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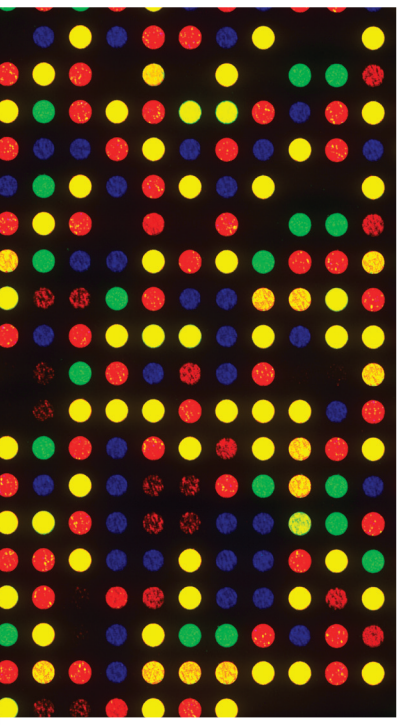
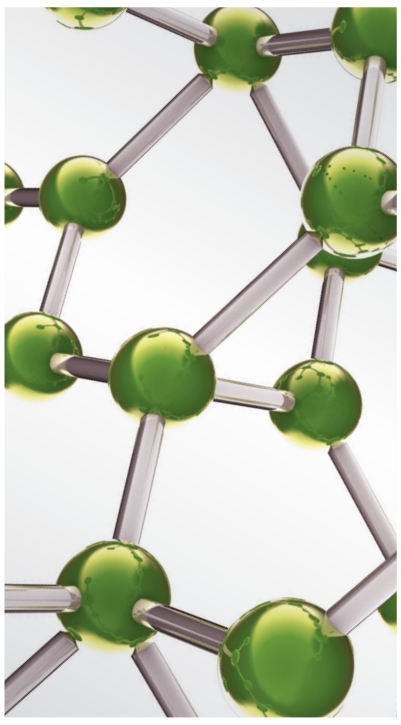
Essential Oils: New Perspectives in Human Health and Wellness

Guest Editors: Fabio Firenzuoli, Vikas Jaitak, Gyorgyi Horvath, Imaël Henri Nestor Bassolé, William N. Setzer, and Luigi Gori



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Editorial Board

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Editorial

Essential Oils: New Perspectives in Human Health and Wellness

Fabio Firenzuoli,[1] Vikas Jaitak,[2] Gyorgyi Horvath,[3] Imaël Henri Nestor Bassolé,[4] William N. Setzer,[5] and Luigi Gori[1]

[1] *Center for Integrative Medicine, Referring Center for Phytotherapy, Tuscany Region, Careggi University Hospital, School of Medicine of Florence, 50139 Florence, Italy*
[2] *Centre for Chemical and Pharmaceutical Sciences, Central University of Punjab Bathinda, Punjab 151001, India*
[3] *Institute of Pharmacognosy, Medical School, University of Pécs, Pécs 7624, Hungary*
[4] *Life and Earth Sciences Training and Research Unit, University of Ouagadougou, Ouagadougou, Burkina Faso*
[5] *Department of Chemistry, University of Alabama in Huntsville, Huntsville, AL 35899, USA*

Correspondence should be addressed to Fabio Firenzuoli; fabio.firenzuoli@unifi.it

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Essential oils are natural products, derived from aromatic plants, traditionally used all over the world for disinfection, as anti-inflammatory, relaxing, and stimulating substances, and with potential and modern exploitation in clinical medicine. The earliest recorded mention of the techniques and methods used to produce essential oils is believed to be that of Ibn al-Baitar (1188–1248). The chemical composition of these essential oils varies widely depending upon the geographical location, botanical origin, genetics, bacterial endophytes, and extraction techniques. Essential oils are commonly used in food and cosmetic industries. They can be used as natural alternatives to synthetic preparations to prevent and treat infectious diseases. They are used traditionally to treat other diseases like respiratory tract, digestive system, gynecological, andrological, endocrine, cardiovascular, nervous system, and skin infections. Many of them have shown anticancer activities, too.

For this special issue we received seventeen papers, confirming the interest shown by the scientific community on a great number of old and new issues still open about essential oil, particularly chemical characterization and standard methods to control quality; human clinical and experimental pharmacology and toxicology of essential oils; safety; pharmacological actions and interactions; new biomedical targets of the biological responses; clinical assays to pharmaceutical products with essential oils in biotechnology; nanotechnology and nanomedicine. We have selected seven papers, with the aim of showing some progress made by the pharmaceutical point of view and the new perspectives of pharmacological research to facilitate their transition into clinical practice.

In the study *"Hepatoprotective effect of pretreatment with Thymus vulgaris essential oil in experimental model of acetaminophen-induced injury,"* R. Grespan et al. investigated the hepatoprotective effect of *Thymus vulgaris* essential oil on acetaminophen-induced hepatic damage in mice. *Thymus vulgaris* is used popularly such as its antiseptic, carminative, and antimicrobial effects, and this study is very interesting for new possible clinical applications. The positive results are confirmed by reduction of the serum marker enzymes aspartate aminotransferase (AST), alanine aminotransferase (ALT), and myeloperoxidase (MPO) and by histopathological analysis. Acute liver damage caused by acetaminophen overdose is a significant clinical problem and could benefit from new therapeutic strategies.

In the paper entitled *"Exploring the anti-Burkholderia cepacia complex activity of essential oils: a preliminary analysis,"* I. Maida et al. have checked the ability of the essential oils extracted from six different aromatic plants to inhibit the growth of 18 known species of the *Burkholderia cepacia* complex (Bcc), an opportunistic human pathogen that can cause severe infection in immunocompromised patients, especially those affected by cystic fibrosis (CF) and who are often resistant to multiple antibiotics. *Eugenia caryophyllata*, *Origanum vulgare,* and *Thymus vulgaris* essential oils were particularly active versus the Bcc strains, including those

exhibiting a high degree or resistance to ciprofloxacin, active toward both environmental and clinical strains isolated from CF patients.

N. El Abed et al. in the work *"Chemical composition, antioxidant and antimicrobial activities of Thymus capitata essential oil with its preservative effect against Listeria monocytogenes inoculated in minced beef meat"* evaluated the preservative effect of *Thymus capitata* essential oil (TCEO) against *Listeria monocytogenes* inoculated in minced beef meat. The antioxidant activity was assessed in vitro by using both the DPPH and the ABTS assays. The essential oil was evaluated for its antimicrobial activity using disc agar diffusion and microdilution methods. The results demonstrated that the minimum inhibition concentration values ranged from 0.32 to 20 mg/mL, and essential oil evaluated in vivo against *Listeria monocytogenes* showed clear and strong inhibitory effect. The application of 0.25 or 1% (v/w) of TCEO to minced beef significantly reduced the *L. monocytogenes* population.

In the paper *"Essential oils for complementary treatment of surgical patients: state of the art"* S. Stea et al. revised the available literature to determine the effectiveness of aromatherapy in surgical patients (to treat anxiety and insomnia, pain and nausea, or to dress wounds). Efficacy studies of lavender or orange and peppermint essential oils, to treat anxiety and nausea, respectively, have shown positive results. Therefore there are encouraging data for the treatment of infections, especially for tea tree oil (TTO). The authors conclude that it is important that the therapeutic use of essential oils be performed in compliance with clinical safety standards.

In the article *"Effect of eugenol on cell surface hydrophobicity, adhesion, and biofilm of Candida tropicalis and Candida dubliniensis isolated from oral cavity of HIV-infected patients"* S. B. de Paula et al. evaluated the effect of eugenol on the adherence properties and biofilm formation capacity of *Candida dubliniensis* and *Candida tropicalis* isolated from the oral cavity of HIV-infected patients. Eugenol showed inhibitory activity against planktonic and sessile cells of *Candida* spp. Scanning electron microscopy demonstrated that eugenol drastically reduced the number of sessile cells on denture material surfaces. The paper corroborates the effectiveness of eugenol against *Candida* species other than *C. albicans*, reinforcing its potential as an antifungal applied to limit both the growth of planktonic cells and biofilm formation on different surfaces.

In the work *"Essential oils loaded in nanosystems: a developing strategy for a successful therapeutic approach"* A. R. Bilia et al. revised the nanoencapsulation of essential oils in drug delivery systems, for their capability of decreasing volatility, improving the stability, water solubility, and efficacy of essential oil-based formulations, by maintenance of therapeutic efficacy. Two categories of nanocarriers can be proposed: polymeric nanoparticulate formulations, extensively studied with significant improvement of the essential oil antimicrobial activity, and lipid carriers, including liposomes, solid lipid nanoparticles, nanostructured lipid particles, and nano/microemulsions.

Lastly, the paper by A. R. Bilia et al. entitled *"Essential oil of Artemisia annua* L.*: an extraordinary component with numerous antimicrobial properties"* describes the qualitative composition and the antimicrobial activities of essential oil of *Artemisia annua* L., a medicinal plant from China, well known and used in the treatment of the chloroquine-resistant and cerebral malaria. The essential oil of *A. annua* is rich in mono- and sesquiterpenes (camphor, germacrene D, artemisia ketone, and 1,8 cineole) exciting a lot of antibacterial and antifungal activities: against gram-positive bacteria (*Enterococcus, Streptococcus, Staphylococcus, Bacillus*, and *Listeria* spp.), gram-negative bacteria (*Escherichia, Shigella, Salmonella, Haemophilus, Klebsiella*, and *Pseudomonas* spp.), and mycetes (*Candida, Saccharomyces*, and *Aspergillus* spp.). The authors believe that this review will serve to prepare the most appropriate microbiological studies also useful for clinical practice.

Fabio Firenzuoli
Vikas Jaitak
Gyorgyi Horvath
Imaël Henri Nestor Bassolé
William N. Setzer
Luigi Gori

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Review Article

Essential Oils Loaded in Nanosystems: A Developing Strategy for a Successful Therapeutic Approach

Anna Rita Bilia,[1] Clizia Guccione,[1] Benedetta Isacchi,[1] Chiara Righeschi,[1] Fabio Firenzuoli,[2] and Maria Camilla Bergonzi[1]

[1] *Department of Chemistry, University of Florence, Via U. Schiff 6, 50019 Sesto Fiorentino, Italy*
[2] *Center for Integrative Medicine, Careggi University Hospital, University of Florence, 50124 Florence, Italy*

Correspondence should be addressed to Anna Rita Bilia; ar.bilia@unifi.it

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Essential oils are complex blends of a variety of volatile molecules such as terpenoids, phenol-derived aromatic components, and aliphatic components having a strong interest in pharmaceutical, sanitary, cosmetic, agricultural, and food industries. Since the middle ages, essential oils have been widely used for bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, and other medicinal properties such as analgesic, sedative, anti-inflammatory, spasmolytic, and locally anaesthetic remedies. In this review their nanoencapsulation in drug delivery systems has been proposed for their capability of decreasing volatility, improving the stability, water solubility, and efficacy of essential oil-based formulations, by maintenance of therapeutic efficacy. Two categories of nanocarriers can be proposed: polymeric nanoparticulate formulations, extensively studied with significant improvement of the essential oil antimicrobial activity, and lipid carriers, including liposomes, solid lipid nanoparticles, nanostructured lipid particles, and nano- and microemulsions. Furthermore, molecular complexes such as cyclodextrin inclusion complexes also represent a valid strategy to increase water solubility and stability and bioavailability and decrease volatility of essential oils.

1. Introduction

Spices have been used since antiquity for their perfume, medicinal and preservative properties and to impart aroma and flavour to food. Hippocrates, the "father of medicine," prescribed perfume fumigations and massages with aromatic oils. Turpentine was known by the Greeks and Romans for its properties against lung diseases and biliary lithiasis. Dioscorides saying the best was the white, clear variety. Pliny, Hippocrates, and Galen favoured its properties too. Venice turpentine was known during the Middle Ages, and the city became one of the principal markets for this medicinal drug [1]. The first distillation of essential oils appeared in the East (India and Persia) [1] more than 2000 years ago and was improved in the 9th century by the Arabs [2]. Nevertheless, the first authentic written account of distillation of essential oil is ascribed to Villanova (ca. 1235–1311), a Catalan physician [1], and only by the 13th century, the essential oils (EOs) were being made by pharmacies and their pharmacological effects were described in pharmacopoeias [2]. By contrast, their use does not appear to have been widespread in Europe until the 16th century; turpentine, juniper wood, rosemary, spike (lavender), clove, mace, nutmeg, anise, and cinnamon became common essential oils. In this century the term "essential oil" was used for the first time by Paracelsus von Hohenheim, who named the effective component of a drug, "Quinta essential" [1]. By the middle of the 20th century, the role of essential oils had been reduced almost entirely to be used in perfumes, cosmetics, and food flavourings: rather in pharmaceutical preparations they still represent an important part of the traditional medicine and several monographs are reported in the official pharmacopoeias. At present ca. 3000 essential oils (EOs) are known, and 10% of them have commercial importance [3] for the pharmaceutical, agronomic, food, sanitary, cosmetic, and perfume industries.

2. EOs Chemical Composition

EOs are volatile, limpid, and rarely coloured liquids, lipid soluble and soluble in organic solvents with a generally lower

density than that of water. They can be synthesized by all plant organs, that is, buds, flowers, leaves, stems, twigs, seeds, fruits, roots, wood, or bark and are stored in secretory cells, cavities, canals, epidermic cells, or glandular trichomes. Constituents are lipophilic and highly volatile secondary plant metabolites, reaching a mass below a molecular weight of 300, that can be physically separated from other plant components or membranous tissue [4].

Nowadays there are several methods for extracting essential oils. These may include use of liquid carbon dioxide or microwaves, low or high pressure distillation employing boiling water or hot steam. As defined by the International Organization for Standardization (ISO), the term "essential oil" is reserved for a "product obtained from vegetable raw material, either by distillation with water or steam, or from the epicarp of citrus fruits by a mechanical process, or by dry distillation" (ISO 9235, 1997), that is, by physical means only. Furthermore, essential oils for medical purposes need to comply with national or international pharmacopoeias.

The chemical profile of the essential oil products differs not only in the number and type of molecules but also in their stereochemical structures, and can be very different according to the selected method of extraction. The extraction product can fluctuate in quality, quantity, and composition according to climate, soil composition, plant organ, age, and vegetative cycle stage [5]. Most of the commercialized essential oils are chemotyped by gas chromatography and mass spectrometry analysis. Analytical monographs have been published (European Pharmacopoeia, ISO, WHO, Council of Europe) to ensure good quality of essential oils. The EOs are generally complex mixtures of volatile organic compounds produced as secondary metabolites in plants; they include hydrocarbons (terpenes and sesquiterpenes) and oxygenated compounds (alcohols, esters, ethers, aldehydes, ketones, lactones, phenols, and phenol ethers) [1].

Generally EOs contain about 20–60 components up to more than 100 single substances, at quite different concentrations; two or three are major components at fairly high concentrations (20–70%) compared to others components present in trace amounts. For example, carvacrol (30%) and thymol (27%) are the major components of the *Origanum* species essential oil.

Generally, these major components determine the biological properties of the essential oils. The components include different groups of distinct biosynthetical origin. The main group is composed of terpenoids, phenylpropanoids, and short-chain aliphatic hydrocarbon derivatives, which are all characterized by low molecular weight. Representative structures are depicted in Figure 1.

Terpenes are made from combinations of several 5-carbon-base (C5) units called isoprene and form structurally and functionally different classes. The biosynthesis of the terpenes consists of synthesis of the isopentenyl diphosphate (IPP) precursor, repetitive addition of IPPs to form the prenyldiphosphate precursor of the various classes of terpenes, modification of the allylic prenyldiphosphate by terpene specific synthetases to form the terpene skeleton, and, finally, secondary enzymatic modification (redox reaction) of the skeleton to attribute functional properties to the different terpenes. Terpenoids derive from the C5-building blocks isopentenyl diphosphate (IPP) and its isomer dimethylallyl diphosphate (DMAPP) and are generally represented by monoterpenes (C10) and sesquiterpenes (C15), while hemiterpenes (C5) are quite rare [6]. Terpenes containing oxygen in the form of hydroxyl, ether, aldehyde, ketone, or carboxylic moieties are called terpenoids.

The monoterpenes (Figure 1) are formed from the coupling of two isoprene units (C10). They are the most representative molecules constituting 90% of the essential oils and allow a great variety of structures. They consist of several functions including acyclic hydrocarbons (myrcene and ocimene); monocyclic hydrocarbons (limonene, terpinenes, p-cymene, and phellandrenes); bicyclic hydrocarbons (pinenes, camphene, and sabinene); acyclic alcohols (geraniol, linalool, citronellol, lavandulol, and nerol); monocyclic alcohols (menthol, α-terpineol, and carveol); bicyclic alcohols (borneol, fenchol, chrysanthenol, and thuyan-3-ol); acyclic aldehydes (geranial, neral, and citronellal); acyclic ketone (tegetone), monocyclic ketone (menthones, carvone, pulegone, and piperitone); bicyclic ketone (camphor, fenchone, thuyone, and pinocarvone); acyclic esters (linalyl acetate or propionate and citronellyl acetate); monocyclic esters (menthyl or α-terpinyl acetate); bicyclic esters (isobornyl acetate); ethers (1,8-cineole and menthofuran); peroxides (ascaridole); and phenols (thymol, carvacrol).

The sesquiterpenes are formed from the assembly of three isoprene units (C15). The extension of the chain increases the number of cyclisations which allows a great variety of structures (Figure 1). Also sesquiterpenes include hydrocarbons (azulene, β-bisabolene, cadinenes, β-caryophyllene, farnesenes, and zingiberene); alcohols (bisabolol, β-nerolidol, farnesol, β-santalol, and patchoulol); ketones (germacrone, β-vetinone, and turmerones); and epoxide (caryophyllene oxide and humulene epoxides).

Other aromatic molecules are phenylpropanoids formed via the shikimic acid pathway leading to phenylalanine [6] and occurring less frequently than the terpenes.

Aromatic compounds originated from the shikimate pathway (phenylpropanoids, Figure 1) comprise aldehydes (cinnamaldehyde); alcohols (cinnamic alcohol); phenols (chavicol and eugenol); methoxy derivatives (anethole, estragole, and methyleugenols); methylenedioxy compounds (apiole, myristicin, and safrole).

