research has been planned with the aim to connect a project in progress by *Centre for the International Development Cooperation of the University of Ferrara* and PhD targets, studying Rwandese Avocado fixed oil and Amazonian essential oils, i.e. Curcuma, Yerba luisa, Basil and Ginger. The main target was to find in these natural derived products some interesting healthy information exploitable as economic local sources. As a consequence, the PhD research has been focused on optimization of extracting procedures, chemical analyses (HPLTC, GC-MS, HPLC-DAD, NMR) and bioactivity assays regarding health efficacy (antioxidant, antimicrobial, mutagen-protective) and safety (cytotoxicity, mutagenic) proprieties of the above-mentioned plant derived products.

The general outline of the research is summarized in the diagram below (Fig. 3), which will be further discussed in the "Material and Methods" and "Results and Discussion" sections.

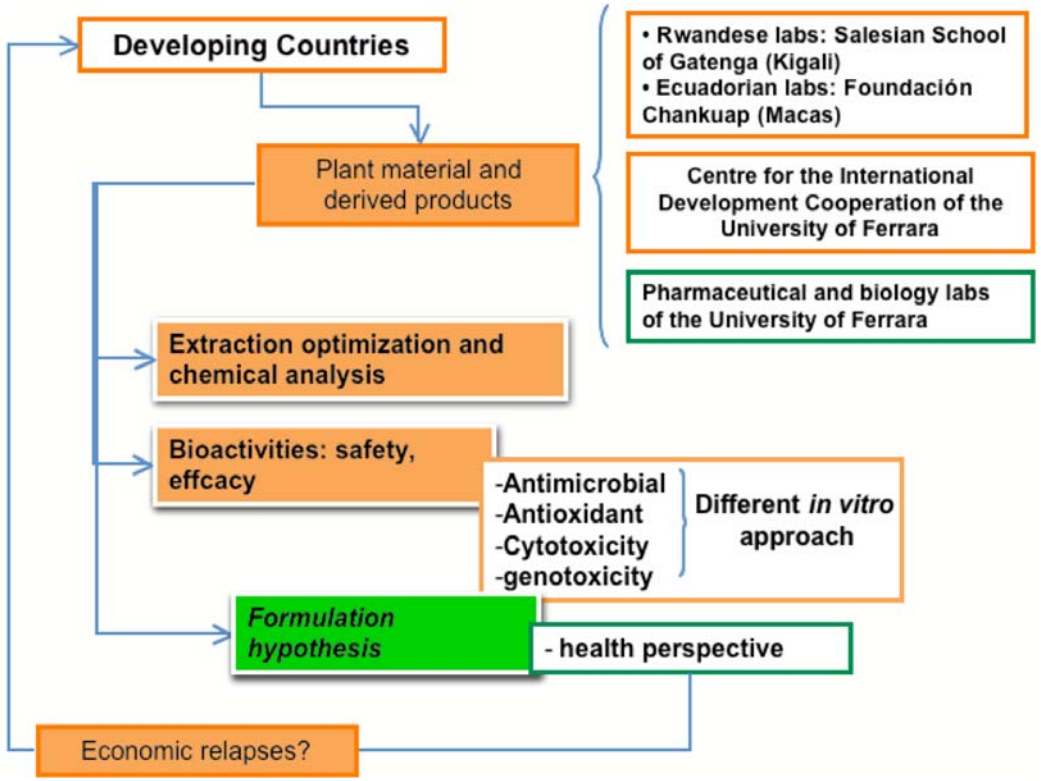


Fig. 3 The scheme reports the general outlines of this PhD research.

The research has been performed following:

- Pharmaceutical biology approach based on:
    a. Extractions (Steam Distillation [SD], Supercritical Fluid Extraction [SFE], Ultrasound Assisted Extraction [UAE]) and phytochemical characterization (^{1}H-NMR, GC-FID, GC-MS, HPTLC, HPLC, UV).
    b. Bioactivity assays to shed a light on health efficacy and safety of phytocomplexes, fractions and pure compounds.

Taking the acquired results in consideration, it has been explored the possibility to prepare simple formulations (i.e. essential oil mixed in a carrier oil) in which the proved bioactivities of the plant derivates would be maintained with health efficacy.

The described PhD research has been performed following the three scheduled steps below, each as a consequence of the previous.

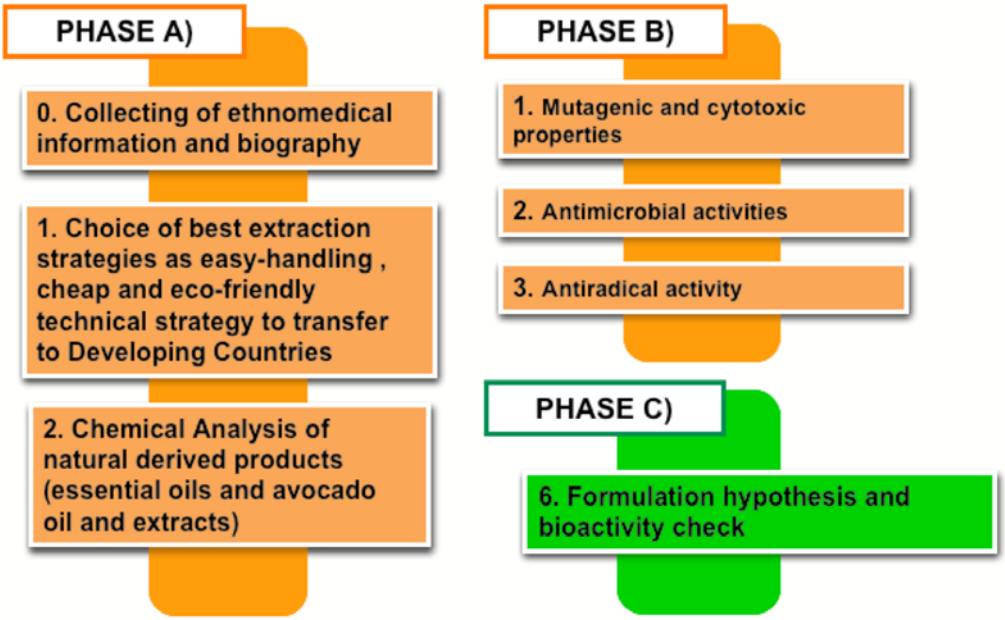


Fig. 4 Research phases.

BOTANICAL AND PHARMACOGNOSTIC DESCRIPTION OF THE STUDIED PLANT SOURCES

1 AMAZONIAN BASIL

Scientific names: *Ocimum micranthum* Wild, *Ocimum campechianum* Mill.

Family: *Lamiaceae*

Part Used: Herb.

Common names: Amazonian basil, wild sweet basil, wild mosquito plant, ocimum, least basil, Peruvian basil, spice basil, alfavaca-do-campo, manjericao, alfavaca, and estoraque.

1.1 Description

The family Lamiaceae has nearly 4,000 species worldwide, and the genus *Ocimum* contains about 160 species. *O. micranhtum* is native to the lowlands of Central and South America and the West Indies.

The plant is a strongly aromatic annual herb, varying in height from 40 to 58 cm. The wide leaf is light green, serrated, and ovate to ovate-lanceolate, 2-5 cm long, in shape, and if slightly bruised exhales a delightful typical herbaceous scent. Its stamens are whitish pink, and its nutlets are ellipsoid in shape and purplish to dark brown.

1.2 Uses

The plant species is known for numerous ethnomedicinal uses. Ethnopharmacologic and *in vitro* study analysis examine the antimicrobial, cardiovascular, and antioxidant activity of the essential oils (Sacchetti et al., 2004 and reference therein). No clinical trials are available.

Traditional/Ethnobotanical uses: Basil, i.e. plants belonging to *Ocimum* genus, employed as spice and for healthy plant source, have many cultural meanings linked to different Countries. In Europe, basil is related to culinary uses, ancient religious rituals both with holy and demoniac meanings. In South America is a less known variety of basil, native of the South and

Central American tropics, known as “Albahaca de campo” or “Albahaca silvestre” or “Albahaca de monte” and widely used by indigenous population both for culinary and medicinal purposes. In fact, besides as a spice, *O. micranthum* is traditionally used for its therapeutic properties against cough, bronchitis and general infections, or as anti-inflammatory, antipyretic, to treat conjunctivitis and even as diuretic and emmenagogue (Sacchetti et al., 2004; Naranjo, Escaleras, 1995).

1.3 Chemical components

Several studies describe the chemistry of the species, primarily the composition and functional properties of the essential oil.

Three major chemical classes occurring in *Ocimum* species: phenylpropanoids, monoterpenes, and sesquiterpenes. In particular, sesquiterpenes vary widely among *Ocimum* species.

More than 31 compounds have been identified in the essential oil of *O. micranthum*. The plant produces pale yellow viscous oil with a green odor, typical middle notes[1], upon hydrodistillation. The main components in the essential oil are eugenol, β-caryophyllene, and β-elemene, with variation related to climate region (Sacchetti et al., 2004 and reference therein). The essential oil of plants from India contains eugenol, 1,8-cineole, β-caryophyllene, and γ-elemene. The oil from Brazilian plants contains eugenol, β-caryophyllene, and elemicin as main components. Through hydrodistillation, the essential oil yield of *Ocimum micranthum* is the highest in the leaves and flowers; some studies document the species as having the highest total oil content (Sacchetti et al., 2004).

[1] Fragrance notes: Perfume is described as having three sets of notes: I. Top notes or head notes, thats are the scents perceived immediately; II. Middle notes or heart notes, emerges just prior to when the top notes dissipate; III. Base notes, that’s appears close to the departure of the middle notes. [wikipedia: perfume]

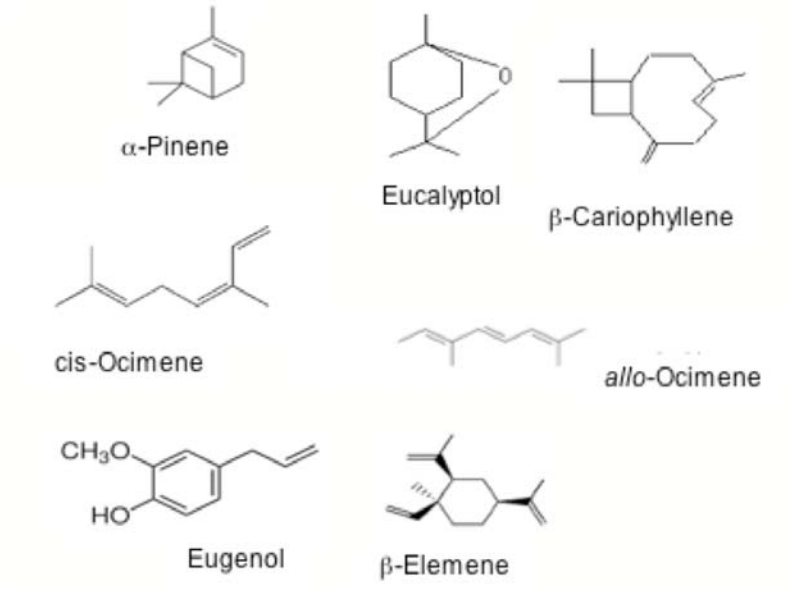


Fig. 5 Chemical structures of essential oils (EO) principal components

1.4 Potential health effects

Ethnopharmacologic and *in vitro* studies about the plant have been performed searching chemical evidence of health effects and pharmacological, toxicological activities.

Antimicrobial activity: According to results obtained to disk-diffusion method, *O. micranthum* essential oil has antimicrobial activity against gram-positive (*Enterococcus faecalis*) and gram-negative bacteria (*Escherichia coli*, *Pseudomonas aeruginosa*). The essential oil also has dose-dependent activity against food-related yeasts and contaminating bacteria. Extracts have antiprotozoal activity against *Trypanosoma cruzi*, possibly because of the plant's polyphenolic compounds, flavonoids, and lignans. (Sacchetti et al., 2004; Navarro et al., 2003).

Cardiovascular activity: *O. micranthum* was not vasoactive on rat aortic smooth muscle; however, the species may have antihemorrhagic properties. According to the results *in vivo* and *in vitro* study, this species moderately neutralizes hemorrhagic activity of pit viper venom within the genus *Bothrops* from Antioquia and Choco, northwestern Colombia. (Slish et al., 1999; Otero et al., 2000).

Other pharmacological activity: *In vitro* analyses report antioxidant and anti-inflammatory activity with suggested (but not proved) interesting applicative perspective. (Lino et al., 2005).

2 CURCUMA

Scientific names: *Curcuma longa* L., *Curcuma domestica* Valeton.

Family: *Zingiberaceae*

Part Used: Dried rhizome.

Common names: Curcuma, Turmeric, Indian saffron, Haldi

2.1 Description:

Perennial plant, member of the ginger family, turmeric is cultivated in particular throughout tropical Asia, India, and China. The plant grows to a height of 0.9 to 1.5 m and bears large, oblong leaves and funnel-shaped, dull yellow flowers, three or five together surrounded by bracteolae. It has a thick rhizome, which is yellowish on the outside and deep orange or reddish brown inside. The lateral rhizomes contain more yellow coloring than the bulb. The dried primary bulb and secondary lateral rhizomes are collected, cleaned, boiled, and dried for use in medicinal and food preparations. The major active principle is curcumin. It has a peculiar fragrant odor and a bitterish, slightly acrid taste, like ginger, exciting warmth in the mouth and colouring the saliva yellow.

2.2 Uses

Traditional/Ethnobotanical uses Turmeric has a warm, bitter taste and is used extensively as a food flavoring and colorant; it is a primary component of curry powders and some mustards. The spice has a long tradition in Asian medicine to treat problems ranging from flatulence to hemorrhage. Uses to

treat ringworm, as a poultice, for pain, and in the management of jaundice and hepatitis has been documented (Jayaprakasha et al., 2005).

In Ayurvedic practices, and also in South American traditional medicine, turmeric is used as an anti-inflammatory agent and remedy for gastrointestinal discomfort associated with irritable bowel syndrome and other digestive disorders. Some may use turmeric in skin creams as an antiseptic agent for cuts, burns and bruises. It is popular as a tea in Okinawa, Japan (Jayaprakasha et al., 2005).

Turmeric is used as a spice in curry powders and mustard. It has been investigated in **clinical trials** for the treatment and prevention of cancers, particularly of the gastrointestinal tract, and for treatment of colitis, Alzheimer and Huntington diseases. Turmeric (coded as E100 when used as a food additive) is used to protect food products from photo-degradation. The oleoresin is used for oil-containing products. The curcumin/polysorbate solution or curcumin powder dissolved in alcohol is used for water-containing products. Over-coloring, such as in pickles, relishes, and mustard, is sometimes used to compensate for fading. (Jayaprakasha et al., 2005 and reference therein).

Curcuma has its mean cultural roots in India, as religious spice, heath crude drug, and cooking relish. In Europe its use has been imported and it is known as Indian saffron because of its main employing as saffron cooking spice substitute.

2.3 Chemical components

Turmeric contains up to 5% essential oil and up to 5% curcumin, a polyphenol. Phytochemical investigations of the plants, revealed a typical phenylpropanoids patterns, among which curcumin, demethoxycurcumin and bisdemethoxycurcumin are the most abundant. Turmerone and carvacrol have been reported as the most constituents of rhizome essential oil of yellow and red varieties of Bangladesh grown *C. longa*; alfa-phellandrene and terpinolene as the predominant constituents of leaf oil of south-west Nigerian grown *C. longa* (Usman et al., 2009).

C. longa, derived from plants cultivated in Amazonian Ecuador, showed a notable amount of α- and β-turmerone (19.8 and 7.35%) and was found to

be rich in monoterpenes, such as α-phellandrene (20.4%), 1,8 cineole (10.3%) and terpinolene (6.19%) (Sacchetti et al., 2005).

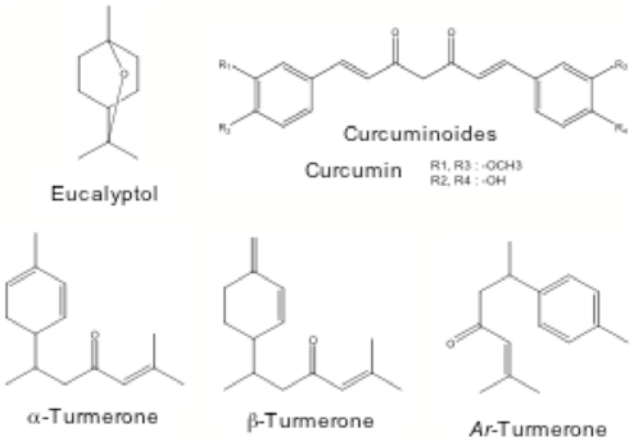


Fig. 7 Chemical structures of essentia oil's principal components.

2.4 Potential health effects

Turmeric has been used historically as a component of Indian Ayurvedic medicine since 1900 BC to treat a wide variety of aliments. Research in the latter half of the 20th century has identified curcumin as responsible for most of the biological activity of turmeric. *In vitro* and *in vivo* studies have suggested a wide range of potential therapeutic or preventive effects associated with curcumin. At present, these effects have not been confirmed in humans. However, numerous clinical trials in humans were in progress, studying the effect of curcumin on various diseases, including multiple myeloma, pancreatic cancer, myelodysplastic syndromes, colon cancer, psoriasis, and Alzheimer's disease (Aggarwal et al., 2007; Hatcher et al.,2008).

Antioxidant activity: According to results using DPPH free radical *C. longa* shows weak radical scavenging actions similar to *Cymbopogon citratus* and higher than *Zingiber officinale* (Sacchetti et al., 2005).

Other healthy bioactivities: *In vitro* and *in vivo* studies have suggested that curcumin may have antitumoral properties (Aggarwal et al., 2006). In addition it may be effective in treating malaria, prevention of cervical cancer, and may interfere with the replication of the Human Immunodeficiency Virus (HIV) by interfering with P300/CREB-binding protein (CBP). It is also

hepatoprotective. In 2008, a study at Michigan State University showed low concentrations of curcumin interfere with Herpes simplex virus-1 (HSV-1) replication. The same study showed curcumin inhibited the recruitment of RNA polymerase II to viral DNA, thus inhibiting its transcription. This effect was shown to be independent of effect on histone acetyltransferase activities of p300/CBP. A previous (1999) study performed at the University of Cincinnati indicated curcumin is significantly associated with protection from infection by HSV-2 in animal models of intravaginal infections (Kutluay et al. 2008 and references cited).

Fig. 8 An example of Amazonian Curcuma Essential oil producted in Ecuador (Foundación Chankuap)

3 GINGER

Scientific names: *Zingiber officinale* Roscoe

Family: *Zingiberaceae*

Part Used: Rhizome.

Common names: ginger, ginger root, black ginger, zenzero, jengibre

3.1 Description:

Ginger is the rhizome of the plant *Zingiber officinale*, consumed as a delicacy, medicine, or spice. It lends its name to its genus and family (*Zingiberaceae*). Other notable members of this plant family are turmeric, cardamom, and galangal. Naturalized in America but it is original of the East Indies. It is now cultivated in great quantities in Jamaica. The root from the West Indies is considered the best crude drug. Also imported from Africa,

there are several varieties known in commerce: Jamaica or White African is a light-brown color with short rhizome, very pungent; Cochin has a very short rhizome, coated red-grey color; Green Ginger is the immature undried rhizome; Preserved Ginger is made by steeping the root in hot syrup; Ratoon is uncultivated Ginger. The flowering stalk rises directly from the root, ending in an oblong scallop spike; from each spike a white or yellow bloom grows. Ginger flowers have an aromatic smell and the bruised stem a characteristic fragrance, but the root is considered the most useful part of the plant, and must not be used under a year's growth (*Zingiber officinale* information from NPGS/GRIN www.ars-grin.gov, 2008).

3.2 Uses

General uses: There are many traditional uses for ginger, but more recent interests focus on the prevention and management of nausea. Ginger may play a role in osteoarthritic pain and cancer. However, there is limited clinical information to support these uses. Ginger Tea is a hot infusion typically rubefacient (M. Grieve “A Modern Herbal” Paperback 1971).

Traditional/Ethnobotanical uses: Medicinal use of ginger dates back to ancient China and India. References to its use are found in Chinese pharmacopoeias, the Sesruta scriptures of Ayurvedic medicine, and Sanskrit writings. Ginger's culinary properties were discovered in the 13th century, its use became widespread throughout Europe. In the Middle Ages, apothecaries recommended ginger for travel sickness, nausea, hangovers, and flatulence (M. Grieve “A Modern Herbal” Paperback 1971).

Ginger root is traditionally used in cooking and main ingredient in traditional medicine in several cultures, for example in India and is used to spice tea and coffee, or consumed in candied and pickled form. In Western cuisine, ginger is traditionally used mainly in sweet foods (ginger ale, ginger biscuits…), or in ginger-flavored liquor or wine (M. Grieve “A Modern Herbal” Paperback 1971).

Tea brewed from ginger is reputed a common folk remedy for colds. Ginger ale and ginger beer are also drunk as stomach settlers. Ginger water is also known to avoid heat cramps in the United States. In China, "ginger eggs" (scrambled eggs with finely diced ginger root) is a common home remedy for coughing; Chinese also make a kind of dried ginger candy, fermented in

plum juice and sugared, which is also commonly consumed to suppress coughing. Ginger has also been historically used to treat inflammation, which several scientific studies support, though one arthritis trial showed ginger to be no better than a placebo or ibuprofen for treatment of osteoarthritis (M. Grieve “A Modern Herbal” Paperback 1971).

In Sud America Shamans uses ginger roots extracts to interrupt hallucinations caused by the consumption of psychoactive ritual plants. Otherwise local *Curanderos* uses ginger roots to contrast pharyngitis, and arthritis (Naranjo, Escaleras 1995 and references cited.)

3.3 Chemical components

Ginger contains up to 3% of a fragrant essential oil whose main constituents are sesquiterpenoids, with (-)-zingiberene as the main component. Smaller amounts of other sesquiterpenoids (β-sesquiphellandrene, bisabolene and farnesene) and a small monoterpenoid fraction (β-phellandrene, cineole, and citral) have also been identified.

In the Amazonian Jengibre essential oil, the major components were zingiberene (23.9%), β-bisabolene (11.4%) and β-sesquiphellandrene (10.9%) (Sacchetti et al., 2005).

The pungent taste of ginger is due to nonvolatile phenylpropanoid-derived compounds, particularly gingerols and gingerol-derived shogaols, which form from gingerols when ginger is dried or cooked. Zingerone is also produced from gingerols during this process; this compound is less pungent and has a spicy-sweet aroma.

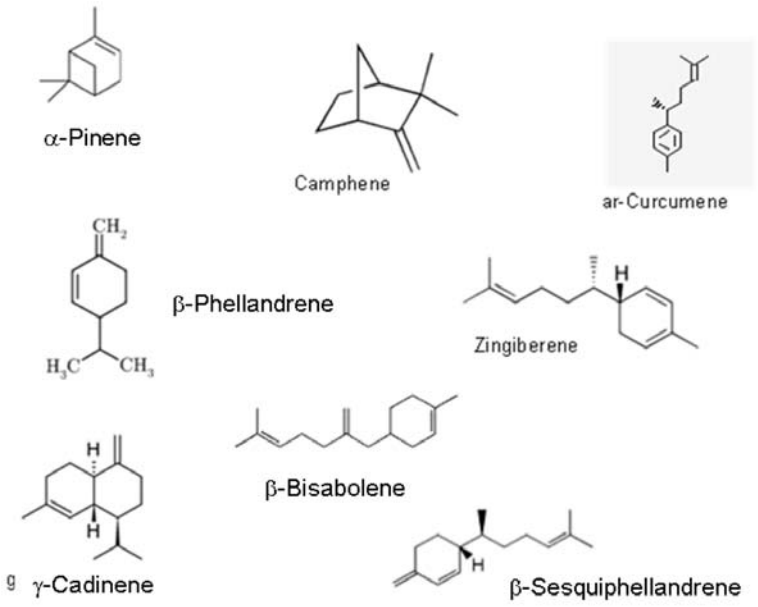


Fig. 9 Chemical structures of essential oil principal components.

3.4 Potential health effects

The characteristic smell and flavor of ginger roots is caused by a mixture of zingerone, shogaols and gingerols, characterizing the 1-3% part, referred to the fresh crude drugs. *In vivo*, the gingerols increase the motility of the gastrointestinal duct showing analgesic, sedative, antipyretic and antibacterial properties. Ginger essential oil has been shown to prevent skin cancer in mice and a study at the University of Michigan demonstrated *in vitro* that gingerols can kill ovarian cancer cells (Choudhury et al.,2010).

The **healthy potentials** of gingerol, a constituent of ginger roots, present a promising future alternative to expensive and toxic therapeutic agents in treatment against gastric lesions and, above all, as effective compound in cancer prevention (Yamahara et al., 1988; Oyagbemi et al., 2010).

Antioxidant activity: Ginger is a source of a large number of important antioxidants that, amongst other activities, reduce lipid oxidation, and shows radical scavenging effects. In particular, ginger essential oils shows anti-oxidant activities similar to the curcuma ones (Sacchetti et al., 2005).

Antimicrobial activity: ginger essential oil showed low activity against some yeasts. In particular this essential oil evidenced best grown inhibition vs. *Schizosaccharomyces pombe* on disk diffusion test (Sacchetti et al., 2005).

Fig. 10 Ginger essential oil producted by Foundación Chankuap (Ecuador)

4 YERBA LUISA

Botanical: *Cymbopogon citratus* (DC. ex Nees).

Family: *Poaceae* (grasses)

Part Used: Herb.

Common names: Yerba luisa, lemon grass, lemongrass, barbed wire grass, silky heads, citronella grass, cha de Dartigalongue, fever grass, Hierba Luisa or Gavati Chaha amongst many others.

Cymbopogon citratus Stapf

4.1 Description

Cymbopogon (lemongrass) is a genus of about 55 species of grasses, (among which the type species is *Cymbopogon citratus*) native to temperate Europe and tropical regions of Asia and Oceania. *Cymbopogon* is a tall, aromatic perennial grass that is native to tropical Asia. *C. citratus* is known as lemongrass in Guatemala, West Indian, and Madagascar. *C. citratus* is cultivated in the West Indies, Central and South America, and tropical regions. The linear leaves can grow up to 90 cm in height and 5 mm in width. Freshly cut and partially dried leaves are used in traditional medicine and as source of the essential oil.

4.2 Uses

Lemongrass is used as a fragrance, flavoring a wide variety of functional foods in folk medicine. However, clinical trials are lacking to support these uses. Some studies have demonstrated antifungal and insecticide efficacy, as well as potential anticarcinogenic activity, while suggested hypotensive and hypoglycemic actions have not been confirmed (Shadab et al.,1992).

Lemongrass is usually employed in infusions of fresh or dried leaves and it is one of the most widely used traditional plants in South American folk medicine. It is used as an antispasmodic, antiemetic, and analgesic, as well as for the treatment of nervous and gastrointestinal disorders and the treatment of fevers. In India it is commonly used as an antitussive, antirheumatic, and antiseptic. It is usually ingested as an infusion made by pouring boiling water on fresh or dried leaves. In Chinese medicine, lemongrass is used in the treatment of headaches, stomach ache, abdominal pain, and rheumatic pain. Lemongrass is an important part of Southeast Asian cuisine, especially as flavoring in Thai food. Other uses include lemongrass crude drug as an astringent, and a fragrance in beauty products (Girón et al., 1991).

4.3 Chemical components

Fresh *C. citratus* grass contains approximately 0.4% volatile oil. The oil contains 65% to 85% citral, a mixture of 2 geometric isomers, geraniol and neral. Related compounds geraniol, geranic acid, and nerolic acid have also been identified. Other compounds found in the oil include myrcene (12% to 25%), diterpenes, methylheptenone, citronellol, linalol, farnesol, other alcohols, aldehydes, linalool, terpineol, and more than a dozen of other minor fragrant components. Geographical variations in the chemical constituents have been noted as generally reported for chemical fingerprinting of plants (Menut et al.2000; Sacchetti et al., 2005).

Nonvolatile components of *C. citratus* consist of luteolins, homo-orientin, chlorogenic acid, caffeic acid, p-coumaric acid, fructose, sucrose, octacosanol (De Matouschek et al., 1991).

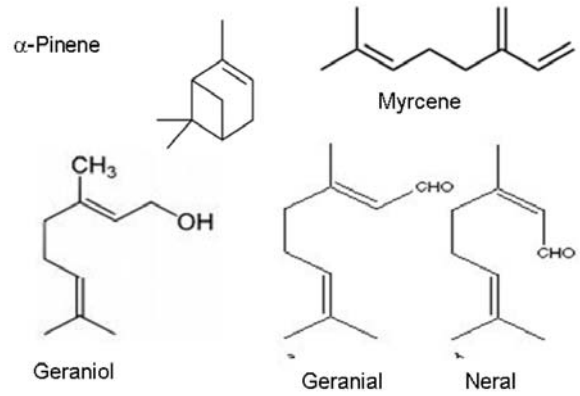


Fig. 11 Chemical structures of EO's principal components.

4.4 Potential health effects

Antimicrobial effects: Antifungal activity of lemongrass oil has been reported several times, mostly against phytopathogens and dermatophytes; its activity against food-spoilage yeasts was scarcely investigated. Geraniol and citral isomers should probably account for such efficacy (Abe et al., 2003). Several reports describe antimicrobial effects of lemongrass, including activity against both gram-positive and gram-negative bacterial pathogens, and fungi (Baratta et al., 1998). The effects are principally due to geraniol (α-citral) and neral (β-citral) (Onawunmi et al., 1984).

Anticarcinogenic and antioxidant effects: Antimutagenic properties of ethanol lemongrass extracts against certain *Salmonella typhymurium* strains have been demonstrated, by Ames test (Vinitketkumnuen et al., 1994) while in other studies, the ethanolic extract was shown to inhibit DNA adduct formation in rat colon but not liver cells (Suaeyun et al., 1997; Vinitketkumnuen et al., 1999). Toxicity and apoptosis-inducing action of the essential oil and the ethanolic extracts against mouse and human leukemia cells, respectively have been described (Kumar et al., 2008). *C. citratus* ethanolic extract exhibited an antioxidant action in mouse skin, leading researchers to suggest its potential use in skin cancer prevention (Nakamura et al., 2003). Yerba luisa essential oil has demonstrated antioxidant and radical-scavenging activity in several other experiments, evidencing its importance as possible healthy ingredient in cosmetics (Sacchetti et al., 2005; Cheel et al., 2005).

Cardiovascular activity: Dose-related hypotensive effects and weak diuretic actions have been demonstrated in rats treated with ethanolic lemongrass extract (Carbajal et al., 1989). *In vitro* ethanolic lemongrass extract reduced the cardiac rate without altering the contractile force (Gazola et al., 2004).

Insecticidal activity: The mosquito-repellent effect of lemongrass essential oil, diluted with different concentration in liquid paraffin, was evaluated in a study using *Aedes aegypti* adult mosquitoes as test insects; interesting applicative perspectives have been attributed to citral content (Oyedele et al., 2002).

5 AVOCADO

Scientific names: *Persea Americana* Mill., *Persea gratissima* Gaerth.

Family: *Lauraceae*.

Part Used: Fruits.

Common names: avocado, alligator pear, aguacate, palta, butter fruit.

5.1 Description

The genus *Persea*, is a member of the laurel family (*Lauraceae*); hence it is related to the cinnamon tree, camphor, and sassafras. *P. americana* Mill. (*P. gratissima* Gaertn.) is the common avocado which has been most widely disseminated throughout the tropics. Avocados vary in habit of growth, being sometimes short and spreading, but more commonly erect, even slender, varying in height from 10 mt to 20 mt. The leaf length varies from 1cm to 20 cm, blades are multiform: lanceolate, elliptic-lanceolate, elliptic, oblong-elliptic, oval, ovate, and obovate, with blunt or acuminate apex, while the base is usually acute or truncate. Flowers, pale green or yellowish green,

are produced in racemes near the ends of the branches, with both stamens and pistils. The fruit is exceedingly variable: the smallest fruits are no larger than plums, while the largest weigh more than 1 kg. The form is commonly pear-shaped, oval, or obo-voidal. Skins varies in color, texture and thickness: some could be yellow-green or almost yellow through many shades of green to crimson, maroon, brown, purple, and almost black; occasionally 5-6 mm thick, hard and woody in texture. The fleshy edible part has buttery consistency, yellow or greenish yellow coloured, of a peculiarly rich nutty flavour in the best varieties, and contains a high percentage of oil. The single large seed is oblate, spherical, conical, or slender. It is covered by two seed-coats, varying in thickness, often adhering closely to one another (Popenoe, 1920).

5.2 Uses

Avocado oil has valuable nutritional properties, include a high level of monounsaturated lipids known for lowering blood cholesterol levels. The oil is also used in cosmetic preparations and its unsaponifiable matter is exploited in various pharmaceutical applications for therapeutic, dermatological and medical uses (Mostert et al., 2007). Avocado usaponifiable fraction, in association with Soya bean unsaponifiables (ASU), has shown anti-osteoarthritic properties (Henrotin et al., 1998; Lesequene et al., 2002).

Avocado fruits and derived fixed oil are largely used as culinary ingredient in sauces and salads. Otherwise avocado oil is used for lubrification and in cosmetics where it is valued for its regenerative and moisturizing properties (Wong et al., 2010). There are no scientific evidences demonstrating healthy traditional application of avocado fruits or its derived products.

Other activities (Biorational pesticides): Pesticides vary in their toxicity and in their potential to cause undesirable ecological impacts. Pest control materials that are relatively non-toxic with few ecological side-effects are sometimes called *bio-rational* pesticides, although there is no official definition of this term. Some, but not all, biorationals are qualify for use on organic farms. Rodriguez-Saona et. al (1996) demonstrated that the avocado oil, extracted from idioblast cells, has insecticide activities against the generalist herbivore *Spodoptera exigua*. (Rodriguez-Saona et al., 1996).

Treatments of osteoarthritis: Avocado unsaponifiable fractions mixed with soyabean ones (ASU), known as Piascledine (Laboratoires Pharmascience, Courbevoie, France), has been used to treat connective tissue diseases (Henrotin et al., 1998), following studies of ASU have demonstrated some anti-osteoarthritic properties: *in vitro* ASU has inhibitory effect on interleukin-1 and stimulated collagene synthesis in articular controcites cultures; *in vivo* ASU significantly prevents the occurrence of lesions of the bruished cartilage in the postcontusive model of osteoarthritis in rabbit (Lequesne et al., 2002).

5.3 Chemical components

Extra virgin avocado oil has a characteristic flavour, high level of monounsaterated fatty acids, and high smoke point (≥250°C), making it a good oil for frying. Cold-pressed avocado oil is brillant emerald green when extracted; the colour is attribuited to high levels of chlorophylls and carotenoids extracted into the oil (Ozdemir et al., 2004).

From a nutritional point of view, avocado is an important and high caloric fruit. Indeed its high content of unsaturated fatty acids is one of its distinguishing characteristics. Moreover, avocado is known to be rich in vitamin E, ascorbic acid, vitamin B6, β-carotene, and potassium (Ozdemir et al., 2004). The minimum oil content necessary for marketing avocado fruit is 8%; after maturation, values greater than 20% can occur. In fact in the period between harvesting and full maturation, when commercial maturity is reached, the oil content increases and change occurs in the oil composition. Concentrations of unsaturated fatty acids increase and those of saturated fatty acids decrease (Ozdemir et al., 2004).

5.3.1 Fatty acids composition

The fatty acids profile is very similar to olive oil, in particular for its high content in oleic acid. A typical avocado oil has 76% monounsaturates (oleic and palmitoleic acids), 12% polyunsaturates (linoleic and linolenic acids), and 12% saturates (palmitic and stearic acids); these values are given as percentage of fatty acid/total fatty acids.

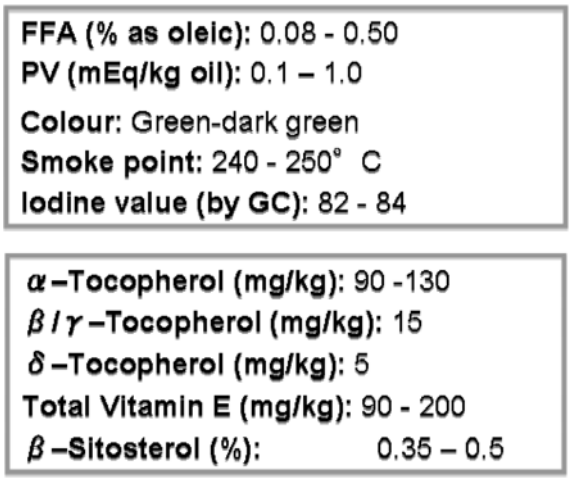


Fig. 12 General characteristic of cold pressed avocado oil (Wong et al., 2010).

The importance of unsaturated fatty acids in foods is well recognized. The classes of unsaturated fatty acids include MUFAs (monounsaturated fatty acids), e.g. oleic acid (18:1) and PUFAs (polyunsaturated fatty acids) of the ω-6 and ω-3 families. The major dietary ω-6 PUFAs include linoleic (18:2), γ-linolenic (18:3), and arachidonic (20:4) acids, whereas major ω-3 PUFAs include α-linolenic (18:3), eicosapentaenoic (20:5), doicosapentaenoic (22:5) and doicosahexaenoic (22:6) acids. Oleic acid is a MUFA (monounsaturated fatty acid) present as a major constituent in avocado oil. Recent researches indicate that MUFA reduces the levels of an oncogene called Her-2/neu (also known as erb-B-2) and is effective in controlling breast cancer cells. High levels of Her-2/neu occur in more than one-fifth of breast cancer patients and are associated with highly aggressive tumors that have a poor prognosis. Further, the presence of oleic acid boosts the effectiveness of trastuzumab (herceptin) and can help to prolong the lives of many such patients. Linoleic acid is also a C-18 polyunsaturated fatty acid with two double bonds. The consumption of long chain ω-3 fatty acids is decreasing the ratio of ω-6 to ω-3 and hence is effective in treating coronary heart diseases, type-2 diabetes, hypertension, immune response disorders and mental illness. The required increase in PUFA intake can be achieved by consumption of PUFA-rich supplements. However, a few problems are encountered in the production, transportation and storage of these fortified foods as PUFAs are extremely susceptible to oxidative deterioration (Logaraj et al., 2008).

5.3.2 Unsaponifiable fraction (UM)

The main fraction of avocado oil unsaponifiables, have been characterised (Farines et al., 1995, and references therein; Gutfinger et al., 1974; Kashman et al., 1969). Considerable quantitative and qualitative differences have been observed in the unsaponifiables of the oil, depending on whether it was extracted from fresh fruit or after drying. In fresh fruit, the unsaponifiables amount to 1-2%, compared with 3-7% in the dried fruit oil (Farines et al., 1995). Some authors have isolated a lipid fractions directly from fresh pulp mainly composed of triols or hydroxyketones, either free or in the form of monoacetates, based on saturated, unsaturated, or polyunsaturated linear aliphatic chains (Farines et al., 1995). Otherwise there is another homogeneous series of compounds with a long aliphatic mono or polyinsaturated chain fixed in alpha to a furyl nucleus (Farines et al., 1995). The chain length always varies from an odd carbon no. of 13 to 17 carbon atoms, always an odd number. This fraction is highly specific for the avocado oil unsaponifiables and can be linked with its pharmacological activities. Squalen (2%), long-chain saturated hydrocarbons (up to 5%), polyols (up to 15%), tocopherols (trace amounts) and sterols (between 4 and 20%), mainly as fltositosterols, are the other components of the avocado unsaponifiables. (Ding et al., 2007 and references cited).

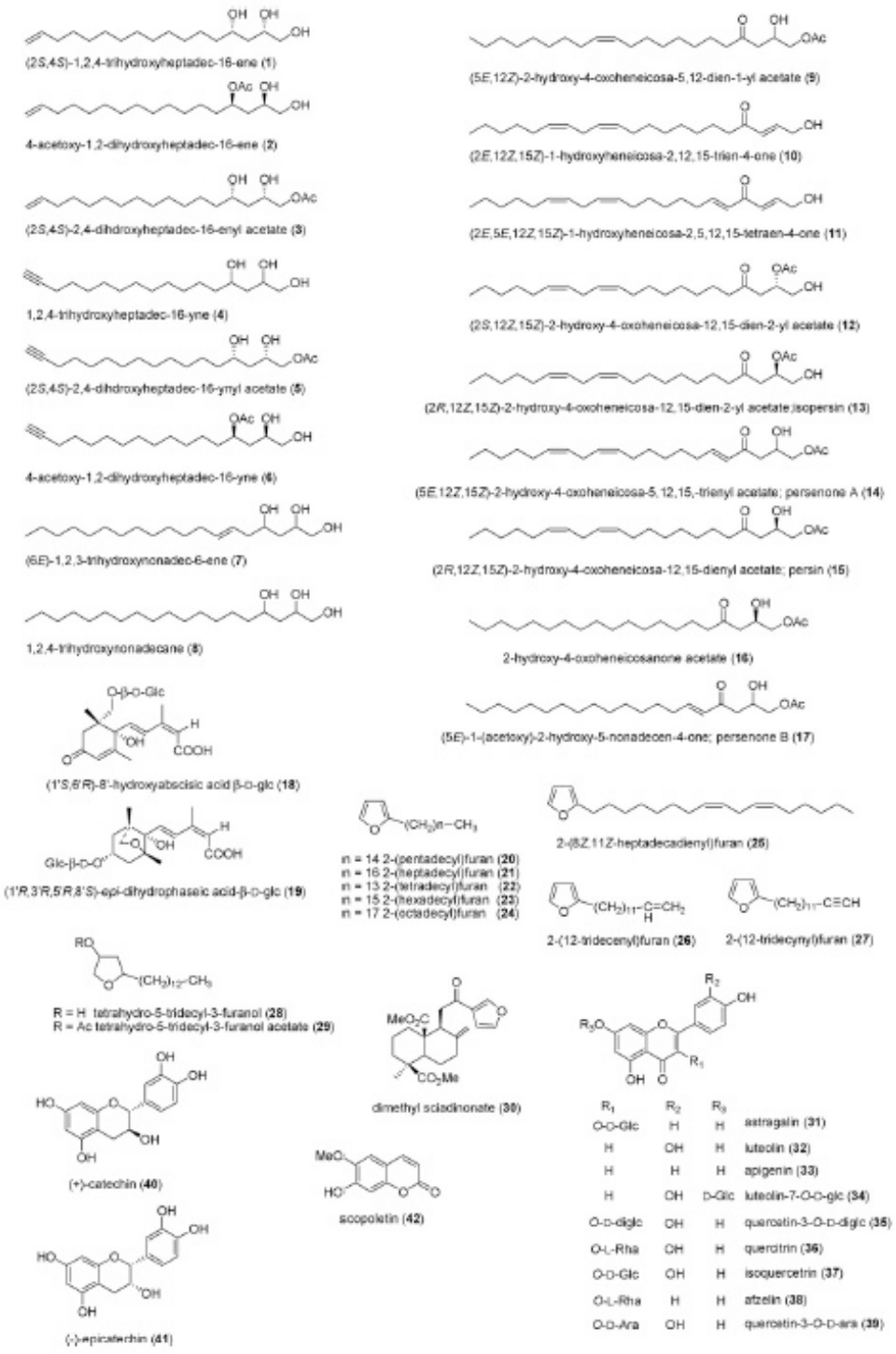
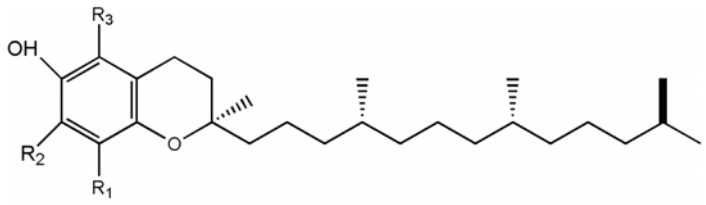


Fig. 13 Structures of secondary metabolite constituents of Avocado (Ding et al., 2007 and references cited).

5.3.3 Tocopherols in Avocado oil and pulp extracts

The main antioxidant in the oil is α-tocopherol, which is present at levels of 70-190 mg/kg oil. β-, γ- nad δ-tocopherols are only present in minor amounts (<10 mg/kg oil). Other nonlipid components present in the oil include chlorophylls (11-19 mg/kg oil) and carotenoids (1,0-3,5 mg/kg oil) (Wong et al., 2010).



Tocopherols	R_1	R_2	R_3
a -tocopherol	$-CH_3$	$-CH_3$	$-CH_3$
γ -tocopherol	$-CH_3$	$-CH_3$	-H
δ -tocopherol	$-CH_3$	-H	-H

Fig. 14 Chemical structures of tocopherol compounds (Hu et al., 2009).

5.3.4 Sterols content (SC)

Avocado oil is suitable for preventing the human body from accumulating the undesirable low-density lipoprotein (LDL) cholesterol and promotes healthy high-density lipoprotein (HDL) cholesterol accumulation, which is beneficial to the heart. Studies also prove that the presence of β-sitosterol in avocado oil helps in relieving the symptoms of prostate enlargement amongst men, besides lowering the cholesterol build-up (Logaraj et al., 2008).

5.3.5 Polyphenols contained in Avocado oils and pulp extracts

Polyphenols are a class of natural organic chemicals generally divided into hydrolyzable tannins (gallic acid esters of glucose and other sugars or cyclitols) and phenylpropanoids (cumarins, lignins, flavonoids, and condensed tannins). Polyphenols have antioxidant activity, in particular they have the ability to scavenge free radicals, and react with oxygen species (i.e. hydrogen peroxide). Consuming dietary polyphenols may be associated

with beneficial effects in higher animal species such as reduction in inflammatory effects, coronary disease, including specific medical results into the pathways of improved endothelial health via downregulation of oxidative LDL. Other beneficial health effects, but are not yet proved scientifically in humans, are anti-aging consequences such as slowing the process of skin wrinkling. For some of the side-benefits (such as prevention of peripheral artery disease), further research is continuing to clarify the role of polyphenol antioxidants may have (Ferrazzano et al., 2011). There is not much knowledge about the total phenolic content and antioxidant capacities among avocados from different strains and cultivars. Avocado seeds and peels of different cultivars demonstrated to contain high levels of procyanidins as major phenolic compounds and antioxidants. Procyanidins in avocados were predominantly B-type with A-type as minor components (Wang et al., 2010).

5.4 Potential health effects

Based on its fatty acid characterization and the presence of phytochemicals, such as tocopherols and phytosterols, extra virgin cold-pressed avocado oil is considered to be an healthfully oil.

Anti-carcinogenic activity: Studies on avocados showed that they contained potentially anti-carcinogenic lipophilic components such as carotenoids (Ding et al., 2007). The lipophilic extract of avocado inhibited prostate cancer cell growth (Lu et al., 2005), induced apoptosis in human breast cancer cells (Butt et al., 2006), and suppressed liver injury (Kawagishi et al., 2001).

MATERIAL AND METHODS

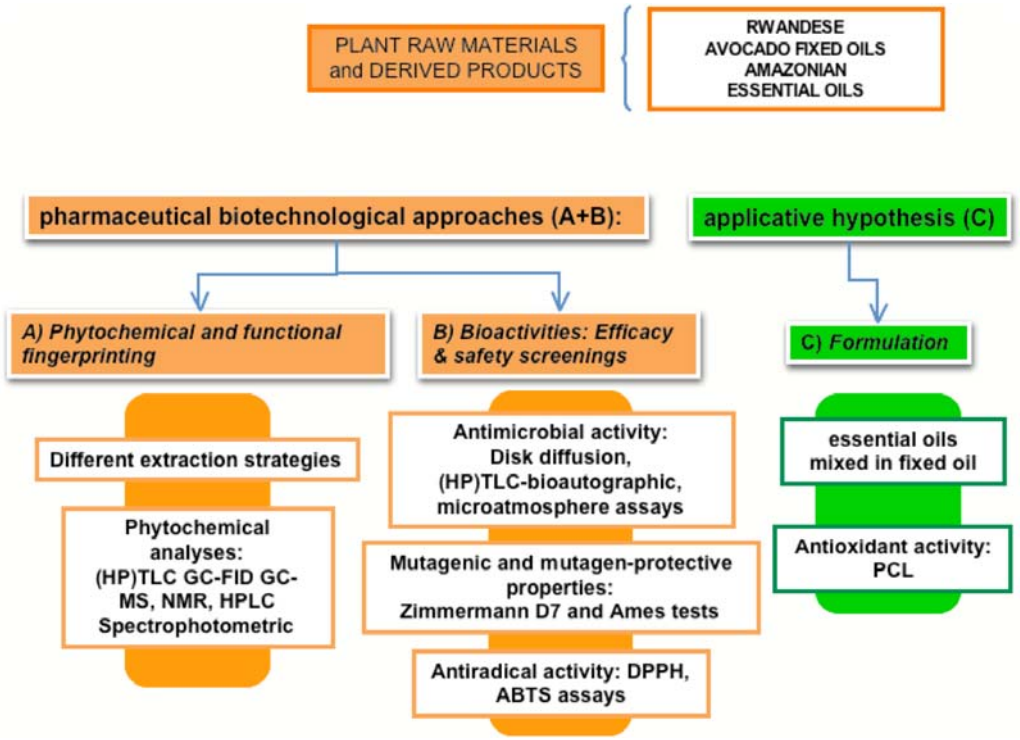


Fig. 15 Research plan scheme.

A) Phytochemical and functional fingerprinting

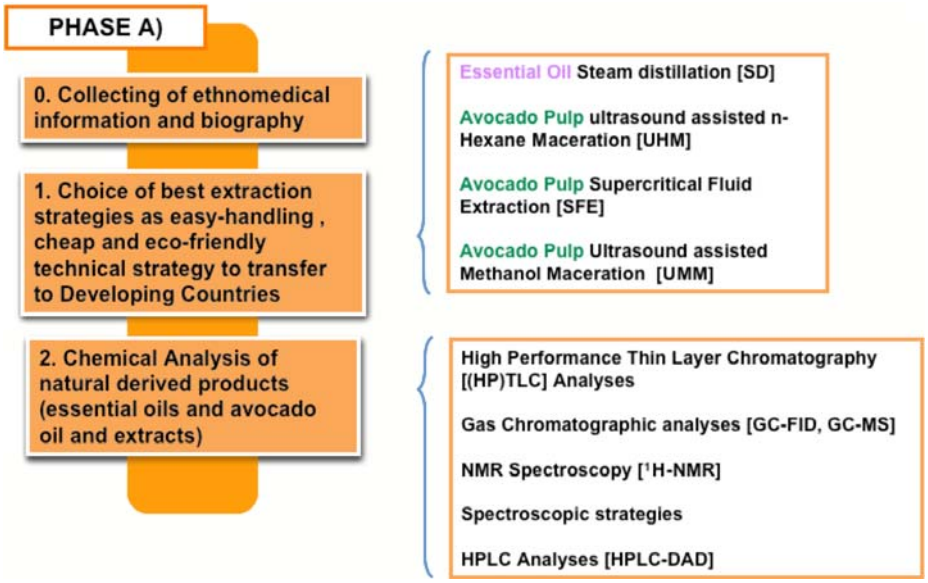


Fig. 16 Research phase A diagram

A) 1 Extraction strategies

A) 1.1 Essential oils

Fig. 17 processing of Amazonian fresh plants

Essential oils derived from Ecuadorian Amazonian locally cultivated plants; extracts were obtained via steam distillation as pure Essential Oils (EO) from: *Curcuma longa*, *Cymbopogon citratus*, *Zingiber officinale*, *Ocimum micranthum*. *Thymus vulgaris* EO, thymol chemotype, employed as reference, was purchased from Extrasynthese (Genay, France); only in last phase of this PhD project has been employed, as positive control, commercial *Melaleuca alternifolia* essential oil, chemotye 4-terpineol, purchased from Jason (Australia). Fresh plant parts, harvested at balsamic time (adult plants), have been subjected to steam distillation in Foundación Chankuap, a local organization linked to *Centre for the International*

Development Cooperation of the University of Ferrara, committed on biodiversity valorisation and market exploitation of local sources. Essential oil was isolated by a 3 h steam distillation of 7 kg of fresh plants in a stainless steel distiller equipped with a commercial Clevenger apparatus, issued in Fundaciòn Chankuap (Macas, Ecuador). Essential oil yield was determined on a volume to dry weight basis. The values for essential oil yield of three distinct distillations corresponding to the three different samplings were averaged. The essential oil samples were stored in glass vials with teflon-sealed caps at -18,0 ± 0,5 °C in the absence of light.

A) 1.2 Avocado Fruits

Avocado fruits (*Persea americana* Mill.) were purchased in Kigali (Rwanda), by a seller of organic products from neighbouring cultivations in order to minimize variable affecting the quality of the fruits and the research to be performed; the plant horticultural used was subjected to the standard processing chain of Avocado. Because of scarce scientific researches concerning pulp derived products and of the possible market exploitation of the pulp fixed oil and other extracts, the only avocado pulp has been considered.

Avocado fruits were stored at 4°C in a fridge for 4 days, until ripeness. Each fruit was peeled, seed removed and flesh pooled. The pulp was immediately freeze dried and kept frozen at -20 °C. The commercial cold pressed Avocado oil (ACO), employed as reference, was purchased from Pietro Brisighello fu E. srl (Padova, Italy); and it was a moisture from different Countries.

A) 1.2.1 Extraction procedures: Avocado pulp.

Avocado's pulp was processed with several strategies in extracts characterized under chemical and biological point of view.

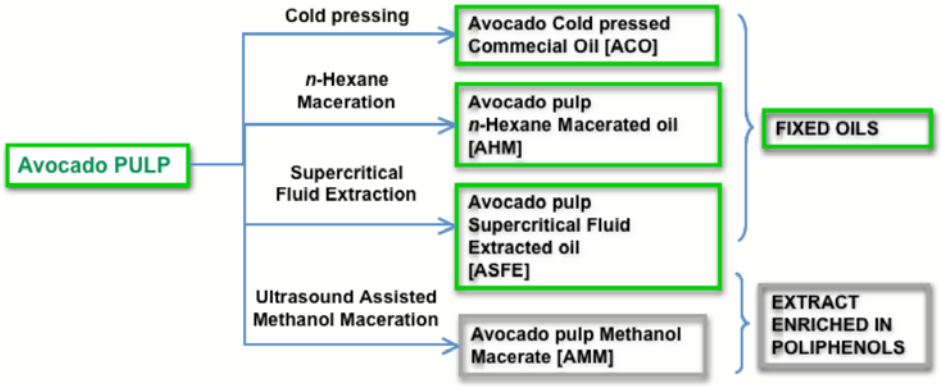


Fig. 18 Avocado pulp extracts processing

Avocado is highly caloric fruit and its high content of unsaturated fatty acids is one of its different distinguishing characteristics. Moreover, avocado is rich in vitamin E, ascorbic acid, vitamin B6, β-carotene, and potassium (Ozdemir et al., 2004). The minimum oil content necessary for marketing cultivated avocado fruit is 8% (w/w). After maturation, values greater than 20% can occur. These values occur in the period after harvesting, when commercial maturity is reached, the oil content increases and change occurring in oil composition. In particular, in harvesting period concentrations of unsaturated fatty acids increase and those of saturated fatty acids decrease (Ozdemir et al., 2004). The fatty acid composition and usaponifiable fraction have been evaluated in extracts obtained by supercritical CO_2 extraction (now called ASFE) and by n-hexane maceration in ultrasound assisted condition (AHM). An enriched fraction of each Avocado's derivates, obtained by defatting process (Lee et al., 2008), has been performed to evaluate the total polyphenols content and the linked antioxidant activities, with the aim to determine possible functional components making avocado pulp a Rwandese plant source useful for new healthy markets.

A) 1.2.2 Freeze-drying of pulp

Avocado pulp contains about 80% water and 20% pulp (w/w). Pulp have been homogenized using an Omni Mixer Homogenizer (Omni International Inc.), and then freeze-dried in a Edwards equipped with vacuum pump XDS10. The drying was conducted at 10^{-1} mbar of vacuum with a drying temperature of -60°C. The total time taken for drying the material was 36h. Freeze-dried material, in appearance a powder, has been then stored at -18,0 ± 0,5°C until extractions.

A) 1.2.3 Hexane Ultrasound Assisted Maceration

Extracts has been obtained with hexane ultrasound assisted maceration, as suggested in ISO/DIS 5509.1998 method. Extracts have been obtained by placing 1 g of *Persea Americana* dried pulp in 10 ml of hexane for 40 min with sonication in Ultrasonik 104x (Incofar) apparatus. Then, the samples have been filtered and centrifuged for 20 min at 3000 rpm. The supernatant have been recovered, dried with rotavapor, and stored at -20°C until GC analysis. Each extraction was performed in triplicate. For qualitative control of the main chemicals occurring in the extracts, samples were deposited as single spot on a (HP)TLC plate (Merck) and eluted with a mixture of toluene/dietthylether/acetic acid (70:30:1). Standard of β-sitosterol (Sigma-Aldrich®) were used to check the presence of components. Components were identified by spraying the plates with a 50% (w/w) aqueous sulphuric acid solution, then heating at 100°C for 10 min (Werman et al., 1996; Oberlies et al., 1998).

A) 1.2.4 Supercritical fluids extraction

Avocado oil is known to contains oleic acid (54–76%) and linoleic acid (11–15.6%) as major components. The supercritical CO_2 extraction (SFE) of this oil provides the maximum yield of unsaturated fatty acids leaving pure extracts without any traces of solvents (Logaraj et al. 2008). Supercritical carbon dioxide extraction of avocado's freeze-dried puree was performed following the method suggested by Logaraj, 2008. The extraction was carried out by using a supercritical CO_2 extractor (Model Fedegari Autoclavi Spa, Italy). The oil was extracted by using 100g of avocado dried pulp at °T

50°C, and °P 300-350 bars. The extraction was completed over a period of 1 h and about 55% of oil has been obtained from the sample (dry basis). For qualitative control the same TLC method described above for hexane maceration has been adopted (Werman et al., 1996).

A) 1.2.5 Ultrasound assisted methanolic maceration

A method inspired by Wagner and Bladt on “Plant Drug Analysis” (Wagner, Bladt, 2009), has been adopted for extracts to be analyzed for total polyphenols content. This extraction strategy is confirmed also by Marìa Garcia-Alonso (Garcia-Alonso et al., 2004) which investigated antioxidant activities of several fruits included avocado. Avocado dried pulp was extracted with solvents for three times. Avocado dried pulp (200 g) was placed in 1000 ml of methanol and subjected to ultrasound treatment for 30 min (UltraSonik 104x, Ney dental International) in the dark at a constant temperature of 25 °C. The sonication was performed with the aim to obtain a better interaction between matrices and solvent. The extracts obtained were then centrifuged (7000 rpm for 20 min) employing an Heraeus LaboFuge GL centrifuge to completely remove the exhausted flour. The supernatants were recovered, dried in a rotavapor, collected and weighed. For the qualitative control was used the same TLC strategy applied on hexane extractions (Werman et al., 1996).

A) 1.2.6 Preparation of hexane/80% ethanolic extracts from the 4 Avocado derivates

Preparation with the hexane/80% ethanol from each avocado oil sample has been carried out according to the method described by Lee (Lee et al., 2008 and references cited), with minor modification. Each avocado extract (15 g) has been dissolved in 50 ml of hexane and the solutions as been extracted with 60 ml of 80% aqueous ethanol. The extracted samples has been concentrated in a vacuum evaporator at 40°C and completely dried using a freeze drier. The hexane80% aqueous extracts have been weighted and used for antioxidant activity assays and total polyhenols determination.

A) 2 Phytochemical and functional fingerprintig

The research has been performed as following:

- Phytochemical characterization: 1H-NMR, GC-FID, GC-MS, HPTLC, HPLC-DAD.
- Bioactivity assays to shed a light on efficacy and safety of phytocomplexes, fractions and pure compounds.