Nitrogenous or sulphured components such as glucosinolates or isothiocyanate derivatives (garlic and mustard oils) are also characteristic secondary metabolites of diverse aromatic plants or of processed, grilled, or roasted products. In addition, some essential oils contain photoactive molecules like coumarins and furocoumarins (*Citrus aurantium* ssp. *bergamia* essential oil contains psoralens) and short-chain aliphatic substances such as 3-octanone and methyl nonyl ketone (Figure 1).

3. Limits and Challenges for the Rational Clinical Use of Essential Oils

The most recent applications of EOs include being as antioxidants and preservatives in food [7], incorporated

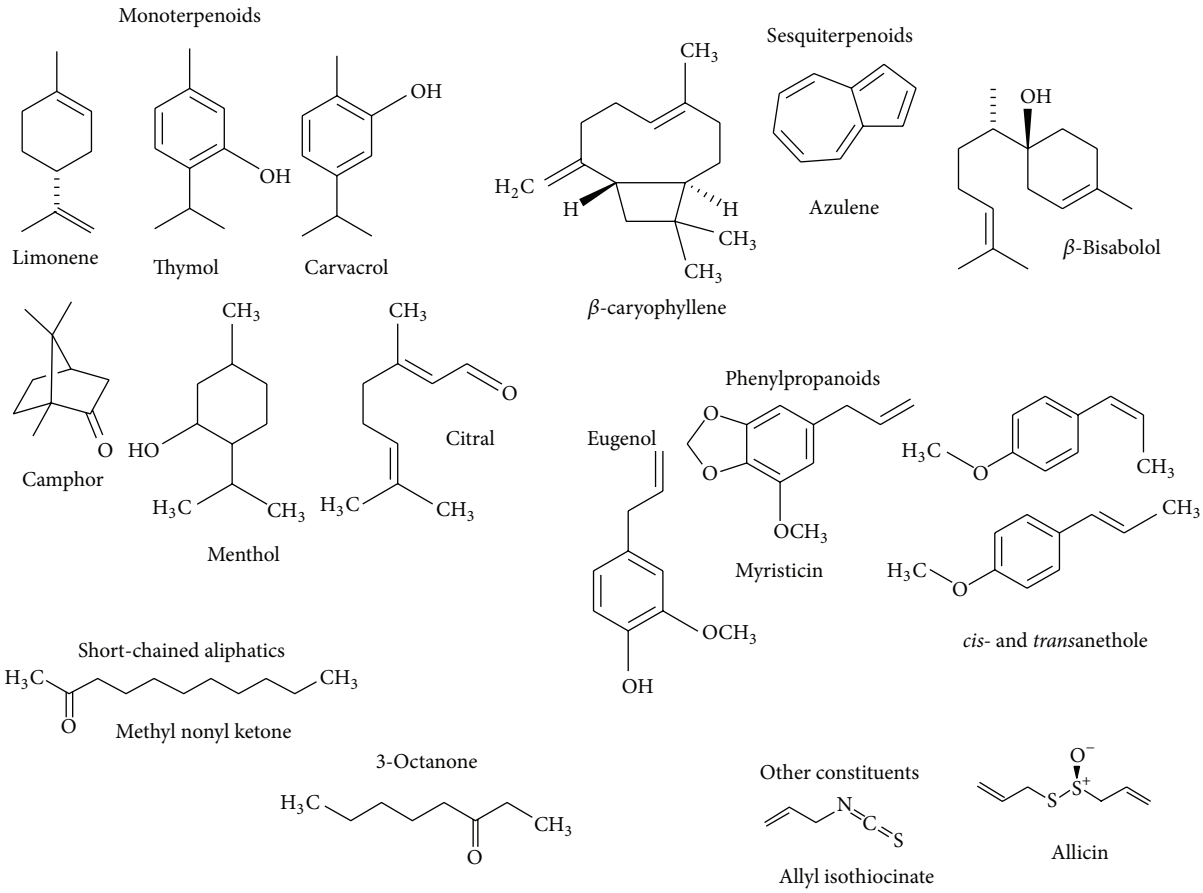

Figure 1: Representative structures typical of essential oils.

into foodstuff packaging material [8], and application as plant and crop protectants [9]. Traditionally, essential oils have been used for many biological properties including bactericidal, virucidal, fungicidal, antiparasitical, insecticidal, and other medicinal properties such as analgesic, sedative, anti-inflammatory, spasmolytic, and locally anesthetic remedies [9–11].

At present, promising approaches have been reported using essential oils or components thereof in medicinal products for human or veterinary use [12]. The most effective way to use most EOs is by external application, as gargles and mouthwashes or inhalation; rarely they are used orally even if generally regarded as safe (GRAS) to ingest. In this case of oral administration they are generally diluted with milk, soy milk, or olive oil. Topical application is generally safe; the oil is diluted in a formulation but sometimes can give skin reactions and in particular some oils (specifically citrus oils) are UV sensitive and may cause irritation or darkening of skin upon exposure to sunlight up to 4 days after application.

In case of inhalation when using strong oils, limit time in immediate vicinity of an essential oil diffuser as the concentrated vapours may cause eye irritation, some of them are not recommended for diffusing or direct inhalation.

There is adequate evidence suggesting that although essential oils are metabolized quickly, their distribution throughout the body is considered to be relatively high.

Most essential oil components are metabolized and either eliminated by the kidneys in the form of polar compounds following limited phase I enzyme metabolism by conjugation with glucuronate or sulfate or exhaled via the lungs as CO_2. For example, after oral administration of (−)-menthol, 35% of the original menthol content was excreted renally as menthol glucuronide [13, 14]. The same happens with thymol, carvacrol, limonene, and eugenol. After their oral administration, sulphate and glucuronide forms have been detected in urine and in plasma, respectively [15, 16]. The fast metabolism and short half-life of active compounds have led to the belief that there is a minimum risk of accumulation in body tissues [17].

EO compounds are small, fat soluble molecules, able to permeate the membranes including the skin before being captured by the microcirculation and drained into the systemic circulation, which reaches all targets organs [9, 18]. In general, the respiratory tract offers the most rapid way of entry followed by the dermal pathway [19]. Topically, aromatherapy EOs can sometimes cause irritation of the skin, especially if

the oils are not diluted. Some oils, such as bergamot oil, can also cause photosensitization and induce malignant change. Applying excessive amounts of highly concentrated oils to a large surface of the skin or on broken skin can result in significant systemic absorption and increase the chance of serious side effects, such as convulsions because EOs are permeation enhancers.

Besides the high volatility, EOs can easily decompose, owing to direct exposure to heat, humidity, light, or oxygen. A recent manuscript has reviewed the factors influencing essential oil stability; specific knowledge on the chemical composition and properties of essential oil is fundamental for an adequate use [20].

Degradation of EOs constituents is due to oxidation, isomerization, cyclization, or dehydrogenation reactions, triggered either enzymatically or chemically [21], strongly influenced by the conditions during processing and storage of the plant material, upon distillation, and in the course of subsequent handling of the oil itself [22]. Furthermore, besides organoleptic alterations and viscosity changes, some aged essential oils as well as oxidized terpenoids have revealed skin-sensitizing capacities [23] leading to a hypersensitivity reaction synonymous to allergic contact dermatitis [24].

4. Nanoencapsulation Technology

Encapsulation of bioactive compounds represents a feasible and efficient approach to modulate drug release, increase the physical stability of the active substances, protect them from the interactions with the environment, decrease their volatility, enhance their bioactivity, reduce toxicity, and improve patient compliance and convenience [25].

A significantly large part of current literature on the encapsulation of EOs deals with micrometric size capsules, which are used for the protection of the active compounds against environmental factors (e.g., oxygen, light, moisture, and pH), to decrease oil volatility and to transform the oil into a powder. Encapsulation in nanometric particles is an alternative for overcoming these problems but additionally, due to the subcellular size, may increase the cellular absorption mechanisms and increasing bioefficacy.

Nanosystems applied to the skin are used to facilitate local therapies even if it is still under discussion of the mechanisms of penetration trough skin. It is accepted that topical drug delivery with nanoparticles targets the nanoparticles into the deeper layers of skin and generally they do not reach the viable epidermis. Only where the cheratine barrier is compromised, however, such as in aged or diseased skin, an enhanced particle penetration occurs. The use of nanoparticles provides a sustained and slow release of the active constituents; nanoparticles represent a reservoir. In addition, nanoparticles can interact with skin at a cellular level as adjuvants to enhance immune reactivity for topical vaccine applications.

Hair follicles and furrows were regarded as insignificant as potential routes for drug delivery, covering less than 1% of the human skin surface area, but their complex vascularisation and deep invagination with a thinning stratum corneum

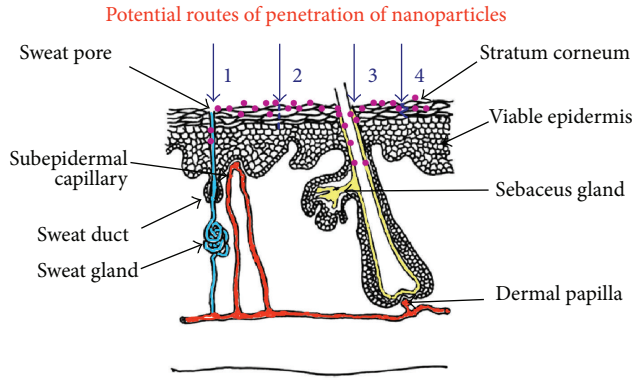

Figure 2: Skin nanoparticle drug delivery takes place in three major sites: stratum corneum surface through intracellular (2) and intercellular (4) penetration, furrows (1), and openings of hair follicles (3). The nanoparticles are shown in violet.

have led to a reappraisal of this view. It has been demonstrated that in particular hair follicles are an efficient reservoir for nanoparticle-based drug delivery and nanoparticles penetration can be increased with massage [26, 27]. Figure 2 shows that the potential sites for skin targeting nanoparticles include the surface of the skin, furrows, and hair follicles.

The alternative routes of administration of EOs are represented by oral intake and inhalation.

Within these routes the nanodelivery systems encounter the mucosal lining of the nasal, lung, oral (sublingual and buccal) cavity, stomach, and gut. Nanocarriers can improve the stability of EOs against enzymatic degradation, achieve desired therapeutic levels in target tissues for the required duration with a lower number of doses, and might ensure an optimal pharmacokinetic profile to meet specific needs. However, the viscous, elastic, and sticky mucus layer that lines all mucosa tissues (even if with different characteristics) has evolved to protect the body by rapidly trapping and removing foreign particles and hydrophobic molecules. As a consequence, mucoadhesion defined as the ability of nanoparticle to adhere to the mucus enhancing drug absorption can represent a valid strategy to enhance the residence time of the nanosystem and enhance absorption and bioavailability of the active constituent because it can facilitate transport across the epithelium. The interaction is generally achieved with natural or synthetic polymers which can form hydrogen bonding and hydrophobic or electrostatic interactions with mucin. The electrostatic interaction is the most effective and it can be achieved using positively charged polymers such as chitosan, being mucin negatively charged [28–32].

Particle size, shape, and surface properties of the nanoparticles play a crucial role in the uptake of nanosized delivery systems across the mucosal membrane. The nanocarriers with particle size of 50–300 nm, positive zeta potential, and hydrophobic surface were found to have preferential uptake as compared to their counterparts [28].

Diverse absorption mechanisms have been established and two have been predominantly used: the paracellular route

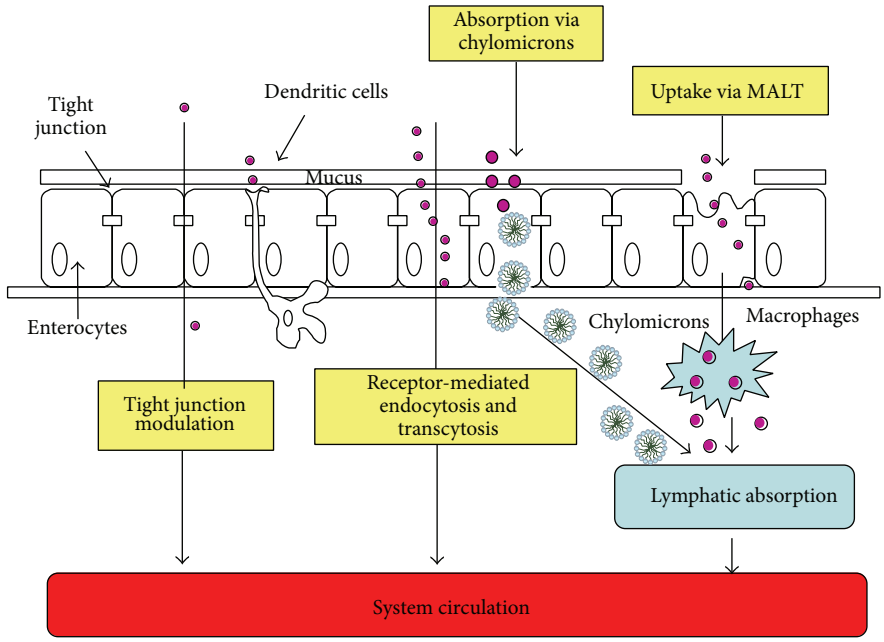

Figure 3: Mechanisms of nanocarriers (violet) enhanced absorption by enteric mucosa.

that is slow and passive and the transport through a lipoidal route and it is also known as the transcellular process which is responsible for the transport of lipophilic drugs that show a rate dependency on their lipophilicity. Drug also crosses cell membranes by an active transport route via carrier-mediated means or transports through the opening of tight junctions interacting with the tight junction proteins [28–32].

For instance the increase in the absorption of nanocarriers by enterocytes is due to tight junction modulation, receptor-mediated endocytosis and transcytosis, phagocytosis via specialized microfold cells (M cells) of the Peyer's patches, and other mucosa associated lymphoid tissues (MALT) and lymphatic absorption via chylomicron uptake mechanism from the enterocytes (mediated by lipase for various lipid-based drug delivery systems) [29]. Mechanism of carriers penetration through enteric mucosa is reported in Figure 3.

5. EO-Loaded Nanodelivery Systems

Nanodelivery systems can be engineered to possess a number of desirable features for therapy, including (i) sustained and controlled release of drugs locally, (ii) deep tissue penetration due to the nanometric size, (iii) cellular uptake and subcellular trafficking, and (iv) protection of cargo therapeutics at both extracellular and intracellular levels.

Nanocarriers can be structured by a great variety of material and designs. This review is focused on the organic nanocarrier systems, characterised by high biodegradability and biocompatibility, and classified in polymer-based nanoparticles and lipid-based nanoparticles. In addition molecular complexes such as inclusion complexes with cyclodextrins are reported. A schematic representation of nanosystem platforms for EOs is reported in Figure 4.

5.1. Polymer-Based Nanocarriers. Polymeric nanocarriers are classified as nanocapsules and nanospheres. Nanocapsules have two compartments: a polymeric wall and a core, which is commonly oily. Nanospheres are matrix systems. The essential oil may be conjugated with the polymer (matrix or wall) or in the oily core.

Biocompatible polymers of synthetic origin include poly-α-cyanoacrylate alkyl esters, polyvinyl alcohol, polylactic acid, polyglycolic acid, and polylactic glycolic acid. The latter is usually divided into two classes: polysaccharides and proteins. Polysaccharides include compounds from plant origin (e.g., pectin, cellulose and its derivatives, starch and its derivatives, arabic gum, carrageenan, and alginate) and polysaccharides from microbial or animal origin (e.g., xanthan gum and chitosan). Proteins are albumin, gelatine, soy proteins, and casein. Nanoparticles made of polysaccharides, due to their unique properties, are promising carriers to deliver and protect the physiological properties of hydrophilic drugs and have been successfully applied as drug delivery systems [33]. As natural biomaterials, polysaccharides are stable, safe, nontoxic, hydrophilic, and biodegradable. In addition, polysaccharides have abundant resources in nature and low cost in their processing. The release of EOs from carriers occurs through one of the following processes: dissolution, desorption of the surface-bound/adsorbed functional ingredient, diffusion through the matrix; matrix erosion including enzyme degradation, and a combination of these processes [34].

Eugenol represents the main constituents of diverse EOs but it is highly volatile, unstable, and sensitive to oxygen, light, and heat during processing, utilization, and storage. Choi et al. [35] reported that encapsulation of eugenol into polycaprolactone nanoparticles could enhance its stability against light oxidation.

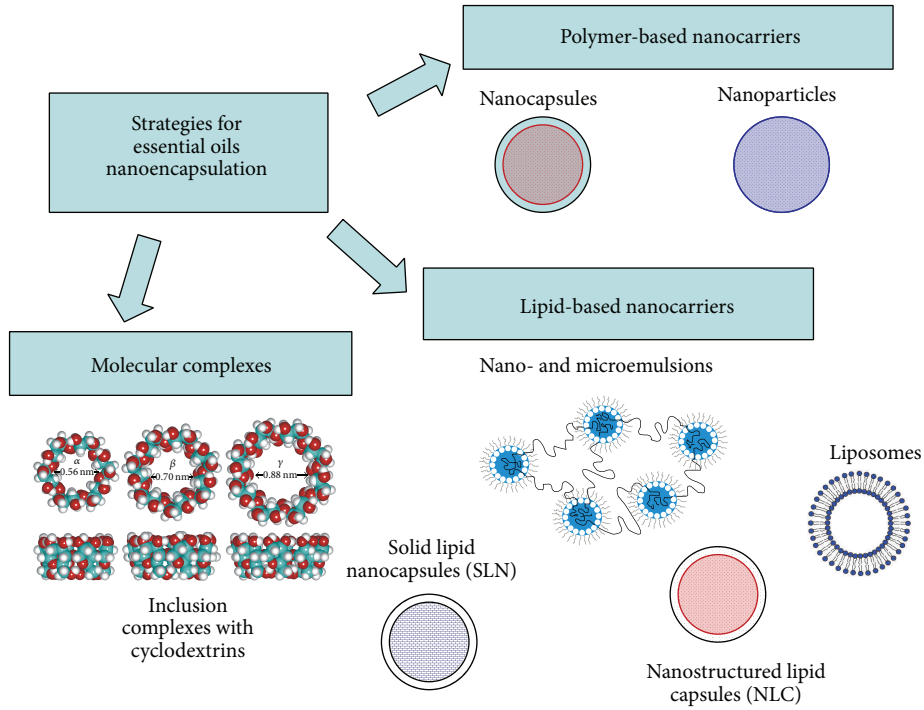

Figure 4: Schematic illustration of nanosystem platforms for essential oils.