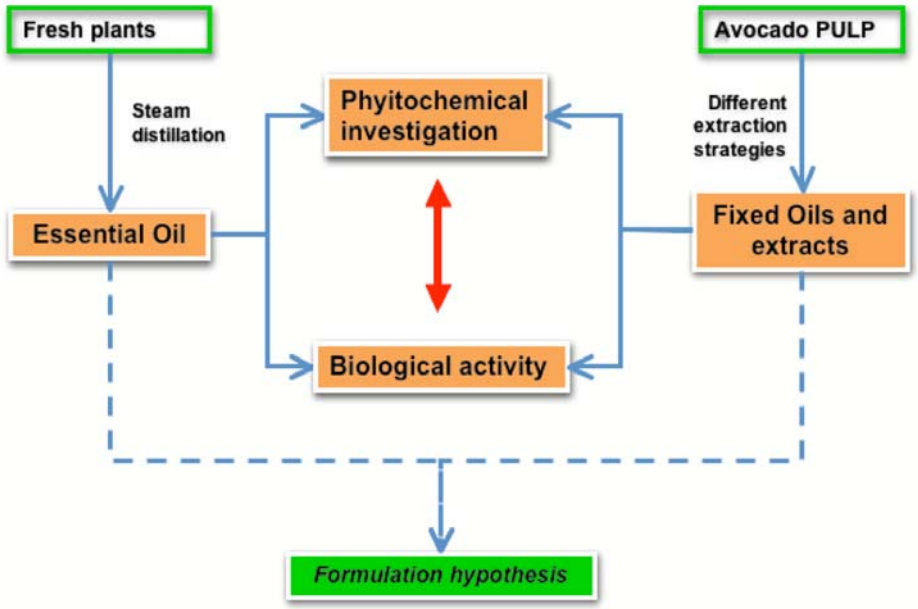


Fig. 19 Scheme from plants to formulations

A) 2.1 High Performance Thin Layer Chromatography (HP)TLC Analysis

As preliminary chemical check of all the Avocado oils and essential oils samples, High Performance Thin Layer Chromatography [(HP)TLC] analysis has been performed. In particular, (HP)TLC has been employed to determine:

In essential oils:

- a qualitative controls of the main terpene compounds;

and in Avocado samples:

- a qualitative control of lipid composition and polyphenols:

(HP)TLC analysis has been then employed as important tool for linking chemical evidences to bioactivity properties through (HP)TLC-bioautographic assays. See operative conditions and bioactivity at B) 2.2 and B) 4 paragraphs.

In fact, High Performance Thin Layer Chromatography (HP)TLC, as an enhanced form of Thin Layer Chromatograph (TLC), is a simple and low-cost technique often used to separate and identify phytocomplexes, their fractions, or pure compounds. Moreover TLC analysis can be applied to compare different phytocomplexes as fast screening qualitative chemical control. Substances can be identified from their so called Rf values. HPTLC plates are characterized by smaller particles (≤10 µm), thinner layers (≤150 µm) and smaller plates (≤10 cm developing distance). In addition, the particle size distribution is narrower than for conventional TLC layers, giving the following general advantages:

A) More resolving power
B) Faster development times
C) Reduced solvent consumption

[Sigma-Aldrich http://www.sigmaaldrich.com]

(HP)TLC plates silica gel 60 F_{254} (Merck) have been exclusively employed.

A) 2.1.1 (HP)TLC for checking the occurring of the main terpenes in Essential Oils

Essential oils composition were checked by (HP)TLC to detecting the main terpene compounds. The method suggested by Wagner, with minor modification, has been adopted (Wagner, Bladt, 2009): on (90x10 mm) TLC plates 10 µl on 6 cm band of each sample have been deposited (i.e. Ginger EO, Basil EO, Yerba luisa EO, Curcuma EO and Thyme EO, as control), and eluted by a toluene/acetic acid/petroleum ether (93:7:20) solution. Detection was performed at first without chemical treatment observing at UV-254, and then monitored at visible light after spraying treatment with a vanillic-sulphuric acid reagent (VS) prepared mixing 50 : 50 1% ethanolic vanillin solution (solution I) with 10% ethanolic sulphuric acid (solution II), and heated at 110 °C (10 min).

A) 2.1.2 (HP)TLC for qualitative controls of sitosterols occurring in the avocado pulp extracts

For the qualitative control of sitosterols occurring in both oil and extract, samples were deposited as single spot with a microsyringe (Hamilton) on a (HP)TLC plate and then eluted with a toluene/diethylether/acetic acid (70:30:1) solution. Standard of β-sitosterol (Sigma-Aldrich®) was used as chemical standard. For detection, the plates have been sprayed with a 50% (w/w) aqueous sulphuric acid solution, and kept at 100°C (10 min) (Werman et al., 1996).

A) 2.1.3 (HP)TLC for qualitative controls of fatty acids contained in Avocado Oils extracts

The Avocado samples have been processed following the method suggested by Platt and Thompson (1992) with minor modifications (Platt, Thompson, 1992). Thin layer chromatography was performed on silica plates (Merck) and developed using a toluene/diethyl ether/acetic acid solution (80:20:1 v/v/v). Palmitic acid free fatty acid was used as reference standard. After brief air-drying, the plate was kept in a closed chamber with crystal iodine. With reference to literature data and to the Rf of the standard palmitic acid, triacylglicerides (TG), diacyglycerides (DG), and monoacylglycerides (MG) have been detected (Chinnasamy et al., 2003; Yoshida et al., 1995).

A) 2.2 Gas Chromatographic analysis

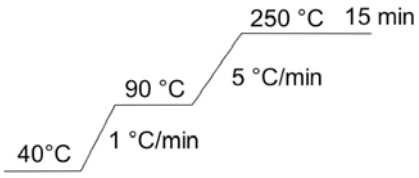
GC analysis have been performed with a Varian GC-3800 gas chromatograph equipped with a Varian FactorFour™ VF-5ms (5% phenyl, 95%dimethylpolsyloxane, 0,25mm x 30mt, 0,10μm), coupled with an MS-4000 mass spectrometer using electron impact (EI) and hooked to NIST library, diversifying the method, step by step, depending on the class of phytochemicals investigated.

D) essential oils: analysis of essential oil constituents (EO)
E) essential oils: headspace analysis (HS)
F) avocado extracts: Total Fatty Acids (TFA)
G) avocado extracts: Unsaponifiable Fraction (UF)

A) 2.2.1 Analysis of Essential Oil constituents (EO): GC-FID, GC-MS

The constituents of the volatile oils have been identified by comparing their GC Retention Indices (RI) and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds, and by matching the MS fragmentation patterns and retention indices with mass-spectra libraries and with those in the literature (Adams, 2007). To determine the KI value of the components, a commercial 24 aliphatic hydrocarbons mixture (Sigma Aldrich) was injected into the GC/MS equipment and analyzed under the same conditions as following reported.

GC-FID (gas chromatography) conditions: injector temperature, 280 °C; FID temperature, 280 °C; carrier gas (Helium), flow rate 1 ml/min and split injection with split ratio 1:40. Oven temperature was initially 45 °C and then raised to 100 °C at a rate of 1 °C/min, then raised to 250 °C at a rate of 5 °C/min and finally held at that temperature for 10 min. 1μl of each sample, dissolved in CH_2Cl_2 (1:100 v/v), was injected. The percentage composition of the EO was computered by the normalization method from the GC peak areas, calculated by means of three injections of each EO, without using correction factors.



MS (mass spectrometry) conditions: ionization voltage, 70 eV; emission current, 40 mA; scan rate, 1 scan/s; mass range, 35–300 Da; ion source temperature, 200 °C. The MS fragmentation pattern was checked with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns with NIST NBS75K mass spectra libraries and with those in the literature (Adams, 2007). The relative amounts of the individual components were obtained from GC analysis, based on peak areas without FID factor correction. The constituents of the volatile oils were also identified by comparing their GC retention indices. A mixture of aliphatic hydrocarbons (C8–C24) in hexane (Sigma–Aldrich, St. Louis, USA) was injected under the above-mentioned temperature programme to calculate the retention indices (RI), using the generalized equation of Van den Dool and Kratz (1963). The results of analysis and GC-MS spectra are shown in tab. 4 in Results and Discussion paragraph.

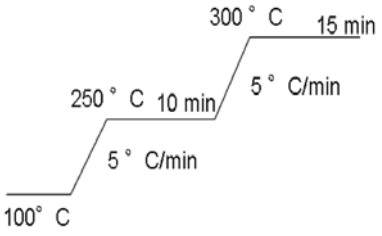
A) 2.2.2 Headspace analysis (HS)-GC-MS of Essential Oils

The chemical composition of volatile fraction of essential oils (HS) have been determined by static headspace analysis in GC-MS under the same conditions above mentioned for the analysis of the whole liquid essential oil. 500 µl of each sample were placed in a 8 ml vial sealed with a crimp top and kept at 37,0 ± 1.0 °C for 1 h. The vapour phase was drawn off with a gas tight syringe and inject into the gas chromatograph. The results and GC-MS spectra are shown in tab. 6 in Results and Discussion paragraph.

A) 2.2.3 Avocado Oil and its total fatty acids composition: GC-FID, GC-MS analysis

The Total Fatty Acid composition (TFA) has been evaluated in extracts obtained through supercritical CO_2 extraction, and extracts obtained by hexane ultrasound assisted maceration. The TFA has been also evaluated in the commercial sample of Avocado oil (i.e. Avocado Oil cold pressed). Each analysis was performed in triplicate. All results are summarized in tab. 7. TFA fractions were investigated as methyl esters of the fatty acid (method ISO/DIS 5509, 1998). The methyl esters were prepared by transmethylation using sodium methoxide in the presence of methyl acetate following the method laid out by Bruni et al. (2001). The constituents were identified by comparing their GC retention times, and MS fragmentation patterns with pure compounds and by matching the MS fragmentation patterns and retention indices with the above mentioned mass spectra libraries and with those in databases.

GC-FID (gas chromatography) conditions: injector temperature, 300 °C; FID temperature, 350 °C; carrier (helium) flow rate, 1,2 ml/min; and split ratio, 1:40. Oven temperature was initially 100 °C and then raised to 250 °C at a rate of 5 °C/min followed by 10 min at 250 °C, at the end raised to 300°C at a rate of 5 °C/min, followed by 10 min at 300 °C. 1 µL of each sample was injected.



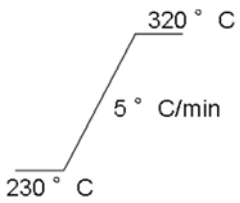
MS (mass spectrometry) conditions: ionization voltage, 70 eV; emission current, 10 µAmp; scan rate, 1 scan/sec; mass range, 29-500 Da; trap

temperature, 150°C, transfer line temperature, 300°C. A mixture of Fatty Acid Standards (C8-C24) in hexane (Sigma-Aldrich, St. Louis, USA) was injected following the above cited scale up program and retention indices computed using the generalized equation by Van den Dool and Kratz (1963).

A) 2.2.4 Analysis for Unsaponifiable Fraction (UF): GC-FID, GC-MS

The UF has been obtained from avocado oils, both Rwandese and commercial samples, following the official method specified in Bruni et al (2001) and REG CEE 2568/91. The yields of UF were 3,32/100 g, 2,14/100 g for Rwandese avocado oil, ASFE and AHM relatively, and 8,52/100 g for the commercial one. The unsaponifiable fraction was silanized at room temperature, as suggested by Bruni et al. (2001), with 2 ml of a silanizing mixture containing pyridine/hexamethyldisilazane/trimethylchlorosilane (5:2:1). After 1 h, the solution was evaporated under a nitrogen flow in a heat bath at 80 °C and then extracted with 0.3 ml of hexane. The conical test tube was placed in ultrasounds bath for 2 min and centrifuged; the supernatant was then withdrawn for injection into GC-FID and GC-MS apparatus.

GC (gas chromatography) conditions for GC-FID and GC-MS: One microliter of the solution was injected into the same apparatus used for TFA analysis under the following conditions: oven temperature was initially 230 °C, the raised to 320 °C at 5 °C/min; carrier gas (He), flux 1,2ml/min, injector temperature 300 °C, and detector temperature 350 °C.



MS (mass spectrometry) conditions: The mass spectra were recorded between 40 and 600 amu at an electron energy of 70 eV; the ion source temperature: 300 °C. Qualitative analysis was based on comparison of retention times and mass spectra with corresponding data in literature (Van Hoed et al., 2006; Damirchi et al., 2005).

A) 2.3 NMR Spectroscopy:

To define the phytochemical and functional fingerprint, ^{1}H-NMR spectroscopic analysis have been employed: in particular, these data confirmed the presence of the main compounds detected by GC-MS (table 4 on results and discussion paragraph), through the chemical shifts were assigned as compiled in table 11. The ^{1}H-NMR spectra were recorded on a Varian Gemini-400 spectrometer operating at 399.97 MHz and at a temperature of 303 K. All of the extracts or reference compounds (25 mg/1.0 ml) were dissolved in proper deuterated solvents ($CDCl_3$) into a 5 mm NMR tube, and the solvent signal was used for spectral calibration (^{1}H 7.26 ppm). ^{1}H spectra were run using a standard pulse sequence “s2pul”, with 45.0 degrees pulse, 3.00 s acquisition time, 8 repetitions, 4000 Hz spectral width, and 0.33 Hz FID resolution. Characteristic resonances of ^{1}H-NMR spectra for standards used were detected according to literature data, and by comparison with chemical shifts observed for single constituent solutions. ^{1}H-NMR signals and their correlations were detected according to literature data.

A) 2.4 Polyphenols in Avocado oil samples through (HP)TLC and spectroscopic strategies

The following samples have been examined:

- Avocado cold pressed commercial oil (ACO)
- Avocado hexane extracts (AHM)
- Avocado supercritical fluid extract (ASFE)
- Avocado methanolic extract (AMM)

All the samples (i.e. AHE, ASFE, AME, ACO) were processed as following for analysis according to Lee (2008), 15 g of avocado samples were dissolved in 50 ml hexane and the solutions were then extracted three times with 20 ml of 80% aqueous ethanol in a separation funnel. The combined extracts were brought to dryness in a vacuum rotary evaporator at 40° C. The residue was dissolved in methanol to obtain the concentration of 10 mg/ml and stored at -20 C until analysis.

A) 2.4.1 (HP)TLC analysis

For polyphenols detection by (HP)TLC a method suggested by Wagner (2009) has been adopted. In particular an aliquot (10 μL) of each hexane/80% ethanol aqueous extract (i.e. EIA.AHM, EIA.ASFE, EIA.ACO, EIA.AMM), dissolved in methanol [60 mg/ml], have been spotted an (HP)TLC silica plate (Merck) and eluted with toluene/ethyl acetate/acetic acid (100:90:10) solution in a chromatographic chamber. To detect flavonoids and coumarines, have been spotted also two standard: the flavonol kaempferol and the coumarin scopoletin (10 μL each one of a 0,1 mg/ml solution); plates were first observed at UV light (365nm), and then sprayed with Natural Products reagent (NP/PEG) (Wagner, Bladt, 2009). Instead, procyanidins were detected with the same elution system suggested by Wagner (Wagner, Bladt, 2009) appropriate for procyanidins detection: ethyl acetate/acetic acid/water (100:20:30/upper phase) solution and spraying VS reagent (see OE TLC method) in place of NP/PEG reagent.

A) 2.4.2 Spectrophotometric assays applied to determine total polyphenols in avocado oils and extracts

Polyphenols, flavonoids and procyanidins were also monitored spectrophotometically following the approaches described below.

An aliquot of the above mentioned EIA. extracts was dissolved in DMSO (10 mg/ml) and used for polyphenols determination.

Folin–Ciocalteau method for total **polyphenols fraction** has adopted as reported with minor proper modifications in Bruni et al. (2006). The total polyphenols content in the avocado hydroalcolic enriched fractions, obtained as above mentioned, was determine as suggested in Bruni et al. (2006). Each extract (0,1 ml) has been diluted with water (7,9 ml), and added to a 0,5 ml Folin-Ciocalteu reagent. After 2 min, 1,5 ml of saturated Na_2CO_3 solution (ca. 20%) was added. The solutions were gently shaken at room temperature in the dark. The reaction was measured after 2 hr at 765 nm, with reference to a control. Gallic acid, at different concentrations ranging from 0 to 5,0 μg/ml, was used as standard for performing the calibration curve assay solution. Results of total phenolic for avocado extracts were

expressed as milligram gallic acid equivalents (GAE) per gram of samples, i.e. oils and methanol macerate (mg GAE/g).

The method suggested by Lamaison (1991) to determine the **flavonoids content** (Lamaison & Carnat, 1991) has been adopted. The total flavonoids in the extracts were checked in accordance to Lamaison (1991): 1 mg of each dried extract has been dissolved in methanol [1 ml] and added to 1 ml of AlCl3•6H2O solution (2%). The solutions were gently shaken at room temperature, in the dark and analyzed. The reaction was measured after 10 min at 394 nm, in comparison with a control. The calibration curve was prepared with a solution of hyperoside (ranging 0 - 60 μg/ml). Results of total phenolic for avocado extracts were expressed as milligram hyperoside equivalents (HE) per gram of samples, i.e. oils and methanol macerate (mg HE/g).

The **total procyanidins** in the extracts have been checked as reported by Porter (1986) with minor modifications: 1 mg of each dried extract has been dissolved in methanol [1 ml] and added to 6 ml of *n*-butanol/chloridric acid solution (95:5), and 0,2 ml of 2% $NH_4Fe(SO_4)_2$•12 H_2O in HCl 2M solution. The solutions were gently shaken at 95° C for 40 min in the dark, then analysed. The reaction was measured, at room temperature, at 550 nm in comparison to blank. Cyanidin chloride solutions (ranging 0 - 60 μg/ml) were used to perform a calibration curve. Results of total phenolic for avocado extracts were expressed as milligram cyaniding chloride equivalents (CClE) per gram of samples, i.e. oils and methanol macerate (mg CClE/g).

A) 2.5 HPLC Analysis of tocopherols

HPLC strategy has been adopted to check the presence of **tocopherols** in Avocado oils (i.e. AHE, ASFE, ACO) and methanolic extracts (AME).

HPLC analysis were performed using a modular Jasco HPLC unit (Tokyo, Japan) which consisted of a PU-2089 pump, an LG-1580-02 ternary gradient unit, a DG-980-503-line degasser; UV detector, sample loop (20μl), columns and mobile phase were settled as requested for the specific analysis. All solvents used were of chromatographic grade (Carlo Erba). Chromatograms were recorded, and the peak areas were determined by integration using dedicated Borwin software (Borwin ver. 1.22, JMBS Developments,

Grenoble, France). For each extract, quali-quantitative analysis was performed in triplicate.

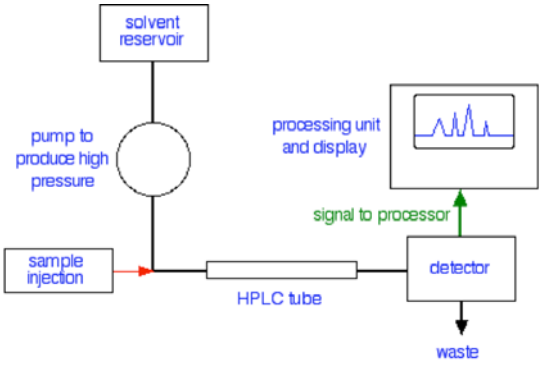
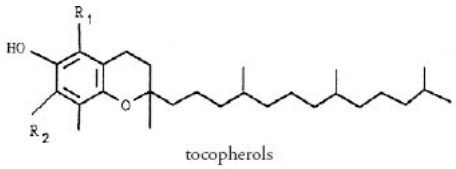


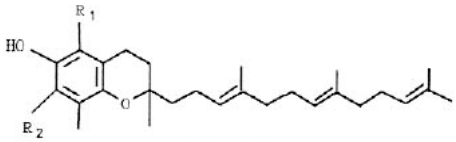
Fig. 20 HPLC apparatus scheme.

Tocopherols content in avocado oils and extract was detected by HPLC as reported by Lozano et al. (1993) and Hu et al. (2009) with minor modification: a 4,6 X 250 mm Lichrosorb Si-60, 5 μm column (Merck) was used. The elution was performed with hexane/isopropanol (0,5%) (Carlo Erba), flow rate: 1 ml/min, room temperature. Tocopherols standards were dissolved in hexane [0,1 mg/ml] and stored at -18°C until injection. Calibration curve was made with α-tocopherol standard solutions. Samples had been previous dissolved in hexane [50μl/ml] and injected immediately in a 20 μl loop; the injection volume was almost 40 μl and the chromatograms were monitored at 295 nm.



tocopherols

R_1	R_2	
CH_3	CH_3	a-tocopherol (a- T)
CH_3	H	b-tocopherol (b- T)
H	CH_3	g-tocopherol (g- T)
H	H	d-tocopherol (d- T)



tocotrienols

R_1	R_2	
CH_3	CH_3	a-tocotrienol (a- T_3)
CH_3	H	b-tocotrienol (b- T_3)
H	CH_3	g-tocotrienol (g- T_3)
H	H	d-tocotrienol (d- T_3)

Fig. 21 Vitamin E isomers: tocopherols and tocotrienols formula. Vitamin E activity is exhibited naturally by eight substances structurally based on tocopherols and tocotrienols. Each vitamer has a different vitamin activity compared with α-tocopherol, which is seen as the primary structure. The preferred analytical method is therefore one that separates and measures all the different vitamins (http://www.fao.org).

B) Bioactivities: Efficacy & safety screenengs

Bioactivities of all the phytocomplexes have been performed following the diagram below (fig 5).

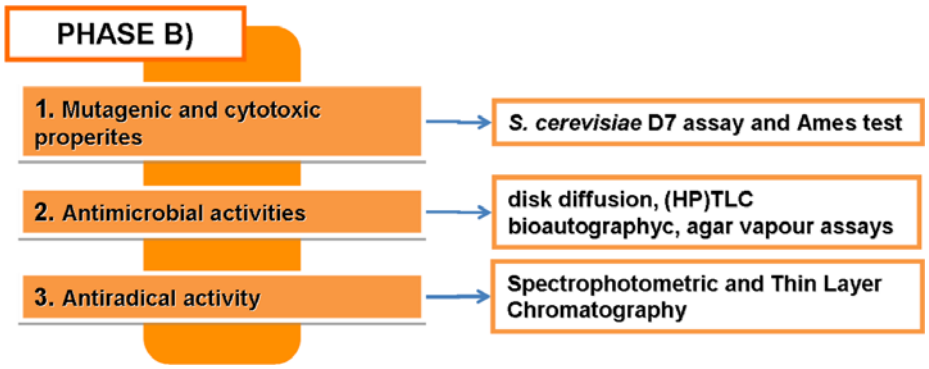


Fig. 22 research phase B diagram

Essential oils has been checked for functional properties evaluating mutagenic and cytotoxic properties with **1.1)** *Saccharomyces cerevisiae* D7 strain (Zimmermann, 1975), and **1.2)** Ames test (Guerrini et al., 2011);

Antibacterial activities, performed through three different approaches: **2.1)** disk diffusion assay (Guerrini et al., 2006; Guerrini et al., 2011), **2.2)** TLC-bioautographyc assay (Rossi et al., 2011; Guerrini et al., 2011) and **2.3)** microatmosphere assay (Maietti et al., in press), has been checked against four Gram +, four Gram – and two Yeasts strain. *In vitro* antioxidant capacity has been cheked through **3.1)** (DPPH•) and **3.2)** (ABTS) assays processed as classical spectrophotometric methods and using TLC bioautographyc approach (Rossi et al., 2011; Guerrini et al., 2011). All the bioactivities were performed comparing all the data with those achieved with appropriate pure synthetic compounds and/or commercial *Thymus vulgaris* and *Melaleuca alternifolia* essential oil, in order to have positive control references with single compounds or comparable phytocomplexes reputed for their functional bioactivities. The use of a phytocomplex known for its chemical and biological properties (for e.g. thyme essential oil) as positive reference results particularly indicative of the real functional efficacy of a tested extract (Sacchetti et al., 2005; Guerrini et al., 2006; Guerrini et al., 2009). Data reported for each assay are the average of three determinations of three independent experiments.

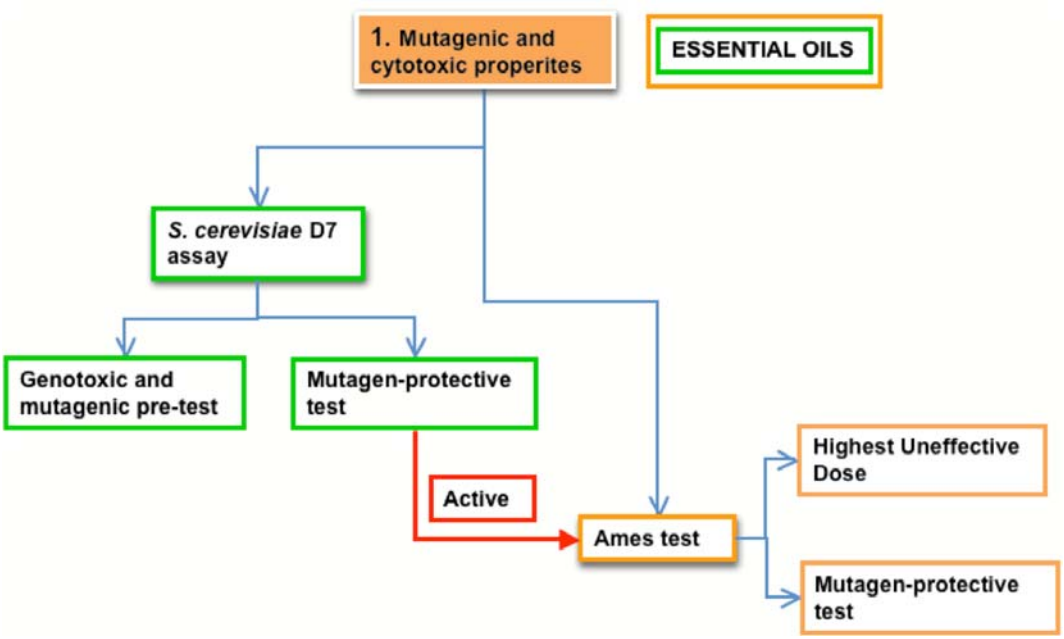


Fig. 23 mutagenic and cytotoxic investigations strategies.

B) 1 Mutagenic and mutagen-protective activity of Amazonian essential oils

The bioactivities have been determined employing short-thermo tests as *Saccharomyces cerevisiae* D7 and Ames test. In light of the wide applicative employ of Essential oils as foods, functional foods, and flavour enhancers, it has been assumed that all the plant derived products would be substantially safe (GRAS: generally recognised as safe). However, as preliminary assay to confirm bibliographic notes and wide use, D7 test has been performed to detect cytotoxicity and mutagenic induction. As main target, however, the determination of mutagen protective properties represented the main focus of this part of the research strategy. In light of this assumption, mutagen protective activities have been monitored closely with both *S. cerevisae* and Ames test, properly modified.

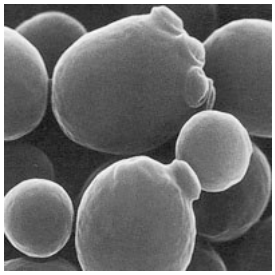


Fig. 24 *Saccharomyces cerevisiae* yeast in the process of asexual cell division known as budding. The new organism remains attached as it grows, separating from the parent organism only when it is mature. Since the reproduction is asexual, the newly created organism is a clone and is genetically identical to the parent organism.

B) 1.1 S. cerevisiae D7 test

Cytotoxicity and mutagenic pre-test was performed on essential oils, employing yeast cells (D7 diploid strain of *S. cerevisiae* ATCC 201137). Complete liquid (YEP), solid (YEPD), and selective media were prepared according to literature (Zimmermann, Rasenberger, 1975; Rossi et al., 2011). Cells from a culture with low spontaneous gene conversion and reverse point mutation frequencies were grown in a liquid medium at 28-30°C, until they reached the stationary growth phase. The yeast cells were pelleted and re-suspended in a volume of 0.1 M sterile potassium phosphate buffer, pH 7.4, to obtain the final mixture of 2 x10^8 cell/ml. The test solutions

(4 ml) were composed of 1 ml of cell suspension, potassium phosphate buffer, dimethylsulphoxide (DMSO) and essential oil. Pure essential oils were progressively diluted, employing DMSO as emulsifier, to have final concentrations of 0,01, 0,025, 0,05, 0,1, 0,25, 0,5, 1,0, 2,5, 5,0, 10,0 mg/plate. The negative control was assessed employing the highest DMSO concentration employed in the essential oil test solutions, while a positive control was set up with Ethyl Methane Sulphonate (EMS) (0.01 mg/plate). The mixture was incubated under shaking for 2 h at 37°C. Then the cells were plated in complete and selective media to ascertain survival, trp- (convertants) and ilv- (revertants). The plates were then incubated at 29 ± 1°C and, after 5 days, the grown colonies were counted to determine the gene conversion at trp locus (trp convertants) and point mutation at ilv locus (ilv revertants) frequencies on the basis of the colonies ratio numbered on selective and complete media. In light of the results achieved with the above-described method, inhibitory effects (i.e. mutagen-protective activities) of each essential oils have been evaluated on gene conversion and reverse point mutation against direct acting mutagen Ethyl Methane Sulphonate (EMS). Starting from a mixture of 2 x 10^8 cell/ml, as described above, experiments were processed in the same way adopted for toxicity and mutagenic evaluation, with the presence of EMS (0.01 mg/plate) included in the test solution. Colony-forming Units (CFU) were assessed after the plates were incubated at 29 ± 1°C and, after 5 days and compared with that of control where no test samples were added. Every genoactivities for each samples were evaluated by visual estimation (colony counting) and integrated by statistical analysis.

B) 1.2 Ames test

The bacteria reversed mutation assay (Ames Test) is used to evaluate the mutagenic properties of the studied plant products. The test uses amino acid-dependent strain of *S. typhymurium* in the absence of an external histidine source, which suppress the cells grow to form colonies. Colony growth is resumed if a reversion of the mutation occurs, allowing the production of histidine to be resumed. Spontaneous reversions occur with each of the strains; mutagenic compounds cause an increase in the number of revertant colonies relative to the background level. Amazonian derived

EO has been tested with Ames test to investigate more closely mutagen-protective activities, already checked with D7 test.

Each essential oil was dissolved in DMSO and tested with *Salmonella typhymurium* strains TA98 and TA100 (100 ml per plate of fresh overnight cultures) with and without the addition of 0.5 ml of a 5% S9 exogenous metabolic activator (S9 mix), using plate incorporation assay. The concentrations of the essential oil samples used were: 0,01, 0,025, 0,05, 0,1, 0,25, 0,5, 1,0 mg/plate. The plate for negative control contained 100 ml of DMSO, with or without S9 mix. The pos. control plates with S9 mix contained 2 mg/plate of 2-aminoantracene for both TA98 and TA100 strains. The pos. control plates without S9 mix contained 2 mg/plate of 2-nitrofluorene for TA98 strain and 1 mg/plate of NaN3 for TA100 strain. A sample was considered mutagenic when the observed number of colonies was at least twofold over the spontaneous level of revertants (Maron, Ames, 1983). The colonies were counted manually after 48 h of incubation at 37±1°C using a Colony Counter 560 Suntex (Antibioticos, Italy). Lyophilized post-mitochondrial supernatant S9 mix (Aroclor 1254-induced, Sprague-Dawley male rat liver in 0.154m KCl soln.), commonly used for the activation of promutagens to mutagenic metabolites, was purchased from Molecular Toxicology, Inc. (Boone, NC, USA) and stored at -80±2°C.

The inhibitory effect of each essential oil samples (0,01, 0,025, 0,05, 0,1, 0,25, 0,5, 1,0 mg/plate) on mutagenic activity of directly acting mutagen 2-nitrofluorene (2 mg/plate) and NaN_3 (1 mg/plate) was examined by plate incorporation assay, derived from mutagenicity test using tester strain TA98 and TA100 respectively; the inhibitory effect of each essential oil samples on mutagenic activity of the indirectly acting mutagen 2-aminoanthracene (2 mg/plate) was instead examined by plate incorporation assay, using tester strain TA98 and TA100 with S9 mix, as described in (Guerrini et al., 2011) and (Rossi et al., 2011). The inhibition rate for mutagenic induction was calculated according to the formula: inhibition rate [%]=(A-B)x100/A, where A are revertants in positive control, and B are revertants in the essential oil samples, having subtracted the spontaneous revertants. A critical point, affecting the outcome of the interaction between an antimutagen and a testing bacterial strain, is the overlapping of the cytotoxic and antimutagenic dose concentration. In other words, it is important to confirm that the dose-

dependent disappearance of the mutant colonies is not a result of cell-killing. For this purpose a simple survival assay for the treated bacteria must be performed to evaluate a Highest Uneffective Dose (HUD). To verify the toxicity of the analyzed samples on bacterial cells and evaluate the HUD, a toxicity test was performed (Maron, Ames, 1983). A fresh 15 h culture was diluted to give a $1–2 \cdot 10^4$ bacteria/ml. The test samples at several concentrations (0,0001, 0,00025, 0,0005, 0,001, 0,0025, 0,005, 0,01 mg/plate), diluted in DMSO and mixed with 2 ml of molten top agar, were plated with 0.1 ml of the diluted culture. Histidine/biotin agar plates were enriched with 10 mmol of l-histidine and 0.05 mmol of biotin by incorporating these nutrients into the soft agar overlay. Triplicate plates were poured for each dose of soln. The Colony-Forming Units (CFU) were assessed after the plates were incubated at 37°C for 48 h and compared with that of control, where no test samples were added. HUD for each samples with and without metabolic activation was evaluated by visual estimation (colony counting) and integrated by statistical analysis.

Statistical Analysis: Relative standard deviations and statistical significance (*Student's t*-test; $p<0.05$) were given, where appropriate, for all data collected. *Student's t*-test ($p<0.05$) combined with HUD comparison was used to interpret the results of significant decrease in the number of *Salmonella* revertants and *Saccharomyces D7* revertants and convertants. When the modulator dose concentration is statistically effective and it ranges below or coincides with the HUD, the samples were considered to present sign of the effect (antimutagenicity). *Student's t*-test was performed also for pharmacological data computations. All computations were made using the statistical software STATISTICA 6.0 (StatSoft Italia srl) (Guerrini et al., 2011).

B) 2 Antimicrobial activities:

Antimicrobial activities were checked for Essential Oils employing different strategies:

- OE disk-diffusion assay
- OE Antibacterial (HP)TLC bioautographic assay
- OE Antibacterial agar vapour method (Headspace's activity)

Strains

gram positive	*Staphylococcus aureus*	ATCC 29213	*S.a.*
	Enterococcus faecalis	ATCC 29212	*E.f.*
	Micrococcus luteus	ATCC 9622	*M.l.*
	Listeria grayi	ATCC 19120	*L.g.*
gram negative	*Pseudomonas aeruginosa*	ATCC 17934	*P.a.*
	Klebsiella oxytoca	ATCC 29516	*K.o.*
	Escherichia coli	ATCC 4350	*E.c.*
	Proteus vulgaris	ATCC 6361	*P.v.*
yeast	*Candida albicans*	ATCC 48274	*C.a.*
	Saccharoimyces cerevisiae	ATCC 2365	*S.c.*

Tab. 1 strains employed in antimicrobial analysis

In previously research the biological activity against yeasts has been determined by employing the standard disk diffusion technique (Sacchetti et al., 2005 and references cited). Now antifungal and antibacterial activities were more closely evaluated on 4 Gram negative bacteria *Pseudomonas aeruginosa* ATCC 17934 (*P.a.*), *Klebsiella oxytoca* ATCC 29516 (*K.o.*), *Escherichia coli* ATCC 4350 (*E.c.*), *Proteus vulgaris* ATCC 6361 (*P.o.*); 4 Gram positive ones *Staphylococcus aureus* subsp. Aureus ATCC 29213 (*S.a.*), *Enterococcus faecalis* ATCC 29212 (*E.f.*), *Micrococcus luteus* ATCC 9622 (*M.l.*), and 2 yeasts: *Candida albicans* ATCC 48274 (*C.a.*) and

Saccharomyces cerevisiae ATCC 2365 (*S.c.*). The strains were cultured in nutrient agar, Tryptic soy agar and Yepd following the suggestions given by ATCC protocols.

B) 2.1 OE disk-diffusion assay

Antimicrobial activity tests of *C. longa*, *C. citratus*, *Z. officinale*, *O. micranthum* essential oils were checked. In addition, with the aim to investigate more closely the role of single chemicals, pure 4-terpineol, β-caryophyllen, eugenol, geraniol, β-pinene, citral, germacrene D and 1,8-cineole (Sigma-Aldrich) were also tested with disk-diffusion assay. Mother cultures of each micro-organism were set up 24 h before the assays in order to reach the stationary phase of growth. The tests were assessed by inoculating Petri dishes from the mother cultures with proper sterile media, with the aim of obtaining the micro-organism concentration of 10^5 – 10^6 colony forming units (CFU)/ml in bacteria and yeast respectively. An aliquot of dimethylsulfoxide (DMSO; Sigma–Aldrich) was added to the essential oils and pure chemicals in order to obtain a 0.01–0.75 mg/ml concentration range. Serial dilutions of the DMSO/essential oil solution were deposited on sterile paper discs (6 mm diameter, Difco) which were subsequently placed in the centre of the inoculated Petri dishes. Therefore, the Petri dishes were then incubated at 37°C for 3-5 days and the growth inhibition zone diameter (IZD) was measured to the nearest mm. The lowest concentration of each DMSO/essential oil solution deposited on the sterile paper disc showing a clear zone of inhibition was taken as the minimum inhibitory concentration (MIC) (Sacchetti et al., 2005 and references therein). Controls were set up with DMSO in amounts corresponding to the highest quantity present in the test solution.

B) 2.2 OE Antibacterial activity: (HP)TLC-bioautographic assay

Antibacterial activity tests were carried out by bioautographic method on high performance thin layer chromatography [(HP)TLC] plate (Rossi et al., 2011). Antibacterial and Antifungal activity of *C. longa*, *C. citratus*, *Z. officinale*, *O. micranthum* essential oils were checked to determine if particular chemical classes of the essential oil are mainly responsible of the antibacterial activity of the whole phytocomplex. (HP)TLC plates prepared as above described (paragraph A) 2.1.1 (HP)TLC for checking the occurring of

the main terpenes in Essential Oils) were treated as reported in Rossi et al. (2011) and Guerrini et al. (2011), for strain inoculum and antimicrobial activity detection. After a period of 24h to strain growth, the plates were monitored and bands with a zone of inhibition registered.

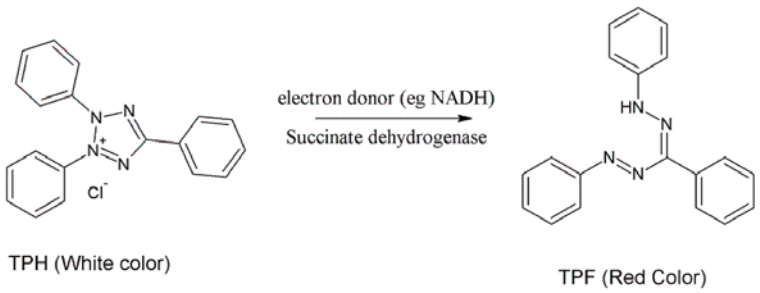


Fig. 25 TTC reaction: The solution of TTC (2,3,5-triphenyltetrazolium chloride) is a redox indicator used to differentiate between metabolically active and inactive tissues. The white compound TPH (2,3,5-triphenyltetrazolium chloride) is enzymatically reduced to red TPF (1,3,5-triphenylformazan) in living tissues due to the activity of various dehydrogenases (enzymes important in oxidation of organic compounds and thus cellular metabolism), while it remains as white TTC in areas of necrosis since these enzymes have been either denatured or degraded.

B) 2.3 OE Antimicrobial agar vapour method (Headspace's activity)

Biological activity of each Amazonian essential oils against the above mentioned bacterial and yeast strains was performed by means of the agar vapour method (Maietti et al., in press). The strains considered were the same used with previously described antimocrbial activities tests. They were grown in Petri plates (90 mm) supplemented with 15 ml/plate of PDA, inoculated with 6 mm plugs from stationary phase cultures. The plates were then incubated for an appropriate period to strains growth. Successively, sterilized filter paper discs (diameter 9.0 mm) were adsorbed with 10 µl of each essential oil samples at several concentrations ranged from 10% to 100% in DMSO, and placed inside the upper lid of each plate, at a distance of about 4mm from the strain. Plates were kept in an inverted position, tightly sealed with parafilm, and incubated for 3-5 days at 26,0 ± 1.0 °C. Blanks served as negative control. Commercial *T. vulgaris* essential oil was prepared as above described for other EO samples and considered as phytocomplex positive control reference. Three replicates were made for

each treatment. After seven days the results were collected as radial growth inhibition and the results expressed as the essential oil amount which determined the 50% growth inhibition of each fungal strain (IC50).

B) 3 Antioxidant activity spectrophotometric assays

Antioxidant activity has been performed on the following samples:

- Amazonian Essential oils: *Ocimum micranthum, Curcuma longa, Cimbopogon citratus, Zingiber officinale*
- Chemical standards: β-caryophyllene, β-pinene, 1,8-cineole
- Avocado hexane extract (AHM), avocado supercritical fluid extract (ASFE), avocado methanolic extract (AMM), avocado commercial oil (ACO).

The bioactivity has been performed with DPPH• and ABTS methods and checked spectrophotometrically and through (HP)TLC bioautography.

B) 3.1 Spectrophotometric DPPH• assay

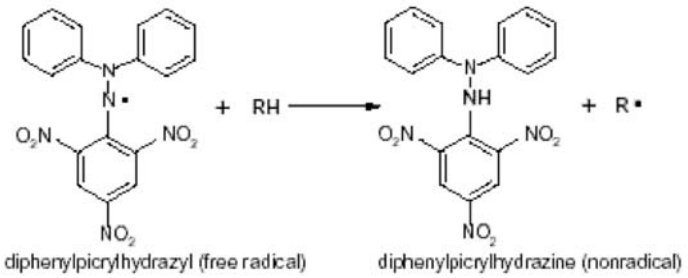


Fig. 26 DPPH• reaction. DPPH$^{\bullet}$ is a stable pink coloured free radical, when it is scavenged, DPPH•-H change its colour into white.

Essential Oils: An aliquot of each essential oil (100 μl) was added at 2,9 ml of DPPH• (Sigma-Aldrich) ethanolic solution The mixture was shaken vigorously and kept in the dark for 30 min at room temperature. Sample absorbance was measured at 517 nm with UV/VIS spectrophotometer (ThermoSpectronic Helios γ, Cambridge, U.K.). A blank was assessed as the

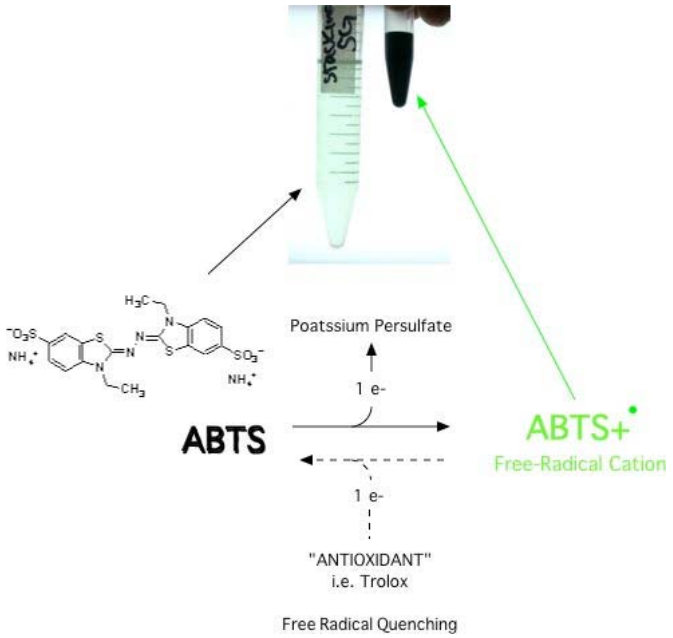
solution assay described above without the essential oils, instead of which distilled H_2O was employed. Butylated hydroxy anisole (BHA), *T. vulgaris* and *Melaleuca alternifolia* essential oils were used as positive controls. The radical-scavenging activities of each sample were calculated according to the following formula for inhibition percentage (Ip) of DPPH•: $Ip=(A_B-A_A)/A_B \times 100$, where A_B and A_A are the absorbance values of the blank sample and of the test sample respectively, after 30 min. Oils and BHA antiradical activity was considered as the concentration providing DPPH• 50% inhibition (IC50), calculated from inhibition curves obtained by plotting inhibition percentage vs. oil concentration (Guerrini et al., 2011). Pure chemicals were tested in the same condition above described.

Avocado Oils and Extracts: Avocado extracts were progressively diluted in DMSO (Merck) to obtain the final concentration of 1,0, 5,0, 10,0, 50,0 and 100,0 mg/ml. An aliquot of each samples (100 µl) was mixed with 900 µl of DPPH• (Sigma-Aldrich) EtOH solution. The mixture was shaken vigorously and kept in the dark for 30 min at room temperature. Sample absorbance was measured at 517 nm with UV/VIS spectrophotometer (ThermoSpectronic Helios γ, Cambridge, U.K.). A blank was assessed as the solution assay described above without the avocado derivates, instead of which distilled H_2O was employed. Butylated hydroxy anisole (BHA) was used as positive controls. The radical-scavenging activities of each sample were calculated according to the following formula for inhibition percentage (Ip) of DPPH•: $Ip = (A_B-A_A)/A_B \times 100$, where A_B and A_A are the absorbance values of the blank sample and of the test sample respectively, after 30 min. Avocado derivates and BHA antiradical activities were considered as the concentration providing DPPH• 50% inhibition (IC50), calculated from inhibition curves obtained by plotting inhibition percentage vs. extracts concentration.

B) 3.2 Spectrophotometric ABTS assay

$ABTS^{+\bullet}$ is also frequently used by the food industry and agricultural researchers to measure the antioxidant capacities of foods. In this assay, ABTS is converted to its radical cation by addition of sodium persulfate. This radical cation is deep green coloured and absorbs light at 734 nm. The $ABTS^{+\bullet}$ radical cation is reactive towards most antioxidants including

phenolics, thiols and Vitamin C. During this reaction, the deep-green ABTS+• radical cation is converted back to its colourless neutral form. The reaction may be monitored spectrophotometrically, with the aim of searching the IC50; or atomizing the solution of ABTS radical cation on (HP)TLC to determine if particular chemical classes are mainly responsible of the whole



phytocomplex showing a clear zone of inhibition (Re et al., 1999).

FIG. ABTS+• reaction. Free radical cation ABTS+•, deep green coloured, when the free radical had been scavenged, changes into its uncoloured neutral form ABTS.

Essential Oils: An aliquot of each essential (100 μl) was added at 900 μl of ABTS (Sigma-Aldrich) EtOH solution The mixture was shaken vigorously and kept in the dark for 60 sec at room temperature. Sample absorbance was measured at 734 nm with UV/VIS spectrophotometer (ThermoSpectronic Helios γ, Cambridge, U.K.). A blank was assessed as the solution assay described above without the essential oil, instead of which distilled H_2O was employed. Butylated hydroxy anisole (BHA) and *T. vulgaris* and *M. alternifolia* essential oil were used as positive controls. The radical-scavenging activities of each sample were calculated according to the following formula for inhibition percentage (Ip) of ABTS: $Ip=(A_B-A_A)/A_B \times 100$, where A_B and A_A are the absorbance values of the blank sample and of the

test sample respectively, after 30 min. Essential oils and BHA antiradical activity was considered as the concentration providing ABTS 50% inhibition (IC50), calculated from inhibition curves obtained by plotting inhibition percentage vs. oil concentration (Scartezzini et al., 2006 and references therein; Wang et al., 1998). Pure chemicals were tested in the same condition above described.

Avocado samples: the method applied was the same used for EO, and Avocado samples concentrations were the same employed for DPPH• test. Avocado derivates samples and BHA antiradical activity was considered as the concentration providing ABTS+• 50% inhibition (IC50), calculated from inhibition curves obtained by plotting inhibition percentage vs. oil concentration.

B) 4 Antioxidant activity (HP)TLC assays

After determination of the antioxidant activity of each samples by spectrophotometric assays, TLC bioautographic assay of radical scavenging activity using the DPPH• and the ABTS+• radicals have been employed to determine active compounds in phytocomplexes.

B) 4.1 DPPH•-(HP)TLC bioautographic assay

Essential Oils: 10 μl of each essential oils solutions (30 mg/ml) in dichloromethane, was applied to (HP)TLC plate of silica gel (high performance thin layer chromatography plates, Merck, silica gel 60, with fluorescence indicator F_{254}) as 10 mm wide bands with Linomat IV (Camag). Then, spots were eluted in a chromatographic chamber with a solvent solution composed of toluene/ethyl acetate/petroleum ether (93:7:20). On the same plate, were deposited also the majority component of each samples as controls. Antiradical compounds appeared as clear white spots against a violet coloured background. (Guerrini et al., 2011; Sacchetti et al., 2005).

B) 4.2 ABTS-(HP)TLC bioautographic assay

The same strategy employed for DPPH•-(HP)TLC bioautographic assay has been applied also to ABTS+• free radical both with EO and Avocado derivates.

Essential Oils: Antiradical ABTS+• activity of *C. longa*, *C. citratus*, *Z. officinale*, *O. micranthum* essential oils, *T. vulgaris* and *M. alternifolia* as positive controls, were checked. Spots were applied and eluted in a chromatographic chamber in the same conditions mentioned above in DPPH-(HP)TLC assays. Antiradical compounds appeared as clear white spots against a green coloured background.

C) Possible formulations (blended essential oils)

Essential oils are concentrated solution which can cause skin irritation if used as it is. Mixed with other oils (i.e. fixed oil) they can be used for cosmetic and/or nutraceutics uses. The oils that essential oils are mixed or diluted with are called carrier oils. Generally a good quality, cold-pressed vegetable oil is the best choice. With the intent of explore a possibility of applicative uses of Rwandese avocado oil and Ecuadorian essential oils for "fair trade" markets, and in light of the results obtained with biological activities investigation, three simple healthy formulation has been prepared mixing an aliquot of avocado oil with essential oil to obtain the final concentration of 1 % (w/w) of EO in carrier oil.

C) 1.1 Preparation of a mixture of essential oil in a carrier oil (blended essential oil)

Three kind of mixture were prepared: 10 g of Avocado oil (ACO) were mixed with 0,1 g of *O. micranthum* essential oil (formulation I); the same aliquot of ACO was mixed also with 0,1 g of *C. citratus* EO (formulation II); and finally with a mixture in same part of the above mentioned essential oils, i.e. Amazonian basil and yerba luisa EOs (formulation III). With the intent to compare biological activity of each formulations (f I, f II, f III), a mixture of avocado oil with well known active essential oils (i.e. *M. alternifolia* EO and *T. vulgaris* EO, formulation IV and formulation V respectively) were prepared too. All the preparations has been stored in the dark at room temperature until analysis.

C) 1.2 Analyses of the antioxidant activities: Photochemiluminescence (PCL) method

The PCL assay, based on the method suggested by Vertuani et al. (2011), has been used to measure the antioxidant activity of extracts with a

Photochem® apparatus (Analytik Jena, Leipzig, Germany) against superoxide anion radicals generated from luminol, a photo-sensitizer, when exposed to UV light (Double Bore® phosphorus lamp, output 351 nm, 3 mWatt/cm2). The antioxidant activity was measured using ACL (Antioxidant Capacity of Liposoluble substance) kits provided by the manufacturer, designed to measure the antioxidant activity of lipophilic compounds (Popov, Lewin, 1994). The luminal reagent and Trolox work solution was freshly prepared according the ACW protocol; the kinetic light emission curve was monitored for 180 seconds and expressed as micromoles of Trolox per gram of formulation (μmol Trolox/g formulation). The areas under the curves were calculated using the PCLsoft control and analysis software. The observed inhibition of the signal was plotted against the concentration of Trolox added to the assay medium. The concentration of the added formulation solution was such that the generated luminescence during the 180 s sampling interval fell within the limits of the standard curve. The formulations were centrifuged (5 min at 16,0°C) prior to analysis. The antioxidant assay was carried out in triplicate for each sample, and 20 µL of the diluted formulations (1:40, v/v) in HPLC-grade methanol was sufficient to correspond to the standard curve. Pure essential oils (*O. micranthum, C. citratus, C. longa, Z. officinale, T. vulgaris* and *M. alternifolia*) were tested in the same condition above described.

RESULTS AND DISCUSSION

Results are described according to the following chart, which deals with functional and pharmaceutical biology approaches. In this PhD context, i.e. from ethnomedicine to laboratory led by international Cooperation projects, the discussion would give a scientific contribute to find new integrated opportunities for human beings progress (i.e. new chemicals for new drugs to treat old and new diseases) with ethical respect to different cultures from different Countries.

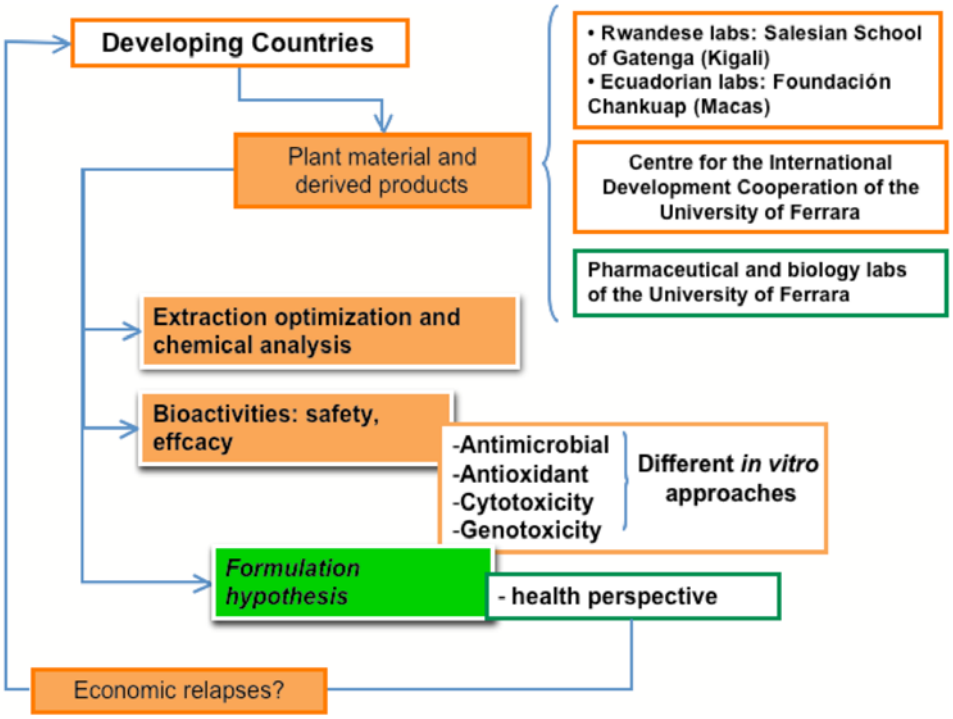


Fig. 26 Schema illustrating the research outline

The research has been performed as described in the scheme below, subdivided into: Pharmaceutical biology approaches based on phytochemical and functional investigations (phases A+B) and health perspective carried out preparing simple formulations (i.e. blended essential oils in avocado oil) in which the proved bioactivities would be maintained with health efficacy.

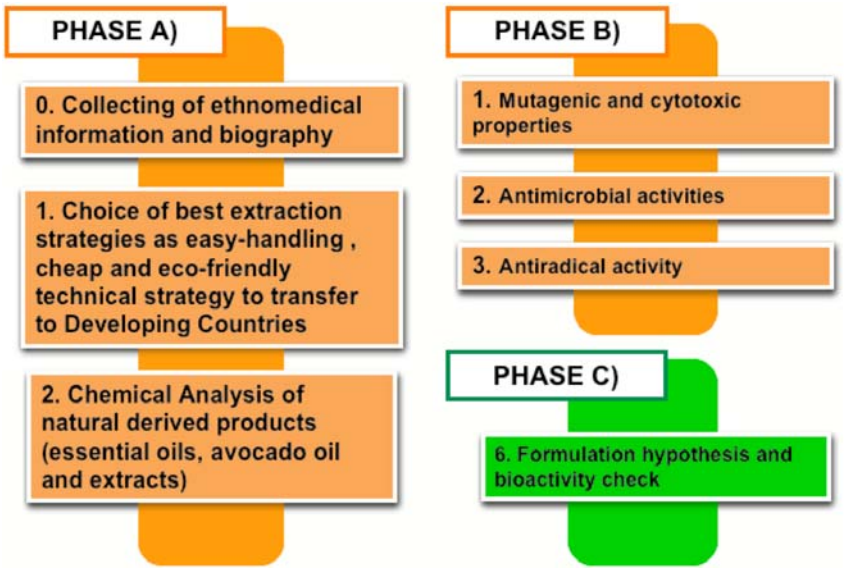


Fig. 27 Research phases subdivision

A) Phytochemical and functional fingerprinting

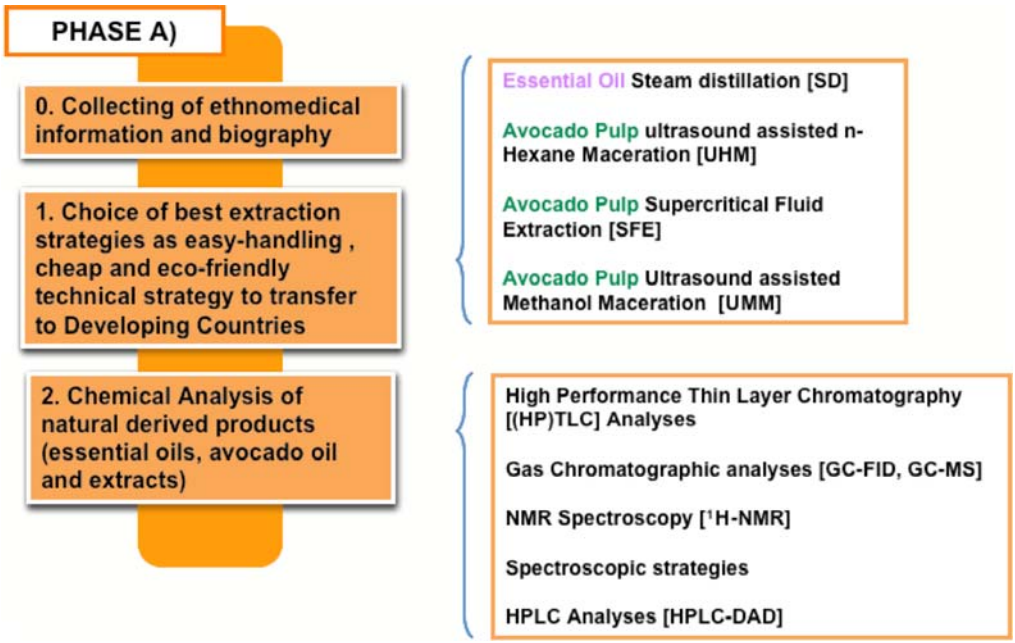


Fig. 28 Research phase A diagram

A) 1 Different extraction strategies

A) 1.1 Essential oils

The plant species considered for their ethno medical importance based to literature and Natives knowledge were*: Curcuma longa, Cymbopogon citratus, Zingiber officinale and Ocimum micranthum*. Fresh crude drugs of

these plants have been processed by steam distillation with a commercial clevenger apparatus, located and set up in Fundación Chankuap (Macas, Ecuador). Essential oils yields are summarized above:

Fresh plant	Part used	Hydro-distillation yield
Basil	Aerial parts	7,0 – 7,8 ml/kg
Curcuma	Rhizome	2,6 - 4,0 ml/kg
Yerba luisa	Aerial part	3,0 - 3,5 ml/kg
Ginger	Rhizome	4,0 - 5,0 ml/kg

Tab. 2 Yields of essential oils obtained by steam distillation with a commercial clevenger apparatus, issued in Fundación Chankuap (Macas, Ecuador).

Hydro-distillation of essential oils is the cheapest technique to recover essential oil from fresh material; it can be used also in poor craft laboratories, such that in Wasakentsa reserve (Ecuadorian Amazon forest) as shown in the figure below.

Fig. 29 Vapour hydro distillation apparatus: on the left, that used at “Centro de Acopio” Foundation Chankuap, Macas; and on the right that used at Wasakentsa reserve, Amazonian forest

A) 1.2 Avocado Fruits

Fruits were purchased from local farmer in Kigali, Rwanda. All fruits have been peeled, seeds removed and pulp freeze-dried. All parts of the fruit have been weighted. Fruits weight ranges 350 – 500 g, seeds 80 – 100 g and peel about 50g. Each fruit has 200 – 300 g of fresh pulp.

A) 1.2.1 Freeze-drying of Avocado plant material

Since water interferes with the efficiency of oil extraction, drying operation of the plant material is a necessary step prior to extraction. Freeze-drying is the preferred method owing to the low temperatures used and minimal exposure of the material to oxygen, it is an efficient technique, probably the most efficient in terms of preserving the shelf-life of natural products or crude-drugs. With reference to the technical equipment of the Rwandese situation, to which the PhD is referred, it is not the best choice because of its high costs; however, it would represent the best investment in light to set up an Avocado processing chains able to produce a quality and competitive fixed oil and derived products. Unfortunately, one of the most frequent problems related to Avocado oil quality, prior to the agronomic and ecological conditions, is the drying process after harvesting. As stated by some Authors, the role of temperature is crucial. In fact, the use of drying conditions set up with temperature around or over 100°C determines a reduction of oil yield and quality (Mostert et al., 2007 and references cited). The yield of pulp after freeze drying was 195 g/kg (19,5%).

A) 1.2.2 Extraction procedures: Avocado pulp

Oil content, fatty acid composition, unsaponifiable fraction and bioactivities of Rwandese avocado fruits have been examined with respect to different extraction procedures as illustrated in the scheme above (fig. 30).