Eugenol has been also encapsulated into chitosan nanoparticles with an average size of less than 100 nm. Loading capacity was 12% and encapsulation efficiency was 20%. The particles had positively charged surface, with a zeta potential value ranging from +16.2 to +33.5 mV. The eugenol-loaded chitosan nanoparticles were thermally stable and could be useful as antioxidants for various thermal processing applications [36].

Chitosan nanoparticles have also been developed with oregano essential oil known for its potent antioxidant and antimicrobial activity. The obtained nanoparticles exhibited a regular distribution and spherical shape with size range of 40–80 nm and the encapsulation efficiency and loading capacity were about 21–47% and 3–8%, respectively, when the initial EO content was 0.1–0.8 g/g chitosan. *In vitro* release studies showed an initial burst effect and followed by a slow drug release [37].

Alginate/cashew gum nanoparticles were prepared via spray-drying to encapsulate *Lippia sidoides* essential oil, rich in thymol which has fungicide and bactericide activities. Cashew gum is a biopolymer extracted from the exudate of *Anacardium occidentale*, a common tree of Brazil's Northeastern region. The gum main chain is composed of galactose (72%), with side-chains of arabinose (4.6%), glucose (14%), rhamnose (3.2%), and uronic acid (4.7%). The averaged sizes of the nanoparticles were in the range 223–399 nm, and zeta potential values ranging from −30 to −36 mV. Encapsulated oil levels varied from 1.9 to 4.4% with an encapsulation efficiency of up to 55%. The *in vitro* release profile showed that between 45 and 95% of oil was released within 30–50 h. The addition of cashew gum to alginate has proven to be able to maximize the hydrophilic character of the polymer matrices, allowing a quicker release at a satisfactory oil loading. Moreover, the oil release profile revealed that the use of alginate in synergy with cashew gum for EO encapsulation presents itself as a potential delivery system with tailored release rate, loading, and encapsulation efficacy [38].

Using the same EO from *Lippia sidoides* nanoparticles made of chitosan (a deacetylated form of chitin, chemically D-glucosamine and N-acetyl-D-glucosamine linked by beta (1–4) linkages) and cashew gum aimed to improve essential oil loading and release profiles. Samples designed using relative ratios, matrix: oil, 10 : 2; gum : chitosan, 1 : 1; and 5% gum concentration, showed high loading (11.8%) and encapsulation efficiency (70%), with average sizes in the range 335–558 nm. *In vitro* release profiles showed that nanoparticles presented slower and sustained release. The nanocarriers presented efficacy against *St. aegypti* larvae, where the mortality rate was related to the loading values and gum : chitosan ratios. In particular, samples gum : chitosan 1 : 1 and gum : chitosan 1 : 10 showed, respectively, 87% and 75% of mortality after 48 h, reaching over 90% of mortality at 72 h. These results showed that the gum-chitosan nanoparticles were designed and present sustained release features [39].

The formation of heat-resistant flavour nanocapsules of jasmine essential oil was achieved by gelatin and arabic gum. Their heat-resistance capability against 80°C was evaluated by both structural characteristics (size, polydispersity index, and zeta potential) and flavour analysis. The results showed that the nanocapsules were stable at 80°C for 7 h, even if the GC-MS revealed that jasmine essential oil began to destroy above 5 h [40].

Thymol loaded in zein (a corn prolamine protein) nanoparticles stabilized with sodium caseinate and chitosan hydrochloride were prepared and characterized. In the absence of sodium caseinate, the particle size and zeta potential of zein nanoparticles were 118.30 nm and +28.10 mV, respectively. The zeta potential of rein nanoparticles after coating with sodium caseinate reversed from positive to negative (in the range of −33.60 to −38.95 mV), while size was around 200 nm. Due to the presence of sodium caseinate, the stabilized zein nanoparticles showed a shift of isoelectric point from 6.18 to 5.05 and had a desirable redispersibility in water at neutral pH after lyophilization. Encapsulated thymol was more effective in suppressing Gram-positive bacterium than unencapsulated thymol for a longer time period. Zein nanoparticles presented a two-phase release profile of the EO. The authors believe that the rapid first phase represents the portion of thymol that was in the external phase of the film; the slower second phase represents thymol that was contained in the zein particles [41].

Polylactic glycolic acid nanocapsules containing eugenol or transcinnamaldehyde both presented a two-phase EO release. The first phase was rapid (under 30 minutes) and approximately 20% of the EO load was detected; the second release phase was prolonged and after 72 hours 64% of eugenol and 87% of transcinnamaldehyde were detected. Considering that PLGA has a low degradation rate, the release was governed mostly by diffusion with a possible influence of polymer swelling and bulk erosion. The first release phase may be attributed to the molecules that are adsorbed to the polymeric wall, while the second release phase represents the EO present in the core of the nanocapsules which diffuses through the polymeric wall [42].

Polylactic glycolic acid nanocapsules containing carvacrol have also been produced. Size was about 209.8 nm, polydispersity was 0.260, zeta potenzial was −18.99, drug loading was 21%, and encapsulation efficiency was 26%. *In vitro* release profile occurred with an initial "burst" release followed by a slower release due to the concentration gradient. Nanoparticles showed a 60% release after 3 h and approach to completeness after 24 h with approximately 95% of carvacrol released. The effect of carvacrol EO antimicrobial activity was enhanced because the nanoparticles significantly altered rheological characteristic of bacterial biofilms potentially facilitating the action of carvacrol [43].

Methyl cellulose/ethyl cellulose polymeric nanoparticles containing thymol attaining the relatively high thymol loading level of 43.53% (weight of encapsulated thymol to weight of the thymol-loaded spheres) were able to reduce and preserve levels of *E. coli* in an oil/water lotion and in a hydrophilic gel, of *P. aeruginosa* in an oil/water lotion and of *S. aureus* in an oil/water lotion and in a water/oil cream. Interestingly, free thymol was also capable of reducing microbiologic levels in these formulations, but the preservation period was shorter except for the *S. aureus* in an oil/water lotion where free thymol maintained low microbial levels for the same period as the nanocapsules. Effective bacterial suppression by encapsulated thymol was also observed when used in cream and aqueous gel formulations [44].

5.2. Lipid-Based Nanocarriers. Lipid-based nanocarriers include micro- and nanometric-scaled emulsions and lipid nanoparticles, roughly divided in liposomes, micelles, niosomes, solid lipid nanoparticles (SLN), and nanostructured lipid carriers (NLC). Liposomes and niosomes are colloidal association of amphiphilic lipids that organize themselves spontaneously in bilayer vesicles and that are suitable with hydrophilic and hydrophobic compounds. SLN and NLC are solid particles at room and human body temperatures that present lipid core, which makes these carriers a proper medium for entrapment of lipophilic compounds, as EO.

As these nanoparticles are composed of lipids and/or phospholipids, they have the ability to interact with several cell types. So, these carriers can been seen as alternatives for treatment of microbial infections, due to their capacity of interaction with infected cells. Furthermore, the association of EO with lipid nanoparticles has different objectives, but the main aims are the enhancement of the stability and the solubility in aqueous media of EO, maintenance or even enhancement of their biological activity, and drug targeting.

5.2.1. Micro- and Nanoemulsions. Microemulsions can be defined as homogeneous thermodynamically stable transparent dispersions of two immiscibleliquids stabilized by an interfacial film of surfactants. They have droplet size above 500 nm and require very low energyto formulate emulsion, since they form spontaneously when aqueous, oily, and amphiphilic components are brought intocontact, besides having a lower production cost compared to nanoemulsions. One major drawback to microemulsions isthat formation requires high surfactant concentration, which can cause toxicity when used in pharmaceutical applications.

In contrast nanoemulsions can be prepared using lower surfactant concentrations. Nanoemulsions are fine oil-in-water dispersions, nonequilibrium systems with a spontaneous tendency to separate into the constituent phases. Nevertheless, nanoemulsions may possess a relatively high kinetic stability even for several years, due to their very small size, essentially the consequence of significant steric stabilization between droplets. They have droplet covering the size range of 10–500 nm and also referred to as miniemulsions, ultrafine emulsions, and submicrometer emulsions.

Antimicrobial properties of micro- and nanoemulsion are believed to result from the small size of oil particles that have a high surface tension which can fuse with and subsequently disrupt the membrane of isolated prokaryotic cells, viruses, and eukaryotic cells of fungi but they do not affect eukaryotic cells of higher organisms. A synergistic antimicrobial effect could be afforded by including some substances which possess strong antimicrobial activity into the formula, reducing the amounts of active substances and detergents used for killing microorganisms by the conventional method and the cost of raw materials. Furthermore, irritation caused from detergents in the formula is not likely to happen when they are used in low concentrations.

Encapsulation in nanoemulsion-based delivery systems of two antimicrobial compounds, a terpenes mixture extracted from *Melaleuca alternifolia* and D-limonene, deals

with the issues of formulation and fabrication in order to retain and possibly enhance the antimicrobial activity of the encapsulated compounds.

The nanoemulsions based on food-grade ingredients were investigated by determining the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) for three different classes of microorganisms (*Lactobacillus delbrueckii*, *Saccharomyces cerevisiae*, and *Escherichia coli*). The increase of the antimicrobial activity resulted in dependance on the formulation andmean diameter of the delivery systems as well as on the microorganisms class. Additionally, GC-MS analysis revealed that high intensity processing for nanoemulsion production may affect the chemicalstability of several active compounds.

The results of the accelerated shelf life studies show that for both fruit juices after 2 days, the total inactivation of the initial microbial load of 10^3 CFU/mL was already reached for the terpenes concentrations of 5.0 g/L and 10 g/L. At a terpenes concentration of 1.0 g/L, microorganism growth is delayed by 5 days in orange juice and 2 days in pear juice in comparison to the control [45].

Another study reported the preparation of a self-nanoemulsifying drug delivery system for the oral delivery of zedoary turmeric oil, an essential oil extracted from the dry rhizome of *Curcuma zedoaria*. The optimized formulation consisting of EO, ethyl oleate, Tween 80, Transcutol P (30.8 : 7.7 : 40.5 : 21, w/w), and loaded with 30% drug was prepared. Upon mixing with water, the formulation was rapidly dispersed into fine droplets with a mean size of 68.3 ± 1.6 nm and zeta potential of -41.2 ± 1.3 mV. The active components remained stable in the optimized formulation stored at 25°C for at least 12 months. Following oral administration in rats, both AUC and Cmax of germacrone, a representative bioactive marker of zedoary turmeric oil, increased by 1.7-fold and 2.5-fold, respectively, compared with the unformulated zedoary turmeric oil [46].

5.2.2. Liposomes. Liposomes are one of the most studied colloidal delivery systems; in fact, they were first developed for drug delivery purposes as early as 1970s [47, 48].

Liposomes consist of vesicular self-assembled system comprising of one or more bilayers, usually formed using a phospholipid, surrounding an aqueous core. Liposomes can contain (i) one bilayer forming unilamellar vesicles (ULV), (ii) several concentric bilayers forming multilamellar vesicles, or (iii) nonconcentric bilayers forming multivesicular vesicles (MVV). The size of these structures can be rather small (in the range of 20 nm) or rather large (exceeding 1 μm). Owing to the presence of the hydrophilic compartment and lipophilic palisade, they can be used as carriers for both lipophilic and hydrophilic molecules [48].

Bioactive compounds compartmentalised in liposomes can be protected against degradation and in case of lipophilic compounds, liposomal encapsulation can also lead to increased solubilisation [48].

The effect of liposomal inclusion on the stability and *in vitro* antiherpetic activity of *Santolina insularis* EO was investigated. Vesicles were obtained from hydrogenated soya phosphatydilcholine and cholesterol. Formulations were examined for their stability for over one year monitoring the drug leakage from vesicles and the average size distribution. The stability of the incorporated oil was verified by studying its quali-quantitative composition. The antiviral activity was studied against herpes simplex virus type 1 (HSV-1) by plaque reduction and yield reduction assays. Results showed that *Santolina insularis* EO can be incorporated in high amounts in the prepared liposomes, which successfully prevented its degradation. Moreover, stability studies pointed out that vesicle dispersions were stable for at least one year and neither oil leakage nor vesicle size alteration occurred during this period. Antiviral activity assays demonstrated that *Santolina insularis* essential oil is effective in inactivating HSV-1 and that the activity is principally due to direct virucidal effects. Free EO proved to be more effective than liposomal oil and a different activity was discovered which related to the vesicular structure. The ED(50) values, significantly lower when cells were preincubated with the EO before the virus adsorption, indicate an intracellular mechanism in the antiviral activity of *Santolina insularis* [49].

The effect of liposomal inclusion on the *in vitro* antiherpetic activity of *Artemisia arborescens* L. EO was investigated. In order to study the influence of vesicle structure and composition on the antiviral activity of the vesicle-incorporated oil, multilamellar (MLV) and unilamellar (SUV) positively charged liposomes were prepared. Liposomes were obtained from hydrogenated (P90H) and nonhydrogenated (P90) soy phosphatidylcholine. Formulations were examined for their stability for over one year, monitoring the oil leakage from vesicles and the average size distribution. The antiviral activity was studied against herpes simplex virus type 1 (HSV-1) by a quantitative tetrazolium-based colorimetric method. Results showed that *Artemisia* EO can be incorporated in good amounts in the prepared vesicular dispersions. Stability studies pointed out that vesicle dispersions were very stable for at least six months and neither oil leakage nor vesicle size alteration occurred during this period. After one year of storage oil retention was still good, but vesicle fusion was present. Antiviral assays demonstrated that the liposomal incorporation of *A. arborescens* EO enhanced its *in vitro* antiherpetic activity especially when vesicles were made with P90H. On the contrary, no significant difference in antiviral activity was observed between the free and SUV-incorporated oil. P90H MLV showed a higher activity than P90 MLV (EC50 values of 18.3 and 43.6 μg/mL for P90H MLV and P90MLV, resp.), while no significant differences of the antiviral activity were observed between the free essential oil and SUV vesicles. Incorporation of *A. arborescens* essential oil in multilamellar liposomes greatly improved its activity against intracellular HSV-1 [50].

A modified technique of rapid expansion of supercritical solutions (RESS) was applied to incorporate EO extracted from *Atractylodes macrocephala* Koidz into liposomes. The optimised entrapment efficiency, drug loading, and average particle size of liposomes were found to be 82.18%, 5.18%, and 173 nm, respectively. The physicochemical properties including the entrapment efficiency, dissolution rate, and stability met the characteristic for a pharmaceutical use of the developed formulation [51].

Carvacrol, thymol, p-cymene, and c-terpinene were identified as major constituents and isolated from the EOs from *Origanum dictamnus* L. The above components were successfully encapsulated in phosphatidyl choline-based liposomes and the possible improvement of their antioxidant and antimicrobial activities was tested against four Gram-positive and four Gram-negative bacteria and three human pathogenic fungi, as well as the food-borne pathogen, *Listeria monocytogenes*. In order to investigate any possible synergistic or antagonistic effect between carvacrol/thymol and carvacrol/c-terpinene, the antimicrobial activities of the mixtures were also determined before and after encapsulation in liposomes. All tested compounds presented enhanced antimicrobial activities after the encapsulation [52].

A study examined carvacrol (derivatives) and thymol encapsulated in liposomes to increase their bioavailability and stability. Similarly, the endurance to humidity and UV light was enhanced [53].

5.2.3. SLN Solid Lipid Nanoparticles. Solid lipid nanoparticles (SLN) refer to nanoscale size particles prepared using lipids that remain solid at room temperature (or/and body temperature). The lipid component may comprise of a broad range of lipid and lipid-like molecules such as triacylglycerols or waxes [54]. The diameter of such lipid particles can be also quite small, that is, in the range between 50 nm and 1 μm. Active ingredients can be solubilised homogeneously either in the core of the SLNs or in the outside part [55]. The advantage of SLNs as delivery system for lipophilic active components is reported to lie in the immobilisation of active elements by the solid particle structure leading to an increased chemical protection, less leakage, and sustained release [56].

This physical property allows a better control of both the physical (against recrystallisation) and chemical (against degradation) stability of the delivered constituents.

The effect of SLN incorporation on transdermal delivery and *in vitro* antiherpetic activity of *Artemisia arborescens* EO has been investigated. Two different SLN formulations were prepared using the hot-pressure homogenization technique, Compritol 888 ATO as lipid, and Poloxamer 188 (SLN 1) and Miranol Ultra C32 (SLN 2) as surfactants.

One day after production, the SLN 1 had a size of 223 nm (0.243 polydispersion index) while the particle size of SLN 2 prepared using Miranol Ultra C32 as surfactant was 219 nm (0.301 polydispersion index, PI). The mean particle size of the formulations increased only slightly after two years of storage, indicating a high physical stability of both SLN 1 and SLN 2 formulations. In particular, 2 years after production, SLN 1 and SLN 2 formulations showed a mean size of 242 nm (0.285 PI) and 239 nm (0.321 PI). The PI values were always smaller than 0.350 indicating a fairly narrow size distribution of the particles. Formulations were examined for their stability for two years by monitoring average size distribution and zeta potential values. The antiviral activity of free and SLN incorporated EO was tested *in vitro* against Herpes Simplex Virus-1 (HSV-1), while the effects of essential oil incorporation into SLN on both the permeation through and the accumulation into the skin strata were investigated by using *in vitro* diffusion experiments through newborn pig skin and an almond oil *Artemisia essential* oil solution as a control. Results showed that both SLN formulations were able to entrap the EO in high yields and that the mean particle size increased only slightly after two years of storage, indicating a high physical stability. *In vitro* antiviral assays showed that SLN incorporation did not affect the EO antiherpetic activity. The *in vitro* skin permeation experiments demonstrated the capability of SLN of greatly improving the oil accumulation into the skin, while oil permeation occurred only when the oil was delivered from the control solution [57].

Alhaj and coworkers developed a formulation based on *Nigella sativa* essential oil into solid lipid nanoparticles SLN. SLN formulations were prepared using hydrogenated palm oil Softisan 154 and *N. sativa* essential oil as lipid matrix, sorbitol, and water. Data showed a high physical stability for formulations at various storage temperatures during 3 months of storage. In particular, average diameter of *N. sativa* essential oil loaded SLN did not vary during storage and increased slightly after freeze-drying the SLN dispersions. Therefore, obtained results showed that the studied SLN formulations are suitable carriers in pharmaceutical and cosmetic fields [58].

Frankincense and myrrh are gum resins obtained from the genera *Boswellia* and *Commiphora*, respectively. Both genera belong to the family Burseraceae, which is native to Northeast Africa and the Middle East. Frankincense and myrrh have been used for medical purposes in China and India for thousands of years. Modern pharmacological research has revealed that essential oils are the primary effective components in frankincense and myrrh oil (FMO) that exhibit a broad spectrum of biological activities such as antimicrobial, anti-inflammatory, and antitumor activities. As with other essential oils, the instability and poor water solubility of FMO result in poor oral bioavailability, which limits its clinical application. The components of FMO are sensitive to light, air, and high temperature, and FMO stimulates the gastrointestinal tract, making it unsuitable for oral administration. A study has reported the preparation of solid lipid nanoparticles for the oral delivery of frankincense and myrrh essential oils (FMO). Aqueous dispersions of SLNs were successfully prepared by a high-pressure homogenization method using Compritol 888 ATO as the solid lipid and soybean lecithin and Tween 80 as the surfactants. Round SLNs were with a mean size of 113.3 nm, a zeta potenzial of −16.8 mV, and an encapsulation efficiency of 80.60%. SLN formulation increased the antitumor efficacy of FMO in H22-bearing Kunming mice. Compritol 888 ATO showed reasonable FMO solubilization capacity. The poorly water-soluble drug FMO was efficiently encapsulated into the nanoparticles. Particles prepared under proper formulation conditions were spherical with diameters of 220 nm [59]. Solid lipid nanoparticles (SLNs) of essential oil of *Zataria multiflora* have been developed. The results showed that the encapsulation efficiency was 38.66%. Results of particle size determination showed a mean size of 650 nm and SLNs were spherical as shown by TEM. The DSC curve of sodium dodecyl sulfate, polyethylene glycol, cetyl alcohol, and EO was different from EO containing SLNs, which indicated that the EO

can interact with the matrix of lipid during the preparation of the SLNs. 93.2% of the essential oil was released after 24 h. The results of characterization of the SLNs indicated the potential application of essential oil of *Z. multiflora* loaded SLN as carrier system [60].

5.3. Molecular Complexes. A simple strategy to deliver active ingredients is by physically complexing them with other molecules in order to have a better solubility profile and/or an increase in the chemical stability of the complexed system. In this context a molecular complex is referring to the physical association between a host and a guest (active ingredient) molecule and in the case of EOs the complexes are reported with cyclodextrins (CDs).

Cyclodextrins are natural macrocyclic oligosaccharides well known for having toroid-shaped structures with rigid lipophilic cavities and a hydrophilic outer surface insuring good dissolution of the complex in an aqueous environment. They are able to enclose highly hydrophobic molecules inside their hydrophobic cavity, constituting a true molecular encapsulation [61]. The major advantages of the use of CD-complexation in pharmaceutical applications, foods, cosmetics, and toiletries are protection of the active ingredients against oxidation, light induced reactions, decomposition and thermal decomposition, loss by evaporation and sublimation, and elimination or reduction of undesired tastes/odours, to reduce or prevent gastric-intestinal irritation (mainly due to anti-inflammatory drugs) or ocular disturbances, prevent drug-drug or drug-additive interactions, or even to convert oils and liquid drugs into microcrystalline or amorphous powders and to reduce microbiological contamination, fibres, or the elimination of other undesired components and hygroscopicity [62]. Moreover, formation of inclusion complex (IC) increases the guest's *in vivo* stability against hydrolysis, oxidation, decomposition, and dehydration, consequently increasing bioavailability and bioefficacy. There are three main types of CDs: α-, β-, and γ-cyclodextrins, corresponding to 6, 7, and 8 glucopyranose units linked by α-(1,4) bonds, respectively. The dimensions of the internal cavity are 0.5–0.8 nm and are crucial for the "encapsulation" of guest molecules [63].

In the last years, physicochemical properties and, consequently, the inclusion capacity of the natives' CD have been improved by chemical modification of their hydroxyl groups [64]. Besides natural cyclodextrins, a growing number of semisynthetic derivatives and copolymers have been prepared and are already commercially available. Many of them found use as structural and chiral selectors, with new properties given by the type and number of substituents. The semisynthetic derivatives of cyclodextrins show better solubility in water, can decrease and modulate the release rate of water soluble molecules, are able to enhance the dissolution rate and the inclusion capacity, and also decrease the side effects of some molecules.

The majority of the publications is concerning the encapsulation of essential oils with β-CD and its derivatives: randomly methylated-β-cyclodextrin, hydroxypropyl-β-cyclodextrin, and low methylated-β-cyclodextrin.

The IC of thymol and cinnamaldehyde and β-CD was investigated [65] in order to study the influence of water adsorption by CDs and their complexes on the release of encapsulated compounds. The results showed that β-CD encapsulates efficiently both of them, in a 1 : 1 molar ratio. The ICs were obtained upon mixing the components in aqueous media and subsequent freeze-drying, as confirmed by differential scanning calorimetry. The samples were stored at constant relative humidity, from 22% to 97%, at 25°C. The release of encapsulated compounds was determined following the melting enthalpy of each guest. Water sorption isotherms for β-CD and the complexes showed constant and low water sorption at RH < 80%; then the uptake of water increased abruptly. The amount of sorbed water at each RH was smaller for the complexes than for β-CD. The guest molecules displaced water molecules from inside the cavity of β-CD. No thymol or cinnamaldehyde release was detected at RH < 84%, and it increased abruptly from 84% RH, coincidentally with the abrupt increase of absorbed water. Water sorption significantly affects β-CD complexes stability, which is thus governed by the shape of the water sorption isotherm. The stability studies showed that the inclusion complexes thymol-β-CD and cinnamaldehyde-β-CD remain stable up to 75% RH during long storage times. In fact, the guests released from the β-CD complexes were detectable in the region of the water adsorption isotherm at which a sharp increase of water content occurred (84% RH). These results show the relevance of selecting appropriated storage conditions for hydrophobic flavours encapsulated in β-CD or for predicting the shelf life of functional products formulated with nanoencapsulated compounds [65].

β-Caryophyllene (BCP), a natural sesquiterpene existing in the essential oil of many plants, has exhibited a wide range of biological activities such as antimicrobial, anticarcinogenic, anti-inflammatory, antioxidant, anxiolytic-like, and local anaesthetic effects. However, its volatility and poor water solubility limit its application in pharmaceutical field. Liu and coworkers investigated and compared the oral bioavailability and the pharmacokinetics of free BCP and BCP/β-CD IC after a single oral dose of 50 mg/kg on rats [66]. BCP was rapidly released from inclusion complex and the *in vivo* data showed that BCP/β-CD IC displayed earlier Tmax, higher Cmax and the AUC0-12 h showed approximately 2.6 times higher increase than those of free BCP. The β-CD has significantly increased the oral bioavailability of the drug in rats than free BCP [66].

The essential oil of *Chamomilla recutita* (L.) Rauschert, syn. *Matricaria recutita* L., contains up to 50% (−)-α-bisabolol which contributes to the anti-inflammatory properties of camomile oil. Bisabolol is a very lipophilic substance, with a tendency to oxidise decreasing anti-inflammatory activity ca. 50%. (−)-α-Bisabolol was found to form an inclusion complex with β-CD in solution as well as in the solid state. To investigate molecular associations of β-CD with pure (−)-α-bisabolol or (−)-α-bisabolol as a component of camomile EO, Waleczek et al. undertook phase solubility studies [67]. The complex constant was 273 M^{-1} for the pure (−)-α-bisabolol and 304 M^{-1} for (−)-α-bisabolol as

a constituent of the EO. The intrinsic solubility of pure (−)-α-bisabolol (4.85×10^{-4} M) and (−)-α-bisabolol as a component of the EO (1.82×10^{-4} M) differ significantly. Computer simulation proved an inclusion complex having a stoichiometric composition of 2 : 1 (β-CD : drug) [67].

Thymol is a monoterpene present in Lamiaceae plants, specially oreganos and thymes. Cinnamaldehyde (3-phenyl-2-propenal) represents 65–75% of the cinnamon EO. Thymol and cinnamaldehyde are frequently used as flavours, but they are also becoming increasingly important as naturally occurring antimicrobial, antioxidant, and antiseptic agents. As natural and artificial flavours they are very sensitive to the effects of light, oxygen, humidity, and high temperatures. The study of Hill et al. [68] aimed to elucidate the physicochemical characteristics of essential oils and β-Cyclodextrin (EO-β-CD) inclusion complexes and their resulting antimicrobial activity. Cinnamon bark extract, transcinnamaldehyde, clove bud extract, eugenol, and a 2 : 1 (transcinnamaldehyde : eugenol) mixture were microencapsulated by the freeze-drying method. EO-β-CD complexes were characterized for particle size, morphology, polydispersity index (PI), entrapment efficiency, and phase solubility. All particles showed a spherical shape, smooth surface, no significant differences in size distribution, and strong tendency to agglomerate. The entrapment efficiencies ranged from 41.7 to 84.7%, where pure compounds were higher ($P < 0.05$) than extracts.

The oils and their β-CD complexes were analyzed for their antimicrobial activity against *Salmonella enterica* serovar *Typhimurium* LT2 and *Listeria innocua*. All the samples effectively inhibited bacterial growth within the concentration range tested, except free eugenol. The EO-β-CD complexes were able to inhibit both bacterial strains at lower concentrations than free oils, likely due to their increased water solubility which determined an increased contact between pathogens and essential oils. The cinnamon bark and clove bud olis/β-CD complexes were the most powerful antimicrobials, despite showing the lowest entrapment efficiencies amongst the oils. The results indicate that such EO inclusion complexes could be useful antimicrobial delivery systems with a broad spectrum of application in food systems where Gram-positive and -negative bacteria could present a risk [68].

Garlic (*Allium sativum* L.) is a widely distributed plant and is used throughout the world not only as a spice and a food, but also as a folk medicine, and many of the beneficial health-related biological effects have been attributed to its characteristic organosulphur compounds [69]. Steam distillation is widely used to extract and condense the volatile organosulphur compounds in garlic, and the final oily product is called garlic oil (GO). The compounds of GO mainly are diallyl disulphide, diallyl trisulphide, allyl propyl disulphide, a small quantity of disulphide, and probably diallyl polysulphide [70]. GO is recognised to be more potent than aqueous extracts of garlic and exhibits a wide range of pharmacological properties including antimicrobial, antidiabetic, antimutagenic, and anticarcinogenic effects [71]. However, the application of GO is limited due to its volatility, strong odour, insolubility in water, and low physicochemical stability.

The characterisation of ICs of GO/β-CD was investigated [72]. The calculated apparent stability constant of IC was $1141\ M^{-1}$, and the water solubility of GO was significantly improved. Furthermore, the release rate of GO from the inclusion complex was controlled. The results of this study clearly demonstrated that GO could be efficiently complexed with β-CD to form an inclusion complex by the coprecipitation method in a molar ratio of 1 : 1. The aqueous solubility and stability of GO were significantly increased by inclusion in β-CD [72].

Isothiocyanates (ITCs) are hydrolysis products of sulphur-containing compounds called glucosinolates, which occur naturally in cruciferous vegetables, such as broccoli and cabbage. Mechanical disruption of cruciferous plant tissues releases ITCs, due to the hydrolysis reaction catalysed by myrosinase bound to the cell wall and possesses antimicrobial activities [73]. ITCs, in particular allyl isothiocyanate (AITC), have been extensively studied for their antibacterial effect for food. Inclusion complexation reactions between isothiocyanates (ITCs), namely, allyl isothiocyanate (AITC) and phenyl isothiocyanate (PITC), and randomly methylated β-cyclodextrin (RM-β-CD) were reported [74].

The apparent activation energy of IC dissociation suggested a reduction of volatility and physical stabilisation by inclusion complexation.

RM-β-CD demonstrated a strong solubilising effect on the poorly water-soluble AITC and PITC in the aqueous phase. RM-β-CD was more effective in solubilising PITC though the AITC/RM-β-CD complex had higher apparent stability constants. Despite the fact that a greater amount of AITC was solubilised in the aqueous phase at any given concentration of RM-β-CD, the solid state AITC/RM-β-CD complex showed an inclusion ratio remarkably lower than that of the PITC/RM-β-CD complex. Both of the ITCs may form inclusion complexes with RM-β-CD at guest to host ratios of 1 : 1 and 1 : 2 in the aqueous phase [74].

The inclusion interactions of cyclodextrins (CDs) and β-cyclodextrin polymers with linalool and camphor in *Lavandula angustifolia* EO were investigated in order to prepare novel controlled release systems for the delivery of essential oil used as ambient odours [75].

The complexation behaviour and the retention capacity of α-CD, β-CD, γ-CD, hydroxypropyl-β-cyclodextrin (HPBCD), randomly methylated-β-cyclodextrin (RAMEB), a low methylated-β-cyclodextrin (CRYSMEB), and crosslinked β-CD polymers for linalool and camphor, two major components of *Lavandula angustifolia* EO, were studied. The complexation and the retention capacity of CDs and CD polymers were investigated under solid support or in aqueous media by static headspace gas chromatography. The release profile of aroma from solid support was studied by multiple headspace extraction (MHE). The retention capacity of the CD derivatives was measured in static experiments.

The stability constants with monomeric CD derivatives were determined for standard compounds and for the

compounds in essential oil. The RAMEB showed the higher formation constant both for the standard compounds (833 M^{-1} for linalool and 1194 M^{-1} for camphor) and for the compounds in the in *Lavandula angustifolia* essential oil (1074 M^{-1} for linalool and 2963 M^{-1} for camphor). All studied CDs and CD polymers reduce the volatility of the aroma compounds and stable 1 : 1 inclusion complexes are formed. β-CD is the most versatile CDs for the two guests, leading to greater formation constant and retention ability in aqueous phase [75].

6. Concluding Remarks

EOs have promising potentials for maintaining and promoting health, as well as preventing and potentially treating some diseases. However, the generally low water solubility and stability, together with the high volatility and side effects associated with their use have limited their application in medicine. Nanotechnology is an innovative approach that has potential applications in medicinal and health research. Indeed, nanoparticles are a very attractive tool and are able to solve the major inconvenience of EOs use increasing the chemical stability in the presence of air, light, moisture and, high temperatures, factors which can lead to the rapid evaporation and to the degradation of the active components. In addition, nanocarriers ensure the easier and safer handling of the liquid substances by changing them in solid powders, determining retention of volatile ingredients and taste masking, setting up controlled release and/or consecutive delivery of multiple active ingredients, reducing toxic side effects, improving water solubility of hydrophobic ingredients, and enhancing bioavailability and efficacy.

Nanoencapsulation of EOs in liposomes, solid lipid nanoparticles, nano- and microemulsions, and polymeric nanoparticles represent a promising strategy for overcoming EOs limitations, lowering their dose and increasing long-term safety of these constituents.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Effect of Eugenol on Cell Surface Hydrophobicity, Adhesion, and Biofilm of *Candida tropicalis* and *Candida dubliniensis* Isolated from Oral Cavity of HIV-Infected Patients

Suelen Balero de Paula,[1] Thais Fernanda Bartelli,[1] Vanessa Di Raimo,[1] Jussevania Pereira Santos,[1] Alexandre Tadachi Morey,[1] Marina Andrea Bosini,[1] Celso Vataru Nakamura,[2] Lucy Megumi Yamauchi,[1] and Sueli Fumie Yamada-Ogatta[1]

[1] *Universidade Estadual de Londrina, Centro de Ciências Biológicas, Departamento de Microbiologia, Rodovia Celso Garcia Cid, km 380, 86057-970 Londrina, Brazil*
[2] *Universidade Estadual de Maringá, Centro de Ciências da Saúde, Departamento de Ciências Básicas da Saúde, Maringá, Brazil*

Correspondence should be addressed to Sueli Fumie Yamada-Ogatta; ogatta@uel.br

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Most *Candida* spp. infections are associated with biofilm formation on host surfaces. Cells within these communities display a phenotype resistant to antimicrobials and host defenses, so biofilm-associated infections are difficult to treat, representing a source of reinfections. The present study evaluated the effect of eugenol on the adherence properties and biofilm formation capacity of *Candida dubliniensis* and *Candida tropicalis* isolated from the oral cavity of HIV-infected patients. All isolates were able to form biofilms on different substrate surfaces. Eugenol showed inhibitory activity against planktonic and sessile cells of *Candida* spp. No metabolic activity in biofilm was detected after 24 h of treatment. Scanning electron microscopy demonstrated that eugenol drastically reduced the number of sessile cells on denture material surfaces. Most *Candida* species showed hydrophobic behavior and a significant difference in cell surface hydrophobicity was observed after exposure of planktonic cells to eugenol for 1 h. Eugenol also caused a significant reduction in adhesion of most *Candida* spp. to HEp-2 cells and to polystyrene. These findings corroborate the effectiveness of eugenol against *Candida* species other than *C. albicans*, reinforcing its potential as an antifungal applied to limit both the growth of planktonic cells and biofilm formation on different surfaces.

1. Introduction

The prevalence of oral colonization by *Candida* spp. can vary among different population groups [1], and the presence of these fungi as commensals of human microbiota is one important predisposing factor for candidosis [2]. Adherence of the microorganisms to host cells and tissues is the first event required for initial colonization or establishment of infection. Moreover, the microbial surface contact can trigger various cellular behaviors, including biofilm formation [1], which is also strongly associated with candidosis [3].

Biofilms can be defined as irreversibly surface-attached communities of cells (sessile cells) embedded in a self-produced exopolymeric matrix, displaying a distinctive phenotype compared to their free-floating (planktonic cells) counterparts [4]. Remarkably, sessile cells are less susceptible to a variety of antifungal agents [5–7] and to host defenses [8]. Biofilms are thereby difficult to eradicate, representing a source of reinfections. Consequently, new antifungal agents are urgently needed, particularly those with antibiofilm activities, for effective management of *Candida* spp. infections.