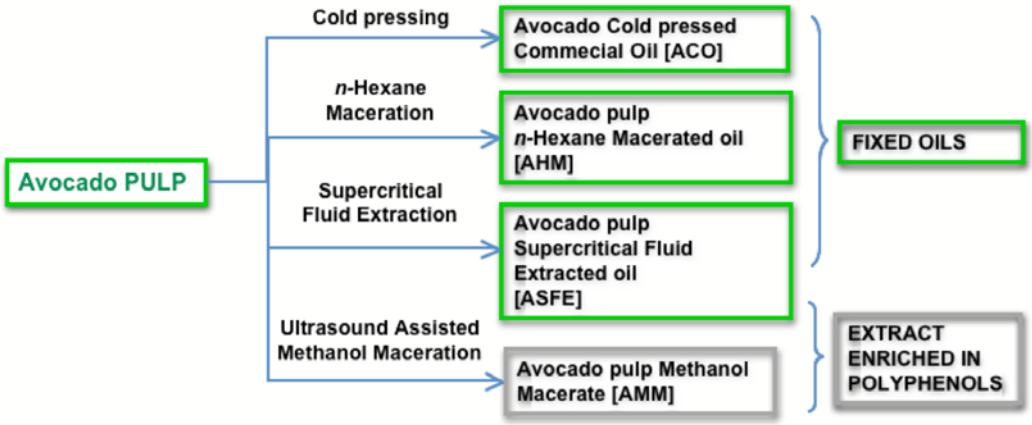


Fig. 30 Avocado pulp extracts.

Maceration in *n*-hexane and supercritical fluid extraction have been compared in terms of yield and oil quality production. The extracts have been then compared for their phytochemical and functional properties to a

commercial cold pressed sample, purchased in Italy. The application of supercritical fluid extraction (SFE), particularly the use of liquid and supercritical carbon dioxide, has received much attention in the food industry in the last few years. This technique offers extraction yields comparable with those obtained by conventional extraction methods using organic solvents. Moreover, carbon dioxide is non-toxic, non-flammable, non-corrosive, biologically safe, cheap and readily available in large quantities with high purity. Since CO_2 also has a relatively low critical pressure (300-350 atm) and critical temperature (50°C), it can be considered an ideal solvent for the treatment of natural products, for example oils, butter and waxes from plant sources (Bruni et al., 2001).

No significant differences on average yield between HM and SFE have been found: hexane maceration of Rwandese cultivar yielded 53% (w/w dry basis) oil from avocado dried pulp, and an average yield of 50% (w/w dry basis) has been obtained using SFE; this difference can be attributed to the lower selectivity of hexane (Mostert et al., 2007). Another difference has been pointed out in fatty acids pattern (see tab 7); in fact SFE extraction yielded a large amount of long-chain fatty acids, i.e. arachidic (C20:0) and lignoceric (C24:4), higher than that detected with hexane maceration and cold press extraction. Similar evidences have been detected for PUFA.

Previous studies have been published on avocado oil extraction procedures (Logaraj et al., 2008; Oberlies et al., 1998). A variety of processes involving extraction with supercritical fluids (SFs) have been developed as promising alternatives to the current separation processes, and industrial applications of SFE using carbon dioxide have increased in the last few years, e.g. decaffeination, the extraction of hops and spices, etc. (Pradhan et al., 2010).

For determination of the content in phenols compounds (i.e. coumarines, flavonoids and procyanidins), an ultrasound assisted maceration of the pulp with methanol has been carried out (AMM yield, 53% of dry basis). The choice of maceration and solvent has been driven by the fact that: 1) maceration is a cheap and simple method; 2) the sonication reduces the extraction time (Bruni et al., 2001); 3) alcoholic solvent (methanol and/or ethanol) allows a good yield of phenols and an enriched unsaponifiable fraction of those chemicals which qualify the oil, butter or waxes of the

functional and healthy properties (Garcia-Alonso et al., 2004; Wagner, Bladt, 2009; Dobiáš et al., 2010; Werman, Neeman, 1987).

To perform bioactivity assay, as previously described in Material and methods paragraph A) 1.2.6, hexane/80% ethanolic extracts (EIA.) of each avocado pulp derivates have been prepared with the following yield: EIA.AHM 1,89 g (yield 12,5 %); EIA.ASFE 0,94 g (yield 6,2 %); EIA.AMM and commercial sample EIA.AOC 0,51 g and 0,56 g (yields 3,38 % and 3,70 %) respectively.

A) 2 Phytochemical and functional fingerprinting

A) 2.1.1 (HP)TLC for checking the occurring of the main terpenes in Essential Oils

High performance thin layer chromatography (HP)TLC is a very sensitive technique to identify volatile organic compounds. Terpene hydrocarbons have been identified with comparison with literature data and with reference to their on Rf value, UV (254 nm) and visible pigmentation after VS reagent treatment (Wagner, Bladt 2009). The results were confirmed by GC and NMR analyses (see chapter A) 2.2.1 and A) 2.3 in Results and discussion paragraph).

Ginger EO shows at R_f 0,6 Turmeric ketones (and at the top a mixture the sesquiterpenes Germacrene D, *ar*-Curcumene, α-Curcumene, trans-Muurola-4(14)-5-diene; Turmeric ketones (β-Turmerone and *ar*-Turmerone) at Rf 0,6 has been detected also in Curcuma EO. Eugenol is the main component of Amazonian Basil EO, as TLC analyses evidenced with peculiar yellow coloured band at Rf 0,5. Yerba Luisa is almost entirely composed by Geraniol and Citral (Geranial + Neral) which have the same Rf 0,5, as blue-violet coloured band and good visibility at UV (254 nm). Standard chemicals were also employed to further confirm the qualitative suggestions given by (HP)TLC.

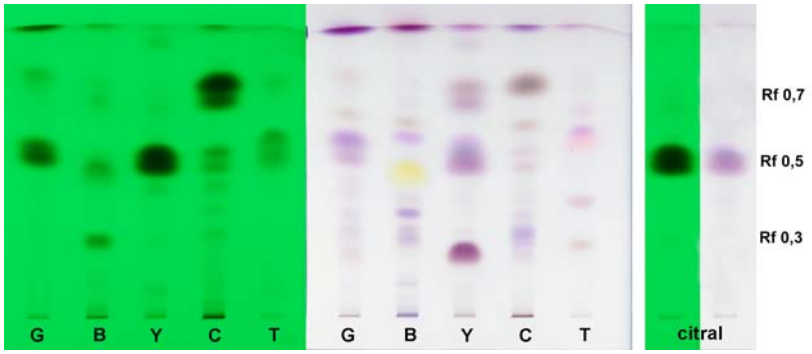


Fig. 32 Main terpenes occurring in Eos samples. Identification of bands has been conducted comparing literature data and evidences emerged from standards elution with (HP)TLC bands (Wagner, Bladt, 2009), the acquired data have been then further confirmed and specified by GC and NMR results.

A) 2.1.2 (HP)TLC for qualitative controls of sitosterol, occurring in the avocado pulp extracts

(HP)TLC of Avocado samples evidenced the presence of sitosterol, and many other bands not identified in this method, but characterized with GC-MS analyses (see chapter A) 2,2,4 in this paragraph). The irregular bands at the top of the eluted (HP)TLC are typical of the samples processed as described (triglycerides).

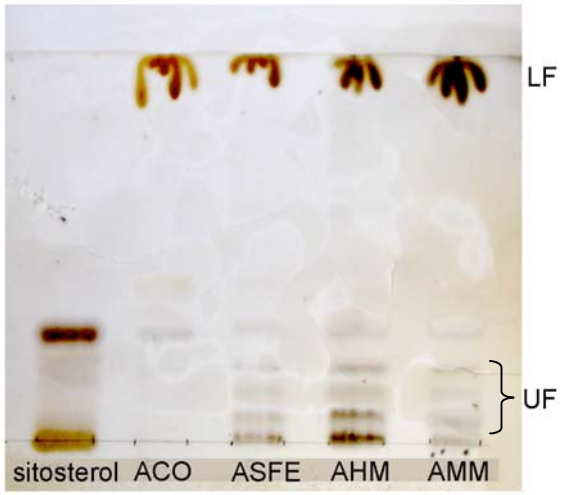


Fig. 33 TLC for qualitative controls of sitosterols and main fraction occurring in the avocado pulp. Note: LF, lipid fraction; UF, unsaponifiable fraction; ACO, avocado commercial oil; ASFE, Avocado oil supercritical CO_2 extracted; AHM, Avocado oil hexane macerated; AMM, Avocado extract methanol macerated.

A) 2.1.3 (HP)TLC for qualitative controls of fatty acids in Avocado Oils extracts

The Avocado samples have been processed following the method suggested by Platt and Thompson (1992) with minor modifications (Platt et al., 1992). With reference to literature data and to the Rf of the standard palmitic acid, triglycerides (TGs), diglycerides (DGs), and monoglycerides (MGs) have been detected (Platt et al., 1992; Chinnasamy et al., 2003; Yoshida et al., 1995). (HP)TLC evidenced the presence of TGs, DGs and MGs in the avocado oil samples, both Rwandese and commercial ones. (HP)TLC showed also the occurring of undefined compounds with lower polarity than DGs, probably characterized by usaponifiable fraction investigated with GC-MS analyses (see chapter A) 2.2.4 in this paragraph).

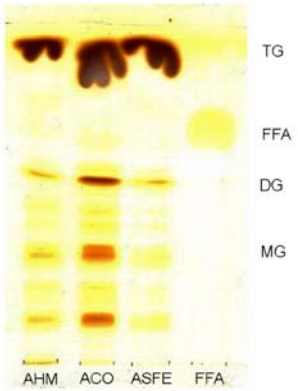


Fig. 34 Thin layer chromatogram of avocado samples and standard. All samples shows typical TG's bands at the solvent front, at Rf 0,5 DG, at Rf 0,3 MGs, and several lipidic substances that have a polarity between DG and the start. Palmitic free fatty acid has been spotted as control. Note: TG, triglycerides, FFA, free fatty acids; DG, diglycerides; MG, monoglycerides; ACO, avocado commercial oil; ASFE, Avocado oil supercritical CO_2 extracted; AHM, Avocado oil hexane macerated; AMM, Avocado extract methanol macerated.

A) 2.2 Gas Chromatographic analysis

A) 2.2.1 Analysis of Essential Oil constituents (EO): GC-FID, GC-MS

Four different Amazonian essential oils and a commercial one (*T. vulgaris*) have been tested. GS-MS analyses allowed the identification of many constituents (approximately 98% of the total considering each EO), listed in Tab. 3. The respective contributes of the major classes of terpenes to the whole phytocomplex have been detected.

Curcuma (*Curcuma longa*) has a sesquiterpene pattern, together with (*Zingiber officinale*); the others EO have a typical monoterpene oxygenated pattern (*Cymbopogon citratus, Ocimum micranthum*), thyme (*Thymus vulgaris*) has a monoterpene pattern, approximately 50% oxygenated. Identification and semi-quantitative analysis has been conducted both comparing retention index with literature and mass spectra (Adams, 2007; Sacchetti et al., 2005).

	Class od Compounds	YERBA	GINGER	CURCUMA	BASIL	THYME
		Area %				
MH	Monoterpene hydrocarbons		**28,09**	**18,74**	3,89	**46,04**
MO	Monoterpenes oxygenated	**79,62**	**24,25**	9,14	**60,99**	**51,28**
A	Alcohols	42,53	6,50	1,07	2,65	4,27
AA	Aliphatics		28,09	16,57	3,89	34,48
AP	Phenolics				50,97	47,01
K	Ketones					
ES	Esters					
AL	Aldehydes	37,63	9,14	0,22		
ET	Ethers		8,61	7,85	7,36	
SH	Sesquit. hydrocarbons	4,55	**39,63**	5,47	**26,42**	0,50
SO	Sesquit. Oxygenated		3,99	**62,90**	4,42	
HC	Hydrocarbons					
HCO	Hydrocarbons oxygenated	13,51				

Tab. 3 Class of compounds occurring in studied essential oils.

The composition is shown in table 4 as percentage. The most abundant components in *Cymbopogon citratus* are geraniol (39,4 %) and citral (14,4% neral and 17,3% geranial); smaller amount of geranyl acetate (8%), citronellal and nerol (4,6% and 2,7% respectively) were detected. This pattern is partial in contrast with those in literature (Sacchetti et al., 2005) where is reported an higher amount of citral (approximately 70%) and myrcene (16%), not detected in these samples. Turmerone compounds were found in *Zingiber officinale* and *Curcuma longa*; 23,4% *ar*-Turmerone, 22,8% α-Turmerone and 15,3% β-Turmerone were detected in *C. longa*, while in *Z. officinale* they were found in smaller quantity (2,1%, > 1% respectively). *O. micranthum* shows a 1,8-cineole chemotype having a composition characterized by eugenol (50%), similar to those reported by Vieira and Simon (2000). *T. vulgaris*, commercial sample adopted as control in all biological tests, is a limonene chemotype (limonene 32%); other compounds detected were Thymol (25%) and Carvacrol (21,9%), *p*-Cymene (11,6%), Linalool and α-Terpineol (1,1% and 2,3% respectively).

N	RT (min)	name	RI	YERBA	GINGER	CURCUMA	BASIL	THYME
				Area %				
1	7,167	*alfa*-Pinene	932		4,16	0,47	tr	0,68
2	7,850	Camphene	946		**14,72**		tr	0,46
3	9,114	Sabinene	969			1,31	tr	
4	9,183	*beta*-Pinene	974		0,47		0,49	
5	10,134	Methyl-5-epten-2-one	981	0,41	0,69			
6	10,234	Myrcene	988		1,80	0,36	0,19	0,79
7	11,020	*alpha*-Phellandrene	1002		0,71	**9,81**		
8	11,775	*alpha*-Terpinene	1014			0,25		0,60
9	12,282	p-Cymene	1020			2,17		**11,55**
10	12,628	Limonene	1024		**5,75**	1,61	0,17	**31,77**
11	12,743	Eucalyptol	1026		8,61	**7,85**	**7,36**	
12	13,555	*cis*-Ocimene	1032				2,87	
13	14,326	*trans*-Ocimene	1044				tr	
14	14,897	*gamma*-Terpinene	1054			0,46		
15	17,185	Terpinolene	1086		0,49	2,30		0,19
16	19,047	Linalool	1095	0,46	0,95	0,26	1,87	1,11
17	21,694	*allo*-Ocimene	1128				0,16	
18	22,230	1-Terpineol	1130					0,19
19	24,384	Citronellal	1148	4,54		0,22		
20	25,475	Borneol	1165		3,59		0,19	0,24
21	25,488	*cis*-Isocitral (isoneral)	1160	0,54				
22	26,468	4-Terpineol	1174			0,34		
23	27,573	*trans*-Isocitrale	1177	0,89				
24	28,383	*alpha*-Terpineol	1186		1,53	0,47	0,59	2,34
25	29,015	*gamma*-Terpineol	1199					0,40
26	30,231	nd					0,31	
27	30,875	nd					0,19	
28	33,072	Nerol	1227	2,64				
29	33,775	Neral	1235	**14,37**	3,98			
30	34,516	nd						0,23
31	35,844	Geraniol	1249	39,43	0,44	tr	tr	
32	37,532	Geranial	1264	17,29	5,16	tr	tr	
33	38,537	Bornyl acetate	1287		0,38			1,96
34	40,685	Thymol	1289			tr		**25,10**
35	41,390	Carvacrol	1298			tr		**21,91**
36	44,080	*delta*-Elemene	1335				tr	
37	47,066	Eugenol	1356	tr			**50,97**	
38	47,561	Neryl acetate	1359	0,52				
39	50,322	*beta*-Elemene	1389		0,35		4,85	

40	50,761	Geranyl acetate	1379	**7,96**				
41	50,862	Isocaryophyillene	1408					0,28
42	51,793	*beta*-Caryophyllene	1417	2,46		0,42	**10,21**	0,22
43	53,385	*alfa*-Caryophyllene	1452	0,41			2,05	
44	53,569	*allo*-Aromadendrene	1458				0,53	
45	53.044	*dehydro*-Aromadendrene	1460				tr	
46	53.539	Cumacrene	1470				tr	
47	54,280	Germacrene D	1484	0,47	1,45			
48	54,578	*ar*-Curcumene	1479		3,80	1,22		
49	54,489	*beta*-Selinene	1489				1,66	
50	54.827	*alpha*-Selinene	1798			tr		
51	54,723	Bicyclogermacrene					4,11	
52	54,765	*trans*-Muurola-4(14),5-diene	1493		2,24			
53	54,983	Germacrene A	1508				2,76	
54	54,989	*alpha*-Zingiberene	1493		**15,45**	1,31		
55	55.151	dehydrocurcumene			tr			
56	55,253	*alpha*-Bisabolene	1506			0,27		
57	55,267	*alpha*-Farnesene	1505		**8,52**			
58	55,388	*delta*-Cadinene	1522	0,56	tr			
59	55.524	nd			tr			
60	55,598	*beta*-Sesquiphellandrene	1521		**6,87**	2,26		
61	56,109	Germacrene B	1559		0,95		0,79	
62	56.257	nd			tr			
63	56,415	*trans*-Nerolidol	1561		0,51			
64	56,514	*alpha*-Cadinene	1537	1,13				
65	56,682	*ar*-Turmerol	1582			1,48		
66	56,522	Spathulenol	1577				4,42	
67	56,890	nd			0,35			
68	57,074	Helifolen-12-ale A	1592			1,60		
69	57,286	nd			1,13			
70	57,385	Apiole	1620	**5,02**				
71	57,631	*beta*-Biotol	1612			2,13		
72	57,523	nd					0,89	
73	57,881	nd					0,69	
74	57,574	nd			0,50			
75	57,827	*beta*-Eudesmol	1649		0,98			
76	57,887	*alpha*-Cadinol	1654	0,49				
77	58,140	nd					0,81	
78	58,146	*ar*-Turmerone	1668		2,06	**23,35**		
79	58,185	*alpha*-Turmerone			0,87	**22,81**		

80	58,625	*beta*-Turmerone			0,56	**15,27**		
81	58,852	nd					0,86	
82	59,555	nd				1,01		

Tab. 4 EO's composition (Adams, 2007; Sacchetti et al., 2005). Compounds, identified on the basis of comparison with MS database spectra, retention index and pure reference chemicals, are listed in order of elution from a Varian FactorFour™ VF-5ms column. RI: Retention index.

A) 2.2.2 Headspace analysis (HS)-GC-MS of Essential Oils

The chemical composition of the vapour phase, called also Head Space (HS), analyzed by HS-GC-MS is shown below (table 5).

	Class od Compounds	YERBA	GINGER	CURCUMA	BASIL	THYME
		Area %				
MH	Monoterpene hydrocarbons	**22,82**	**96,32**	**98,03**	**21,72**	**93,34**
MO	Monoterpenes oxygenated	**66,76**	1,72	0,47	**40,28**	**2,00**
A	Alcohols	20,84	1,15	0,63	9,45	6,59
AA	Aliphatics	20,84	1,00	0,44	2,44	0,32
AP	Phenolics		0,15	0,19	7,01	6,28
K	Ketones	7,11				
ES	Esters					
AL	Aldehydes	40,67	1,26	0,14		
ET	Ethers				30,83	
SH	Sesquit. hydrocarbons		**1,26**	0,37	**9,90**	0,07
SO	Sesquit. Oxygenated			0,66		
HC	Hydrocarbons					
HCO	Hydrocarbons oxygenated					

Tab. 5 Class of compounds detected with HS-GC analyses. All Essential Oils show monoterpene pattern mainly, characterizing the most volatile molecules.

The analysis of the HS composition, and punctual identification of single chemicals, has been conduced in the same way of the previous GC assay, following the method adopted in previous paper (Maietti et al., in press). All the EO tested shows mainly a monoterpene pattern, constituted by the most volatile compounds, pointing out the prevalence of hydrocarbons and oxygenated monoterpenes, with alcohols as most abundant. The relatives percentage of the chemicals occurring are resumed in table 6.

Yerba luisa (*C. citratus*) is the only EO which has only a monoterpene pattern constituted by *cis*- and *trans*-Isocitral (15%, 11%), gamma-terpineol (13%) and citonellal (11%). Ginger (*Z. officinale*) and Curcuma (*C. longa*) vapour phases are composed essentially by monoterpene hydrocarbons (> 96%), camphene (50,4%), α-pinene (16,5%) and limonene (17,2%); α-phellandrene (32,3%) in Ginger in particular and limonene (37,4%) in

Curcuma in particular. Basil is the EO which has demonstrated to have the higher amounts of sesquiterpene: eugenol (7%) and β-caryophyllene (5%) primarily; however the most abundant compounds results monoterpene eucalyptol (31%) and *cis*-ocimene (19,5%). This was an expected evidenced since the vapour phase is characterized by the most volatile compounds, such as monoterpene, while sesquiterpenes were almost present in minor concentration, due to their lower volatility.

N	RT (min)	Compound	RI	YERBA	GINGER	CURCUMA	BASIL	THYME
				Area %				
1	6,944	Tricyclene	921		0,78			
2	7,408	*alpha*-Pinene	932	2,24	**16,47**	**8,28**	4,66	4,55
3	8,096	Camphene	946	3,49	**50,39**		2,51	3,89
4	9,096	Sabinene	969	0,97		9,76	0,26	tr
5	9,506	*beta*-Pinene	974		4,40		10,61	0,30
6	10,134	Methyl-5-hepten-2-one	981	**7,11**				
7	10,527	Myrcene	988		4,36	1,35	3,24	2,38
8	11,388	*alpha*-Phellandrene	1002	0,91	2,25	**32,33**		
9	12,296	*alpha*-Terpinene	1014	0,70				0,23
10	12,768	p-Cymene	1020					**81,46**
11	12,942	Limonene	1024	**5,30**	**17,20**	**37,35**		
12	12,969	Eucalyptol	1026				**30,83**	
13	13,852	*cis*-Ocimene	1032	4,76			**19,49**	
14	14,631	*trans*-Ocimene	1044	**6,64**		0,18	0,29	
15	15,248	*gamma*-Terpinene	1054	2,03		1,65		0,22
16	17,481	Terpinolene	1086	0,54	0,48	**7,13**	0,17	0,30
17	19,31	Linalool	1095	0,78	0,25	0,16	2,11	0,21
18	21,694	*allo*-Ocimene	1128	2,33			**8,43**	
19	24,384	Citronellal	1148	**11,07**				
20	25,831	Borneol	1165	0,97	0,57		0,11	
21	25,488	*cis*-Isocitral (isoneral)	1160	**15,27**				
22	26,468	4-Terpineol	1174	1,87		0,16		
23	27,573	*trans*-Isocitral	1177	**10,89**				
24	28,383	*alpha*-Terpineol	1186	1,16	0,19	0,12	0,22	0,11
25	29,015	*gamma*-Terpineol	1199	**12,92**				
26	30,231	nd		0,61				
27	30,875	nd		0,45				
28	33,072	Nerol	1227	3,15				
29	33,775	Neral	1235	3,45	0,57			
30	34,516	nd		0,40				
31	35,844	Geraniol	1249	tr		0,17		
32	37,532	Geranial	1264	tr	0,70	0,14		

33	40,685	Thymol	1289	tr				4,59
34	41,39	Carvacrol	1298					1,69
35	44,08	*delta*-Elemene	1335				0,18	
36	47,066	Eugenol	1356		0,15	0,19	**7,01**	
37	50,322	*beta*-Elemene	1389		0,07		2,88	
38	50,862	Isocaryophyllene	1408					0,07
39	51,793	*beta*-Caryophyllene	1417		0,06	0,09	4,95	tr
40	53,385	*alpha*-Caryophyllene	1452				0,71	
41	53,569	*allo*-Aromadendrene	1458				0,21	
42	54,578	*ar*-Curcumene	1479		0,14	0,11		
43	54,489	*beta*-Selinene	1489				0,46	
44	54,723	Bicyclogermacrene	1500				0,68	
45	54,765	*trans*-Muurola-4(14)5-diene	1493		0,13			
46	54,989	*alpha*-Zingiberene	1493		0,48	0,09		
47	55,267	*alpha*-Farnesene	1505		0,23			
48	55,598	*beta*-Sesquiphellandrene	1521		0,15	0,08		
49	58,146	*ar*-Turmerone	1668			0,26		
50	58,185	*alpha*-Turmerone				0,23		
51	58,625	*beta*-Turmerone				0,18		

Tab. 6 HS-EO: essential oils vapour phase composition (Adams, 2007 ; Maietti et al., in press). Compounds, identified on the basis of comparison with MS database spectra, retention index and pure reference chemicals, are listed in order of elution from a Varian FactorFour VF-5ms column. RI: Retention index.

A) 2.2.3 Avocado Oil and its total fatty acids composition: GC-FID, GC-MS analysis

The importance of unsaturated fatty acids in foods, functional foods and phytopharmaceuticals, is well known (Monstert et al., 2007; Henrotin et al., 1998; Lesequene et al., 2002; Wong et al., 2010). The classes of unsaturated fatty acids include MUFAs (monounsaturated fatty acids), for e.g. oleic acid (18:1) and PUFAs (polyunsaturated fatty acids) of the ω-6 and ω-3 families. The major dietary ω-6 PUFAs include linoleic (18:2), γ-linolenic (18:3), and arachidonic (20:4) acids, whereas major ω-3 PUFAs include α-linolenic (18:3), *eicosa*-pentaenoic (20:5), *docosa*-pentaenoic (22:5) and *docosa*-hexaenoic (22:6) acids. Oleic acid is a MUFA (monounsaturated fatty acid) present as a major constituent in avocado oil. Recent researches

indicate that MUFA reduces the levels of an oncogene called Her-2/neu (also known as erb-B-2) and it is as cell growth inhibitor in breast cancer tissue. High levels of Her-2/neu occur in more than one-fifth of breast cancer patients with highly aggressive tumors. In addition, it has been demonstrated that the presence of oleic acid positively interact with trastuzumab (herceptin) giving a contribute to the estimated life in much patients. The consumption of long chain ω-3 fatty acids is decreasing the ratio of ω-6 to ω-3 and hence it seems to be effective in treating coronary heart diseases, type-2 diabetes, hypertension, immune response disorders and mental illness. The required increase in PUFA intake can be achieved by consumption of PUFA-rich supplements. However, few problems are encountered in the production, transportation and storage of these fortified foods as PUFAs because of their sensitivity to oxidative deterioration (Logaraj et al., 2008).

The constituents were identified by comparing their GC retention times, and MS fragmentation patterns with pure compounds and by matching the MS fragmentation patterns and retention indices with the above mentioned mass spectra libraries and with those in databases. All results are summarized in table 7.

In general, the fatty acid profile is very similar to olive oil (i.e. high levels of oleic acid; www.aocs.org): in avocado oil extracted by hexane maceration (AHM) we have found 48% of oleic acid; in the supercritical CO_2 extracted sample (ASFE) the oleic acid content was 47,7% and 40,9% in the commercial one (ACO). Palmitoleic, other important MUFA, is present approximately in amount of 10% in AHM and ACO, but in minor amount (8%) in the ASFE sample instead. γ-Linolenic (ω-6) and the isomer α-linolenic (ω-3) acids are present in traces in all samples. As previously described, SFE extraction yielded a large amount of long-chain fatty acids, i.e. arachidic (C20:0) and lignoceric (C24:4), higher than in hexane maceration and cold press extraction. Furthermore, the percentages of PUFA is higher than in cold press and hexane maceration.

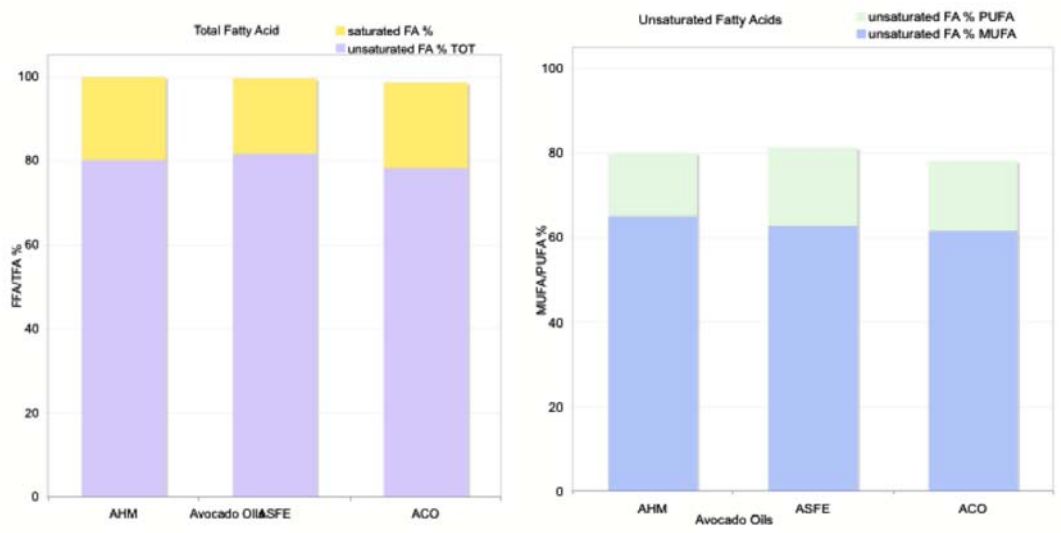
Comparative analysis of TFA fraction is shown in Graph 1 pointing out the differences in composition of each Avocado oils samples.

N	RT (min)	name	Cn:DB	AHM	ASFE	ACO
1	19.349	Palmitoleic ac.	C16:1	**10,23**	**8,06**	**9,72**
2	19.839	Palmitic ac.	C16:0	**18,05**	**16,28**	**19,67**
3	21.080	Margaroleic ac.	C17:1	0,16	0,13	0,07
4	21.255	Margaric ac.	C17:0	0,04	0,04	0,24
5	21,154	nd				0,12
6	21,494	nd				0,53
7	21.720	g-Linolenic ac.	C18:3n6	traces	traces	4,30
8	22.919	Linoleic ac.	C18:2cn6	**15,03**	**18,54**	**12,20**
9	22.974	a-Linolenic ac.	C18:3n3	traces	traces	0,04
10	23.188	Oleic	C18:1cn9	**48,02**	**47,67**	**40,93**
11	23.236	Elaidic ac.	C18:1n9t	**6,46**	**6,71**	**9,55**
12	23.559	Stearic ac.	C18:0	1,48	1,4	0,50
13	24.260	Ethyl Oleate	C18:1 etile	nd	0,26	0,79
14	26.559	cis-11-Eicosenoic	C20:1n9	0,32	0,35	0,57
15	27.006	Arachidic ac.	C20:0	0,21	0,17	nd
16	34.201	Lignoceric ac.	C24:0	nd	0,08	nd

sample	saturated FA %	unsaturated FA %		
		MUFA	PUFA	TOT
AHM	19,78	65,19	15,03	80,22
ASFE	17,97	62,83	18,54	81,72
ACO	20,41	61,63	16,54	78,17

Tab. 7 Total Fatty Acids (TFA) composition in Avocado Oils

A typical avocado oil is defined by M. Wang (2010). It is described to present 76% of monounsaturates (oleic and palmitoleic acids), 12% of polyunsaturates (linoleic and linolenic acids), and 12% of saturates (palmitic and stearic acids). All these values are given as percentage of fatty acid/total fatty acids ratio. Avocado oils tested yielded approximately 60-65% of MUFA and 15-18% of PUFA, differing by those described by Wang (2010).



Graph. 1 Composition in unsaturated and saturated Fatty Acids (FA) in Avocado Oils. Note: AHM, Avocado oil hexane macerated; ASFE, Avocado oil supercritical fluid extracted; ACO, Avocado commercial oil; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids; TFA total fatty acids.

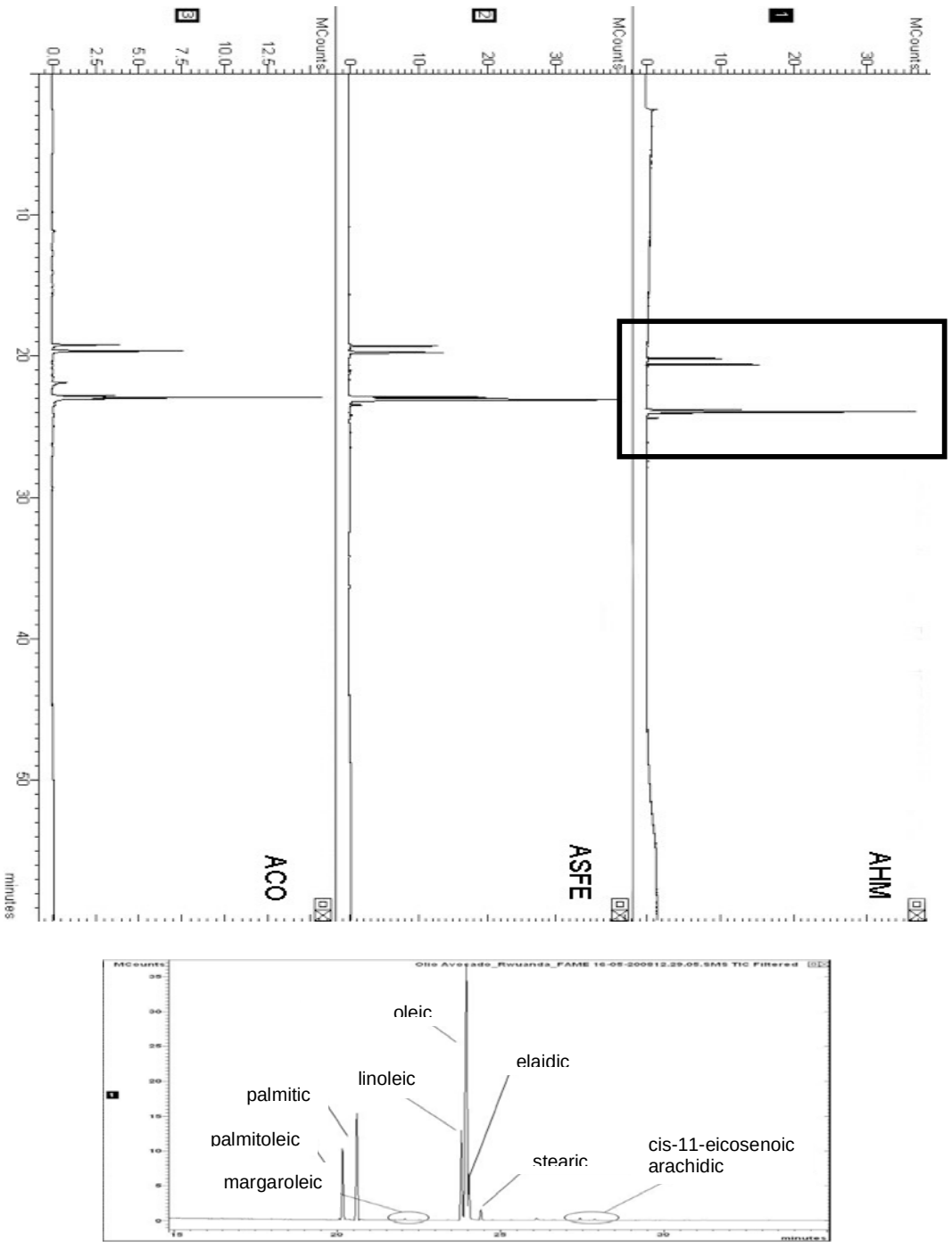


Fig. 35 Avocado oils Total Fatty Acids (TFA) composition. Analyses have been performed with comparison between mass spectra, retention times, libraries and literature data. Note: AHM, Avocado oil hexane macerated; ASFE, Avocado oil supercritical fluid extracted; ACO, Avocado commercial oil.

A) 2.2.4 Analysis for Unsaponifiable Fraction (UF): GC-FID, GC-MS

The unsaponifiable fraction (UF), after saponification with an alkaline hydroxide and extraction by a specified solvent, for e.g. methanol, remains non-volatile under the defined conditions of test (i.e. sterols, higher aliphatic alcohols, tocopherols, pigments) (Dieffenbacher, Pocklington, 1992). UF has a pharmaceutical interest, especially that of avocado oil, well-known in literature as adjuvant in the treatment of connective tissue diseases (Werman, Neeman 1987). In general, UF of Avocado Oils is constituted by hydrocarbons (HC), triglycerides (traces), tocopherols, D5-sterols and D7-sterols (Lozano et al., 1993). In the present work the UF was evaluated in oil obtained through hexane (AHM) and SFE extraction (ASFE), compared with a cold pressed commercial oil (ACO). Supercritical CO_2 extraction yielded an oil with higher unsaponifiable fraction (3,32%) if compared with hexane maceration, but it has been detected in lower amount than that detectable in commercial sample, known to contains very high quantity of UF (8,52%) – data are reported in tab XY.

sample	UF g/100g oil
AHM	2,14
ASFE	3,32
ACO	8,52

Tab. 8 Yields of unsaponifiable extraction. Note: AHM, Avocado oil hexane macerated; ASFE, Avocado oil supercritical fluid extracted; ACO, Avocado commercial oil.

Qualitative analyses have been conduced with comparison between mass spectra, retention times, libraries and literature data (tab 9).

N	RT (min)	name	AHM	ASFE	ACO
				Area (%)	
1	34.242	Squalen	**12,66**	**14,57**	2,04
2	35.333	HC			2,90
3	36.134	*alfa*-sitosterol			**15,95**
4	37.692	nd			1,42
5	37.926	HC			2,18
6	39.678	Campesterol	4,84	**6,14**	**7,25**
7	40.820	*beta*-Sitosterol	**48,80**	**63,07**	**41,44**
8	40.969	D-5-avenasterol	**6,01**	0,82	5,46
9	41.507	Gramisterol	4,87	3,16	3,16
10	41.748	Cycloartenol	3,93		5,32
11	42.583	isomer-24-methylenecycloartenol	4,63	2,94	2,91
12	43.207	Citrostadienol	**14,27**	**9,31**	**9,97**

Tab. 9 GCMS analyses of unsaponifiable of avocado oils coming from: hexane maceration (AHM) and supercritical carbondioxide extraction (ASFE), in comparison with a commercial sample (ACO).

Unsaponifiable gas chromatographic analysis evidenced the presence of phytosterols in large amount, Avocado oil is suitable for preventing human body from accumulating the undesirable low-density lipoprotein (LDL) cholesterol and promoting healthy high-density lipoprotein (HDL), which is beneficial to the heart. Studies also prove that the presence of β-sitosterol in avocado oil helps in relieving the symptoms of prostate enlargement, besides lowering the cholesterol production (Logaraj et al., 2008). Plant sterols occur as free sterols, steryl-esters, steryl-glycosides and and acylated steryl glycosides (Toivo et al., 2001). Therefore, the sample preparation procedure for total sterol determination includes sterols from all possible conjugates; in fact, the most common approach for determination of sterols in foods and plants involves extraction of the total lipid fraction followed by saponifcation, extraction of the non-saponifable matter, possible cleanup of the extract, derivatization of the sterols, and separation and quantification of the sterol derivatives by cromatographyc strategies (Toivo et al., 2001).

β-sitosterol is the most common phytosterol contained in avocado extracts: 63% in ASFE, 49% in AHM and 41% in ACO. The analysis evidenced also the presence of other triterpenoids alcohols, such as cycloartenol and citrostadienol known has part of healthful lipid constituents (Robbins et al., 2011). The presence of campesterol is similar in avocado commercial oil and

Avocado oil extracted with supercritical carbon dioxide (6% and 7% respectively); it is moreover higher than in the hexane-extracted oil. It is however notable the large amount of Δ-5-avenasterol in the AHM sample, comparable with those in commercial oil (6,0% and 5,5% respectively).

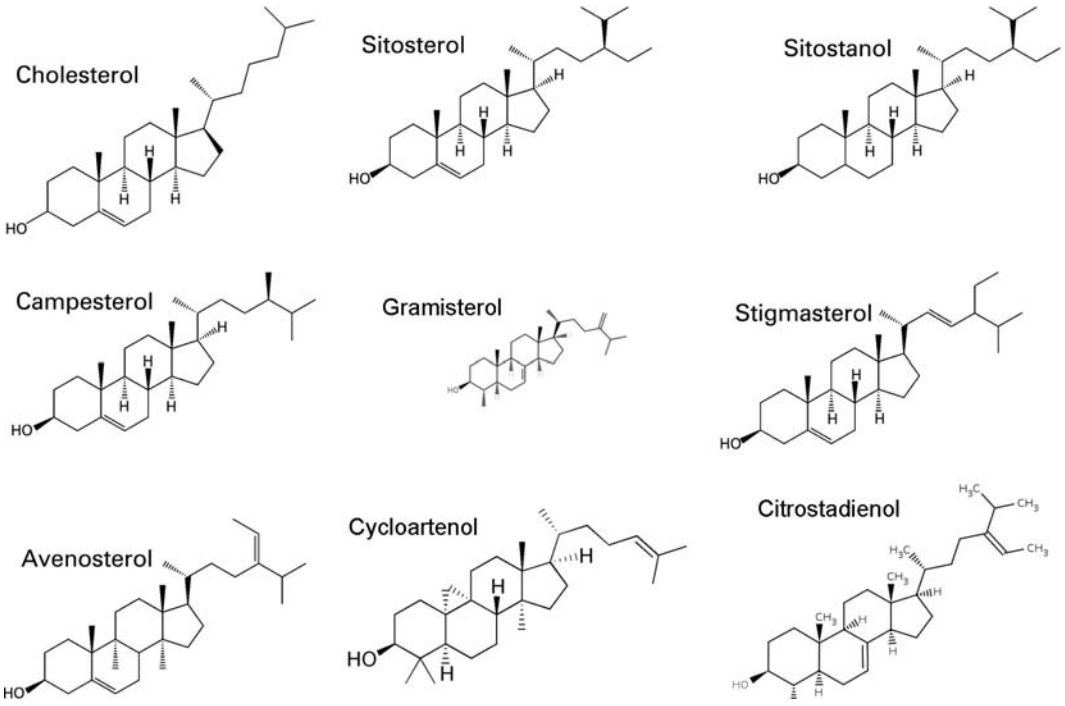


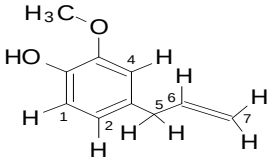
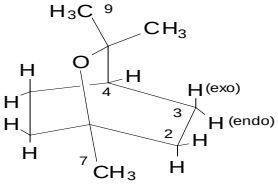
Fig. 36 Phytosterol occurring in avocado oils

A) 2.3 NMR Spectroscopy:

Different chromatographic and spectroscopic methods are currently available for fingerprinting natural derived products and raw material both for quality definition and for fraud detection. In fact, the characterization of complex mixtures of volatile compounds is usually achieved through high-resolution chromatographic or hyphenated techniques as mass spectrometry (MS), enantiomeric and comprehensive two-dimensional (2D) gas chromatography/mass spectrometry (Enantio-GC; GC/GC-MS). During the last decade, high-resolution nuclear magnetic resonance (NMR) emerged as a powerful tool for the fingerprinting of natural extracts, including terpenes, sesquiterpenes, and other volatile compounds, and for assessing the quality of raw materials for food, herbal, and pharmaceutical industries (Guerrini et al., 2011). In the present work ^{1}H-NMR spectroscopic strategies had been employed confirming the presence of the main compound detected by GC-

MS or (HP)-TLC both for essential oils and avocado oils and extracts, and the chemical shifts for identified constituents were assigned (see table 11).

Essential Oils: chemical shifts assignments and multiplicity of mayor constituents identified in essential oil of *O. micranthum, C. citratus, C. longa and Z. officinale* in ^{1}H-NMR spectrum fingerprinting are reported below (tab 11 and fig. 37 to 40). In Basil EO fingerprinting ^{1}HNMR, most significant zone in ^{1}HNMR spectra are in the 5,0 – 7,0 ppm range (aromatic proton of eugenol); the characteristic resonance of the metoxy gruoup of Eugenol is evident at 3,87 ppm followed by the doublet with typical costant coupling of the double bond protons at 3,32 ppm. Signals of 1,8-Cineole (Eucalyptol) are evident in basil EO thanks to its aboundance. The table below showed an example of fingerprinting determinations of the major occurring compounds in EO. Chemical shifts are compared with those in “sdbs database” and standards purchased from Sigma-Aldrich. In *C. citratus* EO ^{1}HNMR two signals at 9,8 – 10,0 ppm corresponding to the aldehydic protons of geranial and neral are very notable. Significant zones in ^{1}HNMR of Yerba luisa EO are in the 4,0 – 6,0 ppm range, where the multiplets of citral (neral and geranial), geraniol and citronellal, and the doulet of geraniol are clearly visible. In the 1,0 – 2,8 ppm range signals attributed at methyl group of citral, geraniol and geranial are overlapped (sdbs databes, Sigma-Aldrich database, Guerrini et al., 2001). For *C. longa* essential, oil characteristics signals can be attributed at H aromatic of *ar*-turmerone at 6,00 – 7,02 ppm range, other signals of α,β-turmerone are overlapped in ranging zone 1,0-3,0 ppm (Yue et al., 2011; Hoi-Shon Lee et al., 2003). Also in *Z. officinale* ^{1}HNMR fingerprinting, aldehydic protons of geranial and neral at round 10 ppm, as the aromatic of *ar*-Curcumene and *ar*-turmerone at 7,1 ppm are clearly visible. Signals between 4,4 – 6,0 ppm range can be attributed to citral, zingiberene and camphene signals. Signals overlapping makes difficult the attribution between 0,8 ppm and 2,8 ppm. However, signals from terminal methyl groups of camphene and citral can be identified at about 1,0 - 1,7 ppm and it is evident the characteristic singlet of eucalyptol methylenic group in 7 position at 1,0 ppm (Bleeker et al. 2011, sdbs database, Sigma-Aldrich database).

Compound	Assignment	δ (H) [ppm]	(multiplicity) [Hz]
Eugenol (i)			
	H ─ C (1)	6,84	(d, J = 8,40)
	H ─ C (2,4)	6,67 - 6,60	(m, 2H)
	H ─ C (6)	5,90 - 6,00	(ψdd J_{Htrans} = 17,04, J_{Hcis} = 10,15)
	H ─ O	5,5	(s)
	H ─ C ($7_{cis, trans}$)	5,03 - 5,10	(m, 2H)
	CH_3 metoxy gruop	3,87	(s, 3H)
	CH_2 (5)	3,32	(d, J = 6,64)
1,8-Cineole (ii)			
	H ─ C (3 exo)	2,02	(m, 2H)
	H ─ C (2 exo)	1,66	(m, 2H)
	H ─ C (3 endo)	1,48	(m, 4H)
	H ─ C (4)	1,43	(m, 1H)
	CH_3 (9 methyl)	1,24	(s, 6H)
	CH_3 (7 methyl)	1,05	(s, 3H)

Tab. 11 Chemical shift of main compounds occurring in *O. micranthum* essential oil. Note: s, singlet; d, doublet; m, multiplet; ψdd, pseudo double doublet.

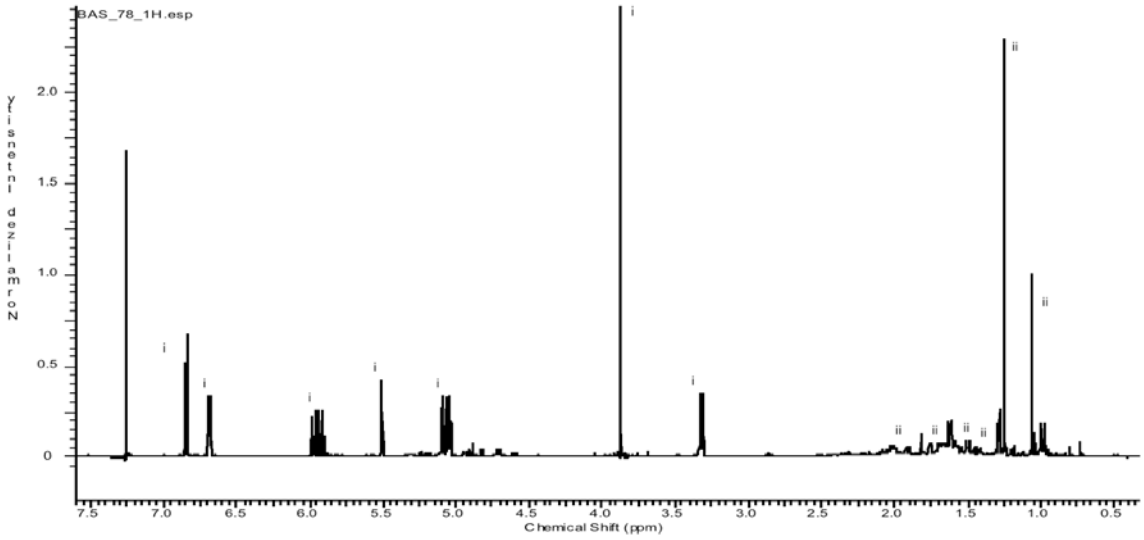


Fig. 37 ^{1}H-NMR of Amazonian Basil essential oil. Note: i: Eugenol signals; ii, 1.8-Cineole signals.

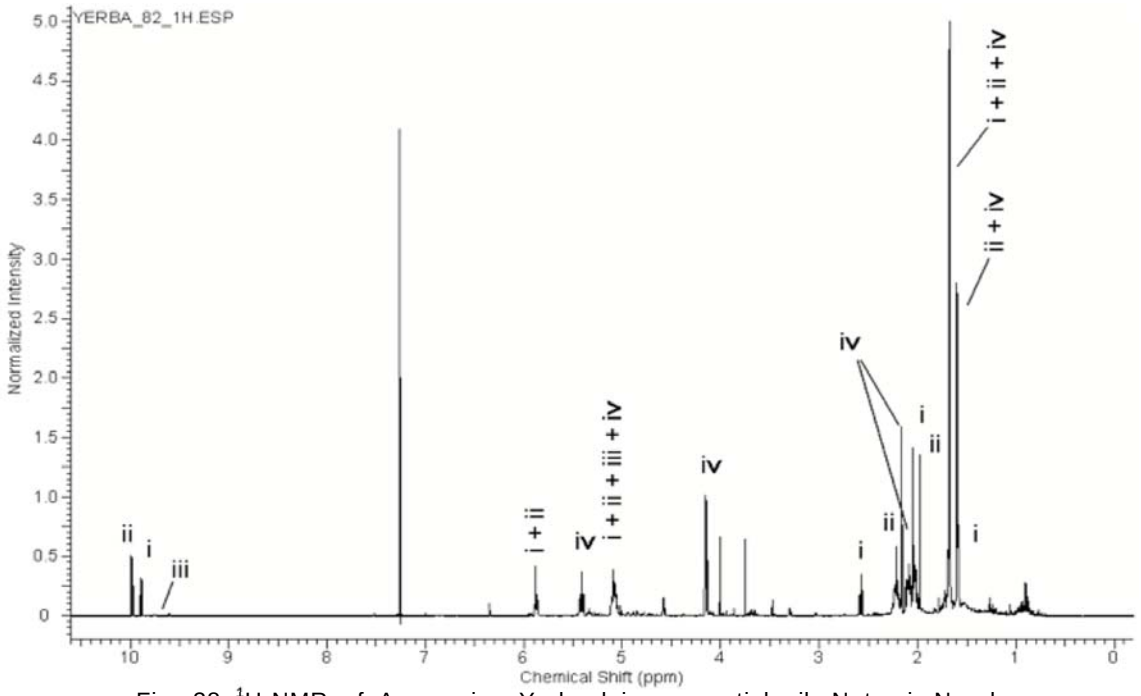


Fig. 38 [1]H-NMR of Amazonian Yerba luisa essential oil. Note: i: Neral signals; ii: geranial, iii: citronellal, iv geraniol, v citral + neral + geraniol.

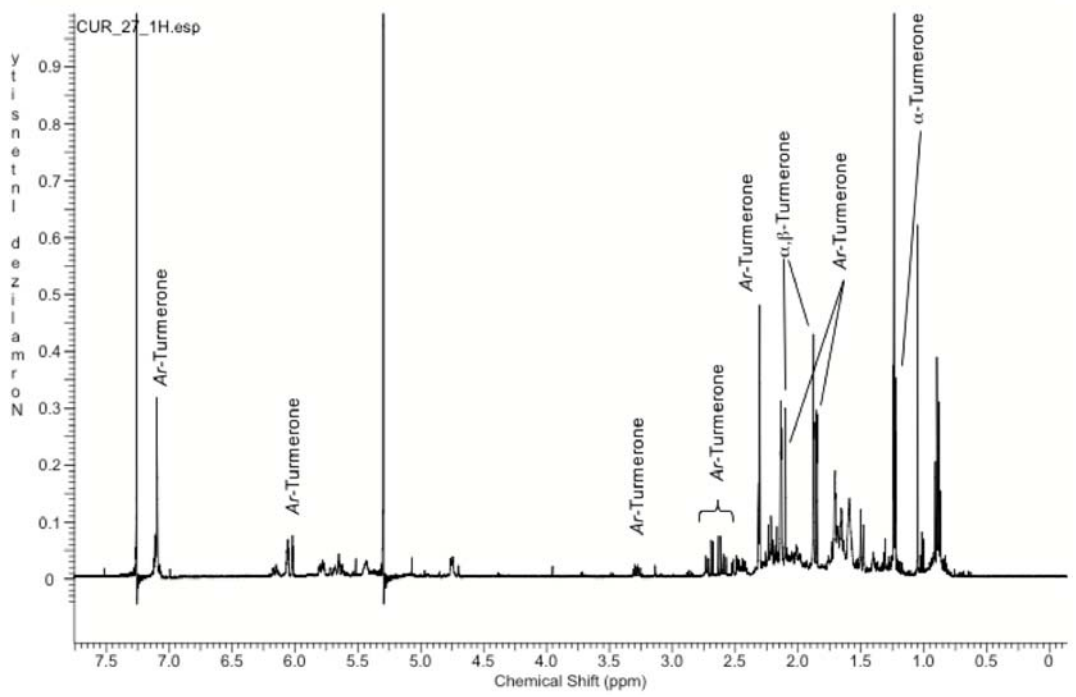


Fig. 39 [1]H-NMR of Amazonian Curcuma essential oil.

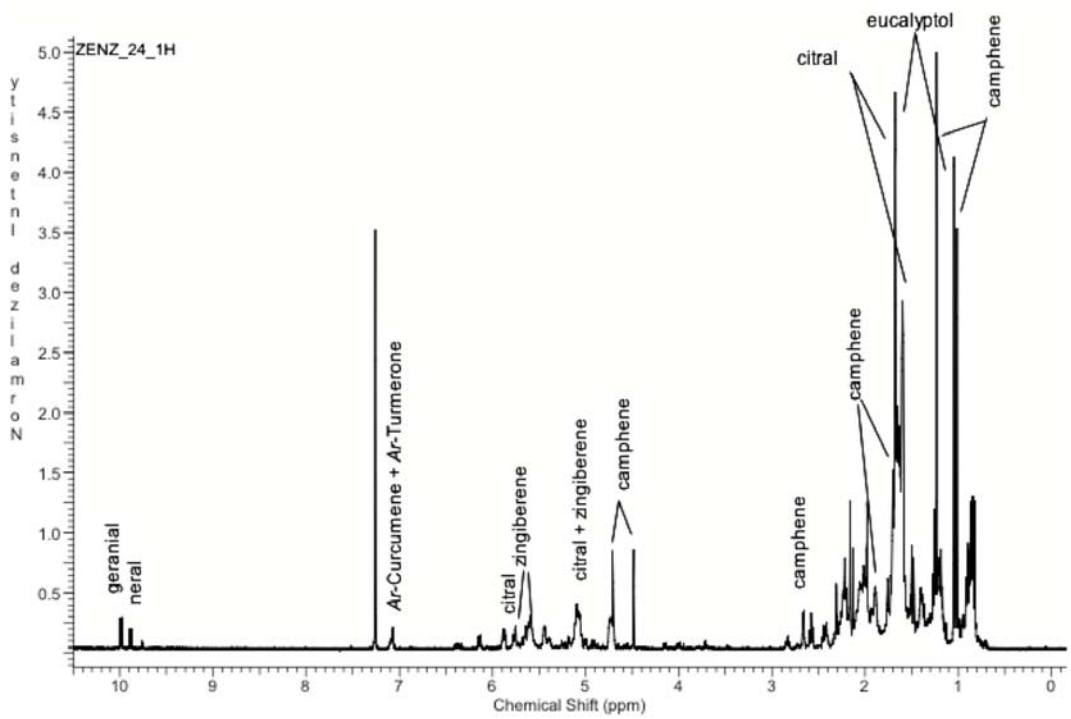
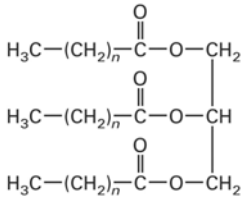


Fig. 40 ^{1}H-NMR of Amazonian Ginger essential oil.

Avocado Oils and Extracts: ^{1}H-NMR fingerprinting of Avocado oils shows a typical signals pattern of tracylglycerol esters. Figure 41 shows ^{1}H NMR spectra of Avocado Oils, i.e. ASFE, AHM, ACO.

The ^{1}HNMR spectrum of the triacylglycerol esters of fatty acids, shows the peaks for the usaturated protons of the longh chain at about 5.3 ppm (multiplets) and two distinct absorptions (4.2 and 4.4 ppm; both are doublets of doublets) of the glycerol CH_2 protons. These assignments have been described in the literature (Lie Ken Jie and Lam, 1995; Sacchi et al., 1997) where also the correlation of these signals to the fatty acid chain linked to the glycerol moiety is discussed. The CH-OOCR protons for the triacylglycerols of fatty acids are detected at 5.2-5.4 ppm in $CDCl_3$. Signals of olefinic protons are visibile at about 5,3 ppm; allylic protons (of linoleic and linolenic) FA give instead signals about at 2,8 ppm. The peak of the methyl group in α to glyceride carbonyl group is detectable at about 2,3 ppm. Typical signals of an ω3 fatty acids, followed by methyl groups in β to the carbonilc is detectable at about 2,0 ppm, overlapped to protons signals of unsaturated fatty acid chains.



Then acylic chains protons have chemical shifts at about 1,3 ppm, and terminal methyl groups at 0,8 ppm.

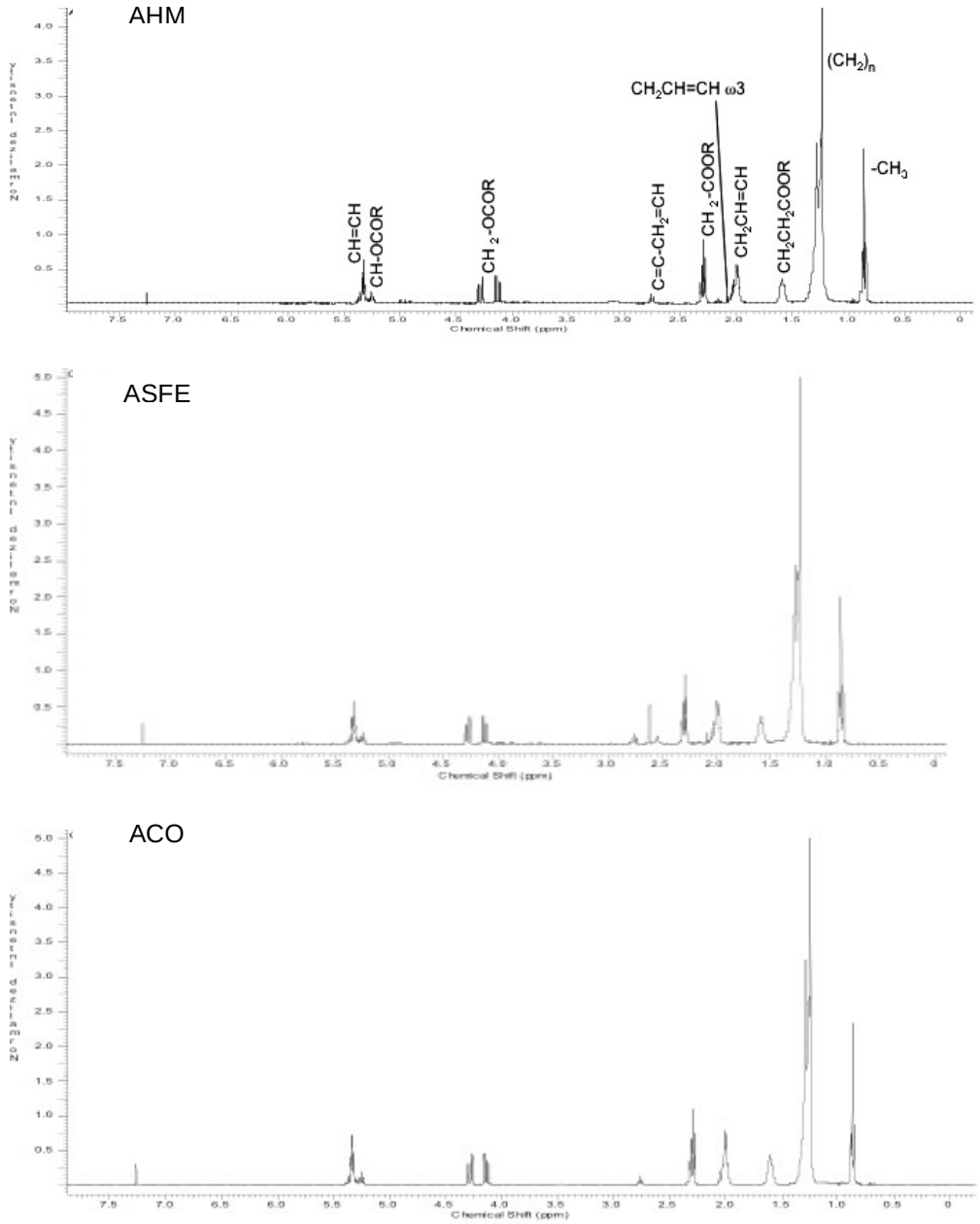


Fig. 41 Chemical shifts of main compounds occurring in avocado oils and extracts. Note: AHM, Avocado oil hexane macerated; ASFE, Avocado oil supercritical fluid extracted; ACO, Avocado commercial oil.