Several researchers have shown the anti-*Candida* biofilm potential of plant-derived compounds such as flavonoids [9] and essential oils [10, 11]. Eugenol is the main active phenylpropanoid component of the essential oil from many aromatic plants [12]. The inhibitory effect of eugenol alone [13–17] and in combination with fluconazole and amphotericin B [18] against planktonic cells of *Candida* spp. has been previously reported. In addition, eugenol can interfere with the initial phases of biofilm formation, as well as with the viability of mature biofilm of *Candida albicans* [10, 11].

Although *C. albicans* continues to be the most common causative agent of candidosis in humans, other species of *Candida* have become a significant cause of such infections [19, 20]. *Candida tropicalis* and *Candida dubliniensis* have been regarded as high biofilm producers, and sessile cells within this community have been found to be resistant to antifungal agents [5–7]. Accordingly, we analyzed the effect of eugenol on the hydrophobicity and adhesion to human epithelial cells and polystyrene of planktonic cells of these species. Moreover, the inhibitory activity against biofilm formation on polystyrene and denture materials was also analyzed.

2. Materials and Methods

2.1. Candida spp. Isolates and Growth Conditions. The *Candida* species used in this study included three *C. dubliniensis* (strains 131, 219, and 248) and three *C. tropicalis* (strains 23, 150, and 176) isolated from the oral cavity of HIV-infected patients. The species identification of oral isolates was carried out by standard mycological methods [21, 22]. Species identification was confirmed by a PCR-based method using specific primers as previously described [23, 24]. *C. tropicalis* ATCC 28707 and *C. dubliniensis* ATCC MYA-646 type strains (kindly provided by FIOCRUZ, Rio de Janeiro, Brazil) were included as quality control. The isolates and strains were stored on Sabouraud dextrose (SD) agar and subcultured monthly. The yeasts were also maintained at −80°C. The study protocol was in accordance with the Ethics Committee of the Universidade Estadual de Londrina (CEP/UEL no. 036/10). Written informed consent was obtained from the patients for the publication of this report.

2.2. Biofilm Formation. *Candida* isolates were cultured in SD broth and incubated at 37°C for 18 h. The yeasts were harvested by centrifugation and washed twice with sterile 0.15 M phosphate-buffered saline, pH 7.2 (PBS), and the cells were counted in hemocytometric chamber (Neubauer Improved Chamber). A 20 μL SD broth suspension of 6×10^5 yeasts was placed into each well of flat-bottomed 96-well microtiter plates (Techno Plastic Products, Switzerland) containing 180 μL of SD broth. The plates were incubated at 37°C for 24 h. Afterwards, the wells were washed once with sterile distilled water, and the metabolic activity of the cells was quantified using the 2,3-bis(2-methoxy-4-nitro-5-sulfo-phenyl)-2H-tetrazolium-5-carboxanilide (XTT)-reduction assay. A 100 μL aliquot of XTT-menadione (0.1 mg/mL XTT, 1 μM menadione, Sigma Chemical Co, USA) was added to each well, and the plates were incubated in the dark for 2 h at 37°C. The XTT formazan product was measured at 490 nm with a microtiter plate reader (Universal Microplate Reader ELx 800, Bio-Tek Instruments, USA) [6]. To analyze the biofilm formation on denture material surfaces, the wells were aseptically coated with polymethylmethacrylate (PMM, OrtoClass, Clássico, Brazil) and ceramic (Noritake, Shofu Dental Corp., USA) before the assay.

2.3. Antifungal Susceptibility Testing. The growth inhibitory effect of eugenol (SSWhite, Brazil) on planktonic cells of *Candida* isolates was determined by broth microdilution assays according to the Clinical and Laboratory Standards Institute [25]. A stock solution of eugenol was prepared in water containing 10% dimethylsulfoxide (DMSO v/v, Sigma Chemical Co., USA). The DMSO final concentration in the assays did not exceed 1.0%. The substance was serially diluted 2-fold in RPMI buffered with MOPS, pH 7.0 (3000–5.85 μg/mL eugenol). Quality control *C. dubliniensis* ATCC MYA-646 and *C. tropicalis* ATCC 28707 and fluconazole (512.0–0.5 μg/mL, Pfizer Central Research, United Kingdom) were included in each experiment. Two wells of each plate served as growth and sterility controls. The minimum inhibitory concentrations (MICs) were determined at total inhibition of visual growth after 24 h incubation compared to untreated planktonic cells. To determine antifungal susceptibilities of sessile cells, biofilms were formed on polystyrene, as described above. After 1 and 24 h of biofilm formation, the medium was aspirated off and each well was washed three times with sterile PBS. A 200 μL aliquot of RPMI 1640 medium containing serial 2-fold dilutions of eugenol and fluconazole was added, and the plates were further incubated for 24 h at 37°C. Controls included antifungal-free wells and biofilm-free wells. Sessile MICs were determined at 100% inhibition ($SMIC_{100}$) compared to antifungal-free control wells using the XTT-reduction assay [6]. To evaluate the time-dependent effect of eugenol, 24 h biofilms of *Candida* species were formed in polystyrene and treated with $SMIC_{100}$ of eugenol as described above. At determined time points (3, 6, 12, and 24 h), the metabolic activity of sessile cells was determined. All experiments were carried out in triplicate on three different occasions.

2.4. Cell Surface Hydrophobicity Determination. The hydrophobicity of untreated and eugenol-treated (0.5 × MIC for 1 h) planktonic cells was determined by the biphasic hydrocarbon/aqueous method according to Anil et al. [26]. Cell surface hydrophobicity (CSH) was expressed as the percentage decrease in optical density of the aqueous phase of the test as compared with the control, where the greater the change in absorbance of the aqueous phase, the more hydrophobic the yeast sample. Each assay was performed on three separate occasions with triplicate determinations each time.

2.5. Adhesion of Yeasts to HEp-2 Cells and Polystyrene. HEp-2 cells (human larynx epidermoid carcinoma) were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco)

Table 1: Metabolic activities of biofilm formed by *Candida dubliniensis* and *Candida tropicalis* on different substrate surfaces.

Isolate	Metabolic activity (OD)[a]		
	Polystyrene	PMM	Ceramic
Candida dubliniensis			
ATCC MYA-646	0.855 ± 0.029	0.711 ± 0.056	0.499 ± 0.055
131	1.045 ± 0.032	0.795 ± 0.058	0.628 ± 0.056
219	0.989 ± 0.033	0.751 ± 0.054	0.566 ± 0.057
248	1.094 ± 0.034	0.810 ± 0.055	0.637 ± 0.056
Candida tropicalis			
ATCC 28707	0.978 ± 0.029	0.745 ± 0.058	0.559 ± 0.056
23	1.136 ± 0.032	0.815 ± 0.056	0.638 ± 0.057
150	1.100 ± 0.034	0.801 ± 0.056	0.635 ± 0.057
176	1.056 ± 0.031	0.786 ± 0.057	0.624 ± 0.054
Mean ± SD	**1.032 ± 0.090***	**0.777 ± 0.037**$^{\#}$	**0.598 ± 0.051**$^{¥}$

[a]Metabolic activity of sessile cells was determined by the XTT-reduction assay. The XTT formazan product was measured at 490 nm.
$^{*,\#,¥}$Means not sharing a symbol differ significantly ($P < 0.05$) between the abiotic surfaces.

supplemented with 10% fetal bovine serum, 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2.5 μg/mL amphotericin B in a humidified 5% CO_2 atmosphere at 37°C. For adhesion assays, HEp-2 cells were seeded in 24-well plates at 4.0×10^5 cells per well and incubated for 18 h. The medium was removed and each well was washed three times with sterile PBS. The fresh culture medium minus the antimicrobials was added and the wells were inoculated with untreated and eugenol-treated *Candida* spp. (0.5 × MIC for 1 h) with approximately 2.0×10^6 cells, and the plates were incubated at 37°C for 2 h in a 5% CO_2 atmosphere. Nonadherent yeasts were removed by washing with sterile PBS. Adherent yeasts were harvested by treatment of the cell monolayers with 1 mL 0.5% (v/v) Triton X-100 (Sigma Chemical Co.) for 10 min on ice. The viable yeasts were enumerated by dilution plating in SD agar. Experiments were carried out in duplicate on three different occasions. The percent adherence was calculated by the equation: % Adherence = $(cfu_{120}/cfu_0) \times 100$, where cfu_{120} refers to adhered cells per mL after 2 h and cfu_0 the initial number of inoculated cells. The adhesion on polystyrene surface was performed as described for biofilm formation with minor modifications. Briefly, untreated and eugenol-treated (0.5 × MIC for 1 h) planktonic cells were placed in each well, and the plates were incubated for 2 h. The metabolic activity of adherent cells was determined using the XTT-reduction assay as described above.

2.6. Scanning Electron Microscopy (SEM). Discs (0.8 cm diameter) of PMM and ceramic were aseptically placed in wells of 24-well tissue culture plates (Techno Plastic Products, Switzerland). A standard inoculum of 3.0×10^6 cells, from overnight culture of the yeast, was prepared in 1 mL of RPMI 1640 medium, pH 7.0, and used to form biofilm on these surfaces. The discs were then immersed in these cell suspension and incubated statically at 37°C for 24 h. Afterwards, nonadherent organisms were removed by washing gently three times with PBS. One milliliter of RPMI containing eugenol ($SMIC_{100}$) was added and the plates were further incubated for 24 h. Biofilms formed on these strips were fixed with 2.5% (v/v) glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) at room temperature. After fixation, the cells were dehydrated with a series of ethanol washes (15, 30, 50, 70, 80, 90, 95, and 100%), critical-point dried in CO_2, coated with gold and examined with a SHIMADZU SS-550 scanning electron microscope.

2.7. Statistical Analysis. The results were evaluated by Student's *t*-test using the software GRAPHPAD PRISM version 5.0 (GRAPHPAD Software, San Diego, CA). *P* values less than 0.05 were considered significant.

3. Results

3.1. Biofilm Formation on Abiotic Surfaces. Biofilm formation by *Candida* species on polystyrene, PMM, and ceramic was monitored using the XTT-reduction assay. All isolates were able to form biofilms on these different substrate surfaces within 24 h, as assessed by the metabolic activity of sessile cells (Table 1). No significant differences in metabolic activities were observed between the strains and isolates in each substrate analyzed. However, a significant difference ($P < 0.05$) was observed between the substrates, where the highest biofilm formation was detected on polystyrene surface, followed by PMM and ceramic. The mean $OD_{490\,nm}$ (optical density at 490 nm ± standard deviation) was 1.032 ± 0.090 for the polystyrene, 0.777 ± 0.037 for PMM, and 0.598 ± 0.051 for ceramic surfaces.

3.2. Antifungal Activity against Planktonic and Sessile Cells. The MICs and SMICs of eugenol and fluconazole for the *Candida* spp. isolates and type strains are reported in Table 2. Planktonic cells of all isolates and the type strain of *C. dubliniensis* were susceptible to fluconazole. However, variation in fluconazole susceptibility was observed for *C. tropicalis*, where the reference type strain (ATCC 28707) and isolate

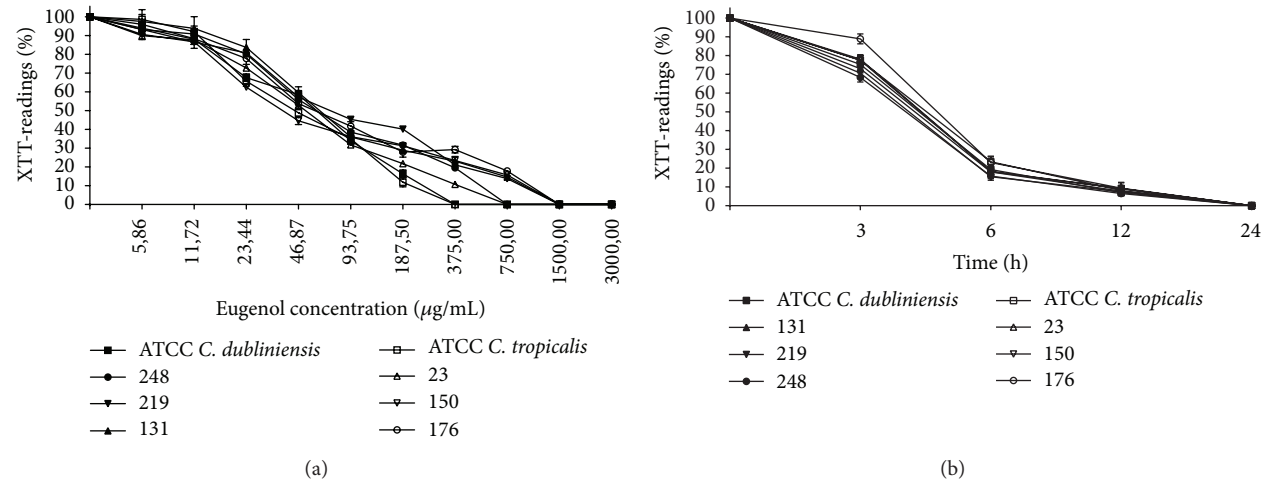


Figure 1: Effect of eugenol on viability of mature biofilm of *Candida dubliniensis* and *Candida tropicalis*. (a) The mature biofilms (24 h) were incubated in the presence of different concentrations (3000.0–5.86 μg/mL) of eugenol for 24 h at 37°C. (b) The mature biofilms were incubated with $SMIC_{100}$ concentrations of eugenol at 37°C and the metabolic activity of sessile cells was assessed at determined time points (3–24 h). Values are expressed as the average percentage of optical density (OD) of wells containing treated biofilms compared to that of control wells (considered to be 100%) for the XTT assays.

176 were resistant, isolate 150 dose-dependently susceptible, and isolate 23 susceptible to fluconazole, according to the CLSI [25] interpretative breakpoints. The biofilm of these *Candida* species exhibited high resistance to fluconazole. The $SMIC_{100}$ of this compound for all isolates and type strains was higher than 512 μg/mL. The MIC values of eugenol for *C. dubliniensis* and *C. tropicalis* planktonic cells ranged from 375 to 750 μg/mL. Trailing growth was observed when fluconazole was tested against all *C. tropicalis* strains, while eugenol completely inhibited the growth of planktonic cells. Eugenol also exhibited an inhibitory effect against mature biofilms of *Candida* species, which appeared to be dose dependent (Figure 1(a)). There was a more than 80% reduction in metabolic activity of 24 h sessile cells with eugenol at concentrations of 187.5 to 750 μg/mL. No metabolic activity was detected at concentrations ranging from 375 to 1500 μg/mL, and these values were considered the $SMIC_{100}$. The inhibitory effect of eugenol against 24 h sessile cells was also time dependent (Figure 1(b)). The reduction in metabolic activity ranged from 11.1 to 31.6%, 76.6 to 85.5%, and 90.6 to 93.5% after incubation in the presence of $SMIC_{100}$ eugenol for 3, 6, and 12 h, respectively. No detectable metabolic activity was observed after 24 h treatment. Eugenol also interfered with biofilm formation, since treatment of 1 h adherent cells resulted in dose-dependent reduction of their metabolic activity (data not shown). The $SMIC_{100}$ for 1 h adherent cells ranged from 375 to 750 μg/mL (Table 2).

3.3. Scanning Electron Microscopy of Candida tropicalis Biofilm on Denture Materials. The effect of eugenol on *C. tropicalis* (isolate 150, MIC of fluconazole = 32 μg/mL) biofilms formed on PMM and ceramic surfaces was monitored by SEM (Figure 2). Mature biofilms of untreated cells of this isolate consisted of a dense network of cells, composed of a heterogeneous layer of yeast, pseudohyphae, and hyphae

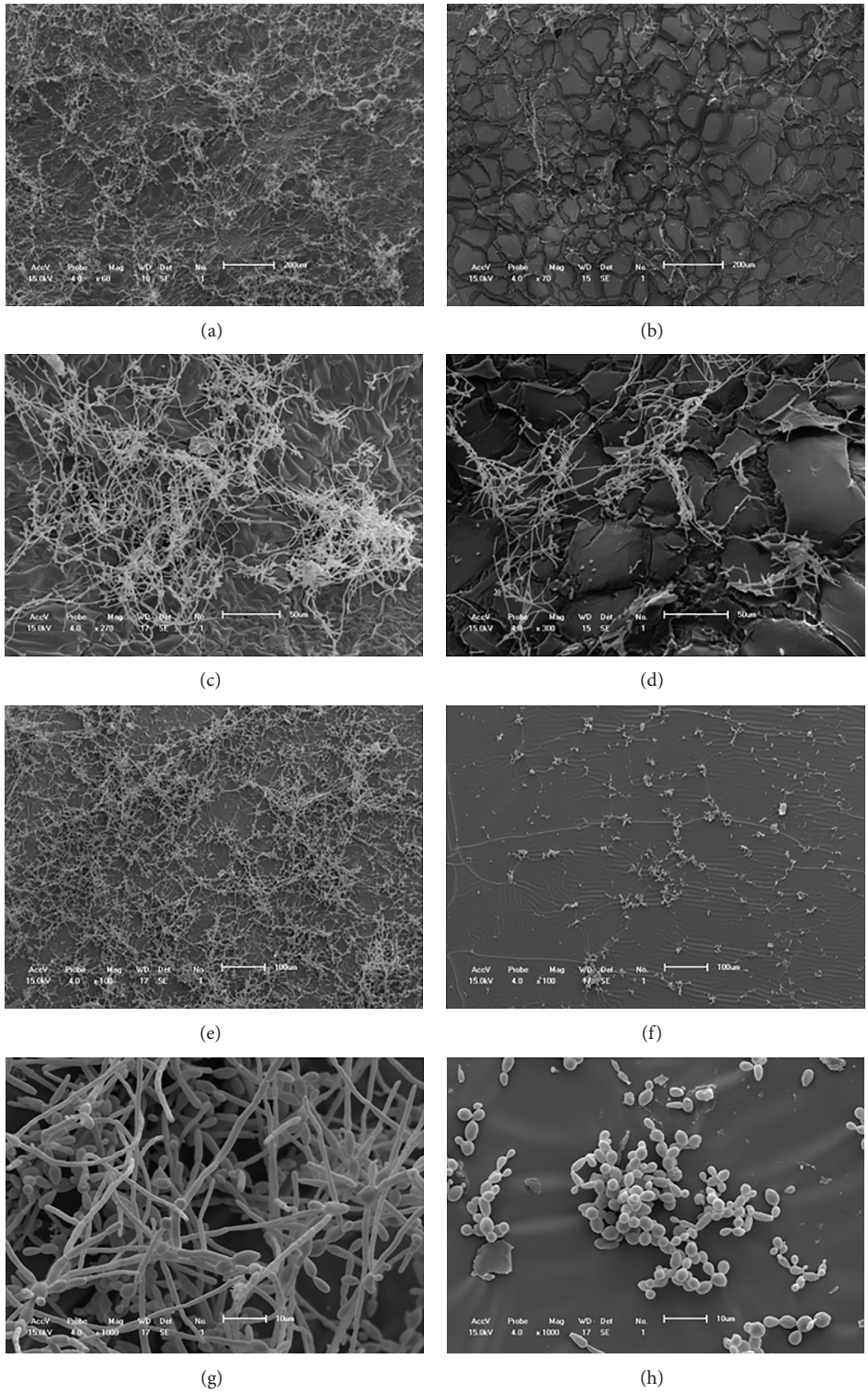
Table 2: Antifungal concentrations of eugenol and fluconazole against planktonic and sessile cells of *Candida dubliniensis* and *Candida tropicalis*.