A) 2.4 Polyphenols in Avocado oil samples (AHM, ASFE, ACO) and methanolic extract (AMM): (HP)TLC and Spectrophotometric assays.

In this study, several strategies to investigate total polyphenols content in avocado pulp oils and extract have been performed. At first, TLC strategy for qualitative investigation of main flavonoids, coumarins and proantocyanidins has been processed. Then, a spectrophotometric investigation (i.e. Folin-Ciocalteu, Total Flavoinds and Total Procyanidins methods, see below) allowed to quantify the total phenolic content. Both the results have been related to biological activities checked (see page XY): in fact antioxidant activity of natural compounds such as phenols, together with tocopherols and carotenoids (also checked in Avocado) are well-know (Garcia-Alonso et al., 2004). Just because of their potential importance as natural antioxidants, phenols are also interesting as possible alternative to synthetic ones for foods and healthy industry (Garcia-Alonso et al., 2004). However, recent views about antioxidant capacity of Avocado fruits have been reported by related literature, pointing out the pulp as the less bioactive part (Wang et al., 2010).

These strategies have been currently applied as bioactivity screening in our labs to investigate natural antioxidant sources as possible affective ingredients and/or phytopharmaceuticals (Sacchetti et al., 106° Congresso SBI Onlus 2011).

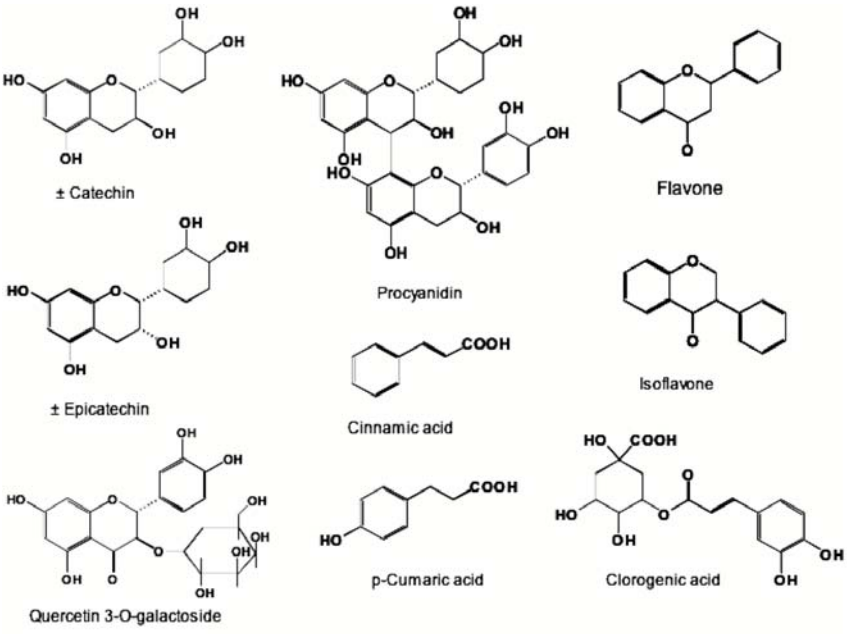


Fig. 42 More common phenolic compounds occurring in Avocado fruits.

A) 2.4.1 (HP)TLC analyses

First step of investigation about phenolic compounds in Rwandese avocado pulp extracts had been conduced by (HP)TLC following the method suggested by Wagner (Wagner, Bladt, 2009), with some modification as reported by Garcia-Alonso (2004) and Lee (2008). Since the high level of fatty acid present in avocado extracts (both oils and methanolic macerate) interferes with phenol detection, all the samples had been defatted (Lee et al., 2008), i.e. hydroalcoholic extract from commercial avocado oil (EIA.ACO), hexane extracted avocado oil (EIA.AHM), supercritical carbon dioxide avocado oil (EIA.ASFE), methanol macerate avocado pulp extract (EIA.AMM). The comparison with standards spotted on TLC allowed to confirm the presence of kaempherol only in the commercial sample, while scopoletin was ubiquitary in different concentration in all samples as it was possible to assume with reference to band size and intensity (fig 43). Our results are in accord with that reported by other Authors, which evidenced the scarce presence of phenolic compound in Avocado oils (unsaponifiable fraction), and lightly higher in seeds and peel extracts. These results are also obviously related to extraction strategies (more or less selective) (Garcia-Alonso et al., 2004; Rodríguez-Carpena et al., 2011; Ding et al., 2007).

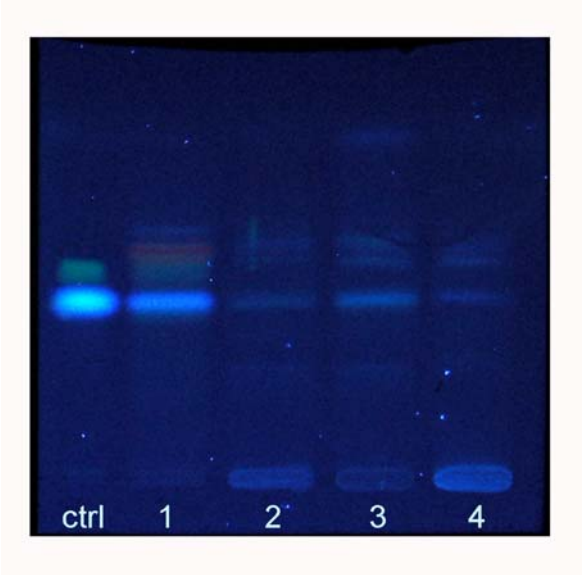


Fig. 43 (HP)TLC for the detection of coumarins and flavonoids. Standard used: kaempferol (green) and scopoletin (light blue) 10 μL of a 0,1 mg/ml solution in CH_2Cl_2; 1) defatted commercial avocado oil; 2) defatted hexane extracted avocado oil; 3) defatted supercritical carbon dioxide extracted avocado oil; 4) defatted methanol macerated avocado pulp extract (10 μL of a 60 mg/ml solution each one).

Proanthocyanidins represent a group of condensed flavan-3-ols that can be found in many plants, in most cases widely known and used as food supplements. Among these plant sources, the most known are for e.g. apples, cinnamon, cocoa beans, grape seed, grape skin, (Souquet et al., 1996) and red wines of *Vitis vinifera* (the common grape). Since their importance as functional chemicals and their increasing use, new plant sources are still investigated for their possible employ in the wide antioxidant market. The research about functional properties of these compounds is also opened to many other important bioactive implications, such as anticancer, anti-inflammatory and immunomodulating agents. As example of new plant sources studied to be employed as food supplements or ingredient in herbal products for their phenols content and for new related bioactivities, the following specie can be cited: green tea and black tea for the antiproliferation capacity of its phenolic fraction (i.e. anticancer plants) (Gupta et al., 2002); bilberry and tomatoes for its anti-inflammatory capacity (Muller et al., 2010); *Ecdysanthera utilis* as immunomodulatory agent (Lie-Chwen et al., 2002). This list to give a mere resume of the of the strong interest about this class of natural compounds. However equally important and subject of many research it is the re-discover of already known plant

sources for new health uses and herbal market proposals. This is one of the reasons that moved us to investigate our Avocado samples under this point of view, with the perspective to employ Avocado in functional formulations. Procianidins detection in Avocado pulp derivates have been carried out at first with TLC method: results are illustrated in figure 44. Procyanidins in avocado derivates has been detected in oligomeric form in accordance to what reported in literature (Wagner, Bladt, 2009; Wang et al., 2010)

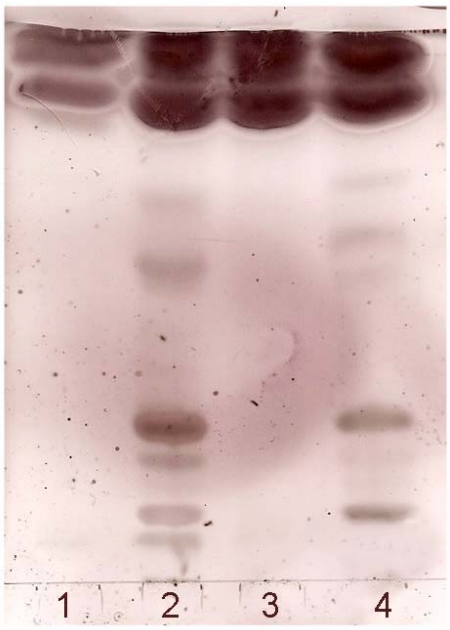
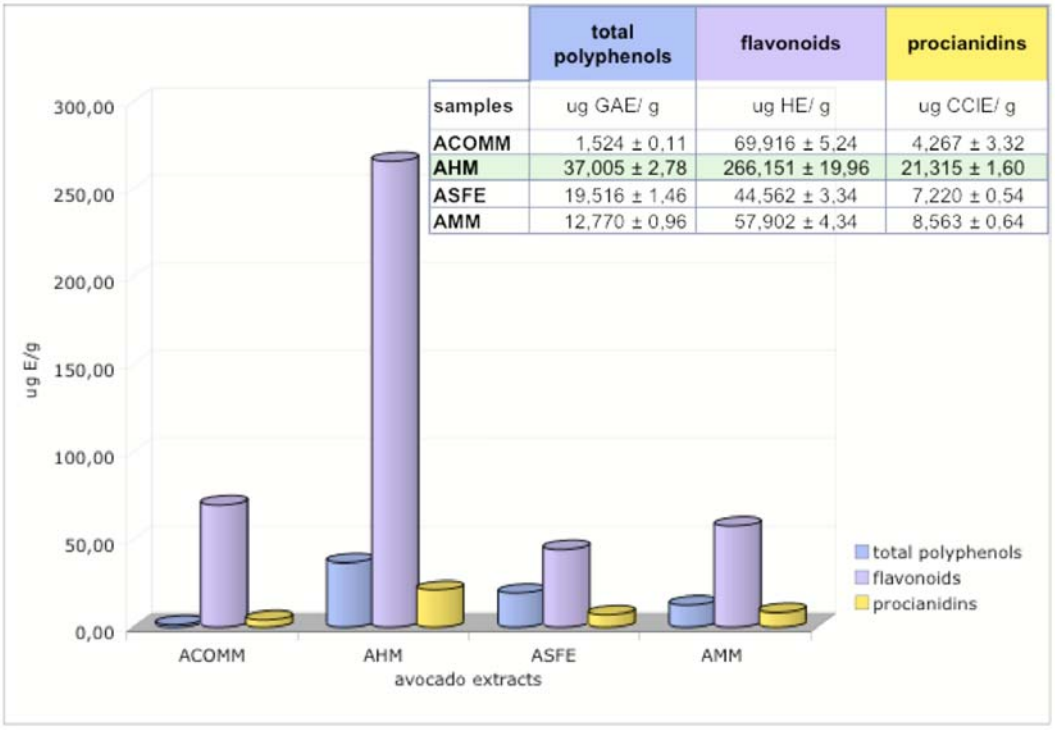


Fig. 44 Procyanidins in Avocado extracts. 1) EIA.ACO, defatted commercial avocado oil; 2) EIA.AHM, defatted hexane extracted avocado oil; 3) EIA.ASFE, defatted supercritical carbon dioxide extracted avocado oil; 4) EIA.AHM, defatted methanol macerated avocado pulp extract (10 µl of a 30 mg/ml solution each one). Rf 0.7-0.8, di- and trimeric procyanidins; Rf 0.5 – 0.7 tri- and tetrameric procyanidins; Rf range 0.05-0.5 tetra- and hexa polymeric procyanidins (wagner, Bladt, 2009).

A) 2.4.2 Spectrophotometric assays applied to determine total poliphenols in avocado oils and extracts

The amount of phenolic compounds in avocado derived products was higher in hexane extracted avocado oil (AHM), both in total polyphenols, flavonoids and procyanidins. Supercritical fluid extracted avocado oil (ASFE) showed medium amounts if compared with other Rwandese avocado derived products. Commercial sample (ACO) showed always phenol lowest amounts. All these results are reported in tab. 12. These results are original with reference to avocado oil, Rwandese in particular, since similar

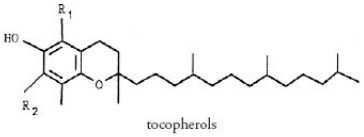
evidences have been already reported about the fresh pulp of different cultivars and varieties differing from that Africans, subject of the present PhD research (Wang et al., 2010).



Tab. 12 Graph. 2 Avocado samples total phenols investigation. Note: GAE, gallic acid equivalent; HE, hyperoside equivalent; CCIE, cyanidin chloride equivalents; ACO, Avocado commercial oil; AHM, Avocado hexane extracted oil; ASFE, Avocado supercritical carbondioxide extracted oil; AMM, Avocado methanol macerated extract.

A) 2.5 HPLC Analysis of Tocopherols

HPLC strategy has been adopted to check the presence Tocopherols in Avocado oils (i.e. AHE, ASFE, ACO) and Avocado extracts (AME).



tocopherols

R_1	R_2	
CH_3	CH_3	a-tocopherol (a- T)
CH_3	H	b-tocopherol (b- T)
H	CH_3	g-tocopherol (g- T)
H	H	d-tocopherol (d- T)

The tocopherols detected in our avocado samples were always in lower amount then that reported in literature, where the total tocopherol content had been estimated varying about 10,2-25,0 mg/100g (Lozano et al., 1993). Even if the saponification needed is known to cause tocopherol depletion, as other oxidizing operative condition (Sacchetti, Bruni, 2006), it is evident that the Rwandese Avocado oil examined revelled a poor tocopherol content, most probably linked to genetic characteristics, environmental, growing and processing condition. Moreover, the only tocopherol detected was the δ-one, in contrast to what reported in literature where the isomer detected were α-tocopherol (2,8 mg/100g) and γ-tocopherol (0,3mg/100g) (Lu et al., 2005). This results does not imply that other isomer are absent, above all because the HPLC analyses present matrix effect that interfered with their detection and peaks at lower retention times with the same UV spectra, probably due to depletion of tocopherols. These interferences could be due to the particular composition of the unsaponifiable fraction of our samples or to problems during analytical process; both the possibilities need to be further investigated. However, a part from δ-tocopherol, clearly separated and quantified, it could be suggested the presence of the α- β- γ isomers. To determine the relative amount of δ-tocopherol had been previously constructed a calibration curve of α-tocopherol and δ-tocopherol verifying the linearity of six concentrations from 1 to 0,001 mg/ml in hexane.

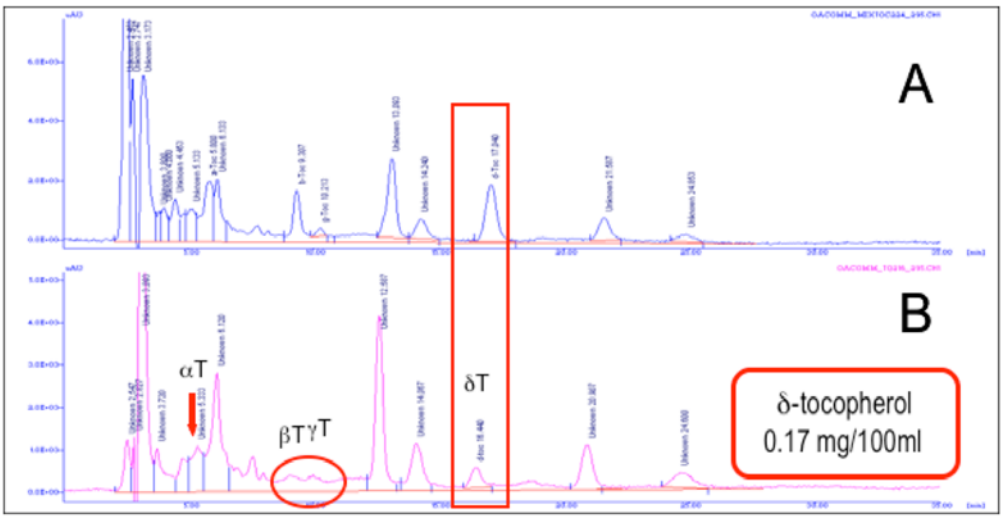


Fig. 44 HPLC Chromatogram of tocopherols analyses. Chromatogram A is referred to a HPLC analyse carried out adding an aliquot of tocopherols mixture as internal standard. Quantification of δ-tocopherols had been achieved indirectly with a calibration standard curve.

B) Bioactivities: Efficacy & safety screenings

Antioxidant properties, antimicrobial activity, mutagenic, antimutagenic capacity and toxicity were processed for essential oil samples, avocado oils and its derivates. All the bioactivities were performed, comparing all the data with those achieved with appropriate pure synthetic compounds and/or commercial *T. vulgaris* essential oil, in order to have positive control references with single compounds or comparable phytocomplexes reputed for their functional bioactivities. In fact, the use of a phytocomplex, known for its chemical and biological properties (e.g. thyme essential oil) as a positive results reference was particularly indicative of the real functional efficacy of a tested extract (e.g. *O. micranthum* eo, *Pelargonium capitatum* eo, Lavander eo, etc) (Sacchetti et al., 2005; Guerrini et al., 2011; Maietti et al., in press). Data reported for each assay are the average of three determinations of three independent experiments.

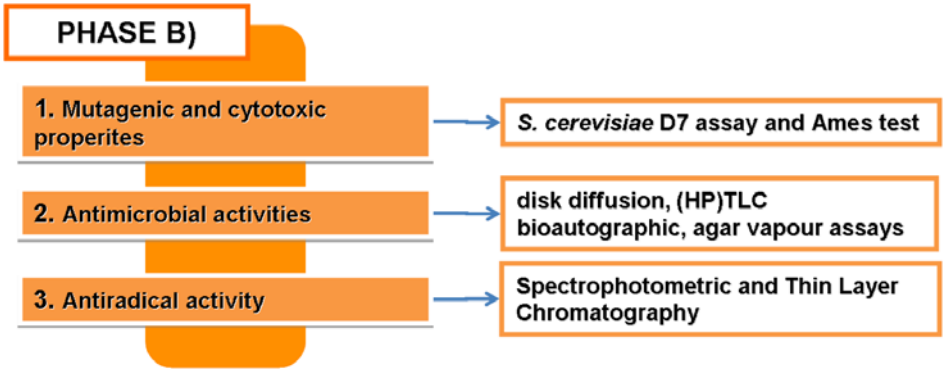


Fig. 45 research phase B diagram

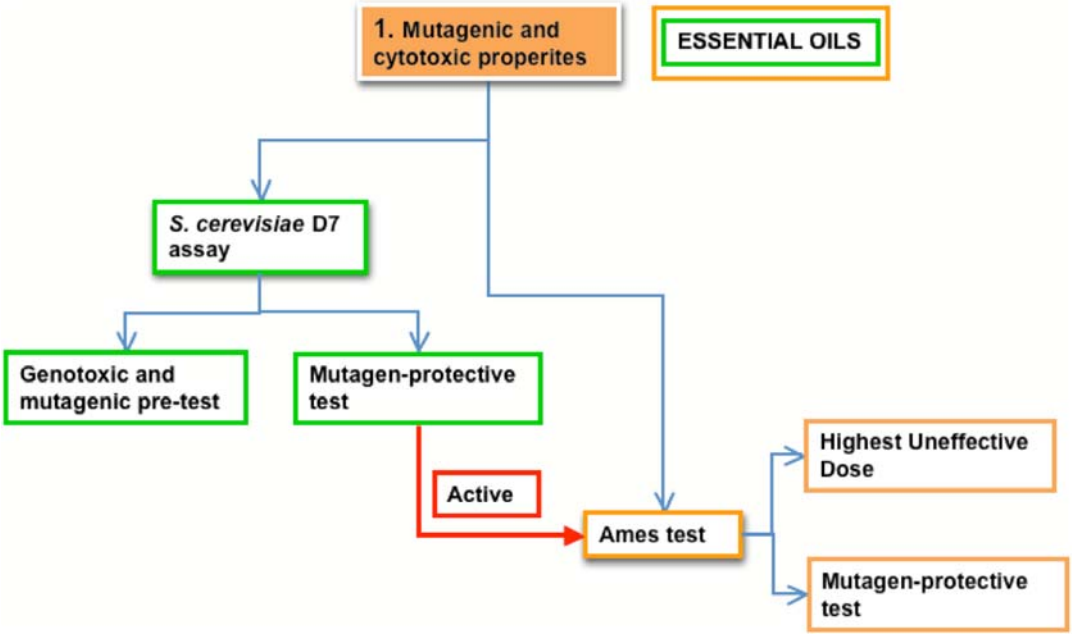


Fig. 1 mutagenic and cytotoxic investigations scheme performed on Amazonian Essential Oils.

B) 1 Mutagenic and mutagen-protective activity of Amazonian essential oils

B) 1.1 S. cerevisiae D7 test

We used the D7 strain of *S. cerevisiae* because it constitutes a rapid and low cost genetic model to investigate simultaneously genotoxic and mutagenic, or mutagen-protective effects of various single compounds and phytocomplexes. In the present PhD, this study was performed both to confirm the safety of *Z. officinale*, *C. longa*, *O. micranthum*, *C. citratus* essential oils, and to evaluate their possible protective effect.

By using plate incorporation pre-test employing *S. cerevisiae* the minimum cytotoxic concentration of each essential oils, with significant statistical effects of 10 % colony strength reduction, was previously assessed: 0,5 mg/plate in ginger eo; 1,0 mg/plate in curcuma EO; yerba luisa and basil EO show higher level of citotoxicity at 0,05 mg/plate and 0,1 mg/plate respectively. Control (thyme eo) shows similar citotoxity (0,05 mg/plate) to that of yerba luisa EO. Data are shown in tab. 13. Genotoxic effects and mutant colonies rate counts were not valuable due to the survival. In all the sample tested, no gene conversion (GCC) and reverse point mutation (MC)

effects were observed at non-cytotoxic concentrations, confirming the safety of these Amazonian essential oils.

mg/plate	GINGER Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6	CURCUMA Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6
DMSO	100,00 ± 9,02	0,75 ± 0,07	0,17 ± 0,02	100,00 ± 4,58	0,71 ± 0,07	0,16 ± 0,02
0,01	97,95 ± 9,82	0,70 ± 0,06	0,21 ± 0,02	98,43 ± 5,51	0,72 ± 0,06	0,19 ± 0,02
0,03	97,26 ± 9,87	0,74 ± 0,07	0,25 ± 0,02	96,47 ± 3,61	0,73 ± 0,07	0,20 ± 0,02
0,05	96,58 ± 8,72	0,78 ± 0,08	0,28 ± 0,03	96,08 ± 3,06	0,78 ± 0,06	0,24 ± 0,02
0,10	96,23 ± 8,14	0,82 ± 0,08	0,26 ± 0,03	95,69 ± 3,51	0,90 ± 0,07	0,29 ± 0,03
0,25	95,89 ± 7,57	0,79 ± 0,07	0,21 ± 0,02	91,76 ± 4,72	0,94 ± 0,08	0,26 ± 0,02
0,50	95,21 ± 6,43	0,76 ± 0,07	0,22 ± 0,02	89,90 ± 4,16	0,83 ± 0,06	0,21 ± 0,02
1,00	94,52 ± 6,29	0,72 ± 0,06	0,18 ± 0,02	90,98 ± 2,52	0,91 ± 0,07	0,17 ± 0,02
2,50	95,55 ± 6,63	0,68 ± 0,06	0,14 ± 0,01	92,94 ± 4,50	0,89 ± 0,08	0,21 ± 0,02
5,00	72,26 ± 6,81	0,71 ± 0,06	0,29 ± 0,03	93,73 ± 3,79	0,84 ± 0,06	0,25 ± 0,02
10,00	6,51 ± 2,08	0,53 ± 0,04	0,53 ± 0,04	54,76 ± 4,58	1,19 ± 0,09	0,38 ± 0,04

mg/plate	BASIL Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6	YERBA Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6
DMSO	100,00 ± 9,65	0,84 ± 0,06	0,42 ± 0,03	100,00 ± 9,65	0,70 ± 0,07	0,35 ± 0,03
0,01	100,35 ± 8,51	0,87 ± 0,07	0,45 ± 0,04	94,49 ± 9,74	0,86 ± 0,08	0,34 ± 0,03
0,03	100,70 ± 8,03	0,80 ± 0,07	0,49 ± 0,05	93,33 ± 9,16	0,90 ± 0,09	0,34 ± 0,04
0,05	98,60 ± 8,43	0,85 ± 0,08	0,53 ± 0,05	92,17 ± 9,00	0,88 ± 0,09	0,31 ± 0,03
0,10	92,63 ± 7,04	0,87 ± 0,09	0,49 ± 0,05	93,04 ± 9,13	0,72 ± 0,07	0,28 ± 0,03
0,25	92,28 ± 7,29	0,91 ± 0,09	0,46 ± 0,05	90,14 ± 8,73	0,74 ± 0,07	0,29 ± 0,03
0,50	90,88 ± 7,63	1,16 ± 0,11	0,31 ± 0,03	82,90 ± 6,66	0,87 ± 0,08	0,42 ± 0,04
1,00	82,81 ± 6,37	1,14 ± 0,12	0,25 ± 0,03	68,41 ± 5,96	0,81 ± 0,07	0,47 ± 0,05
2,50	71,23 ± 6,20	1,13 ± 0,10	0,20 ± 0,02	51,01 ± 5,86	0,68 ± 0,06	0,40 ± 0,04
5,00	2,81 ± 0,58	0,00 ± 0,00	0,15 ± 0,02	26,96 ± 3,89	0,43 ± 0,04	0,22 ± 0,02
10,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00

mg/plate	THYME Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6
DMSO	100,00 ± 9,75	0,75 ± 0,07	0,17 ± 0,02
0,01	93,85 ± 9,48	0,86 ± 0,08	0,24 ± 0,03
0,03	93,02 ± 8,82	0,90 ± 0,09	0,18 ± 0,02
0,05	99,44 ± 9,14	0,87 ± 0,08	0,20 ± 0,03
0,10	96,93 ± 8,13	0,81 ± 0,08	0,29 ± 0,03
0,25	91,06 ± 8,53	0,83 ± 0,07	0,34 ± 0,03
0,50	87,71 ± 6,76	0,83 ± 0,08	0,35 ± 0,04
1,00	67,04 ± 6,56	0,96 ± 0,09	0,21 ± 0,03
2,50	19,27 ± 2,46	0,87 ± 0,08	0,14 ± 0,04
5,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00
10,00	0,00 ± 0,00	0,00 ± 0,00	0,00 ± 0,00

Tab. 13 Cytotoxic and mutagenic pre-test on ginger (*Z. Officinale*), curcuma (*C. longa*), Amazonian basil (*O. micranthum*), yerba luisa (*C. citratus*) EOs, in comparison with commercial thyme (*T. vulgaris*) EO. Note: GCC: gene conversion colony; MC, point mutant colony.

In light of these evidences, inhibitory effects of each essential oils have been evaluated on GCC and MC against direct acting mutagen ethyl methane sulphonate (EMS). The cytotoxicity rate could be change due to the effects of EMS in the media; in fact new minimum cytotoxic concentration were observed both in curcuma EO (2,5 mg/plate) and the control thyme EO (2,5 mg/plate). As previously reported no genoprotective effects could be

assessed where is shown citotoxic action. Antigenotoxic effect of protective chemicals against EMS in *S. cerevisiae* D7 has been explained as the results of protective molecule binding DNA and thus preventing DNA alkylation (Rossi et al., 2011). In general, antigenotoxic effects were observed in all the sample tested (see tab. 14); in particular Anazonian basil EO showed an interesting reduction of colony growth in reverse point mutation plates (MC) yet at low concentrations (27% at 0,01 mg/plate) which persist until the concentration of 0,5 mg/plate. Other essential oils shown modest genoprotective actions at higer concentration than in Amazonian basil EO: curcuma eo, at 1,0 mg/plate was able to protect genoma against point reverse mutation about for 37%; ginger eo started to inhibit growth colony (about 25 %) at 0,5 mg/plate showing dose dependent effects until 2,5 mg/plate (29 % of colony growth inhibition). Yerba luisa EO also showed modest activity on reverse point mutation (MC): at 0,1 mg/plate and 0,25 mg/plate was able to inhibit growing colony for about 15,6 % and 18,3 % (dose dependent effect); on the contrary, it was the only sample which showed protective actions on gene conversion plate (GCC) giving results on 0,1 mg/plate for 71,8 % and 0,25 mg/plate for 75,4%.

mg/plate	EMS (mg/plate)	GINGER Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6	CURCUMA Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6
DMSO	0,01	100,00 ± 7,06	12,66 ± 1,50	40,82 ± 3,83	100,00 ± 7,50	5,77 ± 0,25	29,05 ± 3,79
0,01	0,01	102,22 ± 6,66	12,45 ± 1,64	40,56 ± 3,68	100,61 ± 7,23	5,79 ± 0,26	28,75 ± 3,93
0,03	0,01	100,95 ± 6,51	12,51 ± 1,55	40,75 ± 3,25	99,69 ± 6,66	5,72 ± 0,27	28,86 ± 3,07
0,05	0,01	99,68 ± 6,65	12,60 ± 1,56	40,95 ± 3,81	98,77 ± 6,51	5,68 ± 0,28	28,98 ± 2,94
0,10	0,01	101,27 ± 6,89	12,88 ± 1,87	40,03 ± 3,46	97,85 ± 6,39	5,61 ± 0,26	28,59 ± 2,24
0,25	0,01	102,22 ± 6,77	12,85 ± 1,78	39,35 ± 3,16	99,39 ± 6,51	5,77 ± 0,27	28,99 ± 2,84
0,50	0,01	98,46 ± 6,35	11,94 ± 1,29	**30,85 ± 2,43**	99,08 ± 6,42	6,07 ± 0,34	29,57 ± 2,42
1,00	0,01	99,68 ± 5,66	11,78 ± 1,18	**29,65 ± 2,06**	93,56 ± 6,79	5,61 ± 0,33	**18,30 ± 1,48**
2,50	0,01	96,52 ± 5,63	11,25 ± 1,15	**28,92 ± 2,65**	77,91 ± 5,04	5,20 ± 0,28	16,61 ± 1,86
5,00	0,01	69,94 ± 4,62	13,94 ± 1,24	29,50 ± 2,82	61,66 ± 4,15	6,72 ± 0,31	17,16 ± 1,22
10,00	0,01	7,28 ± 0,58	13,91 ± 1,53	5,22 ± 0,48	21,47 ± 3,81	5,29 ± 0,30	8,14 ± 0,74

mg/plate	EMS (mg/plate)	BASIL Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6	YERBA Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6
DMSO	0,01	100,00 ± 7,29	3,47 ± 0,17	32,06 ± 3,15	100,00 ± 8,08	7,98 ± 0,51	25,52 ± 2,16
0,01	0,01	101,91 ± 7,02	3,56 ± 0,18	**23,41 ± 2,34**	101,44 ± 8,01	7,79 ± 0,46	25,41 ± 2,22
0,03	0,01	97,71 ± 6,86	3,36 ± 0,17	**24,65 ± 2,46**	100,72 ± 9,03	7,38 ± 0,49	24,09 ± 2,43
0,05	0,01	100,00 ± 6,51	3,02 ± 0,15	**25,42 ± 2,55**	102,53 ± 8,31	7,71 ± 0,61	25,42 ± 2,54
0,10	0,01	98,85 ± 6,41	3,17 ± 0,16	**24,52 ± 2,66**	99,28 ± 8,74	**2,25 ± 0,21**	**21,53 ± 2,11**
0,25	0,01	96,56 ± 6,31	3,40 ± 0,16	**22,21 ± 2,41**	94,58 ± 8,29	**1,96 ± 0,15**	**20,83 ± 2,10**
0,50	0,01	93,51 ± 6,13	3,51 ± 0,15	**23,35 ± 2,14**	75,09 ± 7,57	1,20 ± 0,12	19,86 ± 1,82
1,00	0,01	89,31 ± 5,44	3,63 ± 0,16	23,12 ± 2,17	72,92 ± 5,86	0,94 ± 0,09	19,16 ± 1,79
2,50	0,01	85,88 ± 4,93	2,80 ± 0,12	12,12 ± 1,93	65,70 ± 4,93	0,71 ± 0,07	16,87 ± 1,28
5,00	0,01	55,34 ± 4,51	0,76 ± 0,05	9,31 ± 1,42	24,21 ± 4,36	0,58 ± 0,05	6,67 ± 0,82
10,00	0,01	9,54 ± 0,58	0,40 ± 0,02	9,20 ± 1,53	6,64 ± 1,53	0,53 ± 0,03	2,11 ± 0,23

mg/plate	EMS (mg/plate)	THYME Survivals (%)	GCC colonies / 10^5	MC colonies / 10^6
DMSO	0,01	100,00 ± 8,08	9,66 ± 0,51	24,48 ± 2,16
0,01	0,01	105,46 ± 8,01	10,63 ± 0,46	26,81 ± 2,62
0,03	0,01	102,59 ± 9,03	10,20 ± 0,49	28,80 ± 2,73
0,05	0,01	98,28 ± 8,31	10,76 ± 0,61	27,25 ± 2,67
0,10	0,01	91,09 ± 8,74	10,07 ± 0,21	27,10 ± 2,41
0,25	0,01	82,76 ± 8,29	11,08 ± 0,15	26,25 ± 2,39
0,50	0,01	81,03 ± 7,61	10,89 ± 0,12	22,20 ± 1,96
1,00	0,01	66,67 ± 5,86	11,59 ± 0,09	10,22 ± 1,09
2,50	0,01	41,47 ± 4,93	8,48 ± 0,07	7,38 ± 0,88
5,00	0,01	18,68 ± 1,53	6,46 ± 0,05	5,54 ± 0,52
10,00	0,01	2,30 ± 0,00	1,25 ± 0,03	2,50 ± 0,23

Tab. 14 Effect of survival and antigenotoxicity (mutagenesis and gene conversion) in *Saccharomyces cerevisiae* D7 assay from treatment with Ecuadorian essential oils (*Z. officinale*, *C. longa*, *O. micranthum*, *C. citratus*) compared with commercial *T. vulgaris* essential oil. Genotoxic agent employed was ethyl metan sulfonate (EMS) at the concentration of 0,01 mg/plate. Note: circled in red, genoprotective effects; GCC, gene conversion; MC, reverse point mutation.

B) 1.2 Ames test

In light of results obtained with *S. cerevisiae* D7 genoprotective test, mutagen-protective properties, against point and frame shift mutations, of each essential oils has been assayed also on *S. typhimurium* tester strains TA98 and TA100 either with or without S9 metabolic activation.

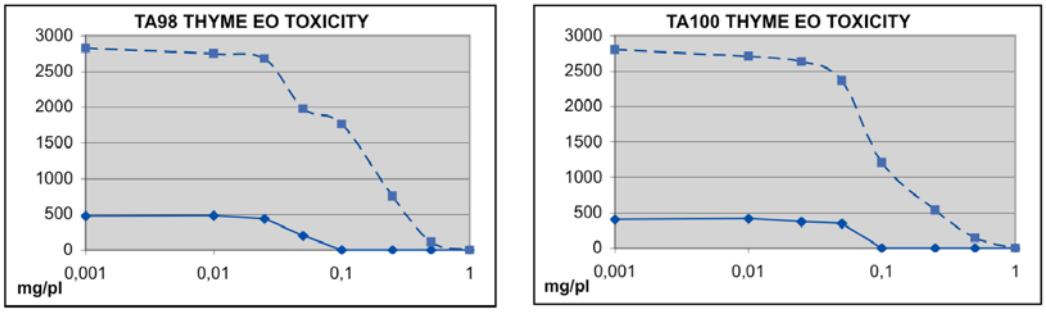
Strains		**Direct acting mutagen**	**Mutation**
S. typhimurium TA98	-S9	2-nitrofluorene (NF)	*frame shift mutation*
	+S9	2-aminoanthracene (AA)	
S. typhimurium TA100	-S9	Sodium azide (NaN_3)	*reverse point mutation*
	+S9	2-aminoanthracene (AA)	

At first, Highest Uneffective Dose (HUD) for toxic effects with or without metabolic activation has been settled for each Amazonian OE (see graphics 3a, 3b, 3c). In particular, according to *t*-test, for TA98 strain Yerba luisa EO demonstrated lowest toxic effects showing HUD at 0,1 mg/plate either with and without metabolic activation; Curcuma and Ginger EOs showed the same HUD at 0,05 mg/plate; Amazonian basil EO, instead showed HUD comparable with those of the control thyme EO (0,025 mg/plate both with and without S9) for TA98 strain without S9 metabolic activation, but quite higher with metabolic activation mix (0,025 mg/plate and 0,05 mg/plate respectively). The toxicity pre-test with TA100 strains gave not the same results in terms of HUD, a part in the case of ginger and thyme EO (HUD 0,05 mg/plate and 0,025 mg/plate, either with and without S9, respectively); instead Yerba luisa EO showed higher HUD than those against TA98 (0,25 mg/plate, with and without S9 activator); HUD for Amazonian basil EO was assessed at 0,025 mg/plate, either ± S9 (the same HUD of the control thyme EO), and Curcuma EO demonstrated the highest HUD at 0,1 mg/plate.

Therefore, decrease of revertants has been tested, and antimutagen activity estimated, with the significant offset differences from revertants in TA98 and TA100 strains ($P < 0,05$), in accord to *t*-test. In detail, Amazonian basil EO inhibited growth cell in *S. typhimurium* TA98 both with and without microsomial activation at 0,01 mg/plate and above, showing dose-dependent response from 17% to 53% in TA98 -S9 strain, and from 12% to 61% in

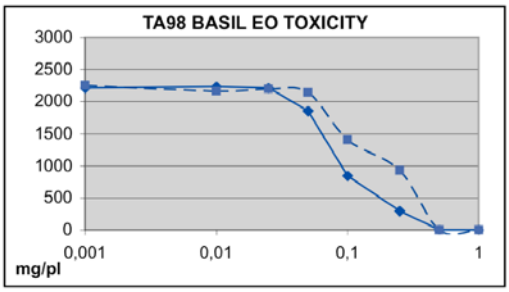
TA98 +S9 strain. Instead Curcuma EO showed interesting dose-dependent activity against TA98 +S9 bacteria at 0,05 mg plate and 0,1 mg/plate (14% and 44% respectively); only Yerba luisa EO showed mutagenprotective action against either against TA98 and TA100 mutant strains, in particular at 0,1 mg/plate inhibited TA98 -S9cell growth for about 14% but the best results has been obtained against TA100 +S9 strain in which case Yerba luisa EO showed dose-dependent growing inhibition from 0,01 mg/plate and above (from 32% to 42%). Moreover, it is not possible to ascribe significant antigenotoxic actions, both on point or frame shift mutations, to *Z. officinale* essential oil and *T. vulgaris* essential oil. Divergence between results in *S. cerevisiae* D7 and Ames test, observed in antimutagenic assays, could be explained by considering the SOS error-prone DNA repair mechanism, which acts only in *S. typhimurium*, in fact *S. cerevisiae* strain does not present an SOS error prone system, is less sensitive to mutagens action and insensitive to the SOS error prone inhibition (Rossi et al., 2011).

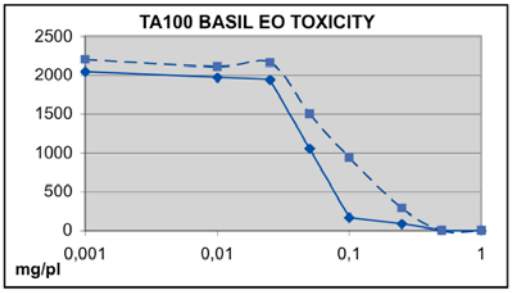
		Revertants His+/plate		Revertants His+/plate	
	mg/plate	TA 98		TA 100	
		- S9	+ S9	- S9	+ S9
THYME	0	480 ± 36	2822 ± 51	402 ± 17	2804 ± 20
	1,0E-02	486 ± 29	2751 ± 42	419 ± 17	2709 ± 31
	2,5E-02	439 ± 52	2678 ± 20	373 ± 12	2636 ± 95
	5,0E-02	203 ± 15	1980 ± 49	351 ± 11	2368 ± 34
	1,0E-01	3 ± 2	1761 ± 35	3 ± 1	1207 ± 73
	2,5E-01	0 ± 0	755 ± 30	0 ± 0	540 ± 19
	5,0E-01	0 ± 0	1115 ± 61	0 ± 0	144 ± 18
	1,0E+00	0 ± 0	0 ± 0	0 ± 0	0 ± 0



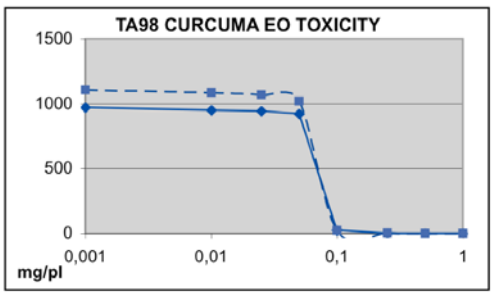
Graph. 3a Toxicity of control Thyme (*Thymus vulgaris*) essential oil tested on *S. typhimurium* TA98 and TA100 with (dotted line) and without (continuous line) S9 metabolic activation.

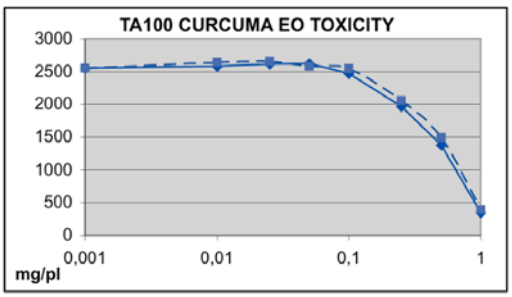
	mg/plate	Revertants His+/plate TA 98 - S9	Revertants His+/plate TA 98 + S9	Revertants His+/plate TA 100 - S9	Revertants His+/plate TA 100 + S9
BASIL	0	2212 ± 54	2554 ± 49	2046 ± 83	2204 ± 58
	1,0E-02	2231 ± 61	2167 ± 53	1975 ± 98	2113 ± 97
	2,5E-02	2214 ± 48	2196 ± 62	1943 ± 95	2166 ± 34
	5,0E-02	1851 ± 85	2146 ± 95	1053 ± 46	1502 ± 44
	1,0E-01	844 ± 39	1403 ± 25	169 ± 24	942 ± 46
	2,5E-01	293 ± 18	925 ± 58	88 ± 38	289 ± 40
	5,0E-01	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	1,0E+00	0 ± 0	0 ± 0	0 ± 0	0 ± 0





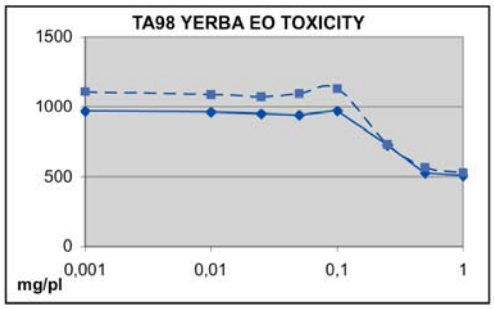
	mg/plate	Revertants His+/plate TA 98 - S9	Revertants His+/plate TA 98 + S9	Revertants His+/plate TA 100 - S9	Revertants His+/plate TA 100 + S9
CURCUMA	0	970 ± 55	1108 ± 27	2553 ± 40	2550 ± 75
	1,0E-02	950 ± 52	1087 ± 71	2578 ± 43	2645 ± 73
	2,5E-02	944 ± 46	1068 ± 42	2613 ± 98	2654 ± 91
	5,0E-02	924 ± 37	1019 ± 14	2625 ± 33	2581 ± 29
	1,0E-01	24 ± 5	25 ± 6	2469 ± 54	2545 ± 53
	2,5E-01	0 ± 0	5 ± 3	1969 ± 56	2055 ± 68
	5,0E-01	0 ± 0	0 ± 0	1379 ± 31	1495 ± 37
	1,0E+00	0 ± 0	0 ± 0	347 ± 39	395 ± 36

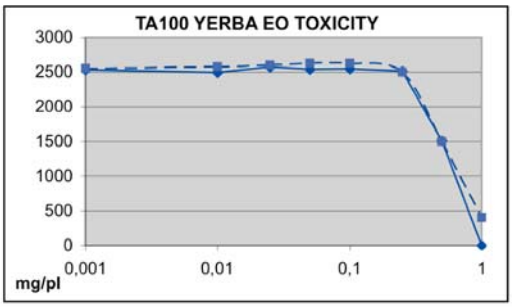




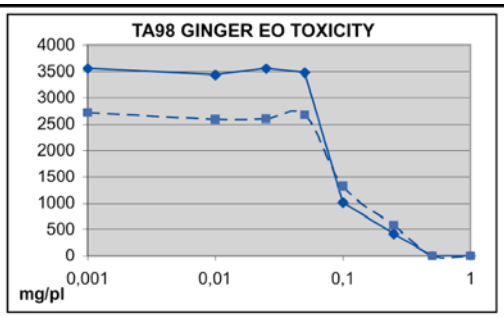
Graph. 3b Toxicity of Amazonian basil (*O. micranthum*) and curcuma (*C. longa*) essential oils tested on *S. typhimurium* TA98 and TA100 with (dotted line) and without (continuous line) S9 metabolic activation.

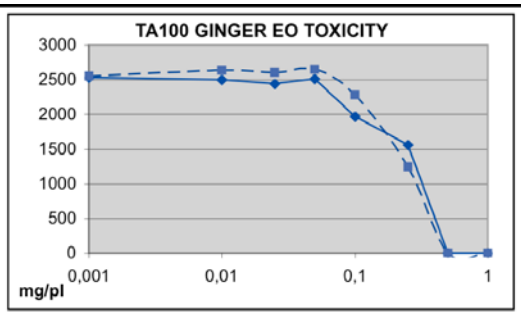
		Revertants His+/plate		Revertants His+/plate	
	mg/plate	TA 98		TA 100	
		- S9	+ S9	- S9	+ S9
YERBA	0	970 ± 55	1108 ± 27	2533 ± 40	2550 ± 75
	1,0E-02	964 ± 52	1088 ± 49	2491 ± 45	2575 ± 56
	2,5E-02	950 ± 36	1072 ± 40	2571 ± 67	2606 ± 57
	5,0E-02	540 ± 21	1095 ± 36	2536 ± 36	2629 ± 72
	1,0E-01	971 ± 19	1130 ± 87	2548 ± 49	2633 ± 69
	2,5E-01	725 ± 16	732 ± 49	2512 ± 30	2503 ± 59
	5,0E-01	525 ± 21	565 ± 39	1512 ± 20	1503 ± 59
	1,0E+00	504 ± 13	528 ± 23	479 ± 49	403 ± 43





		Revertants His+/plate		Revertants His+/plate	
	mg/plate	TA 98		TA 100	
		- S9	+ S9	- S9	+ S9
GINGER	0	3560 ± 59	2719 ± 96	2533 ± 40	2550 ± 75
	1,0E-02	3441 ± 82	2593 ± 66	2499 ± 25	2636 ± 31
	2,5E-02	3556 ± 75	2602 ± 38	2444 ± 92	2604 ± 43
	5,0E-02	3480 ± 69	2680 ± 93	2513 ± 46	2647 ± 40
	1,0E-01	1011 ± 42	1318 ± 34	1968 ± 35	2282 ± 65
	2,5E-01	414 ± 43	578 ± 68	1563 ± 73	1245 ± 87
	5,0E-01	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	1,0E+00	0 ± 0	0 ± 0	0 ± 0	0 ± 0





Graph. 3c Toxicity of Amazonian yerba luisa (*C. citratus*) and ginger (*Z. officinale*) essential oils tested on *S. typhimurium* TA98 and TA100 with (dotted line) and without (continuous line) S9 metabolic activation.

		Revertants His+/plate		Revertants His+/plate	
	mg/plate	TA 98		TA 100	
		- S9	+ S9	- S9	+ S9
BASIL	0	453 ± 37	655 ± 27	1054 ± 28	886 ± 31
	1,0E-02	**396 ± 11**	**578 ± 24**	1001 ± 26	878 ± 44
	2,5E-02	**214 ± 12**	**372 ± 16**	1025 ± 61	834 ± 29
	5,0E-02	181 ± 15	**255 ± 14**	1071 ± 24	710 ± 15
	1,0E-01	162 ± 29	215 ± 14	1067 ± 27	404 ± 10
	2,5E-01	86 ± 13	85 ± 9	901 ± 43	279 ± 24
	5,0E-01	0 ± 0	0 ± 0	589 ± 42	104 ± 17
	1,0E+00	0 ± 0	0 ± 0	123 ± 25	0 ± 0
CURCUMA	0	341 ± 13	1234 ± 25	1161 ± 59	799 ± 73
	1,0E-04	358 ± 32	1212 ± 22	1103 ± 42	832 ± 51
	2,5E-04	337 ± 10	1160 ± 89	1122 ± 18	753 ± 9
	5,0E-04	317 ± 33	1129 ± 43	1126 ± 36	**586 ± 56**
	1,0E-03	250 ± 27	884 ± 22	1136 ± 42	**438 ± 18**
	2,5E-03	164 ± 10	348 ± 39	1139 ± 39	460 ± 32
	5,0E-03	56 ± 9	78 ± 23	841 ± 34	225 ± 48
	1,0E-02	0 ± 0	0 ± 0	274 ± 36	95 ± 15
YERBA	0	507 ± 48	1234 ± 25	1194 ±116	852 ± 67
	1,0E-04	481 ± 42	1257 ± 22	1236 ± 47	**581 ± 81**
	2,5E-04	507 ± 92	1279 ± 89	1212 ± 26	**544 ± 66**
	5,0E-04	538 ± 19	1192 ± 43	1137 ± 33	**536 ± 46**
	1,0E-03	**438 ± 31**	1131 ± 22	1130 ± 54	**509 ± 16**
	2,5E-03	165 ± 9	1049 ± 39	1207 ± 48	**494 ± 29**
	5,0E-03	56 ± 9	265 ± 23	867 ± 29	268 ± 21
	1,0E-02	0 ± 0	0 ± 0	296 ± 33	135 ± 25
GINGER	0	199 ± 18	332 ± 49	777 ± 27	664 ± 21
	1,0E-04	200 ± 17	327 ± 29	779 ± 21	641 ± 47
	2,5E-04	195 ± 24	344 ± 11	789 ± 46	670 ± 17
	5,0E-04	208 ± 10	332 ± 52	813 ± 23	662 ± 15
	1,0E-03	189 ± 10	258 ± 38	826 ± 21	546 ± 25
	2,5E-03	86 ± 13	85 ± 25	690 ± 20	347 ± 23
	5,0E-03	0 ± 0	0 ± 0	342 ± 90	147 ± 25
	1,0E-02	0 ± 0	0 ± 0	72 ± 15	47 ± 8

Tab. 15a Effect of survival and antigenotoxicity (frameshift or point mutation) in *S. typhimurium* Ames test from treatment with Amazonian basil (*O. micranthum)*, Curcuma (*C. longa)*, Yerba luisa (*C. citratus)* and Ginger (*Z. officinale*) essential oils. Genotoxic agent: 2-nitrofluorene, 2-aminoanthracene and sodium azide.

		Revertants His+/plate		Revertants His+/plate	
	mg/plate	TA 98		TA 100	
		- S9	+ S9	- S9	+ S9
THYME	0	609 ± 21	891 ± 29	1053 ± 44	967 ± 55
	1,0E-04	590 ± 49	899 ± 23	1034 ± 24	950 ± 46
	2,5E-04	613 ± 57	842 ± 94	1001 ± 36	929± 48
	5,0E-04	476 ± 24	823 ± 27	988 ± 40	834 ± 55
	1,0E-03	329 ± 23	342 ± 18	1047 ± 93	616 ± 49
	2,5E-03	113 ± 10	182 ± 31	914 ± 77	188 ± 38
	5,0E-03	0 ± 0	0 ± 0	612 ± 27	0 ± 0
	1,0E-02	0 ± 0	0 ± 0	147 ± 44	0 ± 0

Tab. 15b Effect of survival and antigenotoxicity (frameshift or point mutation) in *S. typhimurium* Ames test from treatment with commercial Thyme (*T. vulgaris)* EO, taken as positive control. Genotoxic agent: 2-nitrofluorene, 2-aminoanthracene and sodium azide.

B) 2 Antimicrobial activities:

Antimicrobial activities have been checked for Essential Oils employing different strategies:

- OE disk-diffusion assay
- OE Antibacterial (HP)TLC bioautographic assay
- OE Antibacterial agar vapour method (Headspace's activity)

Strains

gram positive	*Staphylococcus aureus*	ATCC 29213	*S.a.*
	Enterococcus faecalis	ATCC 29212	*E.f.*
	Micrococcus luteus	ATCC 9622	*M.l.*
	Listeria grayi	ATCC 19120	*L.g.*
gram negative	*Pseudomonas aeruginosa*	ATCC 17934	*P.a.*
	Klebsiella oxytoca	ATCC 29516	*K.o.*
	Escherichia coli	ATCC 4350	*E.c.*
	Proteus vulgaris	ATCC 6361	*P.v.*
yeast	*Candida albicans*	ATCC 48274	*C.a.*
	Saccharomyces cerevisiae	ATCC 2365	*S.c.*

Tab. 16 Strains employed for Antimicrobial activities determination on Amazonian essential oils.

In previous research the biological activity against yeasts has been determined by employing the standard disk diffusion technique (Sacchetti et al., 2005 and references cited). Now, antifungal and antibacterial activities were in depth evaluated on gram positive and gram negative bacteria known as non-pathogenic, usually regarded as contaminant, but generally considered as nosocomial pathogens, especially in immunocompromised patients (Guerrini et al., 2011 and references cited). In particular, 4 Gram negative bacteria *Pseudomonas aeruginosa* ATCC 17934 (*P.a.*), *Klebsiella oxytoca* ATCC 29516 (*K.o.*), *Escherichia coli* ATCC 4350 (*E.c.*), *Proteus vulgaris* ATCC 6361 (*P.o.*); 4 Gram positive ones *Staphylococcus aureus* subsp. aureus ATCC 29213 (*S.a.*), *Enterococcus faecalis* ATCC 29212

(*E.f.*), *Micrococcus luteus* ATCC 9622 (*M.l.*), and 2 yeasts: *Candida albicans* ATCC 48274 (*C.a.*) and *Saccharomyces cerevisiae* ATCC 2365 (*S.c.*) have been chosen. The strains were cultured in nutrient agar, tryptic soy agar and Yepd following the suggestions given by ATCC protocols.

B) 2.1 Essential oils disk-diffusion assay

Antimicrobial (antibacterial and antifungal) activities of Amazonian essential oils, compared with that of commercial *T. vulgaris* essential oils, taken as positive control, has been tested giving results particularly indicative of the real functional efficacy of phytocomplexes. An activity of a phytocomplex, essential oils for example, is characterized not only by the simple interaction between an active plant compound and the biological substrate but, more properly, by the synergic activity of more chemical plant compounds that interact in the biological tests. Therefore, to better characterize the functional capacities of a chemically complex plant extract, the evaluation of the bioactivities needs to be compared not only with a single compound as positive control, but also with similar phytocomplex of known functional capacities towards the tested bioassays. Based on this assumption and similar suggestion reported (Guerrini et al., 2011; Maietti et al., in press; and reference therein), *T. vulgaris* essential oil represents a good reference for comparing bioassay results regarding other essential oils, in this case *O. micranthum, C. citratus*, *C. longa*, *Z. officinale* essential oils.

Data collected evidenced that Amazonian basil EO presents the higher antimicrobial activity against all the strains tested. Against gram negative *K.o.*, *E.c.* and *P.a.*, it showed activity comparable with that of the positive control (1,86mg/ml, 4,66 mg/ml, 9,31mg/ml respectively), and slightly higher against *S.c.* (0,93mg/ml vs 1,93mg/ml of Thyme). Yerba luisa EO demonstrated interesting activities against all the strains tested: the bioactivities were comparable with those of the positive control against *S.c.* (1,73mg/ml), and higher against gram negative *E.c* and *P.a* (both 1,73 mg/ml). Ginger EO presented in general modest antimicrobial activities, except against gram negative *P.a.* in which it showed activity 5 times higher than the control (thyme EO). Curcuma EO did instead not shown appreciable activities. In Table 17b are shown antimicrobial activities (MIC) of some standards which are been testing in our labs, and in Tables 17c,d are evidenced the antimicrobial activities of the two most active Amazonian EO

(i.e. Amazonian basil and Yerba luisa EOs) in comparison with those of the chemical detected in GC-MS analyses (Tab. 4). Every difference in activity is due to the whole phytocomplex chemicals action, which could be agonist or antagonist (Maietti et al., in press).

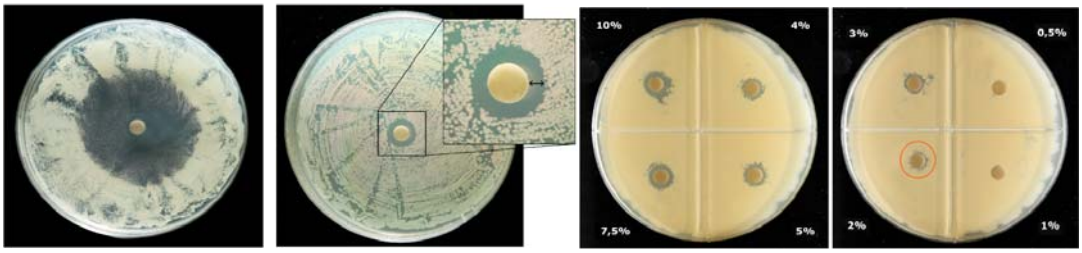


Fig. 47 Growth inhibition zone diameter (IZD) on *Candida albicans*.

		YERBA	GINGER	CURCUMA	BASIL	THYME
				MIC (mg/ml)		
Gram +	*S.a.*	YERBA ±0,60	17,74 ±1,24	448,75 ± 0,61	9,31 ± 0,69	1,93 ± 0,60
	E.f.	8,67 ±0,65	44,35 ±3,32	179,50 ± 0,65	9,31 ± 0,65	1,93 ± 0,65
	L.g.	**1,73 ±0,12**	**8,87 ±0,62**	**8,98 ± 0,12**	**1,86 ± 0,12**	**0,97 ± 0,12**
	M.l.	17,34 ±1,33	44,35 ±3,45	179,50 ± 1,34	9,31 ± 1,33	1,93 ± 1,33
Gram -	*K.o.*	4,34 ±0,30	8,87 ±0,62	89,75 ± 0,30	1,86 ± 0,30	1,93 ± 0,30
	P.v.	8,67 ±0,47	17,74 ±0,97	89,75 ± 0,47	4,66 ± 0,47	1,93 ± 0,47
	Ec.	**1,73 ±0,10**	**8,87 ±0,53**	**44,88 ± 0,11**	**4,66 ± 0,10**	**4,84 ± 0,10**
	P.a.	**1,73 ±0,15**	**1,77 ±0,15**	**179,50 ± 0,15**	**9,31 ± 0,15**	**9,67 ± 0,15**
YEAST	*C.a.*	4,34 ±0,42	17,74 ±1,75	89,75 ± 0,42	4,66 ± 0,42	1,93 ± 0,42
	S.c.	**1,73 ±0,10**	**88,70 ±5,32**	**8,98 ± 0,10**	**0,93 ± 0,10**	**1,93 ± 0,10**

Tab. 17a Antimicrobial (antibacterial and antifungal) activities on Amazonian essential oils compared with those of commercial *T. vulgaris* essential oil. Note: *S.a., Staphylococcus aureus; E.f., Enterococcus faecalis; L.g., Listeria grayi; P.a. Pseudomonas aeruginosa; K.o. Klebsiella oxytoca; E.c., Escherichia coli; P.v., Proteus vulgaris; C.a. Candida albicans; S.c. Saccharomyces cerevisiae.*

		4-terpineol	β-caryophyllen	eugenol	geraniol
		MIC (mg/ml)			
Gram +	*S.a.*	9,34 ±0,70	> 50	10,67 ±0,80	4,49 ±0,33
	E.f.	1,87 ±0,14	> 50	10,67 ±0,82	1,79 ±0,13
	L.g.	4,67 ±0,32	> 50	5,34 ±0,37	1,79 ±0,12
	M.l.	9,34 ±0,46	> 50	10,67 ±0,53	4,49 ±0,22
Gram -	*K.o.*	4,67 ±0,46	> 50	2,13 ±0,21	0,90 ±0,08
	P.v.	9,34 ±0,90	> 50	10,67 ±1,03	4,49 ±0,43
	Ec.	4,67 ±0,40	> 50	5,34 ±0,46	4,49 ±0,39
	P.a.	9,34 ±0,70	> 50	5,34 ±0,40	1,79 ±0,13
YEAST	*C.a.*	9,34 ±0,65	> 50	10,67 ±0,74	4,49 ±0,31
	S.c.	0,47 ±0,02	nd	5,34 ±0,29	1,79 ±0,10
		β-pinene	citral	germacrene D	1,8-cineole
		MIC (mg/ml)			
Gram +	*S.a.*	> 50	4,44 ±0,33	> 50	> 50
	E.f.	8,69 ±0,66	4,44 ±0,34	17,00 ±1,30	0,92 ±0,14
	L.g.	4,35 ±0,30	1,78 ±0,12	> 50	4,61 ±0,32
	M.l.	8,69 ±0,43	4,44 ±0,22	17,00 ±0,85	4,61 ±0,46
Gram -	*K.o.*	4,35 ±0,43	1,78 ±0,17	> 50	4,61 ±0,46
	P.v.	> 50	nd	> 50	nd
	Ec.	17,38 ±1,51	1,78 ±0,15	> 50	9,21 ±0,40
	P.a.	43,45 ±3,25	1,78 ±0,13	> 50	18,42 ±0,70
YEAST	*C.a.*	8,69 ±0,60	4,44 ±0,31	> 50	9,21 ±0,65
	S.c.	0,87 ±0,04	1,78 ±0,06	1,70 ±0,09	46,05 ±0,02

Tab. 17b Antimicrobial (antibacterial and antifungal) activities on some standards tested in our labs. Note: nd, not determinable; *S.a., Staphylococcus aureus; E.f., Enterococcus faecalis; L.g., Listeria grayi; P.a. Pseudomonas aeruginosa; K.o. Klebsiella oxytoca; E.c., Escherichia coli; P.v., Proteus vulgaris; C.a. Candida albicans; S.c. Saccharomyces cerevisiae.*

		BASIL	eugenol	1,8-cineole	β-pinene	geraniol	citral
		MIC (mg/ml)					
Gram +	*S.a.*	9,31 ± 0,69	10,67 ±0,80	> 50	> 50	4,49 ±0,33	4,44 ±0,33
	E.f.	9,31 ± 0,65	10,67 ±0,82	0,92 ±0,14	8,69 ±0,66	1,79 ±0,13	4,44 ±0,34
	L.g.	**1,86 ± 0,12**	5,34 ±0,37	4,61 ±0,32	4,35 ±0,30	1,79 ±0,12	1,78 ±0,12
	M.l.	9,31 ± 1,33	10,67 ±0,53	4,61 ±0,46	8,69 ±0,43	4,49 ±0,22	4,44 ±0,22
Gram -	*K.o.*	1,86 ± 0,30	2,13 ±0,21	4,61 ±0,46	4,35 ±0,43	0,90 ±0,08	1,78 ±0,17
	P.v.	4,66 ± 0,47	10,67 ±1,03	nd	> 50	4,49 ±0,43	nd
	Ec.	**4,66 ± 0,10**	5,34 ±0,46	9,21 ±0,40	17,38 ±1,51	4,49 ±0,39	1,78 ±0,15
	P.a.	**9,31 ± 0,15**	5,34 ±0,40	18,42 ±0,70	43,45 ±3,25	1,79 ±0,13	1,78 ±0,13
YEAST	*C.a.*	4,66 ± 0,42	10,67 ±0,74	9,21 ±0,65	8,69 ±0,60	4,49 ±0,31	4,44 ±0,31
	S.c.	**0,93 ± 0,10**	5,34 ±0,29	46,05 ±0,02	0,87 ±0,04	1,79 ±0,10	1,78 ±0,06

		YERBA	geraniol	citral	germacrene D	eugenol
		MIC (mg/ml)				
Gram +	*S.a.*	8,67 ±0,60	4,49 ±0,33	4,44 ±0,33	> 50	10,67 ±0,80
	E.f.	8,67 ±0,65	1,79 ±0,13	4,44 ±0,34	17,00 ±1,30	10,67 ±0,82
	L.g.	**1,73 ±0,12**	1,79 ±0,12	1,78 ±0,12	> 50	5,34 ±0,37
	M.l.	17,34 ±1,33	4,49 ±0,22	4,44 ±0,22	17,00 ±0,85	10,67 ±0,53
Gram -	*K.o.*	4,34 ±0,30	0,90 ±0,08	1,78 ±0,17	> 50	2,13 ±0,21
	P.v.	8,67 ±0,47	4,49 ±0,43	nd	> 50	10,67 ±1,03
	Ec.	**1,73 ±0,10**	4,49 ±0,39	1,78 ±0,15	> 50	5,34 ±0,46
	P.a.	**1,73 ±0,15**	1,79 ±0,13	1,78 ±0,13	> 50	5,34 ±0,40
YEAST	*C.a.*	4,34 ±0,42	4,49 ±0,31	4,44 ±0,31	> 50	10,67 ±0,74
	S.c.	**1,73 ±0,10**	1,79 ±0,10	1,78 ±0,06	1,70 ±0,09	5,34 ±0,29

Tab. 17a,b Antimicrobial (antibacterial and antifungal) activities on Yerba luisa and Amazonian basil EO in comparison with those of some standards tested in our labs. GC-MS composition percentage detected (see Tab. 4). Amazonian basil: eugenol 51%; 1,8-cineole 7%; β-pinene 0,49%; geraniol, traces; citral, only geranial traces; Yerba luisa: eugenol, traces; geraniol, 39%; citral (neral + geranial), 32%; gremacrene D, 0,47%. Note: *S.a., Staphylococcus aureus; E.f., Enterococcus faecalis; L.g., Listeria grayi; P.a. Pseudomonas aeruginosa; K.o. Klebsiella oxytoca; E.c., Escherichia coli; P.v., Proteus vulgaris; C.a. Candida albicans; S.c. Saccharomyces cerevisiae.*

B) 2.2 OE Antibacterial activity: (HP)TLC-bioautographic assay

Antimicrobial activities characterizing examined phytocomplexes has been evaluated also by a chromatographic technique, i.e. (HP)TLC-bioautographic assay. Also in this case, results have been evaluated in comparison with those of commercial thyme essential oil, taken as analogous phytocomplex. (HP)TLC assay allows determining which fractions would be the most responsible of the antibacterial activity (fig 48). Eluted and TLC treated plates evidenced chemical classes (bands) clearly responsible of the bioactivity with reference to the interaction with bacterial dehydrogenase. Amazonian basil eo has shown higher antimicrobial activities, than other eo, as however evidenced with disk diffusion assay. As general consideration, the most sensitive strains were *L.g., S.a., E.f., P.v., K.o.* and *E,c*,, showing clear bands of active antimicrobial chemicals. *L.g., S.a., P.v., E.f.* seemed to be responsive to all the eos, while *E.c.* and *K.o.* revealed active bands corresponding to Amazonian basil and Yerba luisa eos. *P.a., M.l.* and, always yeasts *C.a.* and *S.c.*, did not present any significant activity. Finally, curcuma eo gave activity results slightly higher than that expressed by disk diffusion assay and this evidence emerges as contrasting conclusion between the two methods. The differences between the two methods can be explained through to the completely different approach of the two research strategies: the disk diffusion assay evaluate the activity of all the chemical compounds together, possible synergic interactions included, while bioautographic assay explores the bioactivity of the single compound excluding possible synergic interactions, both in terms of agonistic or antagonistic synergy (Maietti et all., in press).

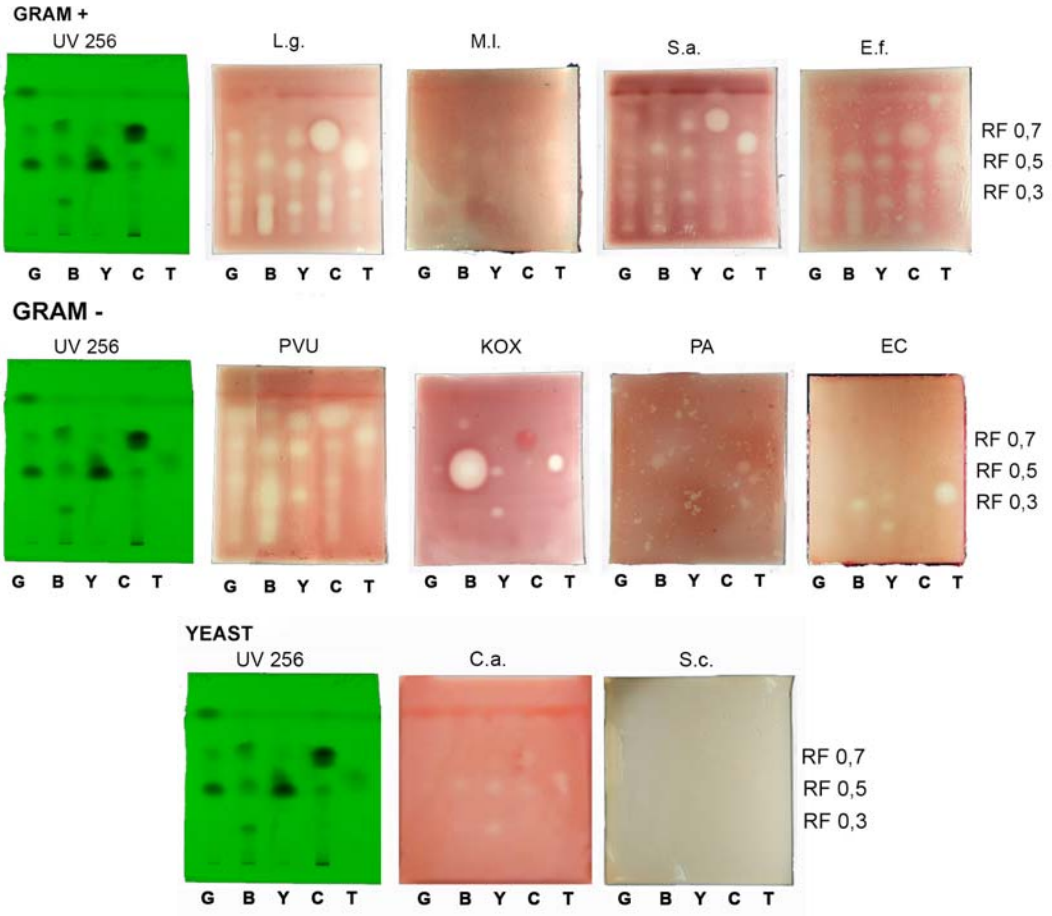


Fig. 48 (HP)TLC-bioautographyc assays performed for antibacterial and antifungal activities of Amazonian essential oils.

To valorise the bioautographic results, it can be evidenced, with reference to the most sensitive microorganism for each essential oil, that the most bioactivity chemicals classes (bands) identified for Amazonian basil and Yerba luisa EO are Eugenol (Rf 0,5), geraniol and geranial (Rf 0,4); ginger eo and curcuma eo are β-turmerone and *ar*-turmerone (Rf 0,75); for the positive control thyme thymol (Rf 0,6). In the figure below are resume all the bioactivities tested on Amazonian basil eo, is notable the strong activity of eugenol (Rf 0,5, yellow coloured and UV visible band), which contrast DPPH radical activity, gram + and gram – bacteria (very strong against *Klebsiella oxytoca*) and, while in lower amount, against the yeast *Candida albicans*. An example of functional application of this very active essential oil would be as

food potential natural preservatives as reported by other authors (Valero et al., 2006 and references cited).

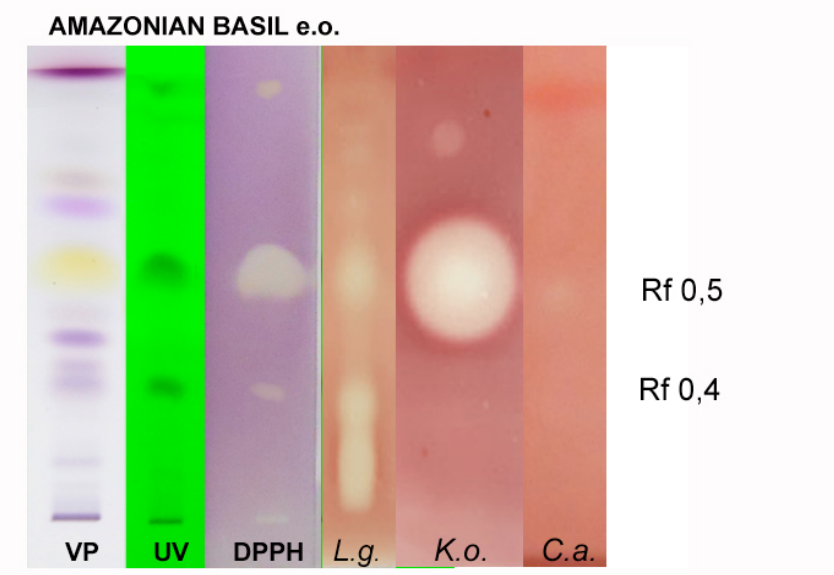


Fig. 49 (HP)TLC bioautographic assay of *O. micranthum* eo illustrating in comparison various bioactivities of Amazonian basil eo fractions. The figure evidenced the most bioactive, both on antioxidant and antimicrobial test, fractions at Rf 0,5 (eugenol) and Rf 0,4 (geraniol and geranial).

B) 2.3 OE Antimicrobial agar vapour method (Headspace's activity)

The above stressed results lead to investigate the antimicrobial properties against the above mentioned bacteria and yeast strains. In particular, the activities of each Amazonian essential oils has been performed on their vapour fractions compared to that of commercial thyme essential oil, since it is reputed a biologically effective phytocomplex and, therefore, it can be considered as a good reference to evaluate the efficacy of analogous extracts (Maietti et al., in press and references cited). The data collected are resumed in the table below (Tab.18). Results reported are expressed as growing colony percentage in comparison with negative control (DMSO). Results obtained revealed a dose dependant correlation, and this evidence can be considered as good index to determine the validity of the method applied. In general, all the essential oil HS fractions exihibited a growth colony inhibition with values ranging from 11% to 78%. As expected, *L.g.* was the most sensitive bacteria even if interesting values have been

determined in *S.a.* cultures, 32% inhibited by Yerba luisa HS fraction. Interesting evidence is that referred to *C.a.,* strongly inhibited by Yerba luisa HS fraction. This latter data needs to be further in-depth investigates because of the contrasting evidenced reported with the previous assays, and of the functional implications that this confirmed result could determine. In general, the bioactivity of each HS essential oil can be attributed to the chemical components identified and detected in HS-GC-MS analysis. In particular, it is reasonable to consider the most abundant compounds (see Tab. 5) detected in each HS essential oil fraction as the most responsible chemicals of the bioactivity, i.e. β-pinene, Eucalyptol and *cis*-Ocimene for Amazonian basil; *cis*- *trans*-isocitral and γ-terpineol for Yerba luisa; α-phellandrene and limonene for curcuma; camphene α-pinene and limonene for Ginger eo.

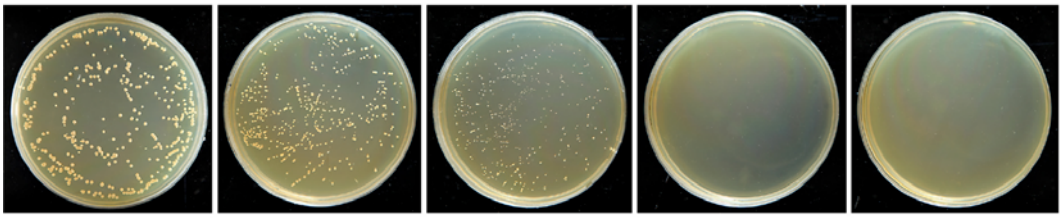


Fig. 50 Antimicrobial activity exploited by Yerba essential oil volatile fraction against *Candida albicans* in comparison with negative control (DMSO, first plate on the left).

		YERBA				GINGER			
	p.e.o. [mg]	8,76	4,38	1,75	0,88	8,87	4,44	1,77	0,89
gram +	*S.a.*	**31**	**19**	**16**	**17**	2	6	-4	0
	E.f.	0	2	-1	4	1	-1	3	-1
	L.g.	**79**	**60**	**44**	**30**	**55**	**35**	**32**	**28**
	M.l.	7	6	5	3	2	-4	-3	-5
gram-	*K.o.*	**10**	6	-1	-7	8	1	-3	-7
	P.v.	**25**	**23**	6	-5	**29**	2	2	5
	Ec.	2	1	5	3	-3	2	3	1
	P.a.	3	-2	2	-1	2	-1	1	-2
YEAST	*C.a.*	**100**	**100**	-6	-5	**36**	2	-5	-8
	S.c.	**100**	**55**	-6	-5	3	-5	-6	-9

		CURCUMA				BASIL			
	p.e.o. [mg]	8,98	4,49	1,80	0,90	9,31	4,66	1,86	0,93
gram +	*S.a.*	10	2	5	6	1	6	3	-4
	E.f.	-1	-5	0	5	1	-5	0	3
	L.g.	**34**	0	-3	-7	**33**	7	5	4
	M.l.	-4	0	-1	-5	0	1	3	4
gram-	*K.o.*	-3	-4	1	3	-5	-4	-2	-5
	P.v.	**17**	1	2	5	3	0	1	4
	Ec.	6	-3	5	0	2	4	3	2
	P.a.	-2	-1	0	-3	3	4	1	-1
YEAST	*C.a.*	4	5	8	9	**16**	**17**	-6	-1
	S.c.	-9	-8	-9	-2	**44**	-3	-8	-6

		THYME			
	p.e.o. [mg]	9,61	4,80	1,92	0,96
gram +	*S.a.*	**35**	**22**	**18**	**14**
	E.f.	**22**	**12**	0	-1
	L.g.	**50**	**19**	**14**	**11**
	M.l.	**10**	4	2	-2
gram-	*K.o.*	-10	-11	2	4
	P.v.	**63**	**36**	**25**	**15**
	Ec.	2	-3	4	3
	P.a.	-2	3	1	-1
YEAST	*C.a.*	**100**	**100**	0	-6
	S.c.	**100**	**83**	**19**	0

Tab. 18 Antimicrobial activity (agar vapour method) of Amazonian essential oils. Data collected are reported as inhibition growing colony percentage with reference to DMSO plate. In bold more interesting results. Note: p.e.o., pure essential oil.

B) 3 Antioxidant activity trough spectrophotometric assays

B) 3.1 Spectrophotometric DPPH and ABTS assays

Essential oils and avocado derivates have been processed through DPPH and ABTS assays in other to evaluate, with two different systems, the radical scavenging activities of the phytocomplexes. The chemical complexity of phytocomplexes, i.e. essential oils and fixed oils, expressed as quality and abundance of compounds, polarity and chemical properties, could lead to scattered bioactivity results depending on the method adopted. Thus, the use of more than a single method is highly advisable (Rossi et al., 2011 and reference cited). With the objective to compare different radical scavenging activities, ensuring a better comparison of the results and covering a wider range of possible herbal applications (i.e. nutraceutics and/or cosmeceutics), DPPH and ABTS tests have been performed. In particular, DPPH test was performed on the whole essential oils, both by spectrophotometric strategy and by (HP)TLC-bioautographic assay. The latter was performed to check the most active fractions of essential oils after TLC plates elution.

Essential Oils: *O. micranthum* eo shows notable radical scavenging activities both against DPPH° and ABTS°+ (DPPH: IC50 = 0,01 mg/ml; ABTS IC50 = 0,001 mg/ml) with values higher than the other EOs object of this PhD, and previous data reported about (Sacchetti et al., 2004). The antioxidant activity of *C. citratus* eo determined by DPPH was almost ten times lower than thyme eo, but two times higher than ginger eo. *C. longa* eo shows the lower antiradical activity (IC50 = 16,3 mg/ml). Otherwise, it is notable the contrasting data about yerba luisa eo and curcuma eo in DPPH and ABTS assays. This evidence can be partially explained with the fact that DPPH and ABTS reflect a different radical scavenging approach, but it needs to be in-depth further investigated with other different approach assays. Finally tea tree oil has been tested as further positive control coupled to thyme. This processing goes toward the need of compare bioactivities of possible new natural products with those of commonly marked analysis. The bioactivity of tea tree oil and eventually of other known phytocomplexes, will be extended also to that antimicrobial in order to

achieve from our data the most realistic suggestions in terms of health uses and marketing about new phytocomplexes.

DPPH° radical scavenging	
sample	IC50 [mg/ml]
ginger oe	5,4780 ± 0,82
basil eo	0,0115 ± 0,00
yerba oe	2,2981 ± 0,34
curcuma oe	16,3193 ± 2,45
tea tree eo	26,0568 ± 3,91
thyme eo	0,2998 ± 0,04
BHA	0,0034 ± 0,00

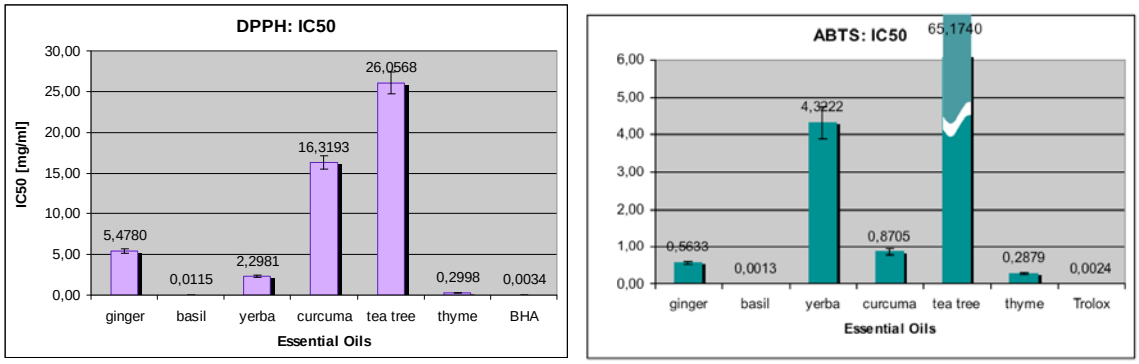
ABTS°+ radical scavenging	
sample	IC50 [mg/ml]
ginger oe	0,5633 ± 0,08
basil eo	0,0013 ± 0,00
yerba oe	4,3222 ± 0,65
curcuma oe	0,8705 ± 0,13
tea tree eo	65,1740 ± 9,78
thyme eo	0,2879 ± 0,04
trolox	0,0024 ± 0,00

Tab. 19a Radical scavenging properties of Amazonian essential oils in comparison with those of commercial Thyme and Tea tree essenial oils (phytocomplexes adopted as positive controls), and Trolox (pure chemical compound adopted as positive control)

DPPH° radical scavenging	
sample	IC50 [mg/ml]
β-caryophyllene	72,1701 ± 3,61
β-pinene	130,1762 ± 12,89
1,8-cineole	405,9768 ± 26,39

ABTS°+ radical scavenging	
sample	IC50 [mg/ml]
β-caryophyllene	13,6051 ± 1,02
β-pinene	123,3980 ± 8,63
1,8-cineole	160,3461 ± 14,43

Tab. 19b Radical scavenging properties of some standards tested in our labs, data not yet published.



Graph. 4 Free radical scavenging activities of ginger, basil, yerba and curcuma eo's. tea tree and thyme eo's has been adopted as natural controls with BHA (buthylated hydroxyl anisole). The values has been expressed as IC50.

Avocado Oils and Extracts: in agreement of total phenolic analysis, and in light of exploration antioxidant properties of healthy formulations using, as carrier, avocado oils, radical scavenging determination has been conduced on avocado oils and methanol pulp extract. The results obtained by DPPH and ABTS assays are summarized in the table below (tab. 20). In general, no significant antioxidant activities have been found: avocado oil hexane macerated (AHM) showed the highest activity on DPPH assay (IC50 = 50,23mg/ml), followed by methanol pulp extract (AMM, which shows IC50 = 65,81 mg/ml). Data collected agreeing with those reported in literature where avocado pulp derivates has not strong antioxidant properties, lower than those of peel and seeds (Wang et al., 2010). This evidence can be also directly related to our data regarding polyphenols detection. ABTS radical scavenging test confirmed the low activity of avocado samples, but unfortunately IC50 has been not determinable due to technical limitation of these strategies (turbidity of samples). While IC50 has been not determined, where possible, inhibition percentage due to 100 μl of undiluted extract has been observed: ACO samples showed highest inhibition values both on DPPH and ABTS (46,66%, 41,03% respectively), ASFE showed 39,94% of inhibition against DPPH radical, AMM and AHM, which showed moderate radical scavenger activity on DPPH assay, give 30,22% and 14,07% inhibition percentage on ABTS assay.

DPPH° radical scavenging		ABTS°+ radical scavenging	
sample	IC50 [mg/ml]	sample	IC50 [mg/ml]
AHM	50,23 ± 0,75	AHM	nd*
ASFE	nd*	ASFE	nd*
AMM	65,81 ± 0,99	AMM	nd*
ACO	nd*	ACO	nd*
BHA	0,0034 ± 0,00	trolox	0,0024 ± 0,00

Tab. 20 Rwandese Avocado derivates radical scavenging properties. nd* = not determined as IC50 (mg/ml), but where possible, determined as inhibition percentages of undiluted samples (100μl): ASFE = DPPH 39,94% of radical inhibition; ACO = DPPH 46,66%, ABTS 41,03% radical inhibition; AHM = ABTS 14,07%; ACO = ABTS 30,22%.

B) 4 Antioxidant activity (HP)TLC assays

(HP)TLC bioautographic antioxidant assays employing DPPH and ABTS as free radicals have been performed on essential oils in order to check and determine chemical classes responsible of the bioactivity (fig 50)

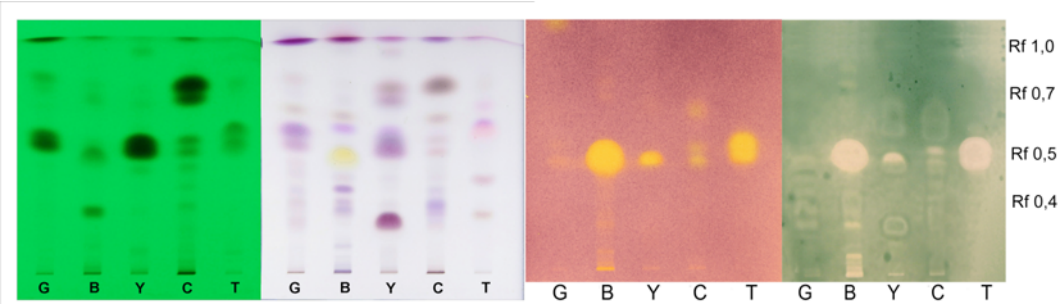


Fig. 50 Antioxidant activities on (HP)TLC bioautographyc assay of Amazonian essential oils. TLC on the left has been sprayed with the DPPH• solution; TLC on the centre with ABTS•+, and the TLC on the right with VP reagent. G, ginger eo; B, basil eo; Y, yerba luisa eo; C curcuma eo; T, thyme eo.

The results about essential oils generally reflected what emerged with previous spectrophotometric investigations. *O. micranthum* eo showed an antioxidant activity comparable with those of the control (*T. vulgaris* eo). Weak antioxidant activity has been checked for *C. citratus* eo and *C. longa* eo, *Z. officinale* eo did not evidenced any clear zone of inhibition, except for Rf = 1. The isolated fractions evidencing antioxidant activity (assayed on DPPH bioautographyc assay) were then isolated and analysed by GC-MS. *O. micranthum* essential oil shows three different areas on TLC with radical inhibition: in particular RF = 0,5 > RF = 0,4 > RF = 1. GCMS analyses revealed β-caryophyllene (35%), β-elemene (26%) and α-selinene (10%) as main compounds in RF = 1. The isolated fraction RF = 0,5, instead, is almost composed by eugenol (91,5%). In the fraction RF = 0,4 eugenol (58%), geraniol (36,5%) and geranial (5%) have been detected. *C. longa* shows two inhibition areas: RF = 0,5 > RF = 0,7. The GCMS analyses of RF = 5 fraction revealed the presence of thymol and *ar*-curcumene as main compounds (37% and 23% respectively) followed by β-caryohyllene and α-selinene (both 20%). *Z. officinale* eo shows only one active area: RF = 1 mainly composed by dehydrourcumene (40%) and *ar*-curcumene (17%). Finally, the weak inhibition area of *C. citratus* eo at RF = 0,4 is composed by

eugenol (99,9%) almost entirely. A large number of studies among antioxidant activities of natural derived products have been published: almost all the authors reported the role of the entirely phytocomplex against oxidative stress, a part for eugenol and geranial. In particular, antiradical mechanism of eugenol had been in-deep investigated and demonstrated (Bortolomeazzi et al., 2010); and recently researchers have focused on antioxidant potential of Geraniol demonstrating its role in oxdative-stress prevention (Chen, Viljoen, 2010 and references cited).

Biological Activity: DPPH antiradical

N	RT (min)	name	RI	YERBA	GINGER	CURCUMA		BASIL		
				Area %						
				Rf 0,4	Rf 1,0	Rf 0,5	Rf 0,7	Rf 0,4	Rf 0,5	Rf 1,0
19	24,384	Citronellal	1148				0,74			
31	35,844	Geraniol	1249				1,20	36,55		
32	37,532	Geranial	1264					5,21		
34	40,685	Thymol	1289			37,43				
37	47,066	Eugenol	1356	99,99				58,23	91,56	
39	50,322	*beta*-Elemene	1389							25,92
42	51,793	*beta*-Caryophyllene	1417			19,80				34,69
44	53,569	*allo*-Aromadendrene	1458							2,46
45	53.044	*dehydro*-Aromadendrene	1460							1,24
46	53.539	Cumacrene	1470							8,19
47	54,280	Germacrene D	1484		4,84					
48	54,578	*ar*-Curcumene	1479		17,10	22,87				
49	54,489	*beta*-Selinene	1489							8,73
50	54.827	*alpha*-Selinene	1798			19,91				10,89
52	54,765	*trans*-Muurola-4(14)5-diene	1493		9,18					
53	54,983	Germacrene A	1508							3,39
55	55.151	dehydrocurcumene			39,93					
58	55,388	*delta*-Cadinene	1522		2,55					
59	55.524	nd			25,01					
61	56,109	Germacrene B	1559							2,16
62	56.257	nd			1,39					
65	56,682	*ar*-Turmerol	1582				4,04			
68	57,074	Helifolen-12-ale A	1592				5,33			
71	57,631	*beta*-Biotol	1612				3,76			
78	58,146	*ar*-Turmerone	1668				3,99			
79	58,185	*alpha*-Turmerone					43,74			
80	58,625	*beta*-Turmerone					33,18			
82	59,555	nd					3,05			
		TOT		100,0	100,0	100,0	99,0	100,0	91,6	97,7

Fig. 21 GC-MS qualitative and semi-quantitative analyses of fraction showing antioxidant activities vs DPPH radical.

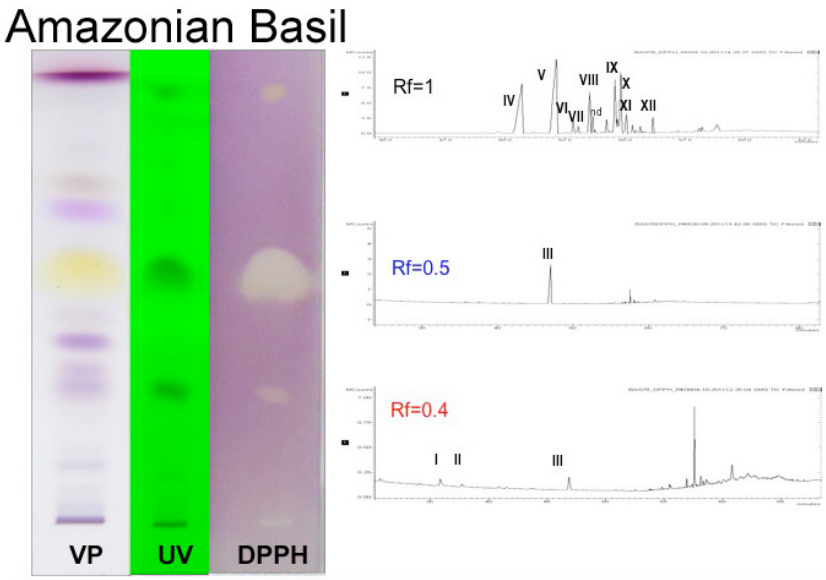


Fig. 51a (HP)TLC bioautographic assay of Amazonian basil eo. Note: VS = vanillic sulphuric acid reagent; UV = ultra violet detection; DPPH = radical scavenging activities. I, geraniol; II, geranial; III, eugenol; IV, β-elemene; V, β-caryophyllene; VI, *allo*-Aromadendrene; VII, dehydro-aromadendrene; VIII, cumacrene, IX, β-selinene; X, α-selinene; XI, germacrene A; XII, germacrene B.

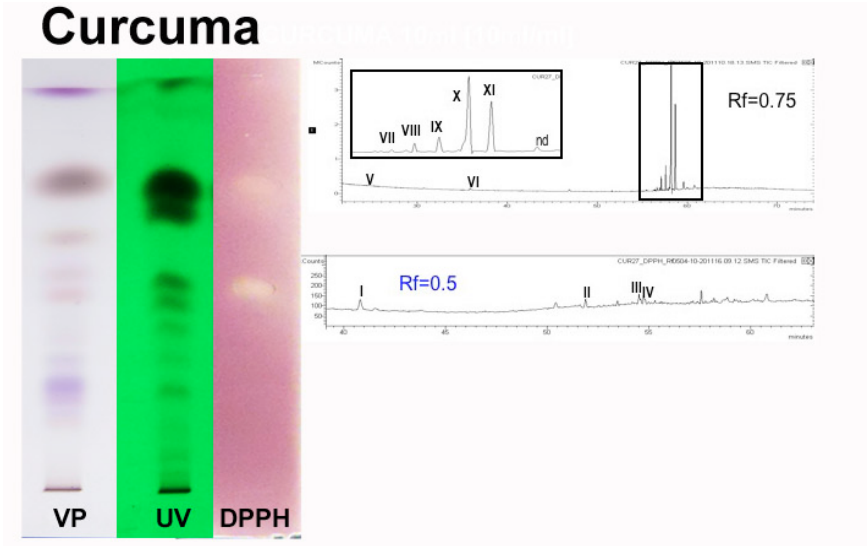


Fig. 51b (HP)TLC bioautographic assay of Curcuma eo. Note: VS = vanillic sulphuric acid reagent; UV = ultra violet detection; DPPH = radical scavenging activities. I, thymol; II, β-caryophyllene; III, ar-curcumene; IV, α-selinene; V, citronellale; VI, geraniol; VII, nd; VIII, heliofolen-12ale A; IX, β-biotol; X, ar-turmerone; XI, β-turmerone.

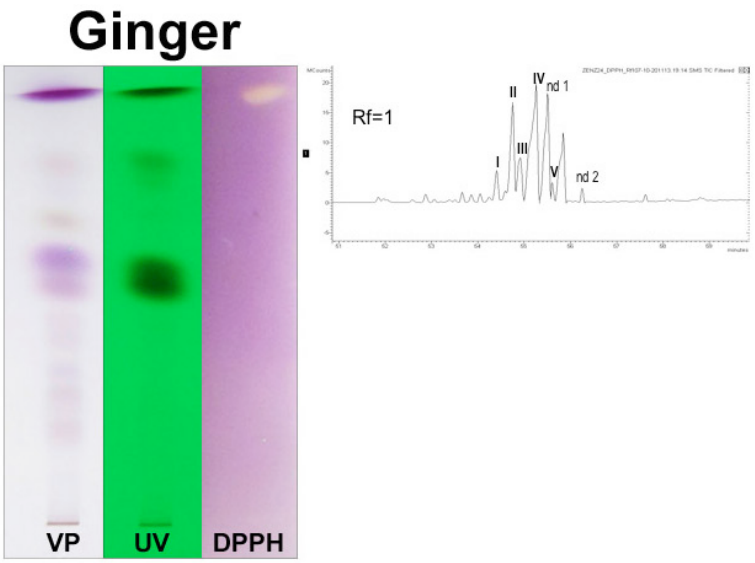


Fig. 51c (HP)TLC bioautographic assay of ginger eo. Note: VS = vanillic sulphuric acid reagent; UV = ultra violet detection; DPPH = radical scavenging activities. I, germacrene D; II, ar-curcumene; III, trans-muurola-4(14)5-diene; IV, α-curcumene; V, δ-ca dinene.

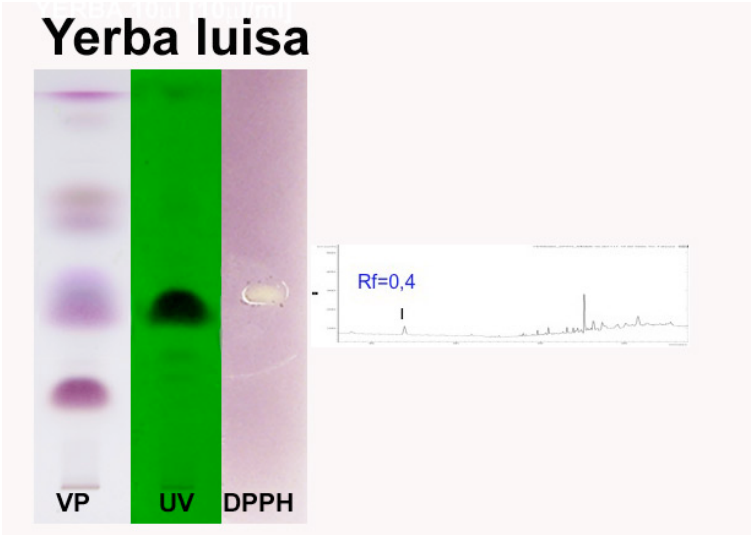


Fig. 51d (HP)TLC bioautographic assay of yerba luisa eo. Note: VS = vanillic sulphuric acid reagent; UV = ultra violet detection; DPPH = radical scavenging activities. I, eugenol.

C) Formulations (blended essential oils)

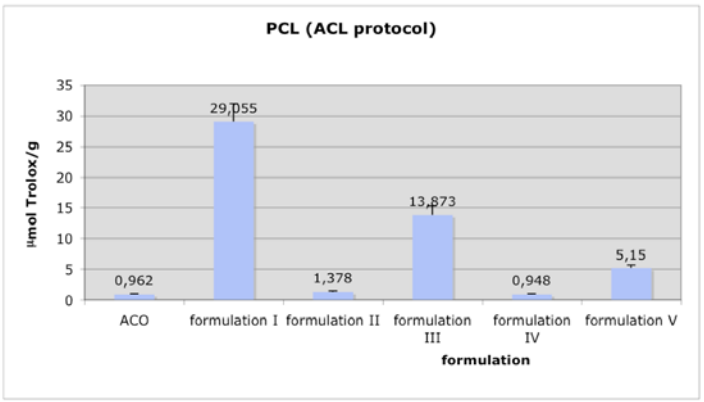
Essential oils are concentrated terpenic phytocomplexes and can cause skin irritation if used as they are. Mixed with other oils, instead, they can be used for cosmetic and/or nutraceutics uses. The fixed oils, used to be mixed with eo, are called carrier oils. Generally a good quality, cold-pressed plant represents the best common choice for herbal market. With the intent to exploring possible applicative uses of Rwandese avocado oil and Ecuadorian essential oils for “fair trade” markets, and in light of the results obtained with biological activities investigation, three simple healthy formulation has been prepared mixing an aliquot of avocado oil with essential oil to obtain the final concentration of 1 % (w/w) of eo in carrier oil. In light of the results obtained with antioxidant preliminary test, the essential oils which demonstrated high antioxidant activities have been chosen: *O. micranthum* essential oil and *C. citratus* essential oil. Since the phytochemical analyses revealed the presence of some allergenic compounds (see tab 22) mentioned by the European Cosmetic Guideline (2003/15/EC), the formulation have been prepared to minimize their concentration in the final product.

Allergenic compounds	YERBA	BASIL
Eugenol	**tr**	**50,97**
Geranial	**17,29**	**tr**
Geraniol	**39,43**	**tr**
Limonene		0,17
Linalool	0,46	1,87
Neral	**14,37**	

Tab. 22 Allergenic compounds occurring in essential oils used for health formulations

Antioxidant activities, of each healthy formulation, measured with PCL method have been compared with those of Tea tree and Thyme essential oils mixture prepared in the same way. The activityhas been measured through PCL thanks to the collaboration of the cosmetic chemistry lab of prof. Stefano Manfredini, University of Ferrara. PCL is an instrumented strategy to check antioxidant activity particularly fitting with complex formulations such as cosmetic ones are (BIBLIO BOOBS). All the formulations have been performed following the indications given by prof. Manfrdini cooworkers and organoleptic (smell) properties emerged in light of

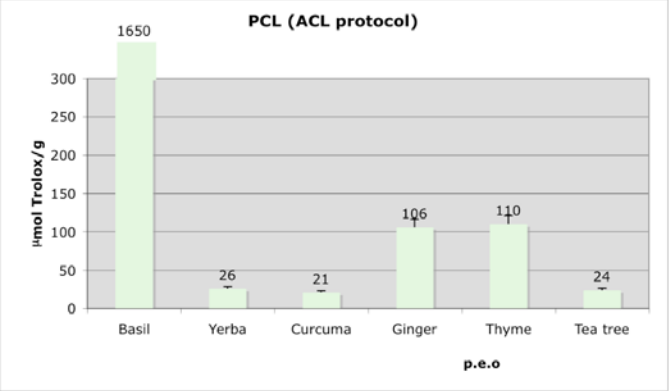
the different mixing. The most interesting results emerged with regard to formulation I, which evidenced values strongly higher than that expressed by controls (formulation IV and V; blended tea tree oil and thyme essential oil, both in avocado commercial fixed oil, respectively). This result reflects what emerged in previous assays about the roles of Avocado oil and essential oils. In fact, *O. micranthum* eo gave always interesting and better results. Formulation III (blended yerba luisa and Amazonian basil essential oils in avocado commercial fixed oil), gave results almost 50% lower but, in light of that emerged about formulation II (blended yerba luisa essential oil in avocado commercial fixed oil), it can be suggested that the bioactivity is almost entirely attributable to Amazonian basil essential oil fraction.



PCL analisy	
formulation	µmol Trolox/g (P ≤ 0.05)
ACO	0,962 ± 0,056
formulation I	29,055 ± 1,802
formulation II	13,78 ± 0,058
formulation III	13,873 ± 0,218
formulation IV	0,948 ± 0,050
formulation V	5,150 ± 0,259

Graph. 5a Tab. 23a results obtained with PCL analyses. OACOMM, avocado commercial oil; formulation I, OACOMM + Basil eo; formulation II OACOMM + Yerba eo; formulation III, OACOMM + Basil and Yerba essential oil (0,5 g each ones); Formulation IV, OACOMM + Tea tree eo; formulation V OACOMM + Thyme eo.

Simultaneously has been tested also Amazonian pure essential oils (i.e. *O. micranthum, C. citratus, Z. officinale and C. longa*) in comparison with commercial samples Thyme and Tea tree (*T. vulgaris* and *M. alternifolia* respectively), data, not already published, are summarized in figure below (Graph. 5b and Table 23b). The results obtained shed light on the interesting antioxidant activity of Amazonian basil and confirm its responsibility in the antioxidant activity showed by the formulation III.



PCL analisy	
OE	µmol Trolox/g (P ≤ 0.05)
Basil	1650,45 ± 11,,03
Yerba	26,65 ± 0,20
Curcuma	21,31 ± 0,11
Ginger	1,06 ± 0,10
Thyme	110,84 ± 8,29
Tea tree	24,84 ± 2,08

Graph. 5b Tab. 23b results obtained with PCL analyses. OACOMM, avocado commercial oil; formulation I, OACOMM + Basil eo; formulation II OACOMM + Yerba eo; formulation III, OACOMM + Basil and Yerba essential oil (0,5 g each ones); Formulation IV, OACOMM + Tea tree eo; formulation V OACOMM + Thyme eo.

Conclusions

My PhD work was split into two general research pathways leading to different goals:

- the valorization through chemical and biotechnological lab research strategies of ethnomedical sources from Rwanda and Ecuadorian Amazonia, in particular, Rwandese Avocado fixed oil and Amazonian Curcuma, Yerba luisa, Basil and Ginger essential oils;
- to help and support non-profit Italian associations to practically set up factory production systems which could give social and economic contributes to Natives in valorizing the biodiversity of the developing Country, exploring the exploitation of cultivated plants and derivates and the possibility to improve their markets, related to Essential, Fixed oils and Formulation (Blended essential oil).

Phytochemical characterization (fingerprinting) developed with different chromatographic techniques (GC-FID, GC-MS, HPLC, HPTLC), and NMR investigation, of phytocomplexes allowed to underline chemodiversity, and investigate possible presence of allergenic compounds limiting formulations.

Biological activities investigations as safety, genoprotective, antimicrobial and antioxidant (radical scaveging) properties of essential oils, linked to their chemical compositions underline the direct correlation between chemical evidences and bioactivities in terms of synergism (agonism or antagonism) of the whole phytocomplex.

Main results:

- Higher oil yields from avocado fruit are obtained with hexane, as solvent extraction than Supercritical CO_2 (SCO_2) method. This result is probably mainly due to the lower selectivity of hexane maceration than SFE, allowing the extraction of higher quantity of different chemicals.
- Total fatty acids amount is higher in avocado oil extracted with hexane than SCO_2 and comparable with that detected in the commercial sample. As expected, oleic acid was however the most abundant fatty acid in the oils.
- Unsaponifiable fraction yield is higher in ASFE, but sitosterol content is similar in both AHM and ASFE. In general, phytosterol pattern is quite different among the different extracts (avenasterol amount is widely higher in AHM than those in ASFE). Notable is the presence of

other triterpenoids alcohol (cycloartenol, gramisterol), which led to considering healthy perspectives of Rwandese avocado oil with respect to related literature (Lu et al., 2005; Wang, Ng, 1999; and references therein)

- Phenolic content, in terms of polyphenols, flavonoids and procyanidins, in general is higher in hexane extracted oil. This result is linked to radical scavenging activity which has been demonstrated higher in the AHM than other samples.
- Amazonian essential oils fingerprinting investigations allowed to point out chemodiversity as source of different health uses of the same plant source; the chemical quality control as main tool to determine identity quality and safety of plant sources through different and complementary methods.
- Genoprotective assays revealed interesting activities in all the essential oils tested, in particular *C. citratus* and *O. micranthum* essential oils showed best results both on *S. cerevisiae* D7 and Ames tests.
- Antimicrobial tests, developed in three different strategies (disk-diffusion (HP)TLC-bioautographyc and agar vapour assays), highlighted the good properties of Amazonian Basil and Yerba luisa essential oils, which demonstrated, in general, higher antimicrobial activities.
- Antioxidant tests, performed as radical scavenging properties investigations, revealed once more the good quality of Amazonian basil essential oil, together with Yerba luisa essential oil suggesting their employ as antioxidands or perseverants.
- At the end, a simple formulation, mixing avocado oils and essential oil has been prepared and tested as prompting antioxidant natural product. Antioxidant properties of formulation I (1% basil) and III (0,5 % basil, 0,5% yerba) were higher than that with the control thyme; on the other hand tea tree oil unexpectedly did not show interesting antioxidant properties.

In conclusion, this PhD research gave to me the possibility to explore situations related to Cooperation and Development projects, not always related to research but to which research could give a precious help. My contribute has been to try to connect modern research to situations far from our research standards. This

starting point of my PhD has led all the research performed and it is the main reason characterizing the scientifically weak evidences sometimes emerged together with interesting aspects related to chemical and biological evidences. However, the main research aspects that I feel to have learnt are the planning of a research strategy and the use of analytical and biological tools to valorize and qualify crude drugs and plant derivates. To stress this important aspects, the paper published and in press to which I contributed as co-author are reported at the end of the present thesis.

Bibliography

Abe SSY, Inoue S, Ishibashi H, Maruyama N, Takizawa T, Oshima H, Yamaghchi H, *Anti-Candida albicans activity of essential oils including lemongrass (Cymbopogon citratus) oil and its component, citral*, Nihon Ishinkin Gakkai zasshi = Japanese Journal of Medical Mycology, 44(4), 285–291, 2003

Adams RP, *Identification of essential Oil Components by Gas Chromatography/Mass Spectrometry*, 4th Edition Allured Publishing Corporation, Carol Stream, Illinois, USA, 2007

Aggarwal BB, Shishodia S, *Molecular targets of dietary agents for prevention and therapy of cancer*, Biochemical Pharmacology, 71(10), 1397-1421, 2006

Aggarwal BB, Sundaram C, Malani N, Ichikawa H, *Curcumin: the Indian solid gold* Advances in Experimental Medicine and Biology, 595, 1-75, 2007

Baratta MT, Damien Dorman HJ, Deans SG, Figueiredo AC, Barroso LG, Ruberto G, *Antimicrobial and antioxidant properties of some commercial essential oils*, Flavour Fragrance Journal, 13, 235-244, 1998

Bleeker PM, Diergaarde PJ, Ament K, Schütz S, Johne B, Dijkink J, Hiemstra H, de Gelder R, de Both MTJ, Sabelis MW, Haring MA, Schuurink RC, *Tomato-produced 7-epizingiberene and R-curcumene act as repellents to whiteflies*, Phytochemistry, 72, 68–73, 2011

Bortolomeazzi R, Verardo G, Lessi A, Callea A, *Formation of dehydrodiisoeugenol and dehydrodieugenol from the reaction of isoeugenol and eugenol with DPPH radical and their role in the radical scavenging activity*, Food Chemistry, 118, 256–265, 2010

Bruni R, Medici A, Guerrini A, Scalia S, Poli F, Muzzoli M, Sacchetti G, *Wild Amaranthus caudatus Seed Oil, a Nutraceutical Resource from Ecuadorian Flora*, J Agricultural and Food Chemistry, 49, 5455-5460, 2001.

Bruni R, Rossi D, Muzzoli M, Romagnoli C, Paganetto G, Besco E, Choquecillo F, Peralta K, Schmitt Lora W, Sacchetti G, *Antimutagenic, antioxidant and antimicrobial properties of Maytenus krukovii bark*, Fitoterapia 77, 538–545, 2006

Butt AJ, Roberts CG, Seawright AA, Oelrichs PB, Macleod JK, Liaw TY, Kavallaris M, Somers-Edgar TJ, Lehrbach GM, Watts CK, Sutherland RL, *A novel plant toxin, persin, with in vivo activity in the mammary gland, induces Bim-dependent apoptosis in human breast cancer cells*, Molecular Cancer Therapeutics, 5(9), 2300–2309, 2006

Carbajal D, Casaco A, Arruzazabala L, Gonzalez R, Tolon Z, *Pharmacological study of Cymbopogon citratus leaves*, J Ethnopharmacology, 25(1), 103-107, 1989

Cheel J, Theoduloz C, Rodriguez J, Schmeda-Hirschmann G, *Free radical scavengers and antioxidants from Lemongrass (Cymbopogon citratus [DC.] Stapf.)*, J Agricultural and Food Chemistry, 53(7), 2511-2517, 2005

Chen W, Viljoen AM, *Geraniol — A review of a commercially important fragrance material, South African Journal of Botany*, 76, 643–651, 2010

Chinnasamy G, Davis PJ, Bal AK, *Seasonal changes in oleosomic lipids and fatty acids of perennial root nodules of beach pea*, J Plant Physiology, 160, 355–365, 2003

Choudhury D, Das A, Bhattacharya A, Chakrabarti G, *Aqueous extract of ginger shows antiproliferative activity through disruption of microtubule network of cancer cells*, Food and Chemical Toxicology, 48(10), 2872-2880, 2010

Daels-Rakotoarison DA, Gressier B, Trotin F, Brunet C, Luyckx M, Dine T, Bailleul F, Cazin M, Cazin JC, *Effects of Rosa canina Fruit Extract on Neutrophil Respiratory Burst*, Phytother. Res. 16, 157–161, 2002

Damirchi SA, Savane GP, Dutta PC, *Sterol Fractions in Hazelnut and Virgin Olive Oils and 4,4'-Dimethylsterols as Possible Markers for Detection of Adulteration of Virgin Olive Oil*, JAOCS 82(10) 717–725, 2005

De Matouschek BV, Stahl-Biskup E, *Phytochemische Untersuchung der nichtflüchtigen Inhaltsstoffe von Cymbopogon citratus (DC.) Stapf (Poaceae)*, Pharmaceutica Acta Helvetiae, 66, 242−245, 1991

Dieffenbacher A, Pocklington WD, *Standard Methods for the Analysis of Oils, Fats and Derivatives*, 1st supplement to the 7th edition Blackwell Science, 1992

Ding H, Chin YW, Kinghorn AD, D'Ambrosia SM, *Chemopreventive characteristics of avocado fruit*, Seminars in Cancer Biology, 17, 386–394, 2007

Farines M, Soulier J, Rancurel A, Montaudoin MG, Leborgne L, *Influence of Avocado Oil Processing on the Nature of Some Unsaponifiable Constituents*, J American Oil Chemists' Society, 72, 473-476, 1995

Ferrazzano GF, Amato I, Ingenito A, Zarrelli A, Pinto G, Pollio A, *Plant phytosterols and their anti-carcinogenic properties: a rewiew*, Molecules, 16(2), 1486-507, 2011

Garcia-Alonso M, S. de Pascual T, Santos-Buelga C, Rivas-Gonzalo JC, *Evaluation of the antioxidant properties of fruits*, Food Chemistry, 84,13-18, 2004

Gazola R, Machado D, Ruggiero C, Singi G, Macedo Alexandre M, *Lippia alba, Melissa officinalis and Cymbopogon citratus: effects of the aqueous extracts on the isolated hearts of rats*, Pharmacological Research, 50(5), 477-480, 2004

Girón LM, Freire V, Alonzo A, Cáceres A*. Ethnobotanical survey of the medicinal flora used by the Caribs of Guatemala*, Journal of Ethnopharmacology, 34 (2-3) 173–187, 1991

Grieve M, A Modern Herbal (Volume 1, A-H): *The Medicinal, Culinary, Cosmetic and Economic Properties, Cultivation and Folk-Lore of Herbs, Grasses, Fungi, Shrubs & Trees with Their Modern Scientific Uses*, Paperback, 1971

Guerrini A, Bruni R, Maietti S, Poli F, Rossi D, Paganetto G, Muzzoli M, Scalvenzi L, Sacchetti G, *Ecuadorian stingless bee (Meliponinae) honey: A chemical and functional profile of an ancient health product*, Food Chemistry, 114, 1413–1420, 2009

Guerrini A, Rossi D, Paganetto G, Tognolini M, Muzzoli M, Romagnoli C, Antognoni F, Vertuani S, Medici A, Bruni A, Useli C, Tamburini E, Bruni R, Sacchetti G, *Chemical Characterization (GC/MS and NMR Fingerprinting) and Bioactivities of South-African Pelargonium capitatum (L.) L Her. (Geraniaceae) Essential Oil*, Chemistry and Biodiversity, 8(4), 624-42, 2011

Guerrini A, Sacchetti G, Muzzoli M, Moreno Rueda G, Medici A, Besco E, Bruni R, *Composition of the Volatile Fraction of Ocotea bofo Kunth (Lauraceae) Calyces by GC-MS and NMR Fingerprinting and Its Antimicrobial and Antioxidant Activity*, Journal of Agricultural and Food Chemistry, 54, 7778-7788, 2006

Guerrini A, Sacchetti G, Rossi D, Paganetto G, Muzzoli M, Andreotti E, Tognolini M, Maldonado ME, Bruni R, *Bioactivities of Piper aduncum L. and Piper obliquum Ruiz & Pavon (Piperaceae) essential oils from Eastern Ecuador*, Environmental Toxicology and Pharmacology, 27(1), 39-48, 2009

Gupta S, Saha B, Giri AK, *Comparative antimutagenic and anticlastogenic effects of greentea and black tea: a review*, Mutation Research/Reviews in Mutation Research, 512(1) 37–65, 2002

Gutfinger T, Letan A, *Studies of unsaponifiables in several vegetable oils*, Lipids, 9(9), 658-663, 1974

Hatcher H, Planalp R, Cho J, Torti FM, Torti SV, *Curcumin: from ancient medicine to current clinical trials, Cellular and molecular life sciences*, 65(11), 1631-1652, 2008

Henrotin YE, Labasse AH, Jaspar JM, De Groote DD, Zheng SX, Guillou GB, Reginster JY, *Effects of Three Avocado/Soybean Unsaponifiable Mixtures on Metalloproteinases, Cytokines and Prostaglandin E2 Production by Human Articular Chondrocytes*, Clinical Rheumatology, 17, 31-39, 1998

Hu JN, Zhu XM, Adhikari P, Li D, Kim IH, Lee KT, *Determination of Tocopherol Contents in Refined Edible Oils Using an HPLC Method*, J Food Science and Nutrition, 14, 260-264. 2009

Jayaprakasha GK, Jagan Mohan RL, Sakariah KK*, Chemistry and biological activities of C. longa*, Trends in Food Science & Technology 16(12), 533-548, 2005

Kashman, Y, Neeman I, Lifshitz A, *New compound from avocado pear*, Tetrahedron 25(18), 4617-4631, 1969

Kawagishi H, Fukumoto Y, Hatakeyama M, He P, Arimoto H, Matsuzawa T, Arimoto Y, Suganuma H, Inakuma T, Sugiyama K, *Liver Injury Suppressing Compounds from Avocado (Persea americana)*, Journal of Agricultural and Food Chemistry, 49(5), 2215–2221, 2001

Kim HJ, Chen F, Wu CQ, Wang X, Chung HY, Jin ZY, *Evaluation of antioxidant activity of Australian tea tree (Melaleuca alternifolia) oil and its components* J Agric Food Chem, 52, 2849– 2854, 2004

Kishimoto K, Urade R, Ogawa T, Moriyama T, *Nondestructive Quantification of Neutral Lipids by Thin-Layer Chromatography and Laser-Fluorescent Scanning: Suitable Methods for "Lipidome" Analysis*, Biochemical and Biophysical Research Communications 281, 657–662, 2001

Kumar A, Malik F, Bhushan S, Sethia VK, Shahia AK, Kaurb J, Tanejaa SC, Qazia GN, Singh J, *An essential oil and its major constituent isointermedeol induce apoptosis by increased expression of mitochondrial cytochrome c and apical death receptors in human leukaemia HL-60 cells*, Chemico-Biological Interaction, 171(3), 332-347, 2008

Kutluay SB, Doroghazi J, Roemer ME, Triezenberg SJ, *Curcumin inhibits herpes simplex virus immediate-early gene expression by a mechanism independent of p300/CBP histone acetyltransferase activity*, Virology 373(2), 239, 2008.

Lamaison & Carnat (1991) Pharm. Acta Helv., 65, 315-320 ######

Lee HS, Choi KJ, Cho KJ, Ahn YJ, *Fungicidal Activity of ar-Turmerone Identified in Curcuma longa Rhizome against Six Phytopathogenic Fungi*, Agric. Chem. Biotechnol. 46(1), 25-28, 2003

Lee OH, Lee BY, Kim YC, Shetty K, Kim YC*, Radical Scavenging-Linked Antioxidant Activity of Ethanolic Extracts of Diverse Types of Extra Virgin Olive Oils*, J Food Science 73(7), 519-525, 2008

Lequesne M, Maheu E, Cadet C, Dreiser RL, *Structural Effect of Avocado/Soybean Unsaponifiables on Joint Space Loss in Osteoarthritis of the Hip*, Arthritis Care & Research 47, 50–58, 2002

Lewin, G.; Popov, I. *Oxidants and antioxidants part B—antioxidative homeostasis: Characterization by means of chemiluminescent technique*, Methods Enzymol, 300, 437-456, 1999

Lie Ken Jie MSF, Lam CC, *^{1}H-Nuclear magnetic resonance spectroscopic studies of saturated, acetylenic and ethylene triacylglycerols*, Chemistry and Physics of Lipids, 77(2), 155-171, 1995

Lie-Chwen L, Yuh-Chi K, Cheng-Jen C, *Immunomodulatory Proanthocyanidins from Ecdysanthera utilis*, J. Nat. Prod., 65 (4) 505–508, 2002

Lino CS, Gomes PB, Lucetti DL, Diógenes JPL, Sousa FCF, Silva MGV, Viana GSB, *Evaluation of antinociceptive and antiinflammatory activities of the essential oil (EO) of Ocimum micranthum Willd. from Northeastern Brazil*, Phytother Res, 19(8), 708-712, 2005

Logaraj TV, Bhattacharya S, Sankar KU, Venkateswaran G, *Rheological behaviour of emulsions of avocado and watermelon oils during storage*, Food Chemistry, 106, 937–943, 2008

Lozano YF , Mayer CD, Bannon C, Gaydoub EM, Unsaponifiable Matter, Total Sterol and Tocopherol Contents of Avocado Oil Varieties, JAOCS, 70(6), 561-565, 1993

Lu QY, Arteaga, JR, Zhang Q, Huerta S, Go VL, Heber D, *Inhibition of prostate cancer cell growth by an avocado extract: Role of lipid-soluble bioactive substances*, J Nutritional Biochemistry, 16(1), 23–30, 2005

Maietti S, Rossi D, Guerrini A, Useli C, Romagnoli C, Poli F, Bruni R, Sacchetti G, *Multivariate analysis approach to the study of chemical and functional properties of chemo-diverse plant derivatives: Lavender essential oils*, Flavour and Fragrance Journal in print

Maron D, Ames BN, *Revised methods for the Salmonella mutagenicity test*, Mutatation Research 113(3-4), 173-215, 1983

Maron DM, Ames BN, *Revised method for the Salmonella mutagenicity test*, Mutat. Res. 113(3-4), 173-215, 1983

Martinez Nieto L, Moreno Romero V, *Sterolic composition of the unsaponifiable fraction of oil of avocado of several varieties*, Grassa y Aceites, 45(6), 402-403, 1994

Menut C, Bessiere JM, Samate D, Djibo, AK, Buchbauer G, Schopper B, *Aromatic plants of tropical west Africa. XI. chemical composition, antioxidant and antiradical properties of the essential oils of three Cymbopogon species from Burkina Faso*, J Essential Oil Research, 12(2), 207–212, 2000

method ISO/DIS 5509, 1998.