Yeast	Eugenol			Fluconazole	
	MIC[a]	SMIC-1[b]	SMIC-24[c]	MIC[d]	SMIC[c]
Candida dubliniensis					
ATCC MYA-646	375	375	375	8	>512
131	750	750	1,500	4	>512
219	375	750	1,500	8	>512
248	375	750	750	4	>512
Candida tropicalis					
ATCC 28707	375	375	375	128	>512
23	375	750	750	8	>512
150	750	750	1,500	32	>512
176	375	750	1,500	64	>512

[a]Minimum inhibitory concentration of the antifungal which resulted in total inhibition of visible planktonic cell growth; [b]Minimum inhibitory concentration of the antifungal which resulted in total reduction in metabolic activity of sessile cells, using the XTT-reduction assay, after 1 h of adhesion; [c]Minimum inhibitory concentration of the antifungal which resulted in total reduction in metabolic activity of sessile cells, using the XTT-reduction assay, after 24 h of biofilm formation; [d]MIC was defined according to CLSI (2008) guidelines for fluconazole broth microdilution assays; The results are expressed as μg/mL.

(Figures 2(a), 2(c), 2(e), and 2(g)). The treatment of biofilms with eugenol drastically reduced the amount of sessile cells of *C. tropicalis* on the denture materials surfaces (Figures 2(b), 2(d), 2(f), and 2(h)).

3.4. Effect of Eugenol on Cell Surface Hydrophobicity and Adhesion to HEp-2 Cells and Polystyrene. To evaluate the effect of eugenol on CSH and adhesion to mammalian cells and polystyrene, planktonic cells of *Candida* species were



(a) (b)

(c) (d)

(e) (f)

(g) (h)

Figure 2: Scanning electron microscopy images of the effect of eugenol on *Candida tropicalis* mature biofilm formed on the surface of polymethylmethacrylate ((a)–(d)) and ceramic ((e)–(h)). Untreated mature biofilms ((a), (c), (e), and (g)) and treated biofilms with eugenol-$SMIC_{100}$ for 24 h ((b), (d), (f), and (h)).

Table 3: Effect of eugenol on cell surface hydrophobicity, and adhesion to human epithelial cells and polystyrene.

Isolate	CSH[a]		Adhesion to HEp-2 cells[b]		Adhesion to polystyrene[c]	
	Untreated	Treated[d]	Untreated	Treated[d]	Untreated	Treated[d]
Candida dubliniensis						
131	$67.97 \pm 5.61^{*}$	39.22 ± 6.97	$92.00 \pm 5.60^{\#}$	35.00 ± 5.27	$0.450 \pm 0.001''$	0.302 ± 0.001
219	$29.48 \pm 2.97^{*}$	15.58 ± 3.16	$45.00 \pm 4.16^{\#}$	14.00 ± 4.53	$0.405 \pm 0.002''$	0.209 ± 0.001
248	$69.20 \pm 9.10^{*}$	16.00 ± 6.11	$90.00 \pm 5.21^{\#}$	30.00 ± 4.73	$0.384 \pm 0.001''$	0.216 ± 0.002
Candida tropicalis						
23	$72.00 \pm 8.22^{*}$	21.00 ± 5.63	$92.00 \pm 5.12^{\#}$	46.00 ± 4.33	$0.397 \pm 0.004''$	0.288 ± 0.003
150	$41.66 \pm 4.72^{*}$	23.75 ± 5.21	$81.00 \pm 5.06^{\#}$	43.00 ± 5.84	$0.335 \pm 0.002''$	0.108 ± 0.001
176	84.59 ± 4.32	81.16 ± 3.19	45.00 ± 3.12	36.00 ± 3.21	0.395 ± 0.005	0.393 ± 0.002

[a]Percentage of cell surface hydrophobicity (CSH) determined by the difference in the optical density (OD) of the aqueous phase between test and control. The greater the change in OD of the aqueous phase, the more hydrophobic the yeast sample is. [b]The percent adherence was calculated by the equation: % Adherence = $(cfu_{120}/cfu_0) \times 100$, where cfu_{120} refers to adhered bacterial cells per mL after 2 h and cfu_0 the initial number of inoculated cells. [c]The metabolic activity of cells was determined by the XTT-reduction assay after 2 h of adhesion on polystyrene surface. [d]Planktonic cells were eugenol-treated for 1 h with 0.5 × MIC before the assay. Significant differences in CSH (∗), adhesion to HEp-2 cells (#) and to polystyrene ($''$) properties when compared to eugenol-treated counterpart cells ($^{*,\#}P < 0.005$; $''P < 0.05$).

exposed to eugenol at a subinhibitory (0.5 × MIC) concentration for 1 h before the assays. Most *Candida* spp. isolates showed hydrophobic behavior as determined by the biphasic hydrocarbon/aqueous method, and the mean relative CSH was 60.82 ± 20.79 ranging from 29.48 ± 2.97 to 84.59 ± 4.32. Except for *C. tropicalis* 176, a significant difference ($P < 0.005$) in CSH of *Candida* species was observed after exposure of planktonic cells to eugenol for 1 h (Table 3). There was a range of 42.3 to 75.1% reduction in the CSH of eugenol-treated cells as compared to untreated counterpart cells. Eugenol also caused a significant reduction in adhesion of most *Candida* species to HEp-2 cells ($P < 0.005$) and to polystyrene ($P < 0.05$). There was no significant difference in the adhesion percentage of isolate 176 of *C. tropicalis* to either surface, although a 20% reduction in adhered cells to mammalian cells was seen after eugenol exposure. The other *Candida* isolates showed a range of 46.9 to 68.9% and 27.4 to 67.8% reduction in adhesion to HEp-2 cells and polystyrene, respectively.

4. Discussion

Eugenol has been widely used in medicine and dentistry due to its antiseptic, antimicrobial, anesthetic, analgesic, antioxidant, anti-inflammatory, and cardiovascular properties [27, 28]. This phenylpropanoid compound has been reported to have antimicrobial activity against planktonic cells of *C. albicans, C. dubliniensis, C. glabrata*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis,* and *C. tropicalis* [13–17, 29]. Moreover, this compound shows *in vitro* synergy with fluconazole and amphotericin B against *C. albicans* [11, 18]. As previously reported [14–17], our results showed that eugenol has fungicidal activity against planktonic cells of *C. tropicalis*, including those classified as fluconazole-resistant and dose-dependent yeasts, and this effect was also observed for *C. dubliniensis*.

Previous studies reported in the literature have focused on determining the antibiofilm activity of eugenol against *C. albicans*. He et al. [10] showed a dose-dependent reduction in metabolic activity of 48 h biofilm formed on a polystyrene surface and treated with eugenol for another 48 h. In the presence of 500 μg/mL and 2000 μg/mL eugenol, 50% ($SMIC_{50}$) and over 80% ($SMIC_{80}$) reduction were detected, respectively. Khan and Ahmad [11] evaluated the effect of phytocompounds (eugenol, cinnamaldehyde, citral, and geraniol) against 48 h biofilm of *C. albicans*, and their results also showed a dose- and time-dependent inhibitory activity for eugenol. The $SMIC_{80}$ after treatment with the compounds for 48 h ranged from 100 to 400 μg/mL. The results obtained in this study showed that eugenol displayed inhibitory activity against biofilms of *C. dubliniensis* and *C. tropicalis*, which, not surprisingly, were highly resistant to fluconazole. Eugenol inhibited biofilm formation, as well as reducing metabolic activity of mature biofilms formed on polystyrene, in a dose-dependent manner. SEM analysis further revealed the reduction in biofilm formed on denture materials (PMM and ceramic).

The mechanisms by which eugenol induces death in *Candida* spp. are not completely understood. This compound caused profound changes in the morphology of planktonic cells and leakage of cytoplasmic constituents, indicating an action on the cell envelope [13, 14]. In fact, several authors have shown that the fungicidal concentration of eugenol against *C. albicans* causes a significant reduction in ergosterol content of the cell [15, 16, 30] and interferes with H^+-ATPase activity [31]. In addition, the extensive damage to the cell membrane [15, 30] may be attributed to oxidative stress mediated by reactive oxygen species [29].

Microbial adherence on the surface of substrates is the initial event of biofilm formation, and the cell envelope mediates the first interaction between the microorganism and the environment. CSH, a nonspecific factor, is considered an important feature that contributes to adherence of *Candida* spp. on different surfaces [32, 33]. Moreover, it has been shown that CSH of planktonic cells of *C. albicans* isolated from different sources correlates positively with biofilm formation on polystyrene [34]. In this study, the presence of eugenol (0.5 × MIC) caused a significant reduction in CSH and adhesion to polystyrene and HEp-2 cells of almost all

planktonic cells of *C. tropicalis* and *C. dubliniensis*. These results suggest that eugenol may interfere with the adhesion properties of *Candida* species. It was previously reported that *C. albicans* adhesion to polystyrene [35] and epithelial cells [36] was reduced after *in vitro* exposure to subinhibitory concentrations of fluconazole, an antifungal that interferes with ergosterol biosynthesis.

Altogether, the findings reported here corroborate the effectiveness of eugenol against planktonic and sessile cells of *Candida* species other than *C. albicans,* reinforcing the potential of this compound as an antifungal, indicating that this phenylpropanoid may have additional beneficial effect in the treatment of local candidiasis. Accordingly, initial *in vivo* studies have demonstrated the safety and efficacy of the topical use of eugenol for the treatment of vaginal [37] and oral [14] candidosis in rats. Further studies are warranted to confirm its efficacy in the prophylaxis and/or treatment of biofilm-associated candidosis in human.

5. Conclusion

The results obtained in this study showed that besides having fungicidal activity, eugenol is capable of changing the CSH and adhesion capacity of planktonic cells of *C. dubliniensis* and *C. tropicalis*. In addition, this phenylpropanoid compound inhibited biofilm formation and mature biofilm formed on polystyrene and denture materials of both *Candida* species.

Conflict of Interests

The authors declare that they have no conflict of interests.

Acknowledgments

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Review Article

Essential Oil of *Artemisia annua* L.: An Extraordinary Component with Numerous Antimicrobial Properties

Anna Rita Bilia,[1] Francesca Santomauro,[2] Cristiana Sacco,[2] Maria Camilla Bergonzi,[1] and Rosa Donato[2]

[1] *Department of Chemistry, University of Florence, Via Ugo Schiff 6, 50019 Sesto Fiorentino, Florence, Italy*
[2] *Department of Health Sciences, University of Florence, Viale G.B. Morgagni 48, 50134 Florence, Italy*

Correspondence should be addressed to Anna Rita Bilia; ar.bilia@unifi.it

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Artemisia annua L. (Asteraceae) is native to China, now naturalised in many other countries, well known as the source of the unique sesquiterpene endoperoxide lactone artemisinin, and used in the treatment of the chloroquine-resistant and cerebral malaria. The essential oil is rich in mono- and sesquiterpenes and represents a by-product with medicinal properties. Besides significant variations in its percentage and composition have been reported (major constituents can be camphor (up to 48%), germacrene D (up to 18.9%), artemisia ketone (up to 68%), and 1,8 cineole (up to 51.5%)), the oil has been subjected to numerous studies supporting exciting antibacterial and antifungal activities. Both gram-positive bacteria (*Enterococcus*, *Streptococcus*, *Staphylococcus*, *Bacillus*, and *Listeria* spp.), and gram-negative bacteria (*Escherichia*, *Shigella*, *Salmonella*, *Haemophilus*, *Klebsiella*, and *Pseudomonas* spp.) and other microorganisms (*Candida*, *Saccharomyces*, and *Aspergillus* spp.) have been investigated. However, the experimental studies performed to date used different methods and diverse microorganisms; as a consequence, a comparative analysis on a quantitative basis is very difficult. The aim of this review is to sum up data on antimicrobial activity of *A. annua* essential oil and its major components to facilitate future approach of microbiological studies in this field.

1. Introduction

Artemisia annua L., a plant belonging to the Asteraceae family, is an annual herb native to China and it grows naturally as a part of steppe vegetation in northern parts of Chatar and Suiyan province in China at 1,000–1,500 m above sea level. This plant can grow up to 2.4 m tall. The stem is cylindrical and branched. Leaves are alternate, dark green, or brownish green. Odour is characteristic and aromatic while the taste is bitter. It is characterized by large panicles of small globulous capitulums (2-3 mm diameter), with whitish involucres, and by pinnatisect leaves which disappear after the blooming period, characterised by small (1-2 mm) pale yellow flowers having a pleasant odour (Figure 1). The Chinese name of the plant is Qinghao (or Qing Hao or Chinghao which means green herb). Other names are wormwood, Chinese wormwood, sweet wormwood, annual wormwood, annual sagewort, annual mugwort, and sweet sagewort. In the USA, it is well known as sweet Annie because after its introduction in the nineteenth century it was used as a preservative and flavouring and its aromatic wreath made a nice addition to potpourris and sachets for linens and the essential oil obtained from the flowering tops is used in the flavouring of vermouth [1]. The plant is now naturalised in many other countries such as Australia, Argentina, Brazil, Bulgaria, France, Hungary, Italy, Spain, Romania, the United States, and the former Yugoslavia [2].

Due to the presence of the unique sesquiterpene endoperoxide lactone artemisinin (Qinghaosu), one of the most important plant-derived drug in the treatment of the chloroquine-resistant and cerebral malarias, the plant is cropped on a large scale in China, Vietnam, Turkey, Iran, Afghanistan, and Australia. In India, it is cultivated on an experimental basis in the Himalayan regions, as well as temperate and subtropical conditions [3].

The essential oil which is rich in mono- and sesquiterpenes represents another source of potential commercial value [4]. Besides significant variations in its percentage and

Figure 1: Picture of *A. annua* flowers (from http://upload.wikimedia.org/wikipedia/commons/5/59/Artemisia_annua_detail.jpeg).

composition have been reported, it has been successfully subjected to numerous studies which mainly concern the antibacterial and antifungal activities. Diverse experimental studies have been reported to date using different methods and testing different microorganisms; therefore, a comparative analysis on a quantitative basis is very difficult. The aim of our review is to sum up data on antimicrobial activity of *A. annua* volatiles and its major components to facilitate future approach of microbiological experimental in this field.

2. Plant Distribution and Yield of the Volatiles

Essential (volatile) oil of *A. annua* can reach yields of 85 kg/ha. It is synthesised by secretory cells, especially of the uppermost foliar portion of the plant (top 1/3 of growth at maturity) which contains almost double number if compared with the lower leaves. It is reported that 35% of the mature leaf surface is covered with capitate glands which contain the terpenoidic volatile constituents. Essential oil from *A. annua* is distributed, with 36% of the total from the upper third of the foliage, 47% from the middle third, and 17% from the lower third, with only trace amounts in the main stem side shoots and roots. The yield of the oil generally ranges between 0.3 and 0.4% but it can reach 4.0% (V/W) from selected genotypes. Several studies have permitted the conclusion that *A. annua* crop could be harvested much before onset of flowering for obtaining high yields of artemisinin and the crop must be allowed to attain maturity to obtain high yields of the essential oil [5, 6].

Yield (herbage and essential oil content) can be increased with added nitrogen and the greatest growth was obtained with 67 kg N/ha. Increasing density of plants tended to increase essential oil production on an area basis, but the highest essential oil yields (85 kg oil/ha) were achieved by the intermediate density at 55,555 plants/ha receiving 67 kg N/ha. Finally the planting date and harvest time can influence the maximum concentration of the produced essential oil [6].

3. Chemical Profile of the Essential Oil

The essential oil, generally obtained by hydrodistillation of the flowering tops, analysed with GC-MS, revealed a great variability both in the qualitative and quantitative composition.

Chemical profile is generally influenced by the harvesting season, fertilizer and the pH of soils, the choice and stage of drying conditions, the geographic location, chemotype or subspecies, and choice of part plant or genotype or extraction method. In Table 1, the main constituents (>4%) of the investigated samples are reported.

Analysis of *A. annua* essential oils revealed the presence of mainly monoterpenoids and sesquiterpenes and the profiles showed great differences in the three main components, artemisia ketone, 1,8-cineole, and camphor, depending on the global phytogeographic origin. Oils can be grouped into the following:

(i) Vietnamese oil with 3.3–21.8% camphor and 0.3–18.9% germacrene D,

(ii) Chinese oil with high content of artemisia ketone (64%),

(iii) Indian oil with 11.5–58.8% of artemisia ketone,

(iv) French oil with 2.8–55% artemisia ketone, 1.2–11.6% 1,8-cineole, and 15% germacrene D,

(v) North American oil with 35.7–68% artemisia ketone and 22.8–31.5% 1,8-cineole,

(vi) Iranian oil with 48% camphor and 9.4% 1,8-cineole.