Moreno Rueda MG, *Biotrasformazioni di terpeni e oli essenziali con batteri e funghi isolati da frutti del genere Citrus della foresta amazzonica (Ecuador)*, 2009

Mostert ME, Botha BM, Du Plessis LM, Duodu KG, *Short Communication Effect of fruit ripeness and method of fruit drying on the extractability of avocado oil with hexane and supercritical carbon dioxide*, J Sci Food Agric 87, 2880–2885, 2007.

Mueller M, Hobiger S, Jungbauer A, *Anti-inflammatory activity of extracts from fruits, herbs and spices*, Food Chemistry 122(4) 987-996, 2010

Nakamura Y, Miyamoto M, Murakami A, Ohigashi H, Osawa T, Uchida K, *A phase II detoxification enzyme inducer from lemongrass: identification of citral and involvement of electrophilic reaction in the enzyme induction*, Biochem Biophys Res Commun, 302(3), 593-600, 2003

Naranjo P, Escaleras R, *La medicina tradicional en el Ecuador*, Biblioteca Ecuatoriana de ciencias, volumen 2, Corporacíon Editora Nacional 1995

Navarro MC, Montilla MP, Cabo MM, Galisteo M, Cáceres A, Morales C, Berger I, *Antibacterial, antiprotozoal and antioxidant activity of five plants used in Izabal for infectious diseases*, Phytother Res, 17, 325-329, 2003

Oberlies NH, Rogers LL, Martin JM, McLaughlin JL, *Cytotoxic and Insecticidal Constituents of the Unripe Fruit of Persea americana*, J Natural Product 61, 781-785, 1998

Onawunmi GO, Yisak WA, Ogunlana EO, *Antibacterial constituents in the essential oil of Cymbopogon citratus (DC.) Stapf.* J Ethnopharmacol, 12(3), 279-286, 1984

Otero R, Nunez V, Barona J, Fonnegra R, Jiménez SL, Osorio RG, Saldarriaga M, Díaz A, *Snakebites and ethnobotany in the northwest region of Colombia. Part III: neutralization of the haemorrhagic effect of Bothrops atrox venom*. J Ethnopharmacol, 73, 233-241, 2000

Oyagbemi AA, Saba AB, Azeez OI, *Molecular targets of [6]-gingerol: Its potential roles in cancer chemoprevention*, BioFactors, 36(3), 169–178, 2010

Oyedele AO, Gbolade AA, Sosan MB, Adewoyin FB, Soyelu OL, Orafidiya OO, *Formulation of an effective mosquito-repellent topical product from lemongrass oil*, Phytomedicine 9(3), 259-262, 2002

Ozdemir F, Ayhan Topuz, *Changes in dry matter, oil content and fatty acids composition of avocado during harvesting time and post-harvesting ripening period* Food Chemistry 86, 79–83, 2004

Platt KA, Thompson WW, *Idioblast oil cells of avocado: distribution, isolation, ultrastructure, histochemistry and biochemistry*, Int J Plant Sci 153(3) 301-310, 1992

Popenoe Wilson, *Manual Of Tropical And Subtropical Fruits*, ed. The Macmillan Company, 1920

Porter LJ, Hrstich LN, Chan BG, *The conversion of procyanidins and prodelphinidins to cyanidin and delphinidin* Phytochemistry, 25(1) 223-230, 1985

Pradhan RC, Meda V,Rout PK, Naik S, Dalai AK, *Supercritical CO_2 extraction of fatty oil from flaxseed and comparison with screw press expression and solvent extraction processes*, J Food Engineering 98, 393–397, 2010

Radice M, *Studi di attività biologica cosmecautica di derivati di specie della zona sud orientale amazzonica ecuadoriana*, 2010

Re R, Pellegrini N, Proteggente A, Pannala A, Rice-Evans C, *Antioxidant activity applying an improved ABTS radical cation decolorization assay*, Free Radical Biology and Medicine 26, 1231–1237, 1999

RE V, Simon JE, *Chemical characterization of basil (Ocimum spp.) found in the markets and used in traditional medicine in Brazil*, Economic Botany 54(2), 207-216, 2000

Regolamento CEE n. 2568/91 della Commissione dell'11 Luglio 1991 relativo alle caratteristiche degli oili di oliva e di sansa d'oliva nonché ai metodi ad essi attinenti

Robbins KS, Shin EC, Shewfelt RL, Eitenmiller RR, Pegg RB, *Update on the healthful lipid constituents of commercially important tree nuts*, J Agricultural and Food Chemistry 59(22), 12083-12092, 2011

Rodríguez-Carpena JG, Morcuende D, Andrade MJ, Kylli P, Estevez M, *Avocado (Persea americana Mill.) Phenolics, In Vitro Antioxidant and Antimicrobial Activities, and Inhibition of Lipid and Protein Oxidation in Porcine Patties*, Journal of Agricultural and Food Chemistry, 59(10), 5625-5635, 2011

Rodriguez-Saona C, Millar JG, Maynard DF, Trumble JT, Novel antifeedant and insecticidal compounds from avocado idioblast cell oil, Journal of Chemical Ecology, 24(5), 867-889, 1998

Rossi D, Guerrini A, Maietti S, Bruni R, Paganetto G, Poli F, Scalvenzi L, Radice M, Saro K, Sacchetti G *Chemical fingerprinting and bioactivity of Amazonian Ecuador Croton lechleri Müll. Arg. (Euphorbiaceae) stem bark essential oil: A new functional food ingredient?*, Food Chemistry 126, 837–848, 2011

Sacchetti G, Bruni R, *Efficiency of Extracting Vitamin E from Plant Sources, The Encyclopedia of Vitamin E,* Edited by Preedy VR, Watson RR, 2007

Sacchetti G, Maietti S, Muzzoli M, Scaglianti M, Manfredini S, Radice M, Bruni R, *Comparative evaluation of 11 essential oils of different origin as functional antioxidants, antiradicals and antimicrobials in foods*, Food Chemistry 91, 2005, 621–632, 2005

Sacchetti G, Medici A, Maietti S, Radice M, Muzzoli M, Manfredini S, Braccioli E, Bruni R, *Composition and Functional Properties of the Essential Oil of Amazonian Basil, Ocimum micranthum Willd., Labiatae in Comparison with Commercial Essential Oils*, J Agric Food Chem, 52, 3486-3491, 2004

Sacchetti G, Rossi D, Maietti S, Grandini A, Poppi I, Ballero M, Guerrini A, *Preliminary phytochemical and biological properties of a Sardinian endemism: Ribes sandalioticum Arrigoni,* Atti del 106° Congresso SBI Onlus, 73, 66-68, 2011

Sacchi R, Addeo F Paolillo L, *^{1}H and ^{13}C NMR of Virgin Olive Oil. An Overview*, Magnetic Resonance in Chemistry, 35, 133-145, 1997

Scalvenzi L, *Amazonian plants from ethnomedicine through pharmaceutical biology approaches: a PhD experience in connecting forest with laboratory*, 2010

Scartezzini P, Antognoni F, Raggi MA, Poli F, Sabbioni C, *Vitamin C content and antioxidant activity of the fruit and of the Ayurvedic preparation of Emblica officinalis Gaertn.*, J Ethnopharmacology104(1-2), 113-118, 2006

Secretary-General Kofi Annan, Bangkok (United Nations Information Services), Tenth United Nations Conference on Trade and Development ("UNCTAD X"), Bangkok, 12 February 2000

Shadab Q, Hanif M, Chaudhary FM, *Antifungal activity by lemongrass essential oils* Pak J Sci Ind Res 35, 246-249, 1992

Slish DF, Ueda H, Arvigo R, Balick MJ, *Ethnobotany in the search for vasoactive herbal medicines* J Ethnopharmacol 66, 159-165, 1999

Souquet J, Cheynier V, Brossaud F, Moutounet M, *Polymeric proanthocyanidins from grape skins* Phytochemistry 43 (2) 509, 1996

Suaeyun R, Kinouchi T, Arimochi H, Vinitketkumnuen U, Ohnishi Y, *Inhibitory effects of lemon grass (Cymbopogon citratus Stapf) on formation of azoxymethane-induced DNA adducts and abberant crypt foci in the rat colon*, Carcinogenesis 18(5) 949-955 1997

Toivo J, Phillips K, Lampi AM, Piironen V, *Determination of sterols in foods: Recovery of free, esterified, and glycosidic Sterols,* J. Fof Food composition and analysis, 14, 631-643, 2001

Usman LA, Hamid AA, George OC, Ameen OM, Muhammad NO, Zubair MF, Lawal A, *Chemical composition of rhizome essential oil of Curcuma longa L. growing in north central Nigeria*, World Jurnal of Chemistry 4(2) 178-181, 2009

Valero D, Valverde JM, Martínez-Romero D, Guillen F, Castillo S, Serrano M, *The combination of modified atmosphere packaging with eugenol or thymol to maintain quality, safety and functional properties of table grapes,* Postharvest Biology and Technology 41 (2006) 317–327

Van del Dool H, Kartz PD, *A generalization of the retention index system including linear temperature programmed gas-liquid partition chromatography*, J Chromatography, 11, 463-47, 1963

Van Hoed V, Depaemelaere G, Vila Ayala J, Santiwattana P, Verhé R, De Greyt W, *Influence of Chemical Refining on the Major and Minor Components of Rice Bran Oil*, JAOCS, 83(4) 315-321, 2006

Vertuani S, Beghelli E, Scalambra E, Malisardi G, Copetti S, Dal Toso R, Baldisserotto A, Manfredini S, *Activity and Stability Studies of Verbascoside, a Novel Antioxidant, in Dermo-Cosmetic and Pharmaceutical Topical Formulations*, Molecules 16, 7068-7080, 2011

Vinitketkumnuen U, Chewonarin T, Dhumtanom P, Lertprasertsuk N, Wild CP, *Aflatoxin-albumin adduct formation after single and multiple doses of aflatoxin B(1) in rats treated with Thai medicinal plants*, Mutat Res 428(1-2) 345-351, 1999

Vinitketkumnuen U, Puatanachokchai R, Kongtawelert P, Lertprasertsuke N, Matsushima T, *Antimutagenicity of lemon grass (Cymbopogon citratus Stapf) to various known mutagens in salmonella mutation assay*. Mutat Res 341(1) 71-75, 1994

Wagner H,S. Bladt *Plant Drug Analysis* ed. Springer-Verlag Berlin Heidelberg New York 2009

Wang W, Bostic TR, Gu L, *Antioxidant capacities, procyanidins and pigments in avocados of different strains and cultivars*, Food Chemistry 122, 1193–1198, 2010

Wang, M, Li J, Rangarajan M, Shao Y, La Voie EJ, Huang TC, Ho CT, *Antioxidant phenolic compounds from sage (Salvia officinalis)* J Agricultural and Food Chemistry, 46, 4869-4873, 1998.

Werman MJ, Mokady S, Neeman I, *A Simple and Sensitive Method for Detecting Avocado Seed Oil in Various Avocado Oils* JAOCS 73, 665-667 1996

Wong M, Requejo-Jackman C, Woolf A, *What is unrefined, extra virgin cold-pressed avocado oil?* http://www.aocs.org, 2010

World Trade Organization (WTO) (2004-09-30). "Continued reforms and technical assistance should help Rwanda in its efforts to achieve a dynamic economy". Trade policy review: Rwanda. Retrieved 2009-02-04

Yamahara J, Mochizuki M, Rong HQ, Matsuda H, Fujimura H, *The anti-ulcer effects in rats of ginger constituents*, J Ethnopharmacol 23(2-3), 299-304, 1988

Yoshida H, Shigezaki J, Takagi S, Kajimoto G, *Varations in the composition of various acyl lipids, tocopherols, and lignans in sesame seed oil roasted in a microwave oven,* J Sci Food Agric (68) 407-415, 1995

Yue GGL, Chan BCL, Hon PM, Lee MYH, Fung KP, Leung PC, Lau CBS, *Evaluation of in vitro anti-proliferative and immunomodulatory activities of compounds isolated from Curcuma longa*, Food and Chemical Toxicology, 48, 2011–2020, 2010

Zimmermann FK, Kern R, Rasenberger H, *A yeast strani for simultaneus detection of induced mitotic crossino over, mitotic gene conversion and reverse mutation*, Mutation Research, 28, 381-388, 1975

Sitography

http://medconditions.net Dictionary of medical conditions terminology

http://orizho.com

http://unstats.un.org/unsd/methods

http://www.fao.org/docrep/008/y4705e/y4705e12.htm

http://www.state.gov/r/pa/ei/bgn/35761.htm Background Note: Ecuador. US Department of State. State.gov. 2010-05-24. Retrieved 2010-06-26

www.ars-grin.gov Curcuma longa information from NPGS/GRIN, 2008

www.atcc.org

www.imf.org International Monetary Found Data

www.wikipedia.org

Chemical Characterization (GC/MS and NMR Fingerprinting) and Bioactivities of South-African *Pelargonium capitatum* (L.) L'Her. (Geraniaceae) Essential Oil

by **Alessandra Guerrini**[a]), **Damiano Rossi**[b]), **Guglielmo Paganetto**[a]), **Massimiliano Tognolini**[c]), **Mariavittoria Muzzoli**[a]), **Carlo Romagnoli**[d]), **Fabiana Antognoni**[c]), **Silvia Vertuani**[f]), **Alessandro Medici**[a]), **Alessandro Bruni**[b]), **Chiara Useli**[a]), **Elena Tamburini**[b]), **Renato Bruni**[g]), and **Gianni Sacchetti***[a])

[a]) Dipartimento di Biologia ed Evoluzione, Sez. Risorse Agrotecnologiche e Farmaceutiche, AgriUnife, Università degli Studi di Ferrara, C.so Ercole I d'Este 32, I-44100 Ferrara (fax: 0039 0532 208561; e-mail: gianni.sacchetti@unife.it)

[b]) AGRIUNIFE Centro di Ateneo per l'Agricoltura di Pianura, Università di Ferrara, Via Conca 73/B, I-44030 Malborghetto di Boara, Ferrara

[c]) Dipartimento di Scienze Farmacologiche, Biologiche e Chimiche Applicate, Università degli Studi di Parma, V.le G.P. Usberti, I-43100 Parma

[d]) Dipartimento del Museo di Paleobiologia e dell'Orto Botanico, Università di Modena e Reggio Emilia, V.le Caduti in Guerra 127, I-41100 Modena

[e]) Dipartimento di Biologia Evoluzionistica e Sperimentale, Università degli Studi di Bologna, Via Irnerio 42, I-40126 Bologna

[f]) Dipartimento di Scienze farmaceutiche, Università degli Studi di Ferrara, Via Fossato di Mortara 17/19, I-44100 Ferrara

[g]) Dip. di Biologia Evolutiva e Funzionale, Università degli Studi di Parma, V.le G.P. Usberti 11A, I-43100 Parma

Chemical fingerprinting of commercial *Pelargonium capitatum* (Geraniaceae) essential oil samples of south African origin was performed by GC, GC/MS, and ^{13}C- and ^{1}H-NMR. Thirty-seven compounds were identified, among which citronellol (32.71%) and geraniol (19.58%) were the most abundant. NMR Spectra of characteristic chemicals were provided. Broad-spectrum bioactivity properties of the oil were evaluated and compared with those of commercial *Thymus vulgaris* essential oil with the aim to obtain a functional profile in terms of efficacy and safety. *P. capitatum* essential oil provides a good performance as antimicrobial, with particular efficacy against *Candida albicans* strains. Antifungal activity performed against dermatophyte and phytopathogen strains revealed the latter as more sensitive, while antibacterial activity was not remarkable against both *Gram*-positive and *Gram*-negative bacteria. *P. capitatum* oil provided a lower antioxidant activity (IC_{50}) than that expressed by thyme essential oil, both in the 1,1-diphenyl-2-picrylhydrazyl (DPPH) and β-carotene bleaching tests. Results in photochemiluminescence (PCL) assay were negligible. To test the safety aspects of *P. capitatum* essential oil, mutagenic and toxicity properties were assayed by *Ames* test, with and without metabolic activation. Possible efficacy of *P. capitatum* essential oil as mutagenic protective agent against NaN_3, 2-nitrofluorene, and 2-aminoanthracene was also assayed, providing interesting and significant antigenotoxic properties.

Introduction. – The genus *Pelargonium* includes *ca.* 270 species, both herbs, shrubs, or undershrubs, the majority of which (*ca.* 80%) are endemic of Southern Africa. The center of biodiversity of the genus is localized in the south-western part of Cape

Province, an area characterized by a distinct Mediterranean-type climate [1][2]. Despite its South African phylogenetic origin, China, Egypt, Madagascar, Algeria, and Morocco are major producers of geranium oil. The main commercial geranium oil type is known as Geranium Bourbon oil from Reunion Island, reputed of the finest quality, while all the other commercial essential oils are from African regions [3–6]. A number of *Pelargonium* species (*i.e.*, *P. graveolens*, *P. capitatum*, *P. zonale*, *P. roseum*, *P. odoratissimum*, and related hybrids) provides a highly-marketed rose-scented essential oil, rich in citronellol and geraniol, obtained by steam distillation of fresh leaves and branches. The oil, mainly known under the generic name of '*geranium oil*', even if obtained from distinct *Pelargonium* species and/or hybrids, is used 'as is' or as base for extraction of commercial rhodinol (a mixure of citronellol, geraniol, and linalool, extensively used as a base for rose, muguet, carnation, appleblossom, and many other perfume types) for manufacturing both superior-quality perfumes and skin-care products. In fact, besides its use as cosmetic ingredient for its fragrance, astringent, and cleansing properties, geranium oil is also highly prized for its functional properties in aromatherapy, and for its antibacterial, antifungal, vulnerary, insect repellant, and skin-cleansing properties, providing at the same time a slight seborrhoic effect [7–11]. As a consequence, this oil represents an interesting raw material both for cosmeceuticals and nutraceuticals (*i.e.*, beverages, candies, and baked goods) [12][13].

Pelargonium capitatum (L.) L'Her., a decumbent or weakly erect plant, with strongly rose-scented and velvet foliage, is one of the earlier species imported to Europe, and its cultivars are at present widespread in warmer climates and selected for their fine scent. In fact, *P. capitatum* is one of the parent species of the Bourbon cultivar, grown in Reunion Island for the production of high-quality rose-scented geranium oil [1]. Rose-scented geranium-oil profile may vary greatly according to geographic origin, time of collection, and cultivar [12]. The market value of the essential oil is determined by terpenoid composition: in particular, besides geraniol, citronellol, linalool, and their esters, isomenthone, the sesquiterpenoid hydrocarbon guaia-6,9-diene, and alcohol 10-*epi*-γ-eudesmol play a key role, as they allow to distinguish between oils of different origin, and different varieties and quality [3][5][6][8][14].

Rose-scented geranium oil is commercially available in different qualities, ranging from the low-quality Chinese oil to the top-quality Bourbon oil [8]. Different chromatographic and spectroscopic methods are currently available for fingerprinting *Pelargonium* fragrances both for quality definition and for fraud detection. In fact, the characterization of complex mixtures of volatile compounds is usually achieved through high-resolution chromatographic or hyphenated techniques as mass spectrometry (MS), enantiomeric and comprehensive two-dimensional (2D) gas chromatography/mass spectrometry (Enantio-GC; GC/GC-MS) [5]. During the last decade, high-resolution nuclear magnetic resonance (NMR) emerged as a powerful tool for the fingerprinting of natural extracts, including terpenes, sesquiterpenes, and other volatile compounds, and for assessing the quality of raw materials for food, herbal, and pharmaceutical industries. Such approach, however, has been scarcely applied to geranium oil fingerprinting, and data regarding a fast and reliable technique as NMR are lacking [5]. Despite the wide range of information attainable with such approach, its widespread use in phytochemical analysis is hindered by the lack of exhaustive information on peak assignment.

In this article, a detailed study of chemical characterization and bioactivity of *Pelargonium capitatum* essential oil of south African origin is reported. Particular emphasis is given to the correlation between chromatographic and NMR data, with the aim to provide a first report of ^{1}H- and ^{13}C-NMR fingerprinting of *P. capitatum* oil. Moreover, to characterize its functional profile both with efficacy and safety parameters; mutagenic/antimutagenic properties, together with antioxidant and antimicrobial activities, have been evaluated for the first time.

Results and Discussion. – 1. *Chemical Composition of the Essential Oil*. The GC and GC/MS analyses of the essential oil allowed identification of 37 compounds, accounting for 94.97% of the total (*Table 1*). According to the rose scent and to the chemical evidences, the composition of the essential oil seems to be in accordance with that of *'Attar of Roses'* essential oils [13]. This suggestion is based on the fact that the most abundant compounds were citronellol (32.71%), geraniol (19.58%), 2-phenylethyl alcohol (9.23%), and citronellyl formate (5.58%). Other compounds, *e.g.*, linalool (3.09%), were all under 5.00%. Among the minority compounds, usually detected in *'Attar of Roses'* oils, guaia-6,9-diene was identified neither by GC and GC/MS, nor NMR. However, although in extremely low quantities, the presence of 10-*epi*-γ-eudesmol (0.27%), a typical marker of the African origin *Pelargonium* species [15], was also checked. 2-Phenylethyl alcohol, not known as a chemical marker of *Pelargonium* essential oils regarding origin and cultivation [13], is instead considered as one of the

Table 1. *Essential Oil Composition of* P. capitatum *Essential Oil*

Entry	Compound[a])	*KI*[b])	RAA[c])	*Entry*	Compound[a])	*KI*	RAA[b])
1	α-Pinene	939	0.20	*20*	Geranial	1267	0.30
2	β-Myrcene	991	0.10	*21*	Citronellyl formate	1274	5.58
3	1,8-Cineole	1031	0.13	*22*	Geranyl formate	1298	1.89
4	(*E*)-Ocimene	1037	0.18	*23*	Citronellyl acetate	1353	0.23
5	(*Z*)-Ocimene	1050	0.87	*24*	Geranyl acetate	1381	0.56
6	Linalool	1097	3.09	*25*	β-Elemene	1391	2.58
7	2-Phenylethanol	1107	9.23	*26*	Diphenyl ether	1398	3.67
8	Rose oxide (*cis*)	1108	1.33	*27*	β-Caryophyllene	1409	0.52
9	Isopulegol	1150	0.19	*28*	α-Guaiene	1440	0.21
10	Menthone	1153	0.19	*29*	Germacrene D	1485	0.27
11	Citronellal	1154	0.19	*30*	Methyl isoeugenol	1492	0.54
12	Isomenthone	1163	0.26	*31*	δ-Cadinene	1523	0.42
13	2-Phenylethyl formate	1176	0.45	*32*	Citronellyl butanoate	1532	1.10
14	α-Terpineol	1189	1.04	*33*	Geranyl butanoate	1564	0.21
15	Dihydrocitronellol	1196	0.45	*34*	10-*epi*-γ-Eudesmol	1624	0.27
16	Linalyl formate	1216	1.01	*35*	Geranyl tiglate	1696	1.19
17	Citronellol	1226	32.71	*36*	Guaiol acetate	1727	2.09
18	Tetrahydrogeraniol	1241	0.58	*37*	(8*S*)-Cedrane-8,14-diol	1890	0.34
19	Geraniol	1253	19.58				
Total							94.97

[a]) Compounds are listed in order of elution from a *SE-52* column. [b]) *Kovats* retention index. [c]) RAA [%] = Relative area percentage (peak area relative to total peak area in %).

most important chemicals responsible for the rose scent and uses of the essential oil [16]. *Pelargonium* essential oils with 2-phenylethyl alcohol abundance similar to that detected in our samples are considered useful as flavor additives in cigarettes, as preservatives in soap preparation, and as antimicrobial agents in ophthalmic solutions [16].

A high degree of variability characterizes *Pelargonium capitatum* essential oils, mainly due to several biochemical pathways and high polyploidy, determining important differences in quality and quantity of the chemicals [3]. In the light of the eight main chemotypes identified with reference to the presence and concentration of citronellyl formate, guaia-6,9-diene, δ-cadinene, 10-*epi*-γ-eudesmol, germacrene D, α-pinene, and caryophyllene epoxide, our samples may be considered as belonging to the sixth chemotype with reference to the key interpretation reported in [3]. Moreover, because of the (linalool + geraniol)/citronellol ratio taken as another tool to phytochemically characterize the *Pelargonium* essential oils, it can be stated that our *P. capitatum* samples show a value of 0.69, consistent with the oil marketed as '*Egyptian oil*' or '*African-type*' quality in general [13].

To define a metabolomic fingerprinting of *P. capitatum* essential oil, ^{1}H- and ^{13}C-NMR spectroscopic analyses were employed confirming the presence of the main compounds detected by GC/MS (*Table 1*), and the chemical shifts for identified constituents were assigned as compiled in *Table 2*. In particular, citronellol, geraniol, 2-phenylethyl alcohol, citronellyl formate, diphenyl ether, linalool, geranyl formate, and β-elemene were identified by 1D spectroscopy (^{1}H and ^{13}C) exhibiting highly diagnostic peaks without signals overlapping. 2D Spectroscopy has been instead employed to correlate vicinal and geminal ^{1}H and ^{13}C signals to the molecular structure of the most abundant compounds (*Figs. 1* and *2*). 1D ^{1}H-NMR Spectrum revealed typical and numerous signals diagnostic for the chemical makeup of H-atoms and, accordingly, of the functional groups typical of the examined molecules: the *doublet* at δ(H) 0.89 ppm and the *multiplet* at 3.66 ppm for citronellol (**1**), the double *doublet* at 4.14 ppm and 5.40 ppm for geraniol (**2**), the *singlet* of aldehydic H-atom at 8.05 and 8.06 ppm for geranyl formate (**8**) and citronellyl formate (**4**), the *triplet*s at 2.87 and 3.85 ppm for 2-phenylethyl alcohol (**3**), the *doublet*s at 5.22 and 5.90 ppm for linalool (**6**), and the *triplet* at 7.08 ppm and the *doublet* at 7.01 ppm for diphenyl ether (**5**). The broader spectral size with respect to ppm intervals of 1D ^{13}C-NMR spectrum, instead, gives more non-overlapped signals attributable to the molecules than the ^{1}H-NMR spectrum. The confirmation of the GC results renders NMR as suitable for the identification, quality control, or fraud detection of *Pelargonium* essential oils. Moreover, these kinds of evidences reinforce the role of non-chromatographic approach as potential tool to discriminate chemotypes, cultivar, and hybrids as already suggested in [17].

2. *Antimicrobial* (Antibacterial and Antifungal) *Activities.* As starting point for tracing a bioactivity profile of *P. capitatum* essential oil, antimicrobial activity was evaluated against different strains of bacteria, yeasts, and fungi with direct or indirect human health implications. The results were then compared with those obtained with *T. vulgaris* essential oil. In fact, the use of thyme essential oil as positive reference in bioassays performed with *P. capitatum* essential oil in the present research led to results particularly indicative of the real functional efficacy of a phytocomplex. The bioactivity

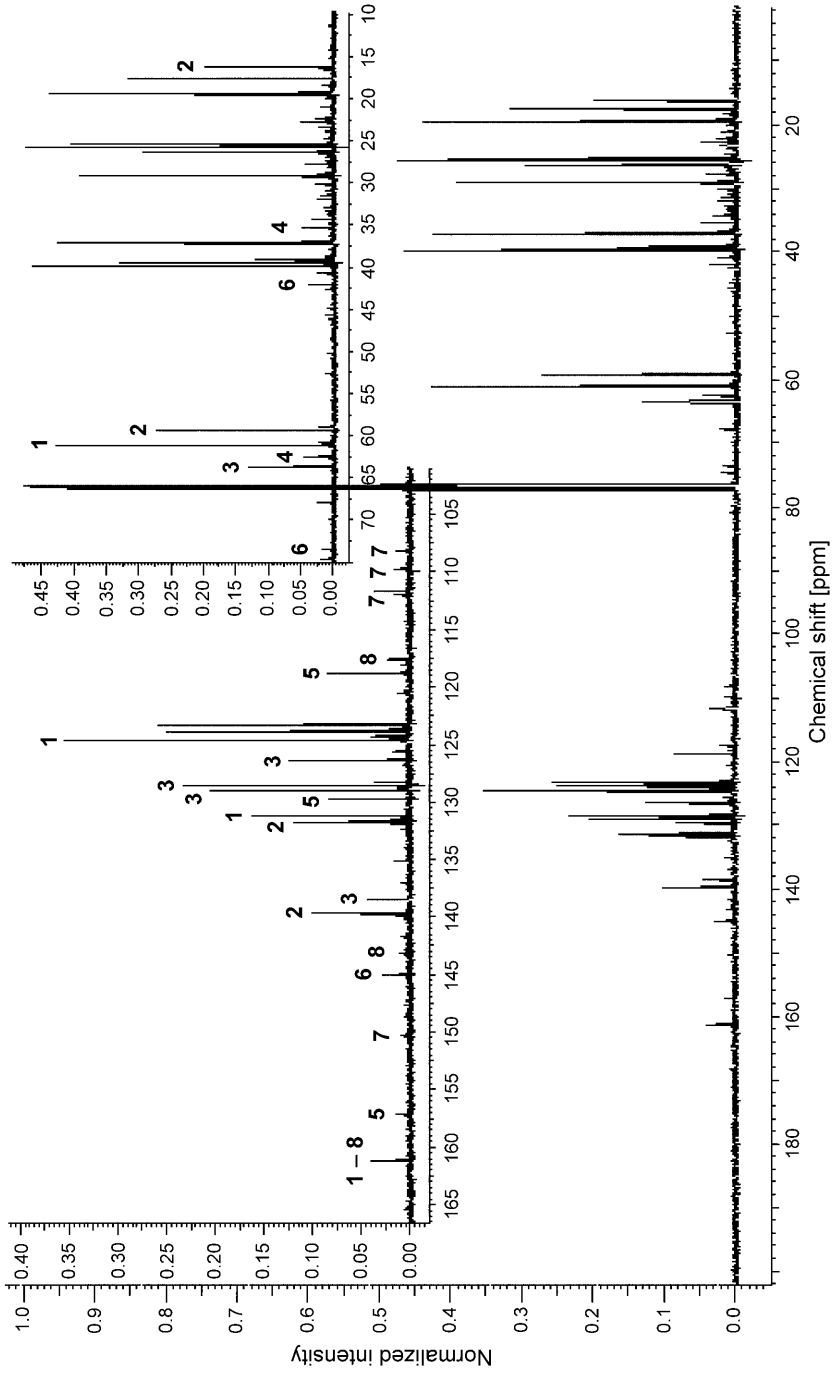


Fig. 1. *^{13}C-NMR Spectrum of* P. capitatum *essential oil*. Peaks of citronellol (**1**), geraniol (**2**), 2-phenylethan-1-ol (**3**), citronellyl formate (**4**), diphenylether (**5**), linalool (**6**), β-elemene (**7**), and geranyl formate (**8**).

Table 2. *Assignments of ^{1}H- and ^{13}C-NMR Chemical Shifts of Constituents Identified in* Pelargonium capitatum *Essential Oil*

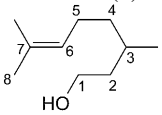
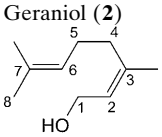
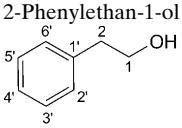
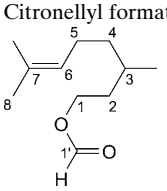
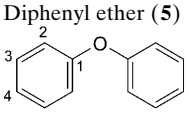
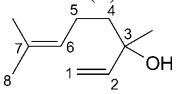
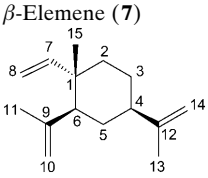
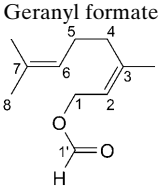
Compound	Assignment	δ(H) [ppm] (multiplicity)	Observed	δ(C) [ppm]
Citronellol (**1**) 	CH_2(1)	3.64–3.68 (*m*)	1D, HETCOR	61.1
	CH_2(2)	1.35–1.42 (*m*), 1.58–1.62 (*m*)	1D, HETCOR	39.7
	H–C(3)	1.57–1.60 (*m*)	1D, HETCOR	29.1
	Me(3)	0.89 (*d*, *J* = 6.6)	1D, HETCOR	19.5
	CH_2(4)	1.17–1.21 (*m*), 1.32–1.36 (*m*)	1D, HETCOR	37.2
	CH_2(5)	1.97–1.99 (*m*)	1D, HETCOR	25.4
	H–C(6)	5.07–5.11 (*m*)	1D, HETCOR	124.7
	C(7)		1D, HMBC	131.3
	Me–C(7)	1.67 (*s*)	1D, HETCOR	25.7
	Me(8)	1.58 (*s*)	1D, HETCOR	17.6
Geraniol (**2**) 	CH_2(1)	4.14 (*d*, *J* = 6.8)	1D, HETCOR	59.4
	CH_2(2)	5.40 (*dd*, *J* = 7.0, 1.4)	1D, HSQC	123.3[a])
	C(3)		1D, HMBC	139.6
	Me–C(3)	1.65 (*s*)	1D, HETCOR	16.2
	CH_2(4)	2.01–2.03 (*m*)	1D, HETCOR	39.5
	CH_2(5)	2.07–2.10 (*m*)	1D, HETCOR	26.4
	H–C(6)	5.07–5.11 (*m*)	1D, HSQC	123.8[a])
	C(7)		1D, HMBC	131.7
	Me–C(7)	1.67 (*s*)	1D, HETCOR	25.4
	Me(8)	1.58 (*s*)	1D, HETCOR	17.6
2-Phenylethan-1-ol (**3**) 	CH_2(1)	3.85 (*t*, *J* = 6.6)	1D, HETCOR	63.7
	CH_2(2)	2.87 (*t*, *J* = 6.6)	1D, HETCOR	39.4
	C(1′)		1D, HMBC	138.5
	H–C(2′,6′)	7.29–7.32 (*m*)	1D, HETCOR, HSQC	128.6
	H–C(3′,5′)	7.22–7.24 (*m*)	1D, HETCOR, HSQC	129.0
	H–C(4′)	7.29–7.32 (*m*)	1D, HETCOR, HSQC	126.4
Citronellyl formate (**4**) 	For *Me*–C(3), CH_2(5), H–C(6), C(7), *Me*–C(7), and C(8), see citronellol.			
	CH_2(1)	4.17–4.21 (*m*)	1D, HSQC	62.4
	CH_2(2)	see citronellol	1D, HMBC	36.9
	H–C(3)	1.57–1.60 (*m*)	1D, HETCOR	29.1
	CH_2(4)	see citronellol	1D, HMBC	35.3
	H–C(1′)	8.05 (*s*)	1D, HETCOR	161.2
Diphenyl ether (**5**) 	C(1)		1D, HMBC	157.3
	4 H–C(2)	7.01 (*d*, *J* = 8.2)	1D, HMBC	118.8
	4 H–C(3)	7.20–7.22 (*m*)	1D, HMBC	129.7
	2 H–C(4)	7.08 (*t*, *J* = 7.4)	1D, HMBC	123.2
Linalool (**6**) 	H_Z–C(1)	5.22 (*d*, *J* = 17.4)	1D, HMBC	111.6
	H_E–C(1)	5.07–5.11 (*m*)	1D	111.6
	H–C(2)	5.90 (*d*, *J* = 17.4)	1D, HMBC	145.0
	C(3)		1D, HMBC	73.4
	Me–C(3)	1.31 (*s*)	1D, HETCOR	27.8
	CH_2(4)	1.57–1.60 (*m*)	1D, HSQC	42.2
	CH_2(5)	2.06–2.08 (*m*)	1D, HETCOR	22.8
	H–C(6)	5.07–5.11 (*m*)	1D, HETCOR	124.4
	C(7)		1D, HMBC	131.4
	Me–C(7)	1.59 (*s*)	1D, HETCOR	17.6
	Me(8)	1.68 (*s*)	1D, HETCOR	25.7

Table 2 (cont.)

Compound	Assignment	δ(H) [ppm] (multiplicity)	Observed	δ(C) [ppm]
β-Elemene (**7**)	C(1)		1D	39.8
	CH_2(2)	1.42–1.44, 1.44–1.46 (2*m*)	1D, HSQC, HMBC	39.9
	CH_2(3)	1.55 (*m*)	1D	33.0
	H–C(4)		1D	52.7
	CH_2(5)	1.56, 1.64 (2*m*)	1D	26.9
	H–C(6)	1.91	1D	45.7
	H–C(7)	5.81 (*dd*, J = 10.6)	1D, HSQC, HMBC	150.4
	CH_2(8)	4.90, 4.89 (2*d*, J = 1.4)	1D, HSQC	109.9
	C(9)		1D	147.7
	CH_2(10)	4.57–4.59, 4.80–4.82 (2*m*)	1D, HSQC	112.0
	Me(11)	1.71	1D	24.8
	C(12)		1D	150.3
	CH_2(13)	4.70–4.72, 4.69–4.70 (2*m*)	1D, HSQC, HMBC	108.2
	Me(14)	1.75 (*s*)	1D, HSQC	21.0
	Me(15)	0.99 (*s*)	1D, HSQC,HMBC	16.6
Geranyl formate (**8**)	For *Me*–C(3), CH_2(4), CH_2(5), H–C(6), C(7), *Me*–C(7), and C(8), see geraniol.			
	CH_2(1)	4.66–4.69 (*m*)	1D, HSQC, HMBC	60.7
	H–C(2)	5.07–5.11 (*m*)	1D, HMBC	117.6
	C(3)		1D	143.0
	H–C(1′)	8.06 (*s*)	1D	161.2

[a]) Assignment in contrast to SDBS database.

of a phytocomplex, an essential oil, for example, is characterized not only by the simple interaction between an active plant compound and the biological substrate but, more properly, by the synergic activity of more chemical plant compounds that interact in the biological tests. Therefore, to better characterize the functional capacities of a chemically complex plant extract, the evaluation of the bioactivities needs to be compared not only with a single compound as positive control, but also with a similar phytocomplex of known functional capacities towards the tested bioassays. Based on this assumption and similar suggestions reported in [18][19], *Thymus vulgaris* essential oil represents a good reference for comparing the bioassay results regarding other essential oils, *i.e.*, *P. capitatum* essential oil in this case. Antibacterial activity was tested against *Gram*-positive and *Gram*-negative bacteria known as non-pathogenic, usually regarded as contaminant, but generally considered as nosocomial pathogens, especially in immuocompromised patients (*Table 3*) [20]. In general, the results did not display a good efficacy of *P. capitatum* essential oil; in fact, the best bioactivity was detected against *Enterococcus faecalis* with *MIC* values (0.42 ± 0.11 mg/ml) similar to the worst one exhibited by thyme essential oil against *K. oxytoca* (0.40 ± 0.1 mg/ml). The evidences are consistent with related literature, which reports a wide diversity in *Pelargonium* sp. and *Geranium* sp. activity against bacteria, due both to the high chemodiversity and to the different kind of extracts employed for assays

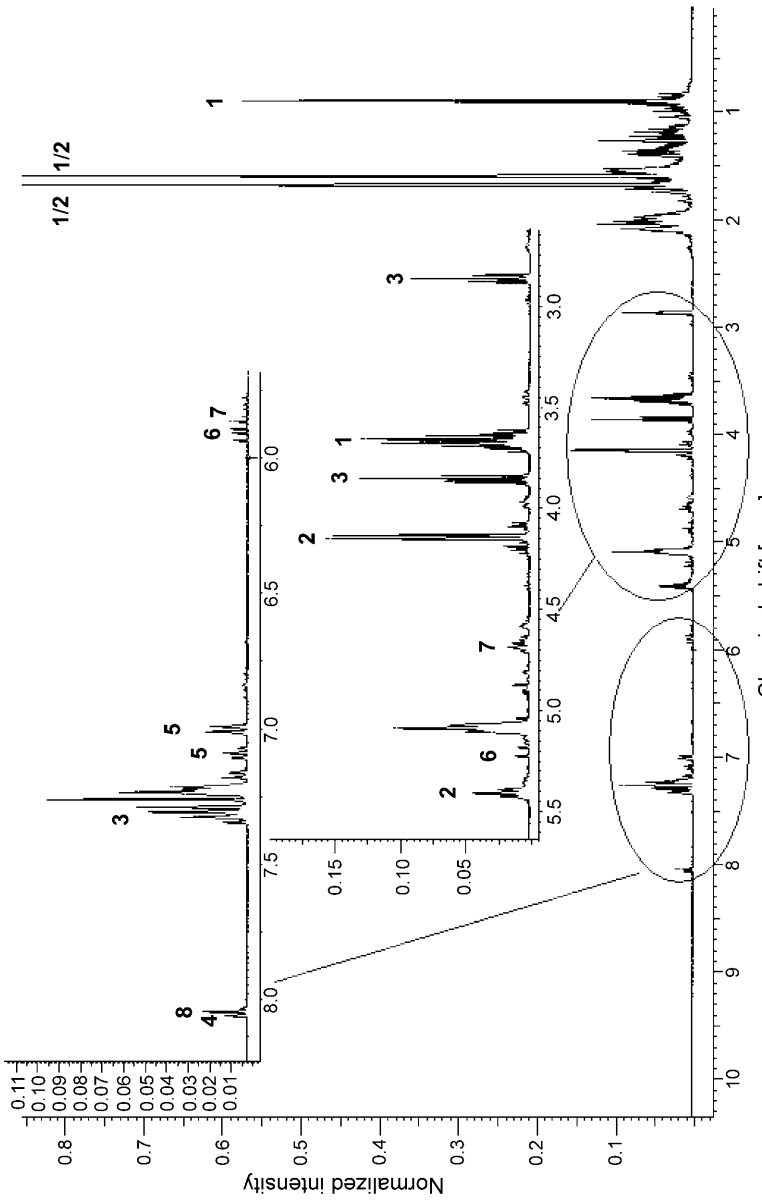


Fig. 2. *^{1}H-NMR Spectrum of* P. capitatum *essential oil*. Peaks of citronellol (**1**), geraniol (**2**), 2-phenylethan-1-ol (**3**), citronellyl formate (**4**), diphenyl ether (**5**), linalool (**6**), β-elemene (**7**), geranyl formate (**8**).

Table 3. *Antimicrobial Activity of* Pelargonium capitatum *Essential Oil Compared with That of Commercial Thyme Essential Oil, Taken as Positive Control*. Bioactivity is expressed as *MIC* (minimum inhibitory concentration; mg/ml ± standard deviation), considered as the lowest concentration of each essential oil showing a clear zone of inhibition (bacteria and yeasts); and as growth-inhibition percentage (fungi; % ± standard deviation) with reference to a untreated mycelium culture for each strain (negative control). Results are derived by triplicate determinations.

		MIC [mg/ml]	
		P. capitatum	*T. vulgaris*
Gram-positive bacteria			
Staphylococcus aureus subsp. *aureus* ATCC 29213		2.21 ± 0.55 (a)[a])	0.11 ± 0.03 (e)
Enterococcus foecalis ATCC 29212		0.42 ± 0.11 (b)	0.11 ± 0.03 (e)
Bacillus subtilis ATCC 7003		2.21 ± 0.55 (a)	0.11 ± 0.03 (e)
Micrococcus luteus ATCC 9622		1.10 ± 0.27 (c)	0.18 ± 0.02 (f)
Gram-negative bacteria			
Escherichia coli ATCC 4350		2.21 ± 0.55 (a)	0.10 ± 0.02 (e)
Pseudomonas aeruginosa ATCC 17934		4.42 ± 1.10 (d)	0.18 ± 0.05 (f)
Klebsiella oxytoca ATCC 29516		4.42 ± 1.10 (d)	0.40 ± 0.10 (b)
Proteus mirabilis ATCC 29852		1.10 ± 0.27 (c)	0.12 ± 0.03 (e)
Yeasts			
Saccharomyces cerevisiae ATCC 2365		0.80 ± 0.22 (a)	0.72 ± 0.20 (a)
Rhodotorula glutinis ATCC 16740		0.85 ± 0.24 (a)	0.72 ± 0.20 (a)
Yarrowia lypolytica ATCC 16617		0.70 ± 0.20 (a)	0.36 ± 0.10 (c)
Schizosaccharomyces pombe ATCC 60232		0.90 ± 0.25 (a)	0.85 ± 0.24 (a)
Candida albicans ATCC 48274		0.60 ± 0.17 (a)	0.65 ± 0.18 (a)
clinically derived CA1		0.50 ± 0.14 (b)	0.55 ± 0.15 (b)
clinically derived CA2		0.30 ± 0.08 (c)	0.35 ± 0.10 (c)
clinically derived CA3		0.25 ± 0.07 (c)	0.30 ± 0.08 (c)
clinically derived CA4		0.30 ± 0.08 (c)	0.35 ± 0.10 (c)
clinically derived CA5		0.45 ± 0.13 (b)	0.50 ± 0.14 (b)
clinically derived CA6		0.55 ± 0.15 (b)	0.60 ± 0.17 (a)
clinically derived CA7		0.50 ± 0.14 (b)	0.60 ± 0.17 (a)
clinically derived CA8		0.60 ± 0.17 (a)	0.70 ± 0.20 (a)
Fungi	p.e.o. [µl][b])	Growth inhibition [%]	
Dermatophytes			
Nannizzia cajetani IHME 3441	0.5	6.3 ± 0.5 (a)	62.4 ± 0.9 (l)
	1.0	10.2 ± 1.5 (b)	75.5 ± 2.0 (c)
	2.5	73.7 ± 1.80 (c)	95.2 ± 1.4 (h)
	5.0	89.5 ± 2.3 (d)	100.0 (e)
	10.0	100.0 (e)	100.0 (e)
Trichophyton mentagrophytes var. *mentagrophytes* CBS 160.66	0.5	6.2 ± 1.2 (a)	55.3 ± 1.6 (m)
	1.0	8.5 ± 0.5 (b)	82.0 ± 2.5 (n)
	2.5	74.4 ± 2.5 (c)	100.0 (e)
	5.0	100.0 (e)	100.0 (e)
	10.0	100.0 (e)	100.0 (e)
Phytopathogens			
Pythium ultimum[c])	0.5	12.6 ± 0.8 (f)	55.3 ± 1.6 (m)
	1.0	15.5 ± 1.8 (g)	70.5 ± 2.0 (c)
	2.5	95.2 ± 3.0 (h)	92.8 ± 2.1 (h)
	5.0	95.5 ± 2.8 (h)	98.8 ± 2.3 (h)
	10.0	97.4 ± 2.5 (h)	100.0 (e)

Table 3 (cont.)

	p.e.o. [μl][b])	Growth inhibition [%]	
		P. capitatum	*T. vulgaris*
Magnaporthe grisea ATCC 64413	0.5	0.9 ± 0.5 (i)	75.4 ± 2.3 (c)
	1.0	2.0 ± 0.5 (i)	85.0 ± 2.0 (n)
	2.5	6.1 ± 1.2 (a)	94.8 ± 1.6 (h)
	5.0	5.1 ± 1.5 (a)	99.5 ± 2.1 (h)
	10.0	8.7 ± 0.8 (b)	100.0 (e)

[a]) Different letters mean significant statistical differences ($p < 0.05$) based on one-way ANOVA (analysis of variance) and LSD (least significant difference) *post hoc Fisher*'s honest significant difference test (statistical computations were performed independently for each different kind of microorganism). [b]) p.e.o. = Pure essential oil. [c]) Kindly supplied by Prof. *G. D'Ercole*, Institute of Plant Pathology, University of Bologna, Italy.

[2][3][6][9][13][21–24]. Particularly promising seems to be, instead, the synergic bioactivity of *Pelargonium* sp. essential oil in presence of synthetic active compounds [25][26].

The tested antifungal activity of the volatile components of *Pelargonium* essential oil samples compared to that of thyme oil against the dermatophytes *Trichophyton mentagrophytes* var. *mentagrophytes* and *Nannizzia cajetani*, and the phytopathogens *Pythium ultimum* and *Magnaporthe grisea* is also reported (*Table 3*). From these data of preliminary relevance, it may be noted, as a general consideration, that dermatophytes seem to be more sensitive than phytopathogens to the treatment with the essential oil of *P. capitatum*. In fact, both dermatophytes showed high inhibition values already at the dose of 2.5 μl, while phytopathogens never reached the total growth inhibition (100%). *M. grisea* exhibited no remarkable growth inhibition at all tested doses, but showed an interesting depigmentation of the mycelium at a dose of 10 μl (data not shown), probably related to functional interaction (disruption?) of cell membranes and cell walls as reported in [27]. In general, if compared to that of the thyme essential oil, the antifungal bioactivity of *P. capitatum* proved to be slightly lower; only for *M. grisea*, the activities were dramatically different. The weak biological activity of *P. capitatum* essential oil against fungi is, in part, confirmed by literature, *i.e.*, the antimicrobial activity of volatile constituents was found comparable to that of most active essential oils only after long exposure [27].

The activity of *P. capitatum* essential oil against yeasts revealed instead remarkable features (*Table 3*) both in terms of *MIC* values in comparison to those of thyme oil, with the sole exception of the *MIC* value obtained against *Y. lypolytica* strongly inhibited by *T. vulgaris*, and of specific antimicrobic properties against all *Candida albicans* strains. These data are of particular relevance because of the constant attention paid from the cosmetic industry towards natural substances with antiseptic properties for treating candidiasis in animals and humans. As indicated for other *Pelargonium* sp. and *Geranium* sp. essential oils [26][28][29], also our *P. capitatum* samples revealed a marked preference against *Candida albicans* strains, both ATCC and clinically isolated, with *MIC* values ranging from 0.25 ± 0.07 to 0.60 ± 0.17 mg/ml. In fact, taking the bioactivity of *P. capitatum* essential oil against *C. albicans* ATCC

48274 strain as reference, the *MIC* was *ca.* 16% higher against *Yarrowia lypolytica* ATCC 16617, 33% higher against *Saccharomyces cerevisiae* ATCC 2365, 25% higher against *Rhodotorula glutinis* ATCC 16740, and *ca.* 50% higher against the less-sensitive *Schizosaccharomyces pombe* ATCC 60232. However, as a general assumption, it could be stated that the low *MIC* values, obtained in particular against all *Candida albicans* strains, are consistent with the suggestion to employ the *Pelargonium* essential oil as *'broad-spectrum activity'* natural product to treat candidiasis induced by microorganisms with different resistance levels.

3. *Antioxidant Properties.* Because of the increasing interest about safety status as well as efficacy of essential oils, which together determine their use also as antioxidizing ingredients in health products for preservative and functional targets [19], *Pelargonium capitatum* essential oil was also tested for its potential as antioxidant (*Table 4*).

Table 4. *Free Radical-Scavenging Activity and Antioxidant Capacity of* Pelargonium capitatum *Essential Oil by 1,1-Diphenyl-2-picrylhydrazyl* (DPPH) *Assay and β-Carotene Bleaching Test, Respectively.* Positive controls: BHA (butylated hydroxyanisole) and *T. vulgaris* essential oil. All values are expressed as IC_{50} [mg/ml]. PCL (photochemiluminescence): antioxidant capacity of *P. capitatum* essential oil compared to that of thyme oil, *Trolox*, and α-tocopherol. The activities are expressed as mmol-equiv. of *Trolox*/g of sample ± standard deviation (sd).

	P. capitatum	*T. vulgaris*	*Trolox*	*α-Tocopherol*	BHA
DPPH Test [mg/ml]	6.92 ± 0.51 (a)[a])	1.24 ± 0.35 (b)	–	–	$3.36\ 10^{-3} \pm 0.1\ 10^{-4}$ (c)
β-Carotene bleaching test [mg/ml]	1.45 ± 0.22 (b)	0.164 ± 0.013 (d)	–	–	$1.34\ 10^{-4} \pm 0.9\ 10^{-5}$ (e)
PCL [mmol-equiv. *Trolox*/g ± sd%]	0.005 ± 0.001 (f)	0.34 ± 0.06 (g)	3.94 ± 0.60 (h)	4.21 ± 0.50 (h)	–

[a]) Different letters mean significant statistical differences ($p < 0.05$) based on one-way ANOVA (analysis of variance) and LSD (least significant difference) *post hoc Fisher*'s honest significant difference test.

The chemical complexity of essential oils, in terms of quality and abundance of compounds, polarity, and chemical behavior, could lead to scattered results, depending on the test employed. Therefore, the use of more than a single method is highly advisable, and for this reason different approaches were evaluated [18][19]. To determine the activity of both hydrophilic and lipophilic species, ensuring a better comparison of the results and covering a wider range of possible applications, DPPH (1,1-diphenyl-2-picrylhydrazyl) test, the β-carotene bleaching test, and the PCL assay were performed (*Table 4*). The three tests and the comparison of the results for *P. capitatum* and thyme essential oil samples allow evaluation of both the primary and the secondary step of oxidation, and the lipid-soluble antioxidant capacity [30]. The comparison with thyme essential oil as positive control, known also for its antioxidant capacity [18][19], enables interpretation of the corresponding results of *P. capitatum* essential oil in a more realistic and applicative perspective. The antioxidant and radical-scavenging efficacy (IC_{50}) of *P. capitatum* essential oil was poor-to-negligible in all the performed essays (*Table 4*). However, besides the weak capacity of our samples, the antioxidant activity of *Pelargonium* sp. and *Geranium* sp. essential oils reported in literature are dated and limited, regarding their correlation to high biodiversity and

chemodiversity of both genera [31][32], in part, also due to their limited bibliographic visibility [33] (article in Chinese). Therefore, our evidences contribute to outline the biological capacity of *Pelargonium* sp. essential oils in the light of its best applicative health perspectives, together with the biodiversity characterization of the *genera*.

4. *Mutagenic and Antimutagenic Properties, and Toxicity.* As part of the efficacy and safety aspects of the *Pelargonium capitatum* essential oil, mutagenic and anti-mutagenic properties were also assayed (*Figs. 3*, *4*, and *5*). In fact, as a general consideration, some evidences of possible genotoxic activities exerted by essential oils are emerging [34], together with evaluation of antimutagenic properties, which are health-relevant [18][35]. By using the plate incorporation assay, no mutagenic activity of *P. capitatum* essential oil was detected when investigated on *S. typhimurium* tester strains TA98 and TA100, either with or without S9 activation (data not shown). *Pelargonium capitatum* essential oil, when tested for mutagen-protective efficacy in the *Ames* Salmonella/microsome assay, has not shown any significant statistical effects of increasing amounts of essential oil on the activity of directly acting mutagens 2-nitrofluorene and NaN_3. Highest Uneffective Dose (HUD) for toxic effect without metabolic activation has been established at $5 \cdot 10^{-2}$ mg/plate for both TA98 and TA100 strains, while the HUD with metabolic activation was checked at $5 \cdot 10^{-1}$ mg/plate (*Fig. 3*). Significant offset differences from revertants of 2-nitrofluorene and NaN_3 ($p < 0.05$) were, according to *t* test, at 1 and 5 mg/plate for 2-nitrofluorene and NaN_3, respectively (*Fig. 4*). Therefore, the decrease of revertants depicted in *Fig. 4* has to be attributed to the toxicity exerted by the essential oil. Different results were obtained

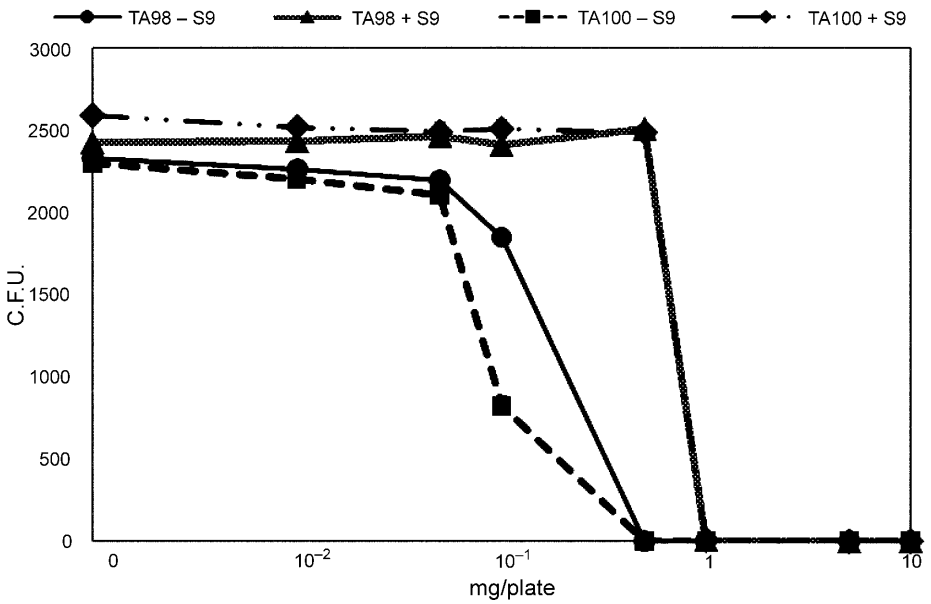


Fig. 3. *Toxicity effect, evaluated as cell-survival reduction at growing concentrations of* Pelargonium capitatum *essential oil, performed to determine cytotoxic Highest Uneffective Dose* (HUD) *on* Ames *TA98 and TA100* S. typhimurium *test strains with and without metabolic activation.* C.F.U.: Colony-forming units.

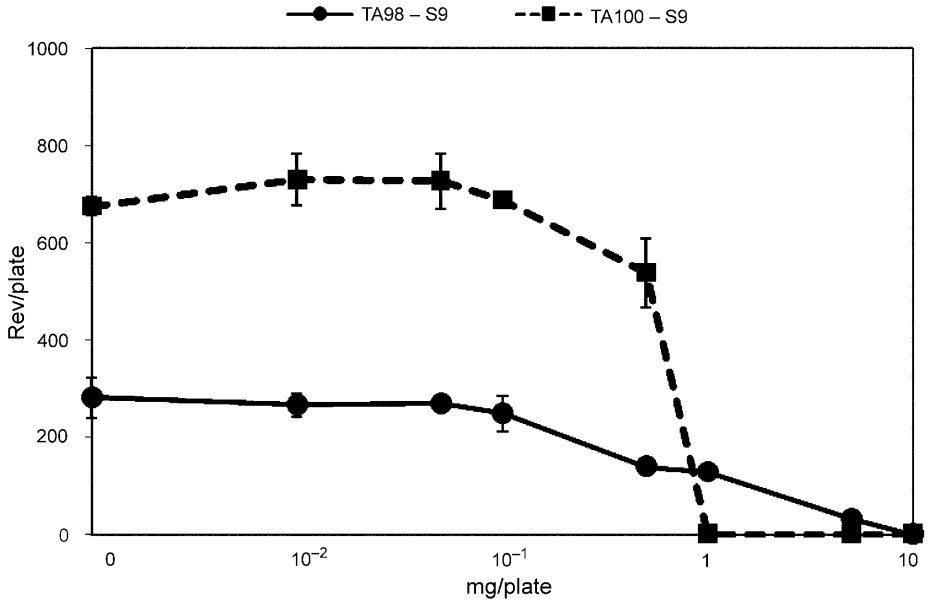


Fig. 4. *Effect of* Pelargonium capitatum *essential oil on mutagenic activity of directly acting mutagen 2-nitrofluorene* (2 μg/plate) *and* NaN_3 (1 μg/plate) *in TA98 and TA100* S. typhimurium *strain without S9 mix*. Effect is observed over HUD of the extract's toxicity; therefore, it cannot be attributed to the oil.

when strains were exposed to the oil in presence of indirectly acting mutagen 2-aminoanthracene, which acts as a genotoxic compound through S9 mix. In this case, oil could induce a statistically significant decrease of the HUD values. The HUD value for toxic effect has been determined as $5 \cdot 10^{-1}$ mg/plate for both TA98 and TA100, and offsets of statistically significant differences for revertants of 2-aminoanthracene ($p < 0.05$) coincide with this value for both strains (*Fig. 5*). Therefore, in this case, we can assume at HUD a significant inhibition rate of 85 and 78% for TA98 and TA100, respectively.

The mechanism by which *P. capitatum* essential oil inhibits the mutagenicity of 2-aminoanthracene is not known. However, some suggestions can be made on the basis of the present data. Since there is an evident difference in the protective activity of the oil against direct and indirect mutagens, it can be assumed that constituents of the essential oil may interact synergically with some specific enzymes in the S9 liver homogenates, which are necessary for activation of chemical mutagens. Thus, the antimutagenic effect of this oil could be explained by the interaction of its constituents with cytochrome P-450 activation system, leading to a reduction of the mutation ratio caused by 2-aminoanthracene, as confirmed by further evidences regarding essential oils [36].

Conclusions. – NMR Characterization of *Pelargonium capitatum* essential oil allowed confirmation of the composition data of the chemotype analyzed by GC and GC/MS, indicating that this analytical approach can be useful to discriminate cultivars,

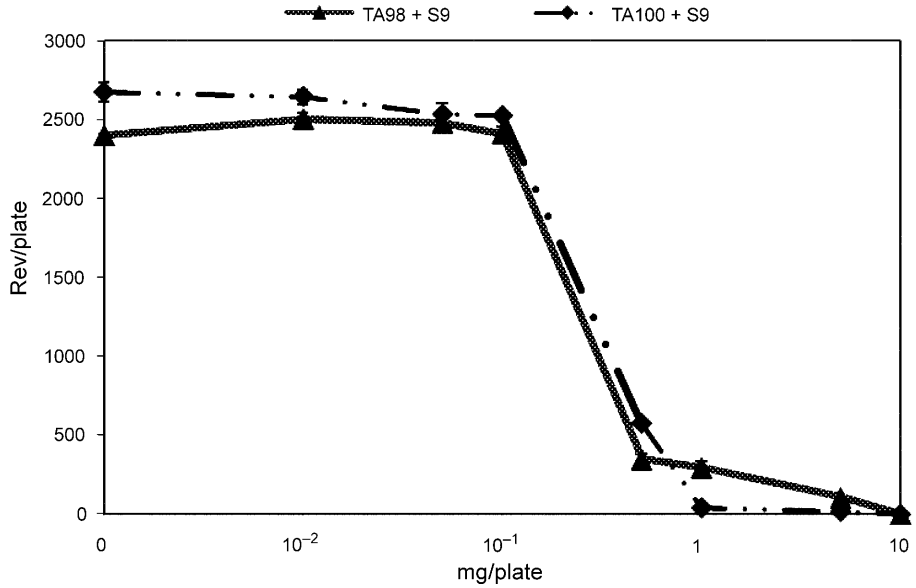


Fig. 5. *Effect of* Pelargonium capitatum *essential oil on the mutagenicity of the indirectly acting mutagen 2-aminoanthracene* (2 μg/plate) *in TA98 and TA100* S. typhimurium *strain with S9 mix*. Effect is observed at concentrations lower than HUD value; therefore, it can be attributed to essential oil.

chemotypes, and hybrids relevant to health as stressed in [17]. Therefore, in the light of these considerations, the achieved fingerprinting data suggest that this non-chromatographic approach may be suitable for the identification, quality control, or fraud detection of *Pelargonium* sp. essential oils, providing their good and fast discrimination. Moreover, the following biological activity profile of the essential oil provided important information regarding antimicrobial properties, against *C. albicans*, in particular, suggesting its use as a functional ingredient of cosmetics and toiletries with particular emphasis for its properties against candidiasis. Regarding the safety of use of *P. capitatum* essential oil, we have shown not only lack of genotoxicity but also a significant antimutagenic effect, which can be considered as an advantage in view of its use in products at contact with the skin for a prolonged time.

The research was financially supported by a grant of the University of Ferrara (FAR 2009) and *MIUR* (Ministero dell'Università e della Ricerca Scientifica), Italy. The Authors wish to thank Dr. *Immacolata Maresca* for skilful technical assistance.

Experimental Part

Essential Oils. South African *Pelargonium capitatum* essential oil samples were purchased from *Biotrade*, Mirandola (MO), Italy. *Thymus vulgaris* essential oil samples (thymol chemotype), used as reference for assessing *P. capitatum* essential oil functional properties, was purchased from *Extrasynthese* (F-Genay). All the essential oil samples were stored in glass vials with *Teflon*-sealed caps at $2.0 \pm 0.5°$ in the dark.

Chemicals. Solvents and pure compounds used as references were purchased from *Extrasynthese* (F-Genay) and *Sigma–Aldrich Italy* (I-Milano), both from *'General'* and *'Flavors and Fragrances'* catalogs. All chemicals employed for antioxidant-activity determination were purchased both from *Sigma–Aldrich Italy* and *Analytikjena* (D-Jena), while microbial culture media were from *Oxoid Italia* (I-Garbagnate).

GC Analysis. Essential-oil samples were analyzed, and the relative peak areas for individual constituents were averaged. The relative percentages were determined using a *ThermoQuest GC-Trace* gas-chromatograph equipped with a FID detector and a *Varian FactorFour VF-5ms* poly-5% phenyl-95%-dimethyl-siloxane-bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.15 μm). Operating conditions were: injector temp., 300°; FID temp., 300°, carrier (He) flow rate, 1 ml/min; and split ratio, 1:50. Oven temp. was initially 55° and then raised to 100° at a rate of 1°/min; then raised to 250° at a rate of 5°/min, and finally held at that temp. for 15 min. One μl of each sample dissolved in CH_2Cl_2 was injected. The percentage composition of the oils was computed by the normalization method from the GC peak areas, without using correction factors.

GC/MS Analysis. Essential oil constituents were then analyzed by a *Varian GC-3800* gas chromatograph equipped with a *Varian MS-4000* mass spectrometer using electron impact (EI) and hooked to NIST library. The constituents of the volatile oils were identified by comparing their GC *Kovats* retention indices (*KI*) and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds, and by matching the MS fragmentation patterns and retention indices with the above mentioned mass-spectra libraries and with those in the literature [37]. To determine the *KI* value of the components, a commercial 24 aliphatic hydrocarbons mixture (*Sigma–Aldrich*) was added to the essential oil before injecting it into the GC/MS equipment and analyzed under the same conditions as above.

The conditions were the same reported for GC analysis, and the same column was used. The MS conditions were: ionization voltage, 70 eV; emission current, 10 μAmp; scan rate, 1 scan/s; mass range, 29–400 Da; trap temp., 150°, transfer line temp., 300°.

NMR Spectroscopy. The ^{1}H-NMR spectrum was recorded on a *Varian Gemini-400* spectrometer at 399.97 MHz and at a temp. of 303 K. The essential oil (15 mg/1.0 ml) was dissolved in $CDCl_3$ into a 5-mm NMR tube, and the solvent signal was used for spectral calibration (^{1}H 7.26 ppm). ^{1}H-NMR Spectrum was run using a standard pulse sequence 's2pul', with 45.0 degrees pulse, 3.00 s acquisition time, 8 repetitions, 6400-Hz spectral width, 0.33 Hz Fid resolution. ^{13}C-NMR Spectrum was recorded at 100.85 MHz and at a temp. of 303 K. The essential oil (60 mg/1 ml) was dissolved in $CDCl_3$. The same spectral calibration as for the ^{1}H-NMR experiments was used. The time domain size was 17 K, spectral width 25063 Hz, Fid resolution 1.43 Hz. DEPT Experiment was acquired at 100.85 MHz and at a temp. of 303 K using a 135 degree decoupler pulse, with 0.7 s acquisition time, 1280 increments, and 25062 Hz for spectral width. For HETCOR, the ^{1}H WALTZ-16 decoupled spectrum was recorded at 399.97 MHz with power of 43 dB *on* during acquisition and *off* during delay, the parameters used were 1.000 s relaxation delay, 0.070 s acquisition time, 32 repetitions and 256 increments, 4000 Hz (^{1}H) and 21186 Hz (^{13}C) for spectral width, and 14.3 Hz (F2) and 7.8 Hz (F1) Fid resolution. For the HSQC experiment, the ^{13}C GARP-1 decoupled spectrum was recorded at 100.58 MHz with power of 50 dB *on* during acquisition and *off* during delay, the parameters used were 1.000 s relaxation delay, 0.150 s acquisition time, 8 repetitions and 256 increments, 3205 Hz (^{1}H) and 17094 Hz (^{13}C) for spectral width, and 6.7 Hz (F2) and 66.8 Hz (F1) Fid resolution. HMBC Experiments were conducted using 1.000 s relaxation delay, 0.150 s acquisition time, 32 repetitions and 256 increments, 4000 Hz (^{1}H) and 20120 Hz (^{13}C) for spectral width, and 6.7 Hz (F2) and 78.6 Hz (F1) Fid resolution.

^{1}H, ^{13}C, DEPT and HETCOR, HSQC, and HMBC spectra were recorded. ^{1}H- and ^{13}C-NMR of citronellol, geraniol, 2-phenylethyl alcohol, linalool, β-elemene, and ^{13}C-NMR of citronellyl formate and geranyl formate were compared with bibliographic data [38–40] and *Aldrich NMR Library* for ACD/Labs version 9.0, pure standards, or pure compound mixture (citronellol, geraniol, linalool), fully characterized for diphenyl ether.

Biological Activities. Antimicrobial (antibacterial and antifungal) activity, antioxidant properties, mutagenic and antimutagenic capacity, and toxicity were evaluated for *P. capitatum* essential oil samples. All of the bioactivities were determined by comparing all data with those obtained with appropriate pure

synthetic compounds and/or commercial *T. vulgaris* essential oil, in order to have positive control references with single compounds or comparable phytocomplexes reputed for their functional bioactivities [18][19]. In fact, the results from the use of a phytocomplex known for its chemical and biological properties (*e.g.*, thyme essential oil) as positive reference are particularly indicative of the real functional efficacy of a tested extract (*e.g.*, *P. capitatum* essential oil) [18][19]. Data reported for each assay are the average of three determinations from three independent experiments.

Antibacterial Activity. Gram-positive (*Staphylococcus aureus* subsp. *aureus* ATCC 29213, *Enterococcus foecalis* ATCC 29212, *Bacillus subtilis* ATCC 7003, and *Micrococcus luteus* ATCC 9622) and *Gram*-negative (*Escherichia coli* ATCC 4350, *Pseudomonas aeruginosa* ATCC 17934, *Klebsiella oxytoca* ATCC 29516, and *Proteus mirabilis* ATCC 29852) bacterial strains were employed. All the assays were performed by employing the standard disk-diffusion technique according to a method described in [17]. The culture media and conditions employed for ATCC strains were in accordance with *American Type Culture Collections* (*ATCC*) protocols. Mother cultures of each microorganism were set up 24 h before the assays in order to reach the stationary phase of growth. The tests were assessed by inoculating from the mother cultures *Petri* dishes with proper sterile media with the aim to obtain the microorganisms concentration of 10^6 CFU/ml. Ten µl of DMSO/essential oil sample solns. were prepared in order to have an assay range comprised between the pure essential oil and a concentration of 0.100 mg/ml, and then deposited on sterile paper disk (6-mm diameter, *Difco*). Negative controls were set up with DMSO in amounts corresponding to the highest quantity present in the test soln., while positive ones were set up with *T. vulgaris* essential oil. The lowest concentration showing a clear zone of inhibition measured after 24 h was taken as the *MIC* (Minimum Inhibitory Concentration).

Antifungal Activity. Bioassays were performed both on phytopathogens (*Pythium ultimum* Trow, kindly supplied by Prof. *G. D'Ercole*, Institute of Plant Pathology, University of Bologna, Italy; and *Magnaporthe grisea* ATCC 64413) and on dermatophytes (*Trichophyton mentagrophytes* var. *mentagrophytes* (Robin) Blanchard, Centraal Bureau Voor Schimmelcultures – CBS 160.66 strain; and *Nannizzia cajetani* Ajello Institute of Hygiene and Epidemiology-Mycology – IHME 3441 strain). Different media were employed for phytopathogens and dermatophytes: the former were cultured on Potato Dextrose Agar (PDA; *Oxoid*), the latter on *Saboraud* Dextrose Agar (SDA; *Oxoid*) in accordance with strain providers and literature [41]. *Petri* dishes were inoculated with 6-mm plugs from mother cultures and upset incubated for 24 h at $26\pm2°$. A sterilized filter paper disk (6.0-mm diameter, *Difco*) was placed in the centre of the lid, and different volumes (0.5, 1.0, 2.5, 5.0 and 10.0 µl) of pure essential oil were added to the paper. Plates were tightly sealed with parafilm, kept in an inverted position, and incubated for 6 d at $26\pm2°$ in the dark. Blanks (DMSO) were taken as neg. control, while pos. controls were set up with *T. vulgaris* essential oil. The growth inhibition was checked at the end of the incubation period and considered as inhibition percentage with reference to the radial growth detected for control cultures. Bioactivity against the yeasts *Rhodotorula glutinis* ATCC 16740, *Schizosaccharomyces pombe* ATCC 60232, *Saccharomyces cerevisiae* ATCC 2365, *Yarrowia lypolitica* ATCC 16617, the human pathogen *Candida albicans* as ATCC 48274, and clinical isolated strains named CA1-8, kindly purchased by Prof. *Roberto Manservigi*, Department Experimental and Diagnostic Medicine, Sect. of Microbiology, University of Ferrara, were also processed. Mother cultures of each yeast strain were set up inoculating 100 ml YEPD (Yeast Extract and Potato Dextrose) liquid medium in 250 sterile flask and incubated in the dark at 30° in order to assess growth curves. From each mother culture at the stationary phase of growth, broth dilutions were made to obtain the strain concentration of 10^5 CFU/ml to inoculate *Petri* dishes with agarized YEPD for bioassays. Then, 10 µl of DMSO/essential oil sample solns. were prepared in order to have an assay range comprised between the pure essential oil and the lowest concentration of 0.100 mg/ml, and then deposited on sterile paper disk (6-mm diameter, *Difco*). The *Petri* dishes were successively incubated at 30° in the dark and checked for evaluating the growth inhibition after 48 h. The lowest concentration of each essential oil showing a clear zone of inhibition was taken as the *MIC*. Negative controls were set up with DMSO in amounts corresponding to the highest quantity present in the test solution (10 µl), while positive ones were assessed with *T. vulgaris* essential oil [19].

Antioxidant Activity. Radical-scavenging and antioxidant properties were evaluated through different assays, namely DPPH (1,1-diphenyl-2-picrylhydrazyl) assay, β-carotene bleaching test, and photochemiluminescence (PCL) according to a method described in [17].

DPPH Assay. An aliquot of essential oil (10 µl) was mixed with 900 µl of 100 mM *Tris*·HCl buffer (pH 7.4), 40 µl of EtOH, and 50 µl of 0.5% (*w*/*w*) *Tween 20* soln. One ml of 0.5 mM DPPH (*Sigma–Aldrich*) EtOH soln. was then added to the mixture. *Tween 20* was used as an oil-in-water emulsifier. The mixture was shaken vigorously and kept in the dark for 30 min at r.t. Sample absorbance was measured at 517 nm with UV/VIS spectrophotometer (*ThermoSpectronic Helios* γ, Cambridge, U.K.). A blank was assessed as the soln. assay described above without the essential oils, instead of which distilled H_2O was employed. Butylated hydroxy anisole (BHA) and *T. vulgaris* essential oil were used as pos. controls. The radical-scavenging activities of each sample were calculated according to the following formula for inhibition percentage (*Ip*) of DPPH: $Ip = (A_B - A_A)/A_B \times 100$, where A_B and A_A are the absorbance values of the blank sample and of the test sample resp., after 30 min. Oil and BHA antiradical activity was considered as the concentration providing DPPH 50% inhibition (IC_{50}), calculated from inhibition curves obtained by plotting inhibition percentage *vs.* oil concentration.

β-Carotene Bleaching Test. Approximately 10 mg of β-carotene (type I synthetic) was dissolved in 10 ml of $CHCl_3$, and 0.2 ml of the soln. was pipetted into a flask containing a boiling mixture of 20 mg of linoleic acid and 200 mg of *Tween 40*. $CHCl_3$ was removed using a rotary evaporator (*Büchi 461*) at 40° for 5 min, and 50 ml of distilled H_2O were slowly added to the residue under vigorous agitation, to form an emulsion. Five ml of the emulsion was added to a tube containing 0.2 ml of the essential oils soln. and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without β-carotene. The tube was placed in a H_2O bath at 50°, and the oxidation of the emulsion was monitored spectophotometrically by measuring absorbance at 470 nm over a 60-min period. Pos. control consisted of BHA and *T. vulgaris* essential oil, whereas neg. control consisted of test emulsion in which the essential oil was replaced by equal amounts of dist. H_2O. The antioxidant activity was expressed as inhibition percentage with reference to the control after a 60-min incubation using the following equation: $AA = 100(DR_C - DR_S)/DR_C$, where AA is the antioxidant activity, DR_C is the degradation rate of the control = $[\ln(a/b)/60]$, DR_S is the degradation rate in the presence of the sample = $[\ln(a/b)/60]$, a is the absorbance at time 0, and b is the absorbance at 60 min. Oil and BHA antioxidant activity was considered as IC_{50}, calculated from inhibition curves obtained by plotting inhibition percentage against oil concentration.

Photochemoluminescence (PCL). PCL of the essential oil was measured with *Photochem* with ACL (Antioxidant Capacity of Liposoluble substance; Analytikjena, D-Jena) kit. A 2.30-ml portion of reagent 1 (solvent and dilution reagent), 200 µl of reagent 2 (buffer soln.), 25 µl of reagent 3 (photosensitizer), and 10 µl of standard (*Trolox* soln. in reagent 1) or sample (essential oil in MeOH) soln. were mixed and measured. A light emission curve was recorded over 130 s, using inhibition as the parameter to evaluate antioxidant potential. The antioxidant capacity was then determined by using the integral under the curve and was expressed as mmol/l of *Trolox* used as standard to obtain a calibration curve. The PCL bioactivity of *P. capitatum* essential oil samples was compared to those expressed by pure *Trolox*, α-tocopherol and *T. vulgaris* essential oil.

Ames *Test: Mutagenic Activity. P. capitatum* essential oil dissolved in DMSO was tested with *Salmonella typhimurium* strains TA98 and TA100 (100 µl per plate of fresh overnight cultures) with and without the addition of 0.5 ml of a 5% S9 exogenous metabolic activator (S9 mix), using plate incorporation assay [42]. The concentrations of the essential oil samples used were 10^{-2}, $5 \cdot 10^{-2}$, 10^{-1}, $5 \cdot 10^{-1}$, 1, 5, 10, 50, 100 mg/plate. The plate for neg. control contained 100 µl of DMSO, with or without S9 mix. The pos. control plates with S9 mix contained 2 µg/plate of 2-aminoantracene for both TA98 and TA100 strains. The pos. control plates without S9 mix contained 2 µg/plate of 2-nitrofluorene for TA98 strain and 1 µg/plate of NaN_3 for TA100 strain. A sample was considered mutagenic when the observed number of colonies was at least twofold over the spontaneous level of revertants [42]. The colonies were counted manually after 48 h of incubation at 37° using a *Colony Counter 560 Suntex* (*Antibioticos*, Italy). Lyophilized post-mitochondrial supernatant S9 mix (*Aroclor 1254*-induced, *Sprague–Dawley* male rat liver in 0.154M KCl soln.), commonly used for the activation of promutagens to mutagenic metabolites, was purchased from *Molecular Toxicology, Inc.* (Boone, NC, USA) and stored at −80°.

Ames *Test: Antimutagenic Activity and Toxicity.* The inhibitory effect of *P. capitatum* essential oil samples (10^{-2}, $5 \cdot 10^{-2}$, 10^{-1}, $5 \cdot 10^{-1}$, 1, 5, 10, 50, 100 mg/plate) on mutagenic activity of directly acting mutagen 2-nitrofluorene (2 µg/plate) and NaN_3 (1 µg/plate) was examined by plate incorporation assay, derived from mutagenicity test as described in [42] with some minor modifications, using tester strain

TA98 and TA100 resp. The inhibitory effect of *P. capitatum* essential oil samples on mutagenic activity of the indirectly acting mutagen 2-aminoanthracene (2 μg/plate) was instead examined by plate incorporation assay, using tester strain TA98 and TA100 with S9 mix. The inhibition rate for mutagenic induction was calculated according to the formula: inhibition rate $[\%] = (A - B) \times 100/A$, where A are revertants in pos. control, and B are revertants in the essential oil samples, having subtracted the spontaneous revertants. A critical point, affecting the outcome of the interaction between an antimutagen and a testing bacterial strain, is the overlapping of the cytotoxic and antimutagenic dose concentration. In other words, it is important to confirm that the dose-dependent disappearance of the mutant colonies is not a result of cell-killing. For this purpose a simple survival assay for the treated bacteria must be performed to evaluate a Highest Uneffective Dose (HUD). To verify the toxicity of the analyzed samples on bacterial cells and evaluate the HUD, a toxicity test was performed [42]. A fresh 15-h culture was diluted to give a $1-2 \times 10^4$ bacteria/ml. The test samples at several concentrations (10^{-2}, $5 \cdot 10^{-2}$, 10^{-1}, $5 \cdot 10^{-1}$, 1, 5, 10, 50, 100 mg/plate), diluted in DMSO and mixed with 2 ml of molten top agar, were plated with 0.1 ml of the diluted culture. Histidine/biotin agar plates were enriched with 10 μmol of L-histidine and 0.05 μmol of biotin by incorporating these nutrients into the soft agar overlay. Triplicate plates were poured for each dose of soln. The colony-forming units (CFU) were assessed after the plates were incubated at 37° for 48 h and compared with that of control where no test samples were added. HUD for *Pelargonium capitatum* extract with and without metabolic activation was evaluated by visual estimation (colony counting) integrated by statistical analyses.

Statistical Analysis. Relative standard deviations and statistical significance (*Student*'s *t*-test; $p < 0.05$) were given, where appropriate, for all data collected. One-way ANOVA and LSD *post hoc Fisher*'s honest significant difference tests were used for comparing the bioactivity of different *P. capitatum* essential oil samples. *Student*'s *t*-test ($p < 0.05$) combined with HUD comparison was used to interpret the results of significant decrease in the number of *Salmonella* revertants. When the modulator dose concentration is statistically effective and it ranges below or coincides with the HUD, the samples were considered to present sign of the effect (antimutagenicity). *Student*'s *t*-test was performed also for pharmacological data computations. All computations were made using the statistical software STATISTICA 6.0 (*StatSoft Italia srl*).

REFERENCES

[1] D. M. Miller, in 'Geranium and Pelargonium, Medicinal and Aromatic Plants – Industrial profiles', Ed. M. Lis-Balchin, Taylor and Francis, London, UK, 2002, p. 49.
[2] H. Kolodziej, *Phytomedicine* **2007**, *14* (*Suppl. 1*), 9.
[3] A. M. Viljoen, J. J. A. van der Walt, J. P. J. Swart, F.-E. Demarne, *J. Essent. Oil Res.* **1995**, *7*, 605.
[4] Icon Group International, Inc., http://www.icongrouponline.com/, 2000.
[5] F. E. Demarne, in 'Geranium and Pelargonium, Medicinal and Aromatic Plants – Industrial profiles', Ed. M. Lis-Balchin, Taylor and Francis, London, UK, 2002, p. 193.
[6] J. Y. Y. Lalli, R. L. Van Zyl, S. F. Van Vuuren, A. M. Viljoen, *S. Afr. J. Bot.* **2008**, *74*, 153.
[7] S. Pattnaik, V. R. Subramanyam, C. R. Kole, *Microbios* **1996**, *86*, 237.
[8] B. R. Rao Rajeswara, P. N. Kaul, G. R. Mallavarapu, S. Ramesh, *Biochem. Syst. Ecol.* **1996**, *24*, 627.
[9] M. Lis-Balchin, G. Buchbauer, K. Ribisch, M.-T. Wenger, *Lett. Appl. Microbiol.* **1998**, *27*, 135.
[10] S. Hart, M. Lis-Balchin, in 'Geranium and Pelargonium, Medicinal and Aromatic Plants – Industrial profiles', Ed. M. Lis-Balchin, Taylor and Francis, London, UK, 2002, p. 116.
[11] M. Hori, *Appl. Entomol. Zool.* **2003**, *38*, 467.
[12] J. A. Duke, M. Beckstrom-Sternberg, in 'Developments in Food Science 34: Spices, Herbs and Edible Fungi', Ed. G. Chavalambous, Elsevier, Amsterdam, 1994, p. 741.
[13] M. Lis-Balchin, in 'Geranium and Pelargonium, Medicinal and Aromatic Plants – Industrial profiles', Ed. M. Lis-Balchin, Taylor and Francis, London, UK, 2002, p. 184.
[14] M. Jalali-Heravi, B. Zekavat, H. Sereshti, *J. Chromatogr., A* **2006**, *1114*, 154.
[15] H. R. Juliani, A. Koroch, J. E. Simon, N. Hitimana, A. Daka, L. Ranarivelo, P. Langenhoven, *J. Essent. Oil Res.* **2006**, *18*, 116.

[16] C. E. Fabre, P. J. Blanc, G. Goma, *Perfum. Flavor.* **1998**, *23*, 43.
[17] A. Guerrini, G. Sacchetti, M. Muzzoli, G. M. Rueda, A. Medici, E. Besco, R. Bruni, *J. Agric. Food Chem.* **2006**, *54*, 7778.
[18] A. Guerrini, G. Sacchetti, D. Rossi, G. Paganetto, M. Muzzoli, E. Andreotti, M. Tognolini, M. E. Maldonado, R. Bruni, *Environ. Toxicol. Pharmacol.* **2009**, *27*, 39.
[19] G. Sacchetti, S. Maietti, M. Muzzoli, M. Scaglianti, S. Manfredini, M. Radice, R. Bruni, *Food Chem.* **2005**, *91*, 621.
[20] R. Maviglia, R. Nestorini, M. A. Pennisi, *Curr. Drug Targets* **2009**, *10*, 895.
[21] S. P. N. Mativandlela, N. Lall, J. J. M. Meyer, *S. Afr. J. Bot.* **2006**, *72*, 232.
[22] Z. Schelz, J. Molnar, J. Hohmann, *Fitoterapia* **2006**, *77*, 279.
[23] V.-G. de Billerbeck, *Phytothérapie* **2007**, *5*, 249.
[24] T. Brendler, B.-E. van Wyk, *J. Ethnopharmacol.* **2008**, *119*, 420.
[25] A. Rosato, C. Vitali, N. De Laurentis, D. Armenise, M. A. Milillo, *Phytomedicine* **2007**, *14*, 727.
[26] A. Rosato, C. Vitali, D. Gallo, L. Balenzano, R. Mallamaci, *Phytomedicine* **2008**, *15*, 635.
[27] S. Inouye, Y. Nishiyama, K. Uchida, Y. Hasumi, H. Yamaguchi, S. Abe, *J. Infect. Chemother.* **2006**, *12*, 349.
[28] E. Uzun, G. Sariyar, A. Adsersen, B. Karakoc, G. Ötük, E. Oktayoğlu, S. Pirildar, *J. Ethnopharmacol.* **2004**, *95*, 287.
[29] A. Schwiertz, C. Duttke, J. Hild, H. J. Müller, *Int. J. Aromather.* **2006**, *16*, 169.
[30] D. Mantle, J. G. Anderton, G. Falkous, M. Barnes, P. Jones, E. K. Perry, *Comp. Biochem. Physiol. B* **1998**, *121*, 385.
[31] H. J. D. Dorman, P. Surai, S. G. Deans, *J. Essent. Oil Res.* **2000**, *12*, 241.
[32] H. J. D. Dorman, S. G. Deans, *J. Essent. Oil Res.* **2004**, *16*, 145.
[33] C.-K. Wang, W.-J. Qu, W. Shun, Y.-J. Li, *Zhongchengyao* **2006**, *28*, 855.
[34] J. R. Lazutka, J. Mierauskienė, G. Slapšytė, V. Dedonytė, *Food Chem. Toxicol.* **2001**, *39*, 485.
[35] F. Bakkali, S. Averbeck, D. Averbeck, M. Idaomar, *Food Chem. Toxicol.* **2008**, *46*, 446.
[36] M. G. Evandri, L. Battinelli, C. Daniele, S. Mastrangelo, P. Bolle, G. Mazzanti, *Food Chem. Toxicol.* **2005**, *43*, 1381.
[37] R. P. Adams, 'Identification of Essential Oil Components by Gas-chromatography/Quadrupole Mass Spectrometry', Allured, Carol Stream IL, USA, 2001.
[38] E. M. Gaydou, R. Faure, J. P. Bianchini, G. Lamaty, O. Rakotonirainy, R. Randriamiharisoa, *J. Agric. Food Chem.* **1984**, *37*, 1032.
[39] K. H. Kubeczka, V. Formáček, 'Essential oils analysis by capillary gas chromatography and carbon-13 NMR spectroscopy', 2nd edn., John Wiley & Sons, 2002.
[40] T. Saito, K. Hayamizu, M. Yanagisawa, O. Yamamoto, 'Spectral Database for Organic Compounds', SDBS (free site organized by National Institute of Advanced Industrial Science and Technology (AIST), Japan) http://riodb01.ibase.aist.go.jp/sdbs/cgi-bin/crey_index.cgi?lang=eng, 2009.
[41] D. Mares, C. Romagnoli, B. Tosi, E. Andreotti, G. Chillemi, F. Poli, *Mycopathologia* **2005**, *160*, 85.
[42] D. Maron, B. N. Ames, *Mutat. Res.* **1983**, *113*, 173.

Received February 9, 2010

Flavour and Fragrance Journal

Multivariate analysis approach to the study of chemical and functional properties of chemo-diverse plant derivatives: Lavender essential oils

Journal:	*Flavour and Fragrance Journal*
Manuscript ID:	Draft
Wiley - Manuscript type:	Research Article
Date Submitted by the Author:	n/a
Complete List of Authors:	Maietti, Silvia; University of Ferrara Rossi, Damiano; University of Ferrara Guerrini, Alessandra; University of Ferrara Useli, Chiara; University of Ferrara Romagnoli, Carlo; University of Modena and Reggio Emilia Poli, Ferruccio; University of Bologna Bruni, Renato; University of Parma Sacchetti, Gianni; University of Ferrara
Keywords:	Lavender essential oils, chemical characterization, biological activity, Principal Component Analysis (PCA), Partial Least Squares regression (PLS)

Multivariate analysis approach to the study of chemical and functional properties of chemo-diverse plant derivatives: Lavender essential oils

Silvia Maietti[a], Damiano Rossi[a], Alessandra Guerrini[a], Chiara Useli[a], Romagnoli Carlo[b], Ferruccio Poli[c], Renato Bruni[d] and Gianni Sacchetti[a,*]

[a]*Dipartimento di Biologia ed Evoluzione – Sez. Risorse Agrotecnologiche e Farmaceutiche - AgriUnife, Università degli Studi di Ferrara, C.so Ercole I d'Este 32, I-44121 Ferrara, Italy;*

[b]*Dipartimento di Biologia, c/o Orto Botanico, Università degli Studi di Modena e Reggio Emilia, V.le Caduti in guerra 127, I-41121 Modena, Italy;*

[c]*Dipartimento di Biologia Evoluzionistica Sperimentale, Università degli Studi di Bologna, Via Irnerio 42, I-40126, Bologna, Italy;*

[d]*Dipartimento di Biologia Evolutiva e Funzionale, V.le G.P. Usberti 11A, I-43100, Università degli Studi di Parma, Italy.*

[*]**Correspondence to:** Gianni Sacchetti, Dipartimento di Biologia ed Evoluzione – Sez. Risorse Agrotecnologiche e Farmaceutiche - AgriUnife, C.so Ercole I d'Este 32, I-44121, Università degli Studi di Ferrara, Italy. Fax 0039 0532 208561; e-mail: gianni.sacchetti@unife.it

***Running title*: Multivariate analyses in studying chemo-diverse plant derivatives**

ABSTRACT: Six lavender essential oils, selected in order to adequately represent the chemodiversity of the *Lavandula* genus (*L. angustifolia*; five *Lavandula* x *hybrida* cultivars: i.e. *Super Z*, *Abrialis*, *RC*, *Alardii*, *Ordinario*), were evaluated from the phytochemical and biological standpoint and the results were elaborated using multivariate data analysis approach. The essential oils were analyzed by gas chromatography (GC-FID), gas chromatography–mass spectrometry (GC-MS) and headspace gas-chromatography (HS-GC-MS). Multivariate analyses (PCA, Principal Component Analysis) identified three main phytochemical clusters among lavender essential oils represented by 1,8-cineole, linalyl-acetate and linalool respectively. Functional properties of the essential oils were checked estimating cytotoxicity and genotoxicity using *Saccharomyces cerevisiae* D7 strain, antifungal activity against three common phytopathogens (*Pythium ultimum*, *Magnaporthe grisea* and *Botrytis cinerea*) performing agar vapour bioassay and antioxidant capacity through 1,1-diphenyl-2-picrylhydrazyl (DPPH) and β-carotene bleaching assays. No mutagenic effects were checked, but multivariate analyses (PLS, Partial Least Squares regression) evidenced that those essential oils belonging to linalool cluster were the most cytotoxic. Antifungal activity against phytopathogens confirmed the predictive results of PLS. The differences emerged among lavender essential oils about the weak antioxidant capacities evidenced a positive relation between the high polarity compounds and DPPH method by PLS. Opposite suggestions emerged with the same kind of compounds and β-carotene. Ketones and esters did not exert interesting antioxidant activity with both the methods. In conclusion, taking lavender essential oils as a pretext, the proposed approach assured the description of the relationship between a phytocomplex, its constituents and bioactivities and allowed to define a comprehensive, predictive approach to which chemical profile provides the best synergic overall effort in terms of applicative perspectives.

Keywords: Lavender essential oils; chemical characterization; biological activity; Principal Component Analysis (PCA); Partial Least Squares regression (PLS).

Introduction

Essential oils are an heterogeneous, complex pool of secondary metabolites, protean both in terms of biosynthetic pathways, chemical structures and functional groups involved, but also extremely changeable in the matter of total production.[1] The extreme plasticity of their yield and chemical composition at intra-generic and often at intra-specific level is the convergent evolutionary result of a number of stimuli, concurring in a synergic way to the modulation of a large number of variables, including biotic and abiotic factors like climate and soil or exposure to parasites and pathogens, to name a few.[2, 3] Given their multiple, simultaneous and interchangeable roles, essential oils represent a paradigm of chemodiversity and can be considered as one of the most striking examples of the versatility of the so-called “swiss army knife” tools available within the Plant Kingdom: multicomponent mixtures of secondary metabolites capable to act in multiple directions according to the specific plant's exigencies.[4] In fact, classes of compounds like simple phenols and mono- and sesquiterpenes, in particular if mixed in proper balance, can fulfil at the same time a plethora of needs: protect plants against predators and pathogens, attract insects for pollination or mutualistic help, defend populations against excessive heat or exposure to oxidative stress, between others. Such physiological and ecological eclecticism translates into the multifaceted biological activities usually ascribed to essential oils and ranging from antimicrobial to antioxidant, from repellent to attractive, from cytotoxic to mutagenic, from safety to efficacy health paradigms.[5] Most of these properties are not put into effect as the consequence of the action of a single highly specific

compound, but rather as the result of synergic efficacy of the whole phytocomplex and are renown for their potential benefit for different areas of human interest too.[6] For example, the widespread and well-rooted use of aromatic spices and essential oils for food preservation or as health promoters give witness of this behaviour, in which the prevention of the onset of oxidative degradation and inflammation goes on side by side with a bactericide or bacteriostatic activity and is a consequence of the simultaneous action of an array of different chemical substances.[7]

Being complex mixtures endowed with complex bioactivities, essential oils are usually evaluated with some degree of reductionism, narrowing down their study just to the relationship between chemical composition and single antimicrobial or antioxidant properties. As a matter of fact, such strategy may constitute a serious constraint when one desires to define a complete assessment of the overall bioactive properties of an essential oil or, in other words, define which chemical profile provides the best overall effort in terms of multiple biological activities at the same time.[5] In fact, in most cases, a comprehensive approach to the vast number of potential properties to be evaluated and the concurrent correlation between them and the phytochemical profile of an oil, is somehow lacking. The results are scattered in a myriad of papers, making difficult to draw sound conclusions and reducing the possibility to extrapolate which chemotype or which cultivar of an essential oil bearing plant is better suited in order to provide the best all-purpose composition.

True Lavender (*Lavandula vera* D.C. or *L. angustifolia* Miller.), lavenders (*L. spica* L., *L. stoechas* L., *L. latifolia* Medikus, etc.) and their lavandin hybrids (*Lavandula x hybrida* Rev.) rank among the most important essential-oil bearing crops, economically-wise. Their essential oils, isolated from flowering tips of a copious number of cultivars and species, are widely used for both functional and aromatic purposes in soaps, cosmetics, perfumes and a steady agro-industrial business has been built around these crops during the last

decades.[8] The quality of lavender and lavandin essential oils is based on the amount of terpenic alcohols (linalool, lavandulol, terpinen-4-ol) and respective acetates, while higher percentages of camphor, 1,8-cineole, β-caryophyllene and other sesquiterpenes are generally disregarded by the market, mainly for off-flavour issues.[9, 10] The high chemodiversity of lavender essential oils and the different corresponding bioactivities need to be considered together with the different efficacy expressed with reference to vapour or solution treatments consistent with possible different most effective applications, such as fumigations in agriculture or topic uses for skin health. As a consequence, an overall assessment of the synergic role of their constituents in the expression of the biological properties is not available. Thus, lavender essential oils may represent a good candidate to test the possible role of multivariate data analysis in the determination of the most prominent contributors to a combination of bioactivities exerted by an essential oil and the possibility to suggest the use of artificial phytocomplexes set up following the data emerged by cross-linking correlations through multivariate analyses of phytochemical and biological activities.

Thus, considering different lavender essential oils as experimental pattern, the aim of the present work is to shed light on different uses that different essential oils should be targeted for, through a multivariate data analysis correlation among chemical evidences of the whole liquid essential oils and of their head space acquired by GC-MS and HS-GC-MS, and *in vitro* bioactivities studied as cytotoxic and mutagenic activity, antioxidant and antifungal properties, in order to assess a possible pattern and suggest which phytochemical profiles and treatments could offer the best overall results.

Materials and methods

Plant material and Essential oils

In the second week of August of 2006, the flowering parts of *L. angustifolia* (coded LA), *L. hybrida,* cv. Ordinario (coded OR), cv. Alardii (coded AL), cv. Abrialis (coded AB), cv. R.C. (coded RC), cv. Super Z (coded SZ), grown at the Herb Garden of Casola Valsenio (Ravenna, Italy) were collected and immediately used as fresh material to obtain essential oils by 2h hydrodistillation with a Clevenger apparatus according to European Pharmacopoeia methods. The essential oil content was determined on a volume to dry weight basis, obtaining the yields reported in Table 1. The essential oil samples were dried over anhydrous sodium sulfate and stored in glass vials with teflon-sealed caps at -18±0.5°C in the absence of light until analysis.

Chemicals

The following solvents and chemicals were from Sigma-Aldrich (Milan, Italy): limonene, cis-Z-ocimene, linalool, 1,8-cineole used as references for GC, GC-MS; an hydrocarbon mixture (C18-C24) for the determination of retention indices; a standard solution of nonane (internal standard) for quantification of the essential oils chemicals; all the chemicals employed for antioxidant assays (see the corresponding paragraph). Microbial culture media (YEP, Yeast Extract Peptone; YEPD, Yeast Extract Peptone Dextrose; PDA, Potato Dextrose Agar) were from Oxoid Italia (Garbagnate, Italy). *Thymus vulgaris* essential oil (thymol chemotype) used as reference for assessing lavender essential oils functional properties (antifungal and antioxidant), was purchased from Extrasynthese (Genay, France). Water was purified by a Milli-Qplus 185 system from Millipore (Milford, MA).

Gas Chromatography

Essential oil samples were analyzed and the relative peak areas for individual constituents averaged. The relative percentages were determined using a ThermoQuest GC-Trace gas-chromatograph equipped with a FID detector and a Varian FactorFour VF-5ms poly-5% phenyl-95%-dimethyl-siloxane bonded phase column (i.d., 0.25 mm; length, 30 m; film thickness, 0.25 µm). Operating conditions were as follows: injector temperature 300°C; FID temperature 300°C, Carrier (Helium) flow rate 1 ml/min and split ratio 1:50. Oven temperature was initially 55°C and then raised to 100°C at a rate of 1°C/min, then raised to 250°C at a rate of 5°C/min and finally held at that temperature for 15 min. Each sample was dissolved in CH_2Cl_2 and 1 µl was injected.

Gas Chromatography - Mass Spectrometry Analysis

Essential oil constituents were then analyzed by a Varian GC-3800 gas chromatograph equipped with a Varian MS-4000 mass spectrometer using electron impact and hooked to NIST and ABREG libraries, dedicated to flavour and fragrance compounds. The constituents of the volatile oils were identified by comparing their GC retention times, LRI and the MS fragmentation pattern with those of other essential oils of known composition, with pure compounds and by matching the MS fragmentation patterns and retention indices with the above mentioned mass spectra libraries and with those in the literature.[11] The GC conditions were the same reported for GC analysis and the same column was used. The MS conditions were as follows: ionization voltage, 70 eV; emission current, 10 µAmp; scan rate, 1 scan/s; mass range, 29-400 Da; trap temperature, 150°C, transfer line temperature, 300°C. A mixture of aliphatic hydrocarbons (C8-C24) in hexane was injected under the above temperature program to calculate the retention indices.[12]

Headspace Gas Chromatography

The chemical composition of volatile fraction of essential oils was determined by static headspace analysis in GC-MS under the same conditions above mentioned. 500 µl of each sample were placed in a 8 ml vial sealed with a crimp top and kept at 26±1.0 °C for 1 h. The vapour phase was drawn off with a gas tight syringe and inject into the gas chromatograph.

Biological Activities

Lavender essential oils were checked for functional properties evaluating mutagenic and cytotoxic properties with *Saccharomyces cerevisiae* D7 strain,[13, 14] antifungal activity performing microatmosphere bioassay against the three phytopathogens *Botrytis cinerea* Micheli (American Type Culture Collection, ATCC 48339**),** *Pythium ultimum* Trow (kindly supplied by Prof. G. D'Ercole, Institute of Plant Pathology, University of Bologna, Italy), *Magnaporthe grisea* **(**American Type Culture Collection, ATCC64413), and *in vitro* antioxidant capacity through 1,1-diphenyl-2-picrylhydrazyl (DPPH) and β-carotene bleaching assays. All the bioactivities were performed comparing all the data with those achieved with appropriate pure synthetic compounds and/or commercial *Thymus vulgaris* essential oil, in order to have positive control references with single compounds or comparable phytocomplexes reputed for their functional bioactivities. The use of a phytocomplex known for its chemical and biological properties (for e.g. thyme essential oil) as positive reference results particularly indicative of the real functional efficacy of a tested extract.[15-18] Data reported for each assay are the average of three determinations of three independent experiments.

Cytotoxicity and mutagenic activity: *Saccharomyces cerevisiae* D7 assay

A toxicity and mutagenic pre-test was performed on lavender essential oils employing yeast cells (D7 diploid strain of *Saccharomyces cerevisiae* ATCC 201137). Complete liquid (YEP), solid (YEPD), and selective media were prepared according to literature.[13, 14] Cells from a culture with low spontaneous gene conversion and reverse point mutation frequencies were grown in a liquid medium at 28±1.0 °C until they reached the stationary growth phase. The yeast cells were pelleted and re-suspended in a volume of 0.1 M sterile potassium phosphate buffer, pH 7.4, to obtain the final mixture of $2x10^8$ cell/ml. The test solutions (4 ml) were composed of 1 ml of cell suspension, potassium phosphate buffer, DMSO and essential oil. Ten microlitres (10.00µl) of each essential oil were used as maximum quantity tested; for each lavender sample, this amount was then progressively diluted employing dimethyl sulfoxide (DMSO) in order to have also test plates with 5.00µl 2.50µl and 1.25µl. Negative control was assessed employing the highest DMSO volume (10.00µl) used in the essential oil test solutions, while positive control was set up with ethyl methane sulfonate (EMS) (0.01 mg/plate). The mixture were incubated under shaking for 2h at 37°C. Then the cells were plated in complete and selective media to ascertain survival, *trp*- (convertants) and *ilv*- (revertants). The plates were then incubated at 28±1.0 °C and, after 5 days, the grown colonies were counted to determine the gene conversion at *trp* locus (*trp* convertants) and point mutation at *ilv* locus (*ilv* revertants) frequencies on the basis of the colonies ratio numbered on selective and complete media.

Antifungal activity: agar vapour assay

Biological activity of lavender essential oils against three phytopathogen fungi was performed by means of the agar vapour method.[19-22] The fungi considered were the phytopathogen *Botrytis cinerea* Micheli ATCC 48339, *Pythium ultimum* Trow, kindly

supplied by Prof. G. D'Ercole (Institute of Vegetal Pathology, University of Bologna, Italy) and *Magnaporthe grisea* ATCC 64413. They were grown in Petri plates (90 mm) supplemented with 15 ml/plate of PDA, inoculated with 6 mm plugs from stationary phase cultures. The plates were then incubated for 24 hours at 26±1.0 °C. Successively, sterilized filter paper discs (diameter 9.0 mm) were absorbed with different volumes of lavender essential oil samples ranged from 0.50 to 25.00 µl, and placed inside the upper lid of each plate, at a distance of about 4mm from the mycelia. Plates were kept in an inverted position, tightly sealed with parafilm, and incubated for seven days at 26±1.0 °C. Blanks served as negative control. Commercial *T. vulgaris* essential oil was prepared as above described for lavender samples and considered as phytocomplex positive control reference. Three replicates were made for each treatment. After seven days the results were collected as radial growth inhibition and the results expressed as the essential oil amount which determined the 50% growth inhibition of each fungal strain (IC_{50}).

Antioxidant activity

Radical scavenging and antioxidant properties of essential oils were performed through different assays, namely DPPH assay, β-carotene bleaching test according to previously described methods.[15-18] This approach permits the antioxidant effectiveness of an essential oil to be more carefully defined, as it is almost impossible to express the antioxidant activity as an absolute value universally recognizable, besides whether expressed by only one kind of assay. All of the data collected for each assay are the average of three determinations of three independent experiments.

DPPH assay. An aliquot of essential oil (10 µl) was mixed with 900 µl of 100 mM Tris-HCl buffer (pH 7.4), 40 µl of ethanol, and 50 µl of 0.5% (w/w) Tween 20 solution. One

milliliter of 0.5 mM 1,1-diphenyl-2-picrylhydrazyl (DPPH, Sigma-Aldrich) ethanol solution was then added to the mixture. Tween 20 was used as an oil-in-water emulsifier. The mixture was shaken vigorously and kept in the dark for 30 min at room temperature. Sample absorbance was measured at 517 nm with UV-vis spectrophotometer (ThermoSpectronic Helios γ, Cambridge, U.K.). A blank was assessed as the solution assay described above without the essential oils, instead of which distilled water was employed. Butylated hydroxyl anisole (BHA) and *T. vulgaris* essential oil were used as positive controls. The radical scavenging activities of each sample were calculated according to the following formula for inhibition percentage (Ip) of DPPH: IpDPPH % = (A_B - A_A)/A_B x 100, where A_B and A_A are the absorbance values of the blank sample and of the test sample respectively, after 30min. Oils and BHA antiradical activity was considered as the concentration providing DPPH 50% inhibition (IC_{50}), calculated from inhibition curves obtained by plotting inhibition percentage against oil concentration.

β-carotene bleaching test. Approximately 10 mg of *β*-carotene (type I synthetic) was dissolved in 10 ml of $CHCl_3$, and 0.2 ml of the solution was pipetted into a flask containing a boiling mixture of 20 mg of linoleic acid and 200 mg of Tween 40. $CHCl_3$ was removed using a rotary evaporator (Büchi 461) at 40°C for 5 min, and 50 ml of distilled water was slowly added to the residue under vigorous agitation, to form an emulsion. Five milliliters of the emulsion was added to a tube containing 0.2 ml of the essential oils solution and the absorbance was immediately measured at 470 nm against a blank, consisting of an emulsion without *β*-carotene. The tube was placed in a water bath at 50°C, and the oxidation of the emulsion was monitored spectophotometrically by measuring absorbance at 470 nm over a 60 min period. Positive control consisted of BHA and *T. vulgaris* essential oil, whereas negative control consisted of test emulsion in which the essential oil was substituted by equal amounts of distilled water. The antioxidant

activity was expressed as inhibition percentage with reference to the control after a 60 min incubation using the following equation: $AA=100(DR_C - DR_S)/DR_C$, where AA is the antioxidant activity, DR_C is the degradation rate of the control =[ln(*a*/*b*)/60], DR_S is the degradation rate in the presence of the sample =[ln(*a*/*b*)/60], *a* is the absorbance at time 0, and *b* is the absorbance at 60 min. Oils and BHA antioxidant activity was considered as IC_{50}, calculated from inhibition curves obtained by plotting inhibition percentage against oil concentration.

Multivariate Data Analysis

Datasets were appropriately transformed for the statistical treatment using the multivariate data analysis software program Simca-P (version 11, Umetrics AB, Umeå, Sweden). Multivariate analysis was carried out using Principal Component Analysis (PCA) and Partial least Squares regression (PLS) to elaborate mutual relationship amongst the quantities of the compounds and classes of components in the essential oils and in the headspace, and to obtain an overview of how the samples were correlated to each other with regard to bioactivities.

PCA gives graphical representations of relationships among samples (liquid and vapour), chemicals and bioactivities. In addition, it reduces the complexity of the data and transforms the original variables into new axes, called principal components (PCs). These PCs are orthogonal in such a way that the data presented in the axes are uncorrelated with each other; PCA expresses as much as possible the total variation of the data in just a few principal components and in a decreasing order with respect to the amount of the variation. In other words, PCA creates a condensed summary of the table, which can be analyzed graphically by means of two plots, the *score plot* and the *loading plot*. The *score plot* is a summary of the relationship among the observations (for e.g., different kinds of

lavender essential oils; t1 and t2 in Fig. 1a) and the *loading plot* a similar summary of the variables (for e.g., compounds; p1 and p2 in Fig. 1b). Score plots represent the projections of the objects (samples) in the planes defined by the PCs (PC1 and PC2), whereas loading plots represent the projections of the original variables in the same planes. PCs define a plane into the multidimensional variable space where it is possible to visualize the structure of the investigated data. The co-ordinate values of the observations on this plane are called *scores*, while the plotting of such a projected configuration is known as a *score plot* (t1 and t2). Objects that are projected close to each other in the score plots have similar characteristics, and, for instance, samples to the right in the score plot have high values for variables placed to the right in the *loading plot* (p1 and p2). The farther a variable is from the axis origin, the more its contribution can be considered a major contribution in the statistical model generated by the principal component analysis. Partial least squares regression (PLS) methods have a theorethical foundation based on perturbation theory of a multivariable system and is aimed at detecting cause-effect relationships.[23]

Results and discussion

Chemical Composition of the Essential Oils

GC-MS analysis of six essential oils, obtained by steam distillation of aerial parts of *Lavandula angustifolia* (coded LA) and of five following *Lavandula x hybrida* cultivars: *L. x hybrida* cultivar Ordinario (coded OR), *L. x hybrida* cultivar Alardii (coded AL), *L. x hybrida* cultivar R.C. (coded RC), *L. x hybrida* cultivar Abrialis (coded AB) and cultivar Super Z (coded SZ), allowed the identification of 75 constituents (approximately 97% of the total), listed in Table 1 along with the respective extraction yields and the relative contribution of

the major classes of secondary metabolites. For a direct comparison with literature, Table 1 reports the essential oils composition expressed as peak area percentage normalized with internal standard and the linear retention index experimentally determined and compared with those reported by the MS library used (Adams, 2001). SZ cultivar provided the best essential oil yield (1.56%) followed by LA (1.50%) and AB (1.53%) cultivars; those yields obtained by other cultivars were instead 19.80% (RC), 11.50% (AL) and 23.00% (OR) lower than that of SZ. As expected, the major constituents were predominantly oxygenated monoterpenes (72.40-86.70%) and alcohols in particular, such as linalool, borneol and 4-terpineol, ethers (for e.g. 1,8-cineole), esters (for e.g. linalyl acetate) and ketones (for e.g. camphor). The sesquiterpene content was quite low (3.60-7.80%) confirming evidences reported by related literature.[8] As purported, the oils provided a good description of the chemodiversity available within the genus, with at least one of the examined samples representative of the major patterns of terpenic secondary metabolism in *Lavandula* sp., attesting how genetic factors of each lavender and lavandin, ecological and technical aspects of their processing affect their chemical composition and functional perspectives.[3, 8, 24, 25] As stated in related literature, the variability of the lavender essential oil composition is mainly determined by genetic factors of each cultivar or hybrids, although the distillation process and other ecological and technical aspects need also to be considered.[3, 24, 25]

In general, lavender essential oils are known to be chemically represented by abundance of linalool, linalyl acetate, minor quantity of lavandulyl acetate, terpinen-4-ol, lavandulol, 1,8-cineole and camphor.[8, 26] This simplistic description does not completely reflect the different uses and efficacies ascribed to lavender essential oils, since tens of minor chemicals contribute to the sensory and health properties of the oils.[8, 25]

The pool of essential oils examined gave the following chemical pattern: AL (*L. x hybrida* cv. Alardii) as essential oil rich in β-pinene and 1,8-cineole, and thus reputed least valuable from the commercial standpoint; OR (*L. x hybrida* cv. Ordinario) as essential oil rich in linalool, borneol and 4-terpineol, but poor in their acetates; RC (*L. x hybrida* cv. RC) essential oil as peculiar essential oil rich in linalool and camphor, but completely devoid of its esters; AB and SZ essential oil samples, as two prototypical essential oil representatives of commercial lavandins, rich in both linalool, linalyl acetate and camphor, one of which also rich in ocimene; *L. angustifolia* essential oil (LA), rich in both linalool, linalyl acetate and 4-terpineol and lacking significant amounts of 1,8-cineole and camphor.

Principal component analyses (PCA) were performed as score plot of chemical composition of the whole essential oils (Fig. 1a; score plot t1=x, t2=y), and as variable loading plot of first two principal components (Fig. 1b; p1=x, p2=y). The first two principal components - score plot and loading plot (Fig. 1a, b) - were representative of more than 74% of the total variance: 55.1% for PC1 and 19.5% for PC2. PCA evidenced three main clusters with reference to the chemical composition of the different essential oils. The cluster represented by the sole AL essential oil samples (Fig. 1a, A), is mainly characterized by 1,8-cineole which is the compound that mostly contributes to PC1 (Fig. 1b). The cluster composed by LA, SZ and AB essential oil samples (Fig. 1a, B) is characterized by linalyl-acetate as most abundant compound which mainly contributes to PC2. Linalool as the most abundant chemical detected in RC and OR essential oil samples characterized the third cluster (Fig. 1a, C). Borneol, cis-β-farnesene, lavandulyl acetate and myrcene (Fig. 1b) are other chemicals responsible of the separation of B and C clusters along PC2 (Fig. 1a), while all the other chemicals close to the origin do not significantly contribute to samples differentiation (Fig. 1b).

Since the essential oil analyses have been always performed by different GC

approaches,[25] analytical methods target has increasingly turned towards the characterization of head spaces volatile components.[20, 21] The analytical approach both to the whole liquid essential oils and their head space leads to better comprehend the role of the different chemicals and their abundance in displaying different efficacy in liquid or vapour treatments for human health, veterinary uses, and/or agricultural applications.[25]

The chemical composition of the vapour phase analyzed by HS-GC-MS (Table 2) detected and identified twenty-two compounds, revealing a different profile if compared with the essential oil as a whole, pointing out the prevalence of hydrocarbons and oxygenated monoterpenes, with alcohols and ethers as most abundant. The vapour phase was in fact enriched with the most volatile compounds, such as α-thujene, α- and β-pinene, while sesquiterpenes were completely absent, likely due to their lower volatility. As exceptions to this result, alcohols were not detected in AL cultivar samples - which showed instead almost equal abundance of oxygenated and ethers monoterpenes - while ethers were checked with low values in LA. LA head-space analyses showed α-pinene (17.02%) and linalool (15.88%) as the most abundant compounds. In all the other samples, instead, 1,8-cineole was checked in highest amounts, ranging from 55.17% in AL cultivar to 25.47% in OR cultivar, followed by linalool. AL cultivar represented the sole exception to this profile since linalool was not detected. In general, the availability of linalool and linalyl acetate almost halved.

Both the chemical evidences regarding the whole essential oils and their head-spaces are of particular relevance, because they give tools to point out and discriminate the compounds involved in bioactivity through direct contact of the essential oil as is and of its volatile fraction, allowing to better relate chemical evidences and functional properties, in particular those regarding flavour and cosmetic industry, those about ecological

allelopathy, health uses and agro-industrial applications (for eg. organic fumigation treatments against phytopathogens, topic treatments for skin health, etc.).[3, 25, 27, 28]

Biological activities

Cytotoxicity and mutagenic properties of the whole liquid essential oil have been assessed vs. *Saccharomyces cerevisiae* D7 strain (Table 3; Fig. 2).[13, 14, 17, 18] Antifungal capacity has been then performed vs. phytopathogens by means of the agar vapour method,[19-22] since vapour treatment methods would better evidence efficacy over solution experimental strategies in limiting filamentous fungi growth and sporulation (Table 4; Fig. 3).[25, 29] Finally, antioxidant capacity has been determined by spectrophotometric DPPH assay and β-carotene bleaching test (Figg. 4, 5). Where appropriate, the data acquired have been compared with pure synthetic compounds and/or commercial *Thymus vulgaris* essential oil, in order to have positive control references with single compounds or comparable phytocomplexes reputed for their functional bioactivities.[15-18] The use of a phytocomplex known for its chemical and biological properties as positive reference (for e.g. thyme essential oil) results particularly indicative of the real functional efficacy of a tested extract. Bioactivities have been then correlated to chemical evidences through multivariate analyses with the aim to report predictive suggestions about applicative uses of the highly chemodiverse lavender essential oils.

Biological activities have been performed with the object to cross-link the data acquired with chemical evidences of liquid and vapour phase of the essential oils, trying to achieve suggestions about the chemicals mainly involved in bioactivities, the presence of possible synergic relations and the possibility to hypothesize the formulation of artificial phytocomplexes, composed only by those compounds really involved in biological expression of the essential oils (Figg. 1-3, 5).

Citotoxic and mutagenic activity

Results obtained from the *Saccharomyces cerevisiae* D7 assay (Table 3) indicates that all the liquid lavender essential oils are citotoxic at the maximum quantity tested (10 µl), in accordance to what expected with reference to related literature.[7] At the tested dose of 5µl, only Alardi (AL) and Abrialis (AB) essential oil samples displayed negative cytotoxic results, while RC samples showed a cytotoxicity of about 40%. LA essential oil samples exhibited a cytotoxicity quantifiable as about 60%, SZ as about 80%, while OR essential oil displayed the strongest cytotoxicity data (Table 3). The correlation between phytochemical composition of the lavender oils and cytotoxic evidences through partial least squares (PLS) regression analyses revealed linalool as the chemical most responsible of the biological activity, while 1,8-cineole as the less cytotoxic compound (Fig. 2), partly agreeing with recent literature results.[7] The evidence regarding mutagenic effect of the oils agree with literature data which moreover stated that characteristic chemicals of lavender essential oils determine a protective effect against mutagenic agents, even if employing a different biological system assay from that of *Saccharomyces cerevisiae* D7 strain.[30] Because of the overlapping of the cytotoxic and genotoxic evidences at 5 and 10 µl, it is not possible to distinguish mutagenic effects (convertants and revertants) from those cytotoxic quantities tested. Therefore, taking 5 µl as the threshold essential oil quantity to determine genotoxic effects excluding those cytotoxic, at 1.25 and 2.50 µl none of the essential oil samples displayed results suggesting mutagenic effects. Not even at 5 µl, dose which does not determine cytotoxic effects for AL and AB essential oils, mutagenic effects have been observed for these two lavender samples (Table 3).

Correlation between chemical characterization and cytotoxic evidences considered with respect to *S. cerevisiae* D7 survivors (Fig. 2), revealed that 1,8-cineole is the

compound less involved in this biological activity. Linalool, instead, seemed to be the most responsible in exerting cytotoxicity, with a possible lower but synergic similar capacity displayed by borneol, 4-terpineol, linalyl acetate and *cis*-β-farnesene, since they are all positively correlated with cytotoxicity. All the other chemicals, plotted close to the origin of the axis, should be considered as non-cytotoxic and without any synergic role in displaying the activity of the essential oils.