The presence of volatile oil is also reported in fruits and roots. Sesquiterpenes are the most abundant chemicals identified in the essential oil of the fruits; in particular, caryophyllene oxide (9.0%), caryophyllene (6.9%), (*E*)-β-farnesene (8.2%), and germacrene D (4.0%) are identified. However, only 52% of the total components were identified [7].

Upon hydrodistillation, the dried roots of *Artemisia annua* L. cultivar Jwarharti, a pleasantly fragrant essential oil, have been obtained with a yield of 0.25%. The oil was rich in sesquiterpenes and oxygenated sesquiterpenes and had *cis*-arteannuic alcohol (25.9%), (*E*)-β-farnesene (6.7%), β-maaliene (6.3%), β-caryophyllene (5.5%), caryophyllene oxide (4.4%), and 2-phenylbenzaldehyde (3.5%) as its major components [8].

Recently, the analysis of aromatic waters, obtained from plants collected at full blooming, showed the presence, among others, of camphor (27.7%), 1,8-cineole (14%), artemisia ketone (10.1%), α-terpineol (6.1%), *trans*-pinocarveol (5.4%), and artemisia alcohol (2%). From plants at the preflowering stage, aromatic waters gave camphor (30.7%), 1,8-cineole (12.8%), artemisia alcohol (11.4%), artemisia ketone (9.5%), alpha-terpineol (5.8%), and *trans*-pinocarveol (3.0%) as the main constituents. The qualitative and quantitative profiles of the two aromatic waters were similar [5].

4. Antimicrobial Activities of the Essential Oils

The essential oil of *Artemisia annua* has been the subject of numerous studies to test the antibacterial and antifungal activity. Tests were carried out both on the whole oil

Table 1: Compounds (>4%) isolated from essential oil of *Artemisia annua* L.

Compound	Country	%	Reference
Artemisia alcohol	China (Cult)	7.5	[20]
	USA-CA	5.2	[21]
	Serbia	4.8	[10]
Artemisia ketone	Not stated	38.0	[22]
	France	52.5	[23]
	Serbia	35.7	[10]
	Egypt	13.9	[15]
	China	2.21	[11]
	Bosnia	30.7	[9]
	USA-CA	35.7	[24]
	China (Cult)	63.9	[20]
	USA-IN	68.5	[25]
	England	61.0	[26]
	Vietnam	0.1–4.4	[27]
	Indian (Cult)	58.8	[28]
	India (Cult)	11.5	[29]
	Turkey	22	[19]
Borneol	Not stated	20.0	[22]
	England	7.0	[26]
	China (Cult)	15.9	[30]
Camphene	Iran	7	[13]
Camphene hydrate	USA-IN	12.0	[25]
Camphor	Vietnam	21.8	[3]
	Serbia	4.2	[10]
	Egypt	5.08	[15]
	France	27.5	[23]
	China (Cult)	21.8	[20]
	Vietnam (Cult)	3.3	[20]
	Bosnia	15.8	[9]
	Iran	1.92	[14]
	Italy	17.6	[5]
	Indian (Cult)	15.75	[28]
	India (Cult)	8.4	[29]
	France	43.5	[12]
	Iran	48	[13]
	Turkey	31	[19]
Trans-Cariophyllene	Egypt	7.73	[15]
β-Caryo phyllene	Italy	9.0	[5]
	Vietnam (Cult)	5.6	[20]
	Vietnam	3.3–8.6	[27]
	China (Cult)	5.98	[30]
	India (Cult)	12.2	[29]
	France	8.9	[12]
Caryophyllene oxide	China	5.13	[11]
Chrysanthenone	Vietnam	1.1–7.3	[27]
	India (Cult)	10.19	[28]
1,8-Cineol	France	11.66	[23]
	Serbia	5.5	[10]
	Egypt	8.13	[15]
	Bosnia	4.8	[9]
	USA-IN	22.8	[25]
	USA-CA	31.5	[21]
	Iran	9.4	[13]
	Iran	11.4	[14]
	Turkey	10	[19]
β-Farnesene	Italy	10.2	[5]
	Vietnam	1.1–12.8	[27]
	Egypt	5.32	[15]
	China (Cult)	12.9	[30]
Germacrene D	Vietnam (Cult)	18.3	[20]
	Italy	21.2	[5]
	Vietnam	0.3–18.9	[27]
	China (Cult)	10.9	[30]
	France	15.6	[12]
α-Guaiene	China (Cult)	4.7	[20]
Linalool	Vietnam	0.1–4.2	[27]
	Iran	8.1	[14]
Linalool acetate	England	10.0	[22]
Myrcene	China (Cult)	5.1	[20]
	USA-CA	4.6	[21]
	Vietnam	0.1–8.5	[27]
α-Pinene	USA-CA	11.2	[21]
	USA-IN	16.0	[25]
	Serbia	16.5	[10]
(*Trans*)-Pinocarveol	France	10.9	[12]
	Serbia	4.8	[10]
Sabinene	France	9.4	[12]
Spathulenol	Iran	4.97	[14]
	Iran	4.9	[13]

(Table 2) and on its principal components such as camphor, 1,8-cineol, α-pinene, and artemisia ketone (Table 3). The main gram-positive bacteria tested with *A. annua* volatiles obtained by hydrodistillation were *Staphylococcus aureus* [9–14], *Enterococcus hirae* [12], *Enterococcus faecalis* [14], *Streptococcus pneumoniae*, *Micrococcus luteus* [9], *Bacillus cereus* [14], *Sarcina lutea* [10], *Bacillus subtilis* [9, 11], *Bacillus thuringiensis* [11], *Bacillus* spp. [14], and *Listeria innocua* [15]. The gram-negative *Escherichia coli* [9, 11–14], *Escherichia coli* UPEC-Uropathogenic [14], *Escherichia coli* ETEC-Enterotoxigenic [16], *Escherichia coli* EPEC-Enteropathogenic [16], *Escherichia coli* EIEC-Enteroinvasive [16], *Escherichia coli* STEC-Shiga-toxin producer [16], *Shigella* sp. [10], *Salmonella enteritidis* [10], *Klebsiella pneumoniae* [10], *Haemophilus influenzae* [9], and *Pseudomonas aeruginosa* [9, 13, 14] were tested. Some strains

Table 2: Tests carried out on the whole oil.

Bacterial strains	Agar disk diffusion	Concentration	Reference	MIC	mg/mL	Reference	MBC	mg/mL	Reference
Gram-positive									
S. aureus	Not active		[12]						
				Active	32	[13]			
	Active	5.00%	[11]	Active	0.0156–0.0313	[11]			
	Active	10 mg/mL	[9]						
				Active	5.0–10.0	[10]	Low activity	>20.0–10.0	[10]
	Active	10 mg/mL	[14]	Active	0.031	[14]	Active	0.031	[14]
E. hirae	Active	0.1 mg/mL	[12]						
E. faecalis	Active		[9]						
	Active	10 mg/mL	[14]	Active	0.026	[14]	Active	0.031	[14]
S. pneumoniae	Active	10 mg/mL	[9]						
M. luteus	Not active		[9]						
B. cereus	Active	10 mg/mL	[14]	Active	0.053	[14]	Active	0.055	[14]
				Low activity	20	[10]	Low activity	20	[10]
B. subtilis	Active	5.00%	[11]	Active	0.00781-0.00781	[11]			
	Active	10 mg/mL	[9]						
B. thuringensis	Active	5.00%	[11]	Active	0.0313–0.0156	[11]			
B. sp.	Active	10 mg/mL	[14]	Active	0.026	[14]	Active	0.053	[14]
L. innocua	Not active		[15]						
Sarcina lutea				Active	2.5	[10]	Active	2.5	[10]
Gram-negative									
E. coli	Not active		[12]						
				Active	64	[13]			
	Active	5.00%	[11]	Active	0.0313-0.0313	[11]			
	Active	10 mg/mL	[9]						
				Low activity	20	[10]	Low activity	20	[10]
	Active	5 mg/mL	[14]	Active	0.017	[14]	Active	0.024	[14]
UPEC	Active	5 mg/mL	[14]	Active	0.026	[14]	Active	0.031	[14]
Shigella sp.				Low activity	20	[10]	Low activity	20	[10]
S. enteritidis				Active	5	[10]	Low activity	20	[10]
K. pneumoniae				Low activity	20	[10]	Low activity	20	[10]
H. influenzae	Active	10 mg/mL	[9]						
P. aeruginosa				Not active		[13]			
	Active	10 mg/mL	[9]						
	Active	10 mg/mL	[14]	Active	0.025	[14]	Active	0.053	[14]
Fungal strains	Agar disk diffusion	Concentration	Reference	MIC	mg/mL	Reference	MFC	mg/mL	Reference
C. albicans	Active	0.2 mg/mL	[12]						
				Active	2	[13]			
				Low activity	20	[10]	Not active	>20	[10]
C. krusei	Active	10 mg/mL	[9]						
S. cerevisiae	Active	0.2 mg/mL	[12]						
				Active	2	[13]			
A. fumigatus				Active	5	[10]	Active	5	[10]

Table 3: Tests on the main components of *A. annua* essential oil.

	Artemisia ketone		α-Pinene		1,8-Cineole		Camphor			α-Terpineol		
	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	MIC (mg/mL)	MBC (mg/mL)	Reference	MIC (%, v/v)	MBC (%, v/v)	Reference
Bacterial strains												
Gram- positive												
S. aureus	0.07–0.15	0.3–0.6	>10	>10	2.5–5	2.5–5	2.5–5	2.5–>10	[10]			
Sarcina lutea	2.5	10	1.25	2.5	0.6	1.25	2.5	2.5	[10]			
B. cereus	0.6	0.6	>10	>10	20	20	10	10	[10]			
Gram- negative												
E. coli	10	10	>10	>10	20	20	>10	>10	[10]			
Shigella sp.	0.6	0.6	>10	>10	10	20	>10	>10	[10]			
S. enteritidis	0.6	10	0.6	5	5	10	>10	>10	[10]			
K. pneumoniae	2.5	2.5	>10	>10	5	5	1.25	5	[10]			
Fungal strains												
C. albicans	10	10	>10	>10	5	20	5	5	[10]	0.25	0.5	[18]
C. glabrata										0.12	0.5	[18]
C. dubliniensis										0.12	0.25	[18]
C. krusei										0.12	0.5	[18]
C. guillermondii										0.12	0.25	[18]
C. parapsilosis										0.06	0.5	[18]
C. tropicalis										0.5	0.5	[18]
A. fumigatus	2.5	2.5	5	5	1.25	2.5	2.5	2.5	[10]			

of yeasts including *Candida albicans* [10, 12, 13], *Candida krusei* [9], and *Saccharomyces cerevisiae* [12, 13] and molds like *Aspergillus fumigatus* [10] were also tested (Table 2).

The main gram-positive bacteria tested with methanol, chloroform, ethanol, hexane, and petroleum ether extracts of *A. annua* were *Staphylococcus aureus* [14, 17], *Enterococcus faecalis* [14], *Micrococcus luteus* [17], *Bacillus cereus* [14, 17], *Bacillus subtilis* [17], *Bacillus pumilus* [17], and *Bacillus* sp. [14]. The gram-negative *Escherichia coli* [14, 17], *Escherichia coli* UPEC [14], *Salmonella typhi* [14, 17], and *Pseudomonas aeruginosa* [14, 17] were tested.

In addition, several single main components were investigated (Table 3), including α-terpineol [18] tested on *C. albicans*, *C. glabrata*, *C. dubliniensis*, *C. guilliermondii*, *C. krusei*, *C. parapsilosis*, and *C. tropicalis*; artemisia ketone, α-pinene, 1,8-cineole, and camphor [10] tested on *C. albicans*, *B. cereus*, *S. aureus*, *S. lutea*, *E. coli*, *K. pneumoniae*, *Ps. aeruginosa*, *S. enteritidis*, *Shigella* sp., and *A. fumigatus*.

The antifungal activity of the essential oil was also evaluated against economically important foliar and soil-borne fungal pathogens of tomato. The essential oil was active against *Sclerotinia sclerotiorum*, *Botrytis cinerea*, *Phytophthora infestans*, and *Verticillim dahliae* [19].

Different methods were used to evaluate the antibacterial and antifungal properties and included agar disk diffusion method [9, 11, 14, 17], minimal inhibition concentration (MIC) [9, 10, 12–14, 16–18], minimal bacterial concentration (MBC) [10, 14], and minimal fungicidal concentration (MFC) [10, 18] as reported in Table 2.

The results related to agar disk diffusion method (Table 2) show that some important pathogens are sensitive to *A. annua* essential oil obtained by hydrodistillation. *S. aureus*, *S. pneumoniae*, *E. coli*, UPEC, *H. influenzae*, *P. aeruginosa*, *C. albicans*, and *C. krusei* were inhibited by the action of the oil. *H. influenzae*, *S. pneumoniae*, and *C. krusei* were more sensitive; their inhibition zones diameters were >60, 50, and 30 mm, respectively. Satisfactory results were also achieved with genus *Bacillus*. On the contrary, *M. luteus* and *L. innocua* were resistant to this essential oil. Since the use of agar disk diffusion method is limited by the hydrophobic nature of most essential oils and plant extracts components that prevents their uniform diffusion through the agar medium, the most authors report the results obtained with MIC and MBC methods.

However, from the literature it is observed that the results obtained by agar disk diffusion method were confirmed by the liquid medium methods (MBC and MIC). At present there is no complete agreement on the concentration of the extracts to be considered active or inactive. Duarte and coworkers [16] proposed a classification to be applied to the extracts based on MIC values; this author considers MIC up to 500 μg/mL as strong inhibitors, MIC between 600 and 1500 μg/mL as moderate inhibitors, and MIC above 1600 μg/mL as weak inhibitors. In recent years, many different microbial species of medical interest have been tested from which emerged encouraging results except in the case of *E. coli* with special pathogenic characters (ETEC, EPEC, EIEC, and STEC) sensitive only at high concentrations of the extracts.

As concerns the results obtained against fungal strains, the data are rather limited. The results are contrasted against *C. albicans* but have to be more explored, while data related to *A. fumigatus* and *C. krusei* are encouraging.

Further studies have been performed with the main components present in *A. annua* essential oil (see Table 3). These studies show that artemisia ketone is the component of the oil that has the greatest antimicrobial activity; in fact, it always turns out to be effective against bacteria and some fungi (*C. albicans* and *A. fumigatus*) at very low concentrations (range 0.07–10 mg/mL). The other compounds tested in the studies have produced variable results; however, it should be emphasized the fact that all the compounds tested by liquid methods were active (range 1.25–5 mg/mL) against *A. fumigatus*, a dangerous microorganism frequently responsible for nosocomial infections in immunosuppressed subjects.

5. Concluding Remarks

During the last decade several authors have evaluated the antimicrobial activity of *Artemisia annua* and some of its main components. The composition of the essential oil shows great differences in the three main characteristic components, namely, artemisia ketone, 1,8-cineole, and camphor, depending on the global phytogeographic origin. Besides the different chemical profiles, artemisia essential oil has revealed strong antimicrobial properties towards numerous bacterial strains, both gram-positive and gram-negative, and diverse fungal strains, including many pathogens. Biological effects are the result of a synergism of all molecules contained in an essential oil, even if it is possible that the activity of the main components is modulated by other minor molecules, but the activity of the isolated constituents is also remarkable. *Artemisia annua* volatile constituents appear to be a resource of many biologically active compounds which will hopefully give new economically important by-product. The good results obtained encourage further researches aiming at a possible application of these substances in food and pharmaceutical and cosmetology fields.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Chemical Composition, Antioxidant and Antimicrobial Activities of *Thymus capitata* Essential Oil with Its Preservative Effect against *Listeria monocytogenes* Inoculated in Minced Beef Meat

Nariman El Abed,[1] Belhassen Kaabi,[2] Mohamed Issam Smaali,[1] Meriem Chabbouh,[3] Kamel Habibi,[4] Mondher Mejri,[4] Mohamed Nejib Marzouki,[1] and Sami Ben Hadj Ahmed[1]

[1] *Laboratory of Protein Engineering and Bioactive Molecules (LIP-MB), Institut National de Sciences Appliquées et de Technologie (INSAT), BP 676, 1080 Tunis Cedex, Tunisia*
[2] *Laboratoire d'Epidémiologie et d'Ecologie des Parasites, Institut Pasteur de Tunis, BP 74, 1002 Tunis Belvédère, Tunisia*
[3] *Institut National Agronomique de Tunisie, 1002 Tunis, Tunisia*
[4] *Institut Supérieur des Etudes Technologiques de Zaghouan, 1121 Mograne, Tunisia*

Correspondence should be addressed to Belhassen Kaabi; belhassen.kaabi@pasteur.rns.tn and Sami Ben Hadj Ahmed; s_benhadj@yahoo.fr

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The chemical composition, antioxidant and antimicrobial activities, and the preservative effect of *Thymus capitata* essential oil against *Listeria monocytogenes* inoculated in minced beef meat were evaluated. The essential oil extracted was chemically analyzed by gas chromatography-mass spectrometry. Nineteen components were identified, of which carvacrol represented (88.89%) of the oil. The antioxidant activity was assessed *in vitro* by using both the DPPH and the ABTS assays. The findings showed that the essential oil exhibited high antioxidant activity, which was comparable to the reference standards (BHT and ascorbic acid) with IC_{50} values of 44.16 and 0.463 μg/mL determined by the free-radical scavenging DPPH and ABTS assays, respectively. Furthermore, the essential oil was evaluated for its antimicrobial activity using disc agar diffusion and microdilution methods. The results demonstrated that the zone of inhibition varied from moderate to strong (15–80 mm) and the minimum inhibition concentration values ranged from 0.32 to 20 mg/mL. In addition, essential oil evaluated *in vivo* against *Listeria monocytogenes* showed clear and strong inhibitory effect. The application of 0.25 or 1% (v/w) essential oil of *T. capitata* to minced beef significantly reduced the *L. monocytogenes* population when compared to those of control samples (P-*value* < 0.01).