Antifungal activities: agar vapour method

The above stressed results, and those regarding cytotoxicity in particular, lead us to investigate the antifungal properties of the essential oils and of linalool and 1,8-cineole as pure chemicals potentially involved in bioactivity (Table 4). PLS has been performed in order to statistically quantify the involvement of the essential oils and their constituents in the biological activity as single components or as synergic mixture (Fig. 3). The most sensitive approach considered to determine the bioactivity against fungi was that of agar vapour method, in light of its predictive capacity to evidence the bioactivity of the vapour phase of phytocomplexes and of the fact that it is reputed the most consistent strategy with possible applicative perspectives, such as fumigations in agriculture.[19, 22, 25, 29-32]

The antifungal activity has been performed *in vitro* against the phytopathogens *B. cinerea* (grey mould), *M. grisea* (rice blast) and *P. ultimum* (roots rot). The activity has been evaluated on the vapour fractions of the lavender essential oils (Table 2) compared to that of linalool. 1,8-cineole was however tested as single compound because of its abundance, even if the previous cytotoxic data evidenced its ineffectiveness against *S. cerevisiae* D7 strain. The antifungal capacity has been also compared to that of commercial *Thymus vulgaris* essential oil, since it is reputed a biologically effective phytocomplex and, therefore, it can be considered as a good reference to evaluate the

efficacy of analogous extracts.[15-18] In general, lavender essential oils expressed a lower antifungal capacity (IC_{50}) than that of thyme essential oil (Table 4). However, *B. cinerea* displayed always the highest sensitivity to all the treatments, followed by *P. ultimum* and *M. grisea*. LA samples were the most active among all lavender essential oils. The differences in bioactivity efficacy among the three fungal strains was important. In fact, taking LA data as key example, *B. cinerea* and *P. ultimum* were 89% and 72% more sensitive than *M. grisea* respectively. The similar sensitivity to treatments of *B. cinerea* and *P. ultimum* is also stressed by the closeness of their IC_{50} values plotted by PLS (Fig. 3). The sensitivity of all the strains to 1,8-cineole did not seem to reflect that of the essential oils as phytocomplexes and, despite its abundance but in accordance to previous PLS data related to cytotoxicity and with the sole exception of LA samples, it did not displayed interesting antifungal activity as single compound. These evidences are however in accordance with related literature data.[33] The activity displayed by the compound linalool was instead consistent with that of the vapour phase of all lavender samples, evidencing its key role in the biological capacity. In particular, it can be noted that the different abundance of linalool in lavender samples (Table 2) is directly proportional with the different activity expressed by the phytocomplexes against the most sensitive *B. cinerea*. It is not possible to state the same suggestion about the other two phytopathogens, since the occurring of 1,8-cineole, limonene and α-pinene with different abundance seem to exert a neutral or negative role on the activity determined by synergic interactions. This suggestion is supported by the low abundance of 1,8-cineole in LA samples and the most effective bioactivity exerted by the whole phytocomplex against all the strains.

Moreover, PLS regression model which relate HS-GC results with antifungal data, confirmed the fact that linalool is the most responsible compound of the antifungal activity, while 1,8-cineole is less involved in the fungi growth inhibition, as clearly evidenced by the

distance between the two compounds in PLS (Fig. 3). The presence of all the other chemicals close and around the origin of the axis represents a limited involvement of all these chemicals in the biological activity likewise. Synergic effects among chemicals in cytotoxicity seem to be very limited since the majority of the compounds is plotted close to the origin of the axes and far from linalool and 1,8-cineole, taking the distance from each compound as representative of the possible interactions.

Antioxidant properties

Antioxidant capacity has been performed on the whole liquid essential oils, following the suggestions given by related literature,[25] hypothesizing their functional use as ingredient of products active by direct contact (for e.g., topic use for skin health, liquid pesticide in agriculture). Even if the target of the research is represented by the correlation between the phytochemical evidences and biological activities in light to point out the more active chemicals and synergies, and the possible predictive role of multivariate analyses in terms of better applicative uses of phytocomplexes, it must be stressed that the antioxidant capacity of lavender samples compared to that of commercial thyme essential oil gave lower values, as however expected.[5, 34] All the essential oils showed very low antioxidant capacities with respect to BHA and *Thymus vulgaris* essential oil, both taken as positive reference (data not shown in Fig. 4). The results of the lavender samples with DPPH and β-carotene bleaching assays seemed to be controversial. In fact, all the lavender essential oils displayed the better IC_{50} values with β-carotene bleaching assay except for AL samples, which showed better IC_{50} with DPPH test (Fig. 4). This result, together with the high diversity in bioactivity expressed by both the tests, is once more illustrative of the different biological expression of the high chemodiversity of lavender essential oils.[8, 35] However, the best results for β-carotene bleaching assay have been achieved by AB

samples, followed respectively by RC, SZ, LA, OR and AL (Fig. 4). For DPPH assay, instead, the best results have been again displayed by AB, but followed respectively by AL, LA, SZ, OR and RC (Fig. 4). The differences emerged with regard to the sensitivity expressed by each lavender sample in both the assays, and to the different efficacy of all the oils in each assay reflects however controversial suggestions.

Trying to clarify these controversial evidences, PLS analyses of the data achieved with both the antioxidant tests related to those regarding chemical analyses (Fig. 5) showed the positive correlation between the high polarity compounds and DPPH method, while negative correlation has been evidenced with apolar chemicals. Opposite suggestions emerged with the same kind of compounds and β-carotene. Ketones and esters, i.e. medium polarity compounds, did not exert, instead, interesting antioxidant activity with both the methods, since PLS showed their point location close and around the origin of the axis (Fig. 5). It is interesting the loading plot position of linalool in PLS diagram, evidencing its involvement as tertiary allylic alcohol in antioxidant capacity with β-carotene assay, generally displayed by low polarity compounds. An analogous interesting exception has been previously pointed out.[31] Satisfactory explanation about this controversial behaviour could be suggested through an in-depth synergic study of the β-carotene bleaching activity involving linalool and compounds mainly responsible of the activity, as emerged by PLS (Fig. 5).

Conclusions

In the present paper, multivariate analyses (PCA) have been performed on GC-MS and HS-GC data of six lavender essential oils evidencing three different chemotypes with reference to linalool, linalyl acetate and 1,8-cineole as most abundant and characterizing chemicals. The multivariate analysis approach (PLS) has been used also to define which

chemotype and compounds can be considered mainly responsible of biological activities, trying also to find possible synergic chemical interactions emerging by the distance among the plotted compounds. Correlations between phytochemical and biological data have been performed with regard to cytotoxicity and mutagenic effects, antifungal and antioxidant properties employing strategies and methods coherent with safety and efficacy approaches and applicative perspectives in agricultural and health fields.

Linalool was always the most effective compound in all the bioassays, while 1,8-cineole was instead the less involved chemical in exerting any tested bioactivity. PLS about cytotoxicity evidenced that a synergic role could be played by linalool, borneol, 4-terpineol, linalyl acetate and *cis*-β-farnesene. Regarding antifungal results computed by PLS, synergic effects seemed to be very limited since the majority of the chemicals was plotted close to the origin of the axes and far from linalool which emerged as the most effective. With reference to antioxidant activity, the data revealed very low efficacy and in some cases controversial results of all the essential oils as liquid phytocomplexes. PLS evidenced high polarity compounds involved in a synergic antioxidant role in DPPH assay (for e.g. linalool, hexil-butanoate, camphene). Opposite conclusions were achieved about the correlation between β-carotene bleaching assays and low polarity compounds (for e.g. bisabolol, lavandulol). The meaning of the loading plot position of linalool in PLS diagram, evidencing its involvement as tertiary allylic alcohol in antioxidant capacity with β-carotene assay, needs to be in-depth further investigated.

In conclusion, lavender essential oils have been represented a pretext to extend the same research approach to other essential oils improving and differentiating the bioactivity assessment using multivariate analyses to verify the possible better applicative uses until to hypothesize the formulation of artificial phytocomplexes with supposed better functional properties given by proved synergic interactions.

Acknowledgements

The research was financially supported by a grant of the University of Ferrara (FAR 2009) and MIUR (Ministero dell'Università e della Ricerca Scientifica), Italy. The Authors wish to thank dott.ssa *Immacolata Maresca* for skillful technical assistance.

The Authors declare that there are no conflicts of interest.

References

[1] A.C. Figuereido, J.G. Barroso, L.G. Pedro, J.J.C. Scheffer, *Flavour Fragr. J.* **2008**, *23*, 213.

[2] N.S. Sangwan, A.H.A. Farooqi, F. Shabih, R.S. Sangwan, *Plant Growth Regul.* **2001**, *34*, 3.

[3] M. Zuzuarte, M.J. Gonçalves, C. Cavaleiro, A.M. Dinis, J.M. Canhoto, L.R. Salgueiro, *Chem. Biodivers.* **2009**, *6*, 1283.

[4] K.S. Gould, *J. Biomed. Biotech.* **2004**, *5*, 314.

[5] A.E. Edris, *Phytother. Res.* **2007**, *21*, 308.

[6] C. Romagnoli, R. Bruni, E. Andreotti, M.K. Rai, C.B. Vicentini, D. Mares, *Protoplasma* **2005**, *225*, 57.

[7] F. Bakkali, S. Averbeck, D. Averbeck, M. Idaomar, *Food Chem. Toxicol.* **2008**, *46*, 446.

[8] M. Lis-Balchin, in *Lavender - The genus Lavandula, Medicinal and Aromatic Plants - Industrial Profiles*, (Ed: M. Lis-Balchin), Taylor & Francis, London, UK, **2002,** pp. 251-262.

[9] J.B. Harborne, C.A. Willams, in *Lavender - The genus Lavandula, Medicinal and Aromatic Plants - Industrial Profiles*, (Ed: M. Lis-Balchin), Taylor & Francis, London, UK, **2002,** pp. 87-91.

[10] K.V. Peter, *Handbook of herbs and spices*, *Vol. 2*, CRC Press – Woodhead Publishing Limited, Cambridge (England), **2004**.

[11] R.P. Adams, *Identification of Essential Oil Components by Gaschromatography/ Quadrupole Mass Spectrometry*, Allured, Carol Stream (IL, USA), **2001**.

[12] B.d'A. Zellner, C. Bicchi, P. Dugo, P. Rubiolo, G. Dugo, L. Mondello, *Flavour Fragr. J.* **2008**, *23*, 297.

[13] F.K. Zimmermann, *Mutat. Res.* **1975**, *31*, 71.

[14] F.K. Zimmermann, R. Kern, H. Rosemberger, *Mutat. Res.* **1975**, *28*, 381.

[15] G. Sacchetti, S. Maietti, M. Muzzoli, M. Scaglianti, S. Manfredini, M. Radice, R. Bruni, *Food Chem.* **2005**, *91*, 621.

[16] A. Guerrini, G. Sacchetti, M. Muzzoli, G.M. Rueda, A. Medici, E. Besco, R. Bruni, *J. Agric. Food Chem.* **2006**, *54*, 7778.

[17] A. Guerrini, R. Bruni, S. Maietti, F. Poli, D. Rossi, G. Paganetto, M. Muzzoli, L. Scalvenzi, G. Sacchetti, *Food Chem.* **2009a**, *114*, 1413.

[18] A. Guerrini, G. Sacchetti, D. Rossi, G. Paganetto, M. Muzzoli, E. Andreotti, M. Tognolini, M.E. Maldonado, R. Bruni, *Environ. Toxicol. Phar.* **2009b**, *27*, 39.

[19] S. Inouye, K. Uchida, H. Yamaguchi, *Mycoses*, **2001**, *44*, 99.

[20] S. Inouye, K. Uchida, S. Abe, *J. Infect. Chemother.* **2006a**, *12*, 210.

[21] S. Inouye, K. Uchida, T. Takizawa, H. Yamaguchi, S. Abe, *J. Infect. Chemother.* **2006b**, *12*, 100.

[22] V. Tullio, A. Nostro, N. Mandras, P. Dugo, G. Banche, M.A. Cannatelli, A.M. Cuffini, V. Alonzo, N.A. Carlone, *J. Appl. Microbiol.* **2007**, *102*, 1544.

[23] L. Eriksson, E. Johansson, N. Kettaneh-Wold, S. Wold, *Multi- and Megavariate Data Analysis. Principles and Applications*. Umetrics Academy, Umea, Sweden, **2001**.

[24] H.M.A. Cavanagh, J.M. Wilkinson, *Phytother. Res.* **2002**, *16*, 301.

[25] H.M.A. Cavanagh, J.M. Wilkinson, *Aust. Infect. Control* **2005**, *10(1)*, 35.

[26] J.A. McGimpsey, N.G. Porter, *Lavender. A Growers guide for commercial production*. New Zealand Institute for Crop and Food Research Limited, Christchurch, New Zealand, **1999**.

[27] J. Popovici, C. Bertrand, E. Bagnarol, M.P. Fernandez, G. Comte, *Nat. Prod. Res.* **2008**, *22(12)*, 1024.

[28] X. Yang, H.T. Zhao, J. Wang, Q. Meng, H. Zhang, L. Yao, Y.C. Zhang, A.J. Dong, Y. Ma, Z.Y. Wang, D.C. Xu, Y. Ding, *J. Med. Plants Res.* **2010**, *4(16)*, 1668.

[29] K.I. Suhr, P.V. Nielsen, *J. App. Microbiol.* **2003**, *94(4)*, 539.

[30] D. Mitic-Culafic, B. Zegura, B. Nikolic, B. Vukovic-Gacic, J. Knezevic-Vukcevic, M. Filipic, *Food Chem. Toxicol.* **2009**, *47,* 260.

[31] G. Ruberto, M.T. Baratta, *Food Chem.* **2000**, *69(2)*, 167.

[32] M.S. Rakotonirainy, B. Lavédrine, *Int. Biodeter. Biodegr.* **2005**, *55*, 141.

[33] S. Kordali, I. Aslan, O. Çalmaşur, A. Cakir, *Ind. Crop. Prod.* **2006**, *23(2)*, 162.

[34] L. Kuan-Hung, Y. Shin-Yin, L. Min-Yi, S. Ming-Chih, Y. Kai-Ts'ung, H. Shih-Ying, 2007. *Food Chem.* **2006**, *105(1)*, 133.

[35] L. Chia-Wen, Y. Chia-wen, W. Sung-Chuan, Y. Kuang-Hway, *J. Food Drug Anal.* **2009**, *17*, 286.

Table 1. Chemical composition of essential oils obtained from *L. angustifolia* and five *Lavandula x hybrida* cultivars. OR: *L. hybrida,* cv. Ordinario; AL: cv. Alardii; AB: cv. Abrialis; RC: cv. R.C.; SZ: cv. Super Z; LA: *L. angustifolia*

No.	Compound[a]	LRI[b]	LRI[c]	OR	AL	AB	RC	SZ	LA
				Relative %[d]					
1	α-Thujene	930	928	0.19	tr.[e]	-[f]	0.90	-	0.17
2	α-Pinene	942	940	0.54	3.39	0.52	0.42	0.32	0.32
3	Camphene	954	953	0.50	0.20	0.45	0.32	0.32	0.17
4	Sabinene	976	975	0.79	2.65	0.92	0.48	0.48	0.07
5	β-Pinene	978	978	tr.	6.57	tr.	tr.	tr.	tr.
6	2,3 dihydro 1,8 cineole	979	978	-	0.14	-	-	-	-
7	1-Octen-3-ol	980	980	0.12	0.11	0.56	0.09	0.08	0.04
8	3-Octanone	982	982	0.20	-	0.26	0.37	0.50	1.56
9	Myrcene	990	989	tr.	3.02	3.54	1.13	2.36	3.09
10	3-Octanol	993	991	0.07	-	-	-	-	0.39
11	α-Phellandrene	1005	1003	0.57	0.14	0.26	0.24	-	0.12
12	α-Terpinene	1008	1007	0.12	0.11	-	0.1	-	0.2
13	Hexyl acetate	1009	1009	-	-	0.42	0.12	1.05	tr.
14	*p*-Cymene	1027	1025	0.25	0.23	-	0.21	-	0.29
15	Limonene	1029	1028	2.03	1.42	1.49	1.23	1.68	0.89
16	1,8 Cineole	1035	1034	8.97	57.52	9.35	8.12	5.09	tr.
17	*cis*-Ocimene	1038	1038	1.84	0.79	1.25	0.16	0.39	0.89
18	*trans*-Ocimene	1054	1052	0.34	0.10	8.56	0.38	0.89	2.41
19	γ-Terpinene	1060	1059	0.43	0.19	0.19	0.24	-	0.49
20	*cis*-Sabinene hydrate	1071	1071	0.23	0.79	0.09	0.07	-	0.34
21	Linalool oxide A	1076	1074	-	-	0.15	0.07	0.13	0.25
22	Linalool oxide B	1088	1087	-	-	-	-	-	0.19
23	Terpinolene	1090	1090	0.69	0.15	0.75	0.37	0.40	0.23
24	Linalool	1097	1095	47.00	0.82	33.47	56.57	34.43	36.15
25	*trans*-Sabinene hydrate	1099	1098	-	0.15	-	-	-	-
26	Propanoic acid hexyl ester	1103	1103	-	-	0.19	-	-	-
27	1-Octen-3-ol acetate	1111	1112	-	-	0.40	-	0.68	0.16
28	Campholenic aldehyde	1126	1125	-	0.07	-	-	-	-
29	Nopinone	1141	1141	-	0.07	-	-	-	-
30	*allo*-Ocimene	1136	1135	0.15	-	2.23	0.22	0.61	1.15
31	*cis*-Sabinol	1145	1143	-	0.28	-	-	-	-
32	Camphor	1150	1149	2.39	2.96	8.34	10.01	8.84	0.98
33	Hexyl isobutanoate	1153	1152	-	-	0.44	0.35	0.44	tr.
34	Verbenol	1155	1146	-	0.14	-	-	-	-

Table 1. *Continued*

No.	Compound[a]	LRI[b]	LRI[c]	OR	AL	AB	RC	SZ	LA
				Relative %[d]					
35	Pinocarvone	1173	1171	-	0.18	-	-	-	-
36	Borneol	1174	1170	13.00	2.78	1.15	1.83	1.60	2.39
37	Lavandulol	1181	1180	0.06	1.49	0.71	0.30	-	-
38	Terpinen-4-ol	1182	1175	8.92	0.48	1.33	4.82	0.63	16.13
39	Cryptone	1184	1183		0.34	-	0.19	-	-
40	α-Terpineol	1191	1190	0.35	3.90	0.69	0.29	0.94	0.26
41	Hexyl butanoate	1193	1193	0.89	-	1.03	2.25	1.94	0.04
42	Myrtenol	1197	1196	-	0.15	-	-	-	-
43	n-Hexyl 2-methyl butanoate	1238	1236	0.17	-	0.21	0.47	0.32	-
44	Isobornyl formate	1240	1239	-	-	-	0.13	-	0.16
45	Hexyl isovalerate	1244	1244	-	-	-	0.23	0.3	-
46	Linalyl acetate	1259	1257	2.87	-	10.34	-	24.36	17.08
47	Bornyl acetate	1291	1289	0.25	-	-	-	0.16	0.12
48	Lavandulyl acetate	1293	1290	0.23	-	2.09	-	1.93	2.50
49	Hexyl tiglate	1335	1333	0.22	-	-	0.11	0.44	-
50	Neryl acetate	1363	1362	-	-	-	-	0.83	0.76
51	Geranyl acetate	1383	1381	-	-	0.63	-	1.51	1.54
52	Daucene	1384	1382	-	-	0.08	tr.	0.06	-
53	β-Cubebene	1387	1386	-	0.58	-	-	-	-
54	β-Bourbonene	1389	1388	-	-	tr.	tr.	0.06	-
55	Hexyl hexanoate	1391	1389	0.16	-	-	0.28	0.18	-
56	Geranyl acetate	1394	1392	-	-	1.29	-	-	-
57	Caryophyllene	1420	1418	0.36	1.18	2.48	2.26	1.47	1.77
58	α-Santalene	1423	1421	-	-	0.52	0.61	0.25	0.19
59	β-Copaene	1433	1431	-	0.38	-	0.10	-	-
60	*trans*-α-Bergamotene	1436	1434	-	-	-	-	0.15	-
61	Bicyclosesquiphellandrene	1437	1435	-	0.48	-	-	-	-
62	Lavandulyl isobutanoate	1440	1438	-	-	0.06	-	-	-
63	Farnesene isomer	1441	1440	-	-	-	-	-	0.18
64	*cis*-β-Farnesene	1444	1443	3.02	0.37	-	-	-	tr.
65	α-Humulene	1455	1454	0.25	-	0.11	0.13	0.07	0.06
66	α-Himalachene	1456	1451	-	0.38	-	-	-	-
67	γ-Acoradiene	1457	1499	-	1.52	-	-	-	-
68	Farnesene isomer	1458	1457	-	-	-	0.14	-	0.08
69	*trans*-β-Farnesene	1460	1459	-	-	0.78	0.97	0.77	5.43
70	Germacrene D	1487	1485	-	-	-	0.39	0.05	-

Table 1. *Continued*

No.	Compound[a]	LRI[b]	LRI[c]	OR	AL	AB	RC	SZ	LA
							Relative %[d]		
71	α-Farnesene	1504	1502	-	-	-	-	0.53	-
72	*cis*-Calamenene	1541	1540	-	0.44	-	-	-	-
73	Lavandulyl Isovalerate	1513	1511	-	-	-	-	0.27	-
74	Caryophyllene oxyde	1586	1584	-	0.43	tr.	-	0.07	0.14
75	α-Bisabolol	1684	1683	-	1.62	-	0.26	0.47	-
	Total[g]			98.17	98.71	97.33	97.93	98.05	99.38
Extraction Yield %				1.20	1.38	1.53	1.25	1.56	1.50
Monoterpenes hydrocarbons				8.44	18.96	20.16	6.4	7.45	10.49
Monoterpenes oxygenated				86.10	72.37	73.2	86.67	86.65	81.04
	-Alcohols			69.75	11.09	38.15	64.04	37.81	56.14
	- Ethers			8.97	57.66	9.35	8.12	5.09	-
	- Esters			4.79	-	17.1	3.94	34.41	22.36
	-Aldehydes			-	0.07	-	-	-	-
	- Ketones			2.59	3.55	8.60	10.57	9.34	2.54
Sesquiterpenes hydrocarbons				3.63	5.33	3.97	4.6	3.41	7.71
Sesquiterpenes oxygenated				-	2.05	-	0.26	0.54	0.14

[a] Compounds are listed in order of elution from a Varian FactorFour VF-5 ms column; [b] LRI (Linear Retention Indeces) calculated on a Varian FactorFour VF-5 ms column; [c] LRI (Linear Retention Indeces) reported in the Adam's Library, 2001 (Allured, IL, USA); [d] Relative percent of peak areas normalized with nonane as internal standard; [e] tr=trace (< 0.02%); [f] dash = not detected; [g] Total amount of the identified compounds

Table 2. Headspace composition of essential oils obtained from *L. angustifolia* and five *Lavandula x hybrida* cultivars. OR: *L. hybrida*, cv. Ordinario; AL: cv. Alardii; AB: cv. Abrialis; RC: cv. R.C.; SZ: cv. Super Z; LA: *L. angustifolia*

No.	Compound[a]	LRI[b]	OR	AL	AB	RC	SZ	LA
					Relative %[c]			
1	1-Methoxy-hexane		-[e]	-	1.32	1.86	2.51	1.54
2	α-Thujene	930	3.36	tr.[d]	-	1.62	-	9.39
3	α-Pinene	942	13.71	11.58	7.25	7.90	10.81	17.02
4	Camphene	954	5.99	0.52	3.83	4.67	8.36	3.53
5	Sabinene	976	1.99	5.47	0.83	1.37	1.64	1.07
6	β-Pinene	978	5.13	13.36	4.46	3.76	5.53	1.11
7	3-Octanone	982	-	-	-	-	-	4.09
8	Myrcene	990	3.69	3.53	1.99	2.36	2.96	4.26
9	α-Terpinene	1008	3.34	-	-	0.99	-	3.10
10	*p*-Cymene	1027	1.86	0.34	-	1.42	-	5.58
11	Limonene	1029	7.58	3.33	3.67	4.53	6.73	2.91
12	β-Phellandrene	1032	-	5.95	-	-	-	1.82
13	1,8-Cineole	1035	25.47	55.17	25.83	30.93	30.25	2.90
14	*cis*-Ocimene	1038	4.95	0.27	7.36	3.18	-	8.03
15	*trans*-Ocimene	1054	0.64	-	22.18	8.40	-	7.37
16	γ-Terpinene	1060	0.66	-	-	-	-	2.21
17	Terpinolene	1090	0.84	-	0.90	-	-	-
18	Linalool	1097	15.30	-	11.02	17.63	14.29	15.88
19	Camphor	1150	1.32	0.48	5.48	6.56	9.02	-
20	Borneol	1174	1.27	-	-	-	-	-
21	Terpinen-4-ol	1182	2.28	-	-	-	-	5.61
22	Linalyl acetate	1259	0.61	-	3.87	2.82	7.90	2.58
	Total[f]		99.99	100.00	99.99	100.00	100.00	100.00
Monoterpenes hydrocarbons			53.74	44.35	53.79	42.06	38.54	68.94
Monoterpenes oxygenated			46.25	55.65	46.20	57.94	61.46	31.06
	-Alcohols		18.85	-	11.02	17.63	14.29	21.49
	- Ethers		25.47	55.17	25.83	30.93	30.25	2.90
	- Esters		0.61	-	3.87	2.82	7.90	2.58
	- Ketones		1.32	0.48	5.48	6.56	9.02	4.09

[a] Compounds are listed in order of elution from a Varian FactorFour VF-5 ms column; [b] LRI (Linear Retention Indices) calculated on a Varian FactorFour VF-5 ms column;[c] Relative area percentage (peak area relative to total peak area percent; [d] tr=trace (< 0.02%); [e] dash = not detected; [f] Total amount of the identified compounds

Table 3. Effect of survival and genotoxicity (mutagenesis and gene conversion) in *Saccharomyces cerevisiae* D7 strain assay from treatment with lavender essential oils. OR: *L. hybrida,* cv. Ordinario; AL: cv. Alardii; AB: cv. Abrialis; RC: cv. R.C.; SZ: cv. Super Z; LA: *L. angustifolia*. Negative control: DMSO (10µ) in 4ml of test solution

Samples	Essential oil (µl)	Survivors (%) ± s.d.	Convertants/10^5 survivors ± s.d.	Revertants/10^6 survivors ± s.d.
	Negative control	100.00	0.65±0.06	0.16±0.03
	1.25	94.83±8.23	0.36±0.05	0.13±0.02
OR	2.50	92.15±6.12	0.36±0.03	0.15±0.04
	5.00	1.42±0.18	n.d.	n.d.
	10.00	0.00	n.d.	n.d.
	Negative control	100.00	0.94±0.09	0.25±0.04
	1.25	97.59±9.19	0.56±0.05	0.31±0.05
AL	2.50	95.11±10.05	0.95±0.08	0.28±0.05
	5.00	94.44±4.32	0.85±0.07	0.21±0.03
	10.00	27.01±1.26	n.d.	n.d.
	Negative control	100.00	0.56±0.08	0.12±0.05
	1.25	100.40±14.35	0.57±0.06	0.12±0.05
AB	2.50	98.73±8.27	0.56±0.05	0.10±0.05
	5.00	97.10±3.64	0.46±0.04	0.15±0.05
	10.00	9.38±0.99	n.d.	n.d.
	Negative control	100.00	0.82±0.08	0.40±0.05
	1.25	97.05±7.33	0.86±0.09	0.44±0.06
RC	2.50	95.29±8.45	0.91±0.09	0.31±0.05
	5.00	60.89±4.79	1.15±0.11	0.32±0.04
	10.00	0.00	n.d.	n.d.
	Negative control	100.00	0.49±0.04	0.13±0.02
	1.25	95.73±3.50	0.47±0.05	0.15±0.02
SZ	2.50	91.63±5.66	0.36±0.04	0.22±0.03
	5.00	20.00±1.83	n.d.	n.d.
	10.00	4.09±0.81	n.d.	n.d.
	Negative control	100.00	0.45±0.04	0.17±0.03
	1.25	100.79±10.01	0.60±0.06	0.10±0.01
LA	2.50	98.20±6.18	0.46±0.05	0.13±0.02
	5.00	38.61±2.48	n.d.	n.d.
	10.00	1.27±0.08	n.d.	n.d.

s.d. = standard deviation

n.d. = not determined

Table 4. Antifungal activity (agar vapour method) of Lavender essential oils against phytopathogens. OR: *L. hybrida,* cv. Ordinario; AL: cv. Alardii; AB: cv. Abrialis; RC: cv. R.C.; SZ: cv. Super Z; LA: *L. angustifolia*. All the values are expressed as IC_{50} (µl/plate) ± standard deviation

Essential oil samples	*Magnaporthe grisea*	*Pythium ultimum*	*Botrytis cinerea*
OR	21.10±1.23	10.15±0.97	2.53±0.18
AL	n.d.[a]	5.57±0.26	12.07±1.10
AB	23.48±2.02	4.65±0.54	4.22±0.22
RC	20.10±1.69	5.12±0.48	1.33±0.08
SZ	12.93±1.01	12.83±0.88	2.29±0.17
LA	13.65±1.05	3.58±0.32	1.72±0.09
Thymus vulgaris	0.38±0.05	0.42±0.05	0.23±0.04
1,8-cineole	n.d.[b]	n.d.[c]	21.60±1.08
Linalool	2.06±0.22	2.50±0.16	1.46±0.07

[a] n.d. = not determined as IC_{50} (µl/plate); 28.9% growth inhibition at the maximum concentration tested (25 µl/plate)
[b] n.d. = not determined as IC_{50} (µl/plate); 23.8% growth inhibition at the maximum concentration tested (25 µl/plate)
[c] n.d. = not determined as IC_{50} (µl/plate); 34.5% growth inhibition at the maximum concentration tested (25 µl/plate)

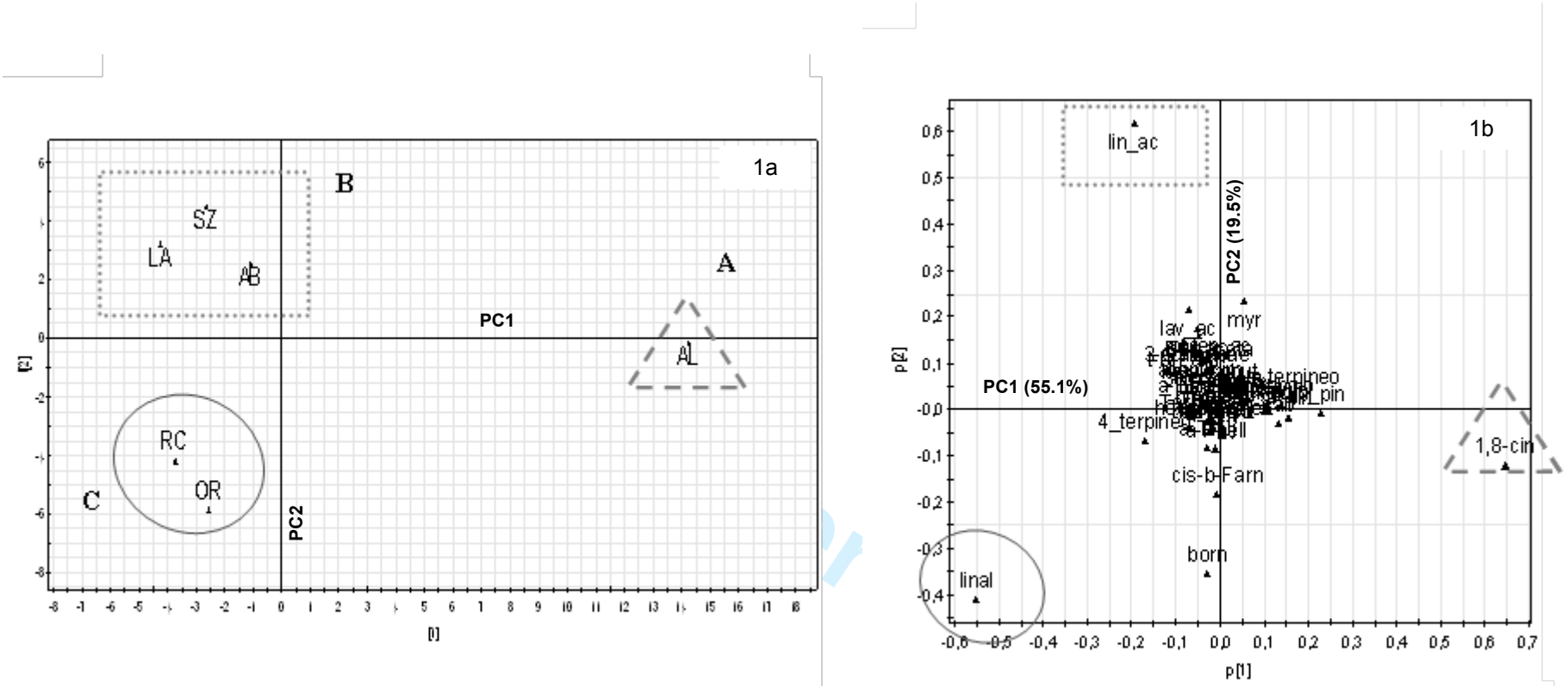


Figure 1. Principal component analysis (PCA) involving compositions of Lavender essential oils; **1a)** score plot diagram of different oils (score plot t1=x, t2=y). OR: *L. hybrida,* cv. Ordinario; AL: cv. Alardii; AB: cv. Abrialis; RC: cv. R.C.; SZ: cv. Super Z; LA: *L. angustifolia*. A, B, C = three main clusters of lavender essential oils; **1b)** variable loading plot diagram of the first two principal components (loading plot p1=x, p2=y).

Cluster A (broken line triangle; sample AL in 1a) is mainly chracterized by 1,8-cineole (1,8-cin in the broken line triangle in 1b) which is the compound that mostly contributes to PC1; Cluster B (dotted rectangle; samples LA, AB, SZ in 1a) is mainly characterized by linalyl-acetate (lin_ac in the dotted rectangle in 1b) which mainly contributes to PC2; Cluster C (circled area; samples RC, OR in 1a) is mainly characterized by linalool (linal in the circled area in 1b). Borneol (born), cis-β-farnesene (cis-b-Farn), lavandulyl acetate (lav ac) and myrcene (myr) (1b) are other chemicals responsible of the separation of B and C clusters along PC2 (1a)

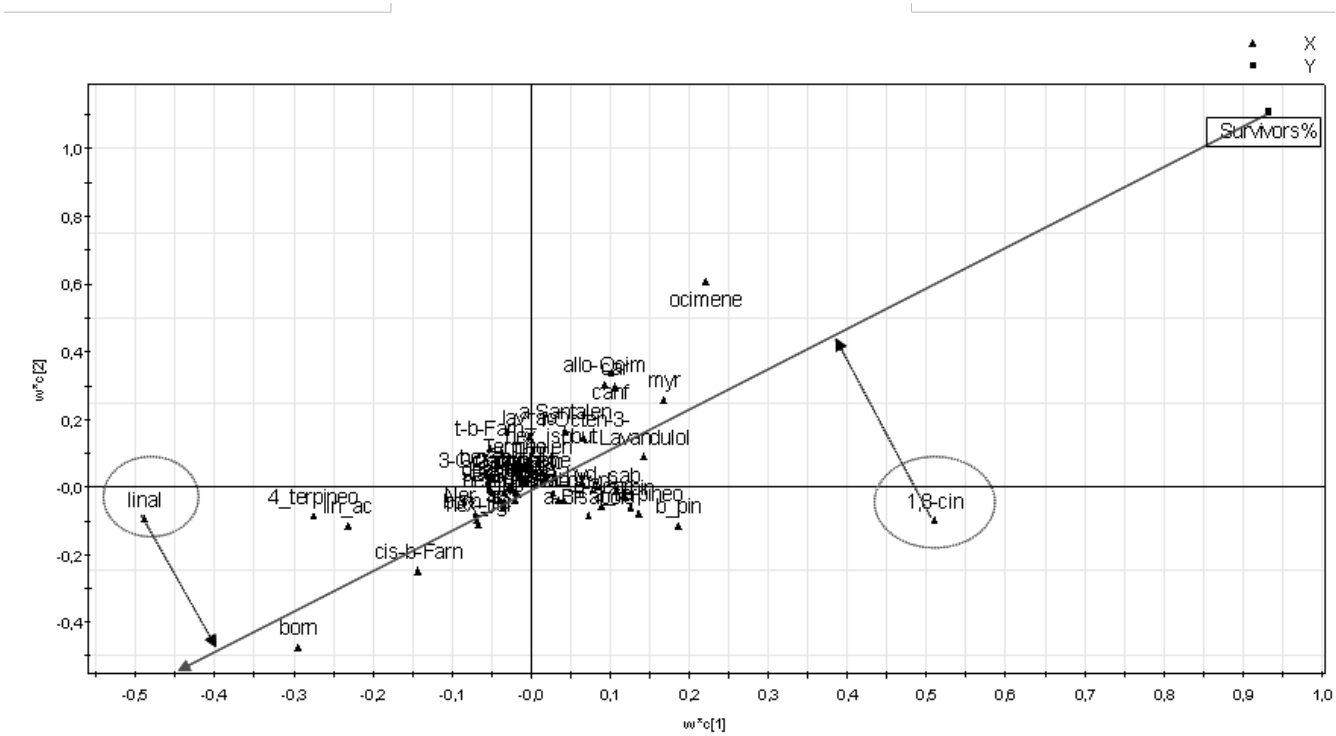


Figure 2. Partial Least Squares regression (PLS) evidencing cytotoxicity against *S. cerevisiae* D7 strain related to Lavender essential oil composition. The diagonal represents the *S. cerevisiae* survivors, and its direction (from the right to the left) indicates their progressive decrease. The orthogonal projection of the plane position of the compounds of the essential oils on this diagonal line gives the involvement of the chemicals in cytotoxicity: more the projection of the chemicals is plotted along the left part of the diagonal, more these compounds are involved in cytotoxicity. Linalool (linal) results the most responsible compound in bioactivity, while 1,8-cineole (1,8-cin) the less involved.

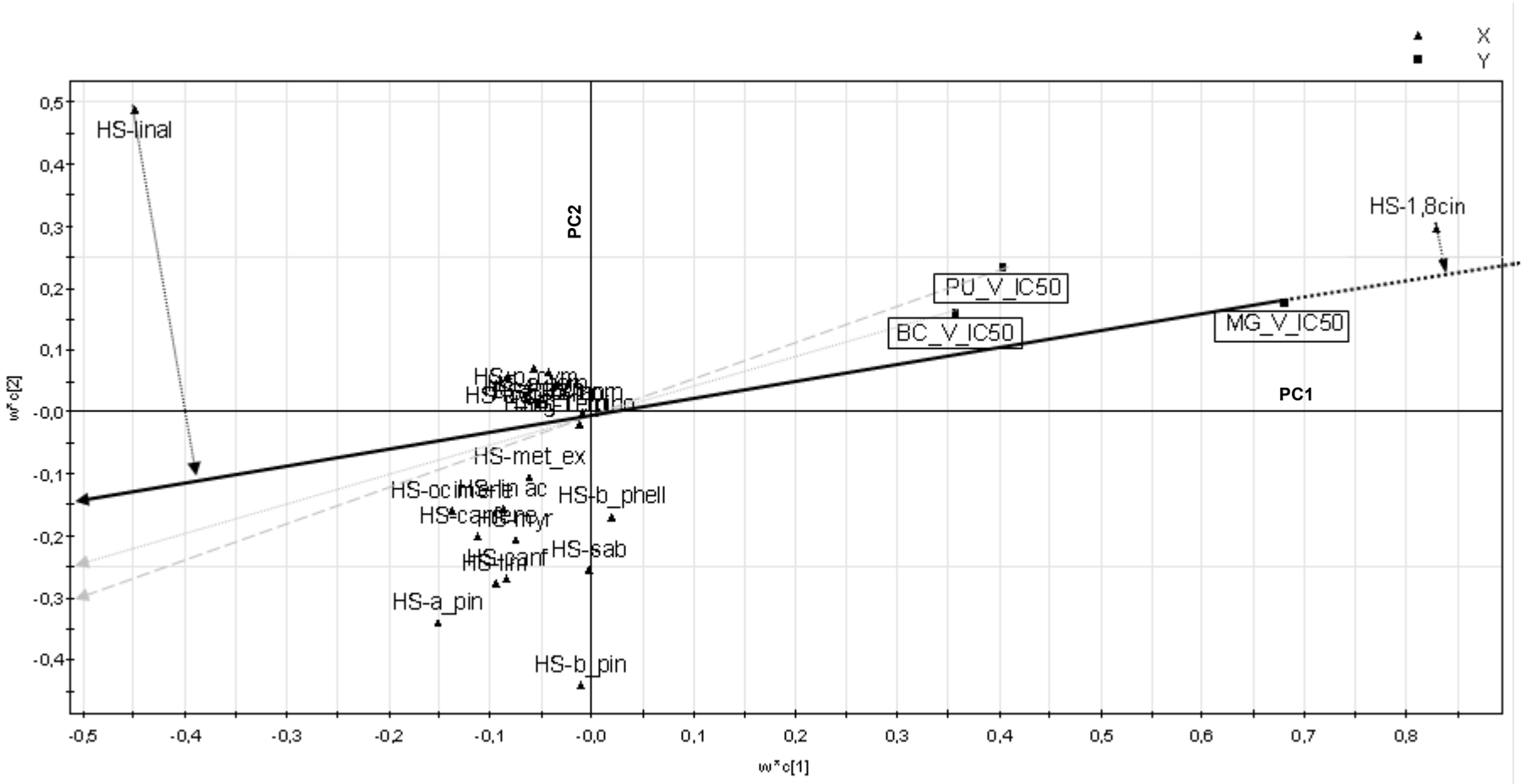


Figure 3. Partial Least Squares (PLS) regression model of antifungal activity, determined with agar vapour assay with reference to volatile chemicals of Lavender essential oils (see Table 2). All compounds are indicated with the prefix HS (Head Space): HS-b_pin: β-pinene; HS-a_pin: α-pinene; HS-linal: linalool; HS-1,8-cin: 1,8-cineole; HS-sab: sabinene; HS-b_phell: β-phellandrene; HS-met_ex: methoxy hexane. PU_V_IC50: IC_{50} variable of *Pythium ultimum* obtained with agar Vapour method; BC_V_IC50: IC_{50} variable of *Botrytis cinerea* obtained with agar Vapour method; MG_V_IC50: IC_{50} variable of *Magnaporthe grisea* obtained with agar Vapour method. The diagonals, starting from the plotted IC_{50} variable of each fungal strain passing through the origin of the axes, indicate a progressive IC_{50} reduction; thus, the orthogonal projections of the plane plotted chemicals on these diagonals suggest, from the right to the left, an increasing antifungal capacity of the essential oil compounds: more the projection of the chemicals is plotted along the left part of the diagonal, more these compounds are involved in antifungal capacity.

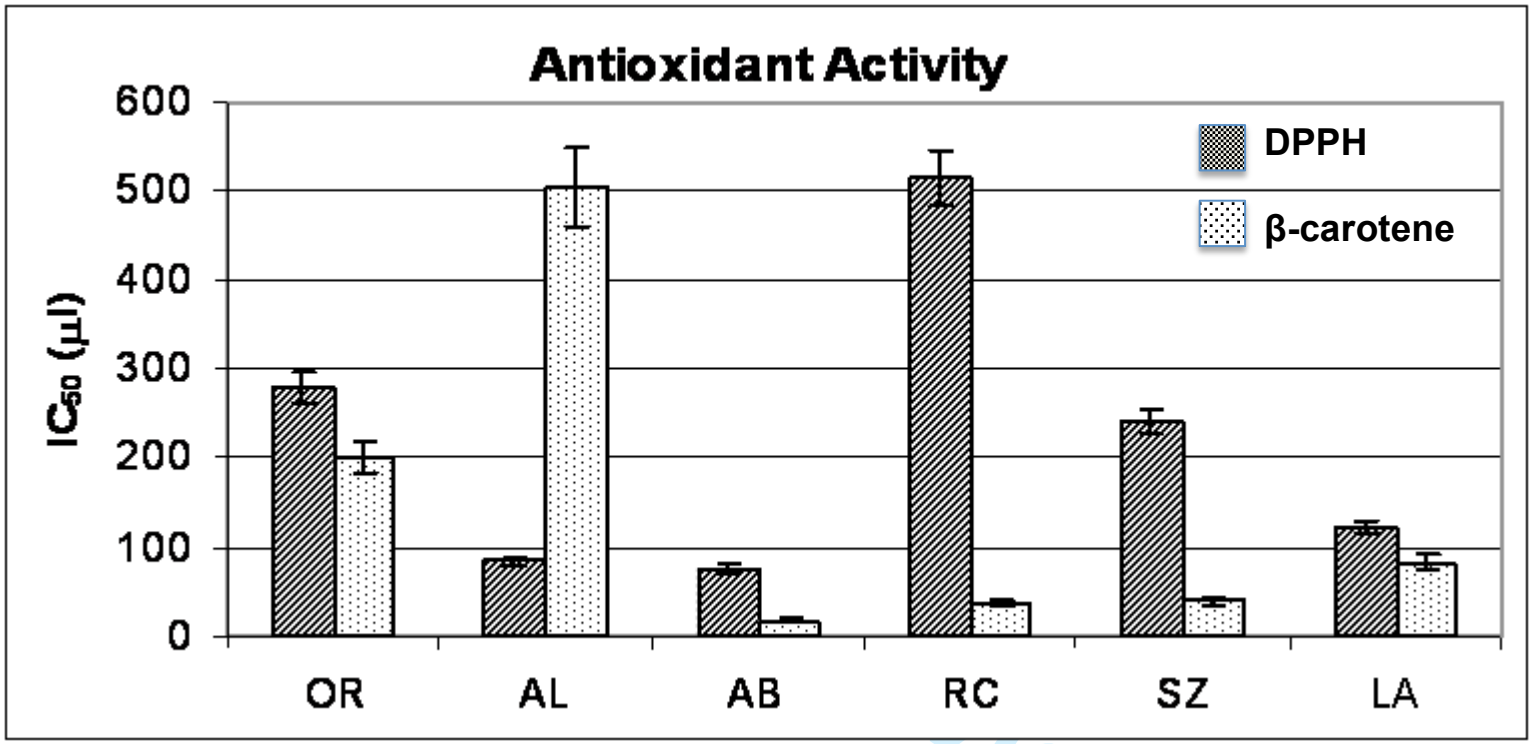


Figure 4. Antioxidant activity (DPPH and β-carotene bleaching assays) of lavender essential oils samples expressed as IC_{50}. OR: *L. hybrida,* cv. Ordinario; AL: cv. Alardii; AB: cv. Abrialis; RC: cv. R.C.; SZ: cv. Super Z; LA: *L. angustifolia*. Results are derived by triplicate determinations; means ± standard deviation.

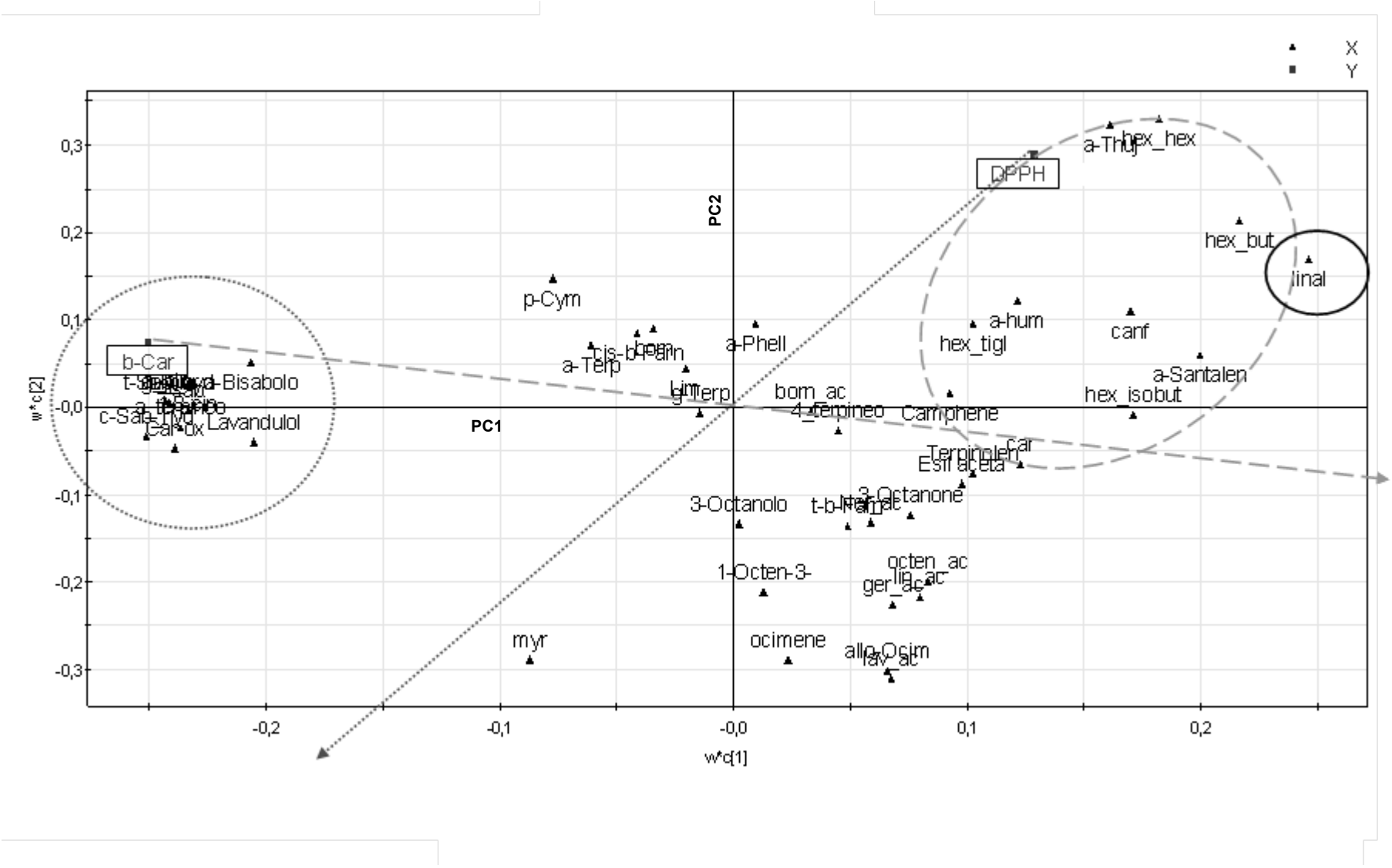


Figure 5. Partial Least Squares (PLS) regression model of antioxidant activity, determined with DPPH and β-carotene bleaching tests computed as IC_{50}. The dotted line diagonal passing through the origin of the axes, directed from the right to the left, starts from the plane position of the IC_{50} variable referred to DPPH assay (DPPH). The broken line diagonal passing through the origin of the axes, directed from the left to the right, starts from the plane position of the IC_{50} variable referred to β-carotene bleaching test (b-Car). The orthogonal projections of the chemicals on these diagonals - far from the starting point of these lines - suggest their involvement in bioactivity. The compounds circled by a dotted line (high polarity compounds) resulted the most involved in exerting antioxidant capacity through DPPH test. The compounds circled by a broken line (apolar chemicals), instead, resulted the most involved in exerting antioxidant capacity through β-carotene bleaching test. Linalool (linal, solid line circle) represents an exception as chemical involved in β-carotene bleaching efficacy as polar tertiary allylic alcohol.

Relazione o comunicazione a congresso

BIOTRASFORMAZIONI DI TERPENI E OLI ESSENZIALI CON BATTERI E FUNGHI ISOLATI DA FRUTTI DEL GENERE CITRUS DELLA FORESTA AMAZZONICA (ECUADOR)

A. Guerrini, G. Moreno Rueda, P. Pedrini, D. Rossi, S. Maietti, A. Grandini, K. Saro, C. Useli, L. Scalvenzi, I. Maresca, M. Muzzoli, G. Sacchetti

Fitomed, Atti del IV Congresso Intersocietà sulle Piante Medicinali Anno: 2010

La letteratura riguardante le biotrasformazioni di oli essenziali è a tutt'oggi molto limitata e per lo più orientata allo studio dei singoli monoterpeni, markers specifici di oli essenziali. Possibili obiettivi di queste applicazioni biotrasformative possono essere la deterpenazione dell'olio, ottenendo come prodotti finali composti ossigenati con caratteristiche più idrofile e proprietà organolettiche differenti, oppure l'attivazione degli oli essenziali, mediante reazioni che modifichino le molecole di partenza, migliorando le capacità bioattive del fitocomplesso. In questo lavoro dalle foglie di *Citrus limon*, *C. aurantifolia, C. aurantium* e *C. paradisi*, specie naturalizzate nell'ambiente dell'Ecuador amazzonico presso le comunità Achuar nei territori Wasakentsa, Pumpuentsa e Sewastian, si sono ottenuti per distillazione in corrente di vapore gli oli essenziali e, dall'epicarpo dei frutti, si sono isolati 61 tipi batterici e 55 tipi fungini che, ad un primo esame macroscopico, potevano essere associati a specie differenti. Si è valutata dapprima la possibile capacità biotrasformativa su singoli composti terpenici, maggiormente rappresentativi degli oli in oggetto e, successivamente, sui ceppi a maggior attività si è proceduto a valutare la biotrasformabilità dell'intero fitocomplesso olio essenziale. Sui singoli terpeni linalolo, limonene, citronellale e sabinene, rappresentativi degli oli essenziali in oggetto, le biotrasformazioni che hanno restituito risultati significativi sono state ripetute su scala preparativa e i prodotti separati per cromatografia e caratterizzati mediante GC-MS, ^{1}H-NMR, ^{13}C-NMR. La biotrasformazione del linalolo sia con tipi fungini che batterici ha restituito principalmente E/Z-furanlinalolo ossido. In particolare, il fungo inizialmente denominato 1B14 ha fornito 2-metil-2-epten-6-one, prodotto di degradazione della catena idrocarburica, mai documentato prima in letteratura come prodotto biotrasformativo del linalolo, come pure i diasteroisomeri del 1-metil-1-(4-metilpentil)-2-ossiranmetanolo (rese 10%-30%) ottenuti da altri 5 tipi fungini. Il sabinene ha fornito come prodotti principali 4-terpineolo e γ-terpinene con il solo tipo fungino denominato 1C22. Nelle biotrasformazioni con (-)-S-citronellale alcuni tipi fungini e un batterio (1B9) hanno restituito come prodotti: (-)-S-citronellolo, (-)-S-acido citronellico, (cis)-p-mentan-3,8-diolo, (trans)-p-mentan-3,8-diolo, isopulegolo e neoisopulegolo. Non si sono ottenuti prodotti di biotrasformazione con limonene. Le biotrasformazioni dell'olio di *C. latifolia*, scelto tra gli altri oli perché presentava il più alto contenuto di citronellale, e l'olio di *C. limon*, per l'alto contenuto di sabinene, con i tipi fungini e batterici maggiormente reattivi sui terpeni, hanno fornito in linea generale i prodotti di biotrasformazione attesi, tranne il limonene-1,2-diolo, derivante dal limonene, non riscontrato nella biotrasformazione del singolo substrato.

Tale risultato è probabilmente da attribuirsi alla funzione elicitoria che altri terpeni, minoritari nell'olio, esercitano sul patrimonio enzimatico sui microrganismi considerati. La collaborazione con il Centraal bureau voor Schimmelcultures (CBS) ha poi consentito di identificare i funghi maggiormente efficaci come *Peniciullum paxilli* Balnier (1C22, 1D6), *Fusarium concentricum* Nirenberg & O'Donnell (2D15), *Fusarium fijikuroi* Nirenberg (1B14), mentre per altri due ceppi (1C5, 1D2) si è sinora identificato con certezza solo il genere (*Fusarium*). Si è infine valutato, in via preliminare, l'attività antiossidante espressa dagli oli essenziali tal quali e dei prodotti della biotrasformazione mediante test del DPPH. Tali risultati, confrontati con l'attività espressa dall'olio essenziale di timo (chemotipo timolo) preso come standard di riferimento, non presentavano differenze significative e degne di nota facendo emergere che l'attività biotrasformativa non aveva in questo caso determinato variazioni delle capacità bioattive dei fitocomplessi.

Relazione o comunicazione a congresso

AZADIRACHTA INDICA: CARATTERIZZAZIONE BIOLOGICO FARMACEUTICA DI UNA PREPARAZIONE ERBORISTICA AYURVEDICA

G. Sacchetti, D. Rossi, S. Maietti, A. Grandini, K. Saro, C. Useli, L. Scalvenzi, I. Maresca, F. Poli, F. Antognoni, A.Guerrini, M. Muzzoli

Atti del IV Congresso Intersocietà sulle Piante Medicinali Anno: 2010

Azadirachta indica A. Juss (Meliaceae) è un albero sempreverde, a crescita rapida che può raggiungere un'altezza di 15-20 metri, nativo del subcontinente indiano e del sud est asiatico. Ogni parte di *A. indica*, conosciuto comunemente come albero del Neem, ha un uso consolidato nella medicina tradizionale ayurvedica come rimedio nei confronti di varie tipologie di disturbi. Nel presente lavoro di ricerca è stata indagata sotto il profilo biologico farmaceutico, mediante tecniche di indagine cromatografiche e spettroscopiche (GC-MS, HPLC-DAD, NMR, spettrofotometria UV-VIS) accoppiate ad evidenze di bioattività, una preparazione erboristica di uso tradizionale, il decotto di corteccia di *Azadirachta indica*, preparato secondo il protocollo tipico della medicina Ayurvedica e mai sino ad ora indagato. A partire da questo fitocomplesso sono state isolate 3 frazioni: a) estratto MeOH/H2O; b) OPF (frazione arricchita in proantocianidine oligomeriche) e c) PPF (frazione arricchita in proantocianidine polimeriche) con lo scopo di risalire ai maggiori componenti responsabili di eventuali profili di bioattività. I componenti principali del decotto sono risultati essere polifenoli pari al 38,60±1,92%; in questa categoria di composti, l'acido gallico rappresentava il 2,60±0,13%, le proantocianidine il 3,90±0,22% mentre gli antrachinoni, mai descritti in letteratura come classe chimica caratterizzante la specie, il 2,60±0,09%. Un'elevata componente zuccherina risultava poi evidente nel fingerprinting NMR. Per quanto riguarda gli estratti, l'OPF ha evidenziato un contenuto più elevato di polifenoli totali (53,60±3,75%), di acido gallico (8,90±0,16%), antrachinoni (16,80± 0,95%), proantocianidine (3,60±0,21%) in particolare di natura oligomerica, la

presenza di catechina (2,80±0,05%) e di gallocatechina in quantità paragonabile, acidi ferulico e caffeico e protocatecuico come componenti minoritari. Uno studio più approfondito degli antrachinoni mediante HPLC-DAD e analisi strutturali con GC-MS e NMR ha consentito di evidenziare la struttura dei più abbondanti: emodin-1-O-?-glucoside, crisofanolo-1-O-?-glucoside, crisofanolo-8-O-?-glucoside e physcion-glicosilato. I saggi biologici per la valutazione dell'attività antiossidante (DPPH, β-carotene bleaching test) hanno evidenziato importanti potenzialità antiradicaliche (espresse come IC50) in tutti i campioni analizzati e, tra questi, l'OPF è risultato il più attivo. Non è stata invece evidenziata alcuna attività "scavenger" nei confronti del radicale superossido. Il saggio del DPPH effettuato su HPTLC ha permesso di evidenziare l'acido protocatecuico, le proantocianidine oligomeriche, l'acido gallico e catechina come le categorie maggiormente responsabili dell'attività. La valutazione delle proprietà antibatteriche con la metodica TLC-bioautoghraphic assay ha confermato come l'estratto OPF sia anche in questo caso il più attivo nei confronti di due batteri Gram positivi (*Staphylococcus aureus* ed *Enterococcus foecalis*); mentre nessuna attività significativa era riscontrata verso batteri Gram negativi. In ogni caso, le molecole potenzialmente responsabili di tale bioattività potrebbero essere l'acido gallico, protocatecuico, physcion-glicosilato, crisofanolo-1-O-?-glucoside, crisofanolo-8-O-?-glucoside in quanto componenti maggioritari delle frazioni isolate da TLC e analizzate mediante HPLC-DAD, GC-MS. Per evidenziare aspetti di sicurezza in termini di citotossicità e mutagenicità è stato condotto un saggio in vitro con un ceppo di *Saccharomyces cerevisiae* (D7), sul solo fitocomplesso decotto che ha dimostrato mancanza di citotossicità e genotossicità nel range di concentrazione testato (0,05 - 25,00 mg/ml), ponendo per contro in evidenza in termini di efficacia significative proprietà mutagenoprotettive già a concentrazioni di 1,00 mg/ml.