1. Introduction

The problems of spoilage and food poisoning, mainly by oxidation processes or by microorganism activity, during production and storage are still concerns for both the food industry and consumers, despite the use of synthetic chemical additives and various preservation methods [1–3]. However, the side effects of some synthetic antioxidants used in food processing, such as butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA), have already been documented. They showed carcinogenic effects in living organisms [4, 5]. Consequently, there has been increasing interest in developing new types of effective and nontoxic natural antioxidant and antimicrobial compounds both to prevent the growth of food- borne and spoiling microbes and to extend the shelf-life of foods [6, 7]. In this context, medicinal and aromatic plants have emerged as an alternative to synthetic products, used not only in traditional medicine

but also in a number of food and pharmaceutical products, due to their high content of phenolic compounds, their nutritional properties, and bioactivity [8].

Thymus capitata is a Mediterranean herb of the Lamiaceae family that grows mainly in northern Tunisia [9]. This species is an aromatic plant, mostly used (fresh or dried) as a spice, in some Tunisian traditional meat dishes, both for its preservative qualities and its savory taste. In Tunisian folk medicine, *Thymus* species are well known as medicinal plants because of their biological and pharmacological properties, which include antiasthmatic, antiseptic, antimycotic, spasmolytic, anti-inflammatory, antimicrobial and, antioxidant activities [9–12]. Recently, *Thymus* species essential oils (EOs) and their components gained increasing importance because of their wide acceptance by consumers and other exploitations and potential multipurpose functional use [9].

Generally, the essential oils (EOs) are aromatic and volatile liquids extracted from plant materials, such as flowers, roots, bark, leaves, seeds, peel, fruits, wood, and whole plant. They are considered to be plant secondary metabolites, which play an important role in plant defense as they often possess antimicrobial and antioxidant properties [13–15]. For these reasons, EOs have been primarily used, in the food industry, as flavoring agents in food system and can be used as natural antimicrobials in food preservation (extending shelf-life) [15, 16] against a wide range of food spoiling microbes.

Previous phytochemical studies of the genus *Thymus* EOs have reported the presence of a number of bioactive compounds, including carvacrol, thymol, *p*-cymene, and γ-terpinene, which have been reported to have many biological activities [3, 9, 11]. Figueiredo et al. in 2008 [11] have demonstrated that EOs of Portuguese *T. capitata* presented great chemical homogeneity characterized by a relatively high amount of carvacrol.

In addition, to our knowledge, there are no published studies that have evaluated the preservative effect of *T. capitata* EO against *L. monocytogenes* in minced meat, the causative agent of listeriosis, one of the most virulent foodborne diseases. Human infection predominantly occurs as a result of occasional contamination of ready-to-eat and raw food products, particularly meat products [17, 18]. Listeriosis has been associated with a mortality rate as high as 30–40% [19]. The ubiquitous prevalence of this pathogen in nature, its ability to proliferate at temperature near 0°C, and its resistance to certain preservatives has resulted in an extensive effort to develop processes to control its growth in foods [20].

Today, different strategies are applied in order to control pathogens in meats, and interest has been focused on the application of EOs as a safe and effective alternative to chemical preservative. Their application in controlling pathogens could reduce the risk of foodborne outbreak and assure consumers safe meat products. The chemical composition and antimicrobial properties of EOs extracted from diverse plant species have been demonstrated using a variety of experimental methods [21, 22].

The purposes of the present work are (i) to evaluate the chemical composition of Tunisian *T. capitata* EOs by GC-MS and compare it to previous published works, (ii) to confirm *in vitro* the antioxidant activity of this EO, and (iii) to assess *in vitro* its antimicrobial activities against a selected group of bacteria strains. Besides, this study was also designed to determine the efficacy of *T. capitata* EO in inhibiting *L. monocytogenes* growth in model minced beef meat during refrigerated storage.

2. Materials and Methods

2.1. Materials and Chemicals. Chemicals: 2,2-diphenyl-2-picrylhydrazyl hydrate (DPPH), 2,6-di-tert-butyl-4-methylphenol (BHT), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), ascorbic acid, dimethyl sulfoxide (DMSO), potassium persulfate, and all reagents were purchased from Sigma (St. Louis, MO, USA), Fluka Chemie (Buchs, Switzerland), and Merck (Nottingham, UK).

2.2. Plant Materials. The aerial parts of *T. capitata* were collected from Zaghouan region (north Tunisia) in June 2010. The samples species were identified and confirmed by a specialist in botany. The freshly cut plants were sorted out and dried in the shade at ambient temperature for two weeks. Dried samples were grounded into powder, packed in paper bags, and stored in the dark in a dry place.

2.3. Preparation of the Essential Oils. The dried powder aerial parts of plant were submitted to hydrodistillation process in a clevenger-type apparatus for 3 hours according to the method recommended in the current European Pharmacopoeia 6.0 in 2008 [23]. The EO collected was then dried over anhydrous sodium sulphate (Na_2SO_4), filtered, and stored at 4°C in the dark for further use.

2.4. Chemical Composition of Essential Oil

2.4.1. Apparatus. GC-MS analysis of the essential oil was carried out with Hewlett Packard 7890 A GC equipped with a 5975 mass selective detector and an HP-5 MS capillary column (30 m × 0.25 mm id, film thickness 0.25 μm). For GC/MS detection, the ion source was set to 230°C with electron ionization energy of 70 eV. Scanning range was varied from 40 to 550 atomic mass units (amu). Helium was used as the carrier gas at a flow rate of 0.8 mL/min. One μL of diluted oil in hexane (1/100, v/v) was injected manually in splitless mode. The oven program temperature was programmed from 60°C to 250°C with a rate of 4°C/min and then held constant for 5 min.

2.4.2. Qualitative and Quantitative Analyses of EO. The identification of the chemical compounds of EO was based on mass spectral library (Wiley 275.L, 8th edition) and/or with standards when available and confirmed by comparison of their GC retention indices either with those of authentic standards injected under the same chromatographic conditions or with data published in the literature, as described by Adams in 2007 [24].

2.5. Quantification of Total Antioxidant Activity. The literature outlines different approaches for the determination of

the antioxidant activities of the plant extracts. Therefore, generally different methodological approaches lead to scattered results, which are hardly comparable and sometimes conflicting [25, 26]. For that reason, we combined two complementary techniques, based on DPPH and ABTS free radical-scavenging activity.

2.5.1. DPPH Radical-Scavenging Assay. Radical-scavenging activity (RSA) of plant extracts against stable DPPH was determined by spectrophotometry. EOs extracts at different concentrations (0.1; 0.25; 0.5; 1; 5; 10; 50; 100; 200 μg/mL) were mixed with the same volume of 0.2 mM methanolic DPPH solution. Samples were kept in the dark for 30 min at room temperature, and absorption-decrease was measured. Absorption of negative control containing the same amount of methanol and DPPH solution was prepared and measured in the same time. The experiment was carried out in triplicate. RSA of extracts was measured by the method described by Brand-Williams et al. in 1995 [27] but slightly modified as shown below:

$$\text{Inhibition } \% = \left[\frac{AB - AA}{AB}\right] \times 100, \quad (1)$$

where AB is AB absorption of blank sample at $t = 0$ min and AA is the tested sample absorption at $t = 30$ min.

The antioxidant activity was also expressed as IC_{50}, which was defined as effective concentration of the sample (in μg/mL) at which 50% of DPPH radicals are scavenged. BHT and ascorbic acid were used as positive control. Each assay was repeated 3 times. The average result and standard deviation were reported.

2.5.2. ABTS Activity. ABTS radical-scavenging activity of EOs was determined according to Re et al. in 1999 [28]. The ABTS solution was diluted with methanol, to absorbance of 0.7 at 734 nm. After the addition of 950 μL of diluted ABTS solution to 50 μL of plant EOs, the mixture was incubated at 37°C for 10 min, and then the absorbance was measured at 734 nm. Tests were carried out in triplicate. Butylated hydroxytoluene (BHT) and ascorbic acid (AA) were used as positive controls.

The ABTS radical-scavenging activity of the sample was calculated by the following equation:

$$\text{Inhibition } (\%) = \left[\frac{\text{Abs Control} - \text{Abs Sample}}{\text{Abs Control}}\right] * 100, \quad (2)$$

where Abs control is the absorbance of ABTS radical + methanol and Abs sample is the absorbance of ABTS radical + sample (EO/standard).

Sample concentration providing 50% inhibition (IC_{50}) was obtained plotting the inhibition percentage against sample concentrations.

2.6. Antimicrobial Screening

2.6.1. Microorganisms and Growth Conditions. The EO was tested against a large panel of microorganisms. Bacteria were obtained from international culture collections ATCC and the local culture collection of *Pasteur Institute of Tunis*. They included 8 Gram-positive bacteria and 16 Gram-negative bacteria (Table 1). The bacterial strains were cultivated in Luria Bertani Medium (LB) (Oxoid Ltd., UK) at 37°C except for *Bacillus* species, which were incubated at 30°C. Working cultures were prepared by inoculating a loopful of each test bacteria in 5 mL of Luria Bertani Medium (LB) (Oxoid Ltd., UK) and incubated at 37°C for 18 hours.

2.6.2. Disc-Diffusion Method. The paper disc-diffusion method was employed for the determination of EO antimicrobial activity [29]. Briefly, suspension in LB of the tested microorganism (0.1 mL of 10^7-10^8 cells per mL) was spread on the solid LB media plates. Paper discs (9 mm in diameter) were individually impregnated with 12 μL of the oil and then placed on the inoculated plates. We did not use the DMSO to facilitate the solubilization of EO in LB-Agar. However, in order to accelerate diffusion of the essential oil, plates were placed at 4°C for 2 hours and were then incubated at 37°C for 24 hours. The diameters of the inhibition zones were measured in millimeters. All tests were performed in duplicate and repeated three times. Streptomycin B (15 μg/mL) and chloramphenicol (30 μg/mL) were used as positive controls.

2.6.3. Determination of the Minimum Inhibitory Concentration. The Minimal Inhibitory Concentrations (MICs) of the EO against the tested microorganisms were determined by the broth microdilution method [30]. All tests were performed in LB, supplemented with DMSO (the highest final concentration 0.1%). Microbial strains were cultured overnight at 37°C and were suspended in LB medium to give a final density of 5×10^5 CFU/mL, which was confirmed by viable counts. Geometric dilutions ranging from 0.039 mg/mL to 20 mg/mL of the EOs were prepared in 96-well microtiter plate (Iwaki brand, Asahi Techno Glass, Japan), including one growth control (LB+DMSO), and one sterility control (LB+DMSO+ test oil). Plates were subsequently incubated under normal atmospheric conditions at 37°C for 24 hours and under vigorous agitation. The wells were then examined for evidence of growth indicated by the presence of white "pellets" on their bottoms. MICs values were determined as the lowest EO concentration that inhibited visible growth of the tested microorganism. The negative controls were set up with DMSO in amounts corresponding to the highest quantity present in the test solution (0.1%). The tests were performed three times.

2.7. Inhibitory Effect of the EO against Listeria Inoculated in Minced Beef Meat. The *in situ* efficacy of the EO was evaluated against *L. monocytogenes* in a minced beef meat model according to the procedure described by Careaga et al. in 2003 [31] but with a slight modification.

Table 1: Bacteria strains used.

Gram-negative bacteria	Gram-positive bacteria
Escherichia coli ATCC 25922	*Enterococcus faecalis* ATCC 11700
Enterobacter cloacae ATCC 13097	*Listeria monocytogenes* ATCC 19118
Proteus mirabilis ATCC 29906	*Staphylococcus aureus* ATCC 6538
Pseudomonas aeruginosa ATCC 27853	*Staphylococcus aureus* ATCC 25923
Pseudomonas aeruginosa ATCC 9027	*Staphylococcus aureus* ATCC 6538
Salmonella enteritidis ATCC 502	*Streptococcus pyogenes* ATCC 12344
Salmonella salamae ATCC 6633	*Bacillus cereus* ATCC 11778
Salmonella typhimurium ATCC 14028	*Bacillus cereus* (food isolate)
Shigella flexneri ATCC 29903	*Bacillus subtilis* (food isolate)
Yersinia enterocolitica ATCC 23715	
Klebsiella oxytoca (clinical isolate)	
Morganella morganii (clinical isolate)	
Pseudomonas aeruginosa (clinical isolate)	
Salmonella anatum (food isolate)	
Shigella sonnei (clinical isolate)	
Vibrio cholerae (clinical isolate)	

2.7.1. Preparation of Meat Beef. Freshly postrigor lean beef muscles were obtained from a slaughter house in Tunis,Tunisia. Each piece was immersed in boiling water for 5 min, in order to reduce the number of the microorganisms attached to the beef muscle surface. The cooked surface of the muscle was eliminated with sterile knives under aseptic conditions.

2.7.2. Treatment of Minced Beef. Prior to minced beef contamination with *Listeria monocytogenes* and the addition of EO, beef muscles were also examined for any contamination by bacteria (aerobic psychrotrophic flora) and the tested pathogens (results not shown). In order to evaluate the antimicrobial activity of *T. capitata* EO in a meat beef sample, the pieces of meat prepared as above were minced in a sterile grinder, and portions of 25 ± 0.1 g were put in high-density polyethylene bags. The meat samples were inoculated with *L. monocytogenes in concentration of* 10^5 CFU/g of meat and mixed homogeneously for 3 min at room temperature to ensure proper distribution of the pathogen. Following homogenization, the *T. capitata* EO was dissolved in 10% DMSO and was subsequently added at different concentrations (0.02; 0.06; 0.1; 1; 1.5; 2 and 3 % (v/w)) to the inoculated samples. To obtain uniform distribution of the added compounds, treated meat samples were then homogenized by means of a Stomacher 400 Seward (London,UK) used at a normal speed for 5 min. All bags containing these samples of meat were stored at 7°C and examined at 0, 3, 6, 9, 12, and 15 days of storage for *L. monocytogenes* enumeration. The untreated samples (controls) were added to sterile water (instead of EO), inoculated with the test bacteria, and stored under the same conditions as the tested samples. Three replicates of each experiment were performed in all cases.

2.7.3. Bacterial Enumeration. A microbiological analysis was performed on the meat, with the aim to assess quantitatively and qualitatively the background microflora. *L. monocytogenes* count was done adding 250 mL of Muller-Hinton broth to the 25 g in the polyethylene bag. The samples were homogenized for one min and incubated at 37°C for 6 hours. From this pre-enrichment, the *L. monocytogenes* was determined by the plate colony count technique. After serial 10-fold dilution with physiological saline solution, 100 μL of each sample was spread onto surfaces of the Muller-Hinton agar medium followed by incubation at 37°C for 24 hours. Sterile saline water was added to the untreated control, inoculated with the test bacteria instead of *T. capitata* EO stored under the same conditions as the other samples.

2.8. Statistical Analysis. The inhibitory concentration 50% (IC50 values) for antioxidant activities was calculated by non-linear regression analysis using the Graphpad Prism version 5.0. The dose-response curve was obtained by plotting the percentage of inhibition versus the concentrations. Correlations between inhibition activity and EO concentration were evaluated using Spearman's correlation test [32]. Statistical significance of the differences between the treated and the control sample means was evaluated by Welch 2-sample t-test. Repeated ANOVA test [33] was used to check overall difference in activity tendency and EO concentration effect. A P value < 0.05 was considered to imply significance; however, corrections for multiple testing were carried out when necessary. All computations were performed using The R software 2.11 version (http://www.r-project.org/).

3. Results and Discussion

3.1. Chemical Composition of the Extracted Essential Oils. Table 2 shows the chemical constituents, their relative percentage of the total chromatogram area and Kovats index of *T. capitata* EO.

GC-MS analysis of the volatile constituents of the EO allowed the identification of 19 compounds representing 98.97% of the total oil. Carvacrol was the major one with 88.98%. The other identified components were minor. These results are in line with those reported by Napoli et al. [34]. The chemical composition of this EO showed that it is rich in oxygen containing monoterpenes (94.98%). Monoterpene hydrocarbons or both sesquiterpene and oxygen containing sesquiterpene were represented at about 2% each. This wealth of oxygen-containing monoterpenes (OM), especially carvacrol, can enhance the value of this EO as an active natural product. The major product carvacrol was described as a strong antibacterial molecule [9, 11] and it is now considered one of the products singled out for their pharmacological

Table 2: Chemical composition of the essential oil isolated from the aerial parts of *Thymus capitata* from Zaghouan region (Tunisia).

Compounds	Retention time	%[a]	RI[b]	Method of identification[c]
1-Octen-3-ol	6.434	0.25	987.179	RI, MS
Beta-myrcene	6.749	0.11	1020.159	RI, MS
α-Terpinen	7.481	0.15	1018.808	RI, MS
p-Cymene	7.699	1.14	1026.317	RI, MS
γ-Terpinene	8.677	0.40	1060.006	RI, MS
Sabinene hydrate	8.946	0.09	1069.27	RI, MS
Linalol	9.902	1.57	1101.99	RI, MS
Borneol	12.070	1.06	1169.64	RI, MS
Terpinen-4-ol	12.431	1.41	1180.90	RI, MS
α-Terpineol	12.866	0.29	1194.47	RI, MS
Trans-dihydrocarvone	13.066	0.11	1200.07	RI, MS
Beta-citral	14.474	0.24	1243.84	RI, MS
Carvone	14.600	0.18	1247.70	RI, MS
Citral	15.481	0.33	1274.69	RI, MS
Thymol	16.202	0.51	1296.78	RI, MS
Carvacrol	16.688	**88.98**	1311.93	RI, MS
Caryophyllene	20.264	0.63	1425	RI, MS
Caryophyllene epoxide	25.191	1.08	1589.88	RI, MS
Dodecyl acrylate	28.144	0.44	1695.47	RI, MS
Total		**98.97**		

(2) Compounds are listed according to their elution on HP-5MS capillary column.
[a]Peak area of essential oil components.
[b]Kovats retention indices relative to C_9–C_{20} *n*-alkanes on the HP-5MS capillary column.
[c]Components were identified based on their KI on HP-5MS capillary column and GC-MS data.

effects. These results are in accordance with previous studies [35, 36], which demonstrated that carvacrol was the main compound of *T. capitata* oils with 75% and 65.8%, respectively.

On the other hand, there are many reports on the chemical composition of other oils isolated from the plants belonging to the genus of *thymus*. Tomaino et al. in 2005 [37] reported that the major constituents of thyme EO were carvacrol, thymol, and *p*-cymene and they can reach the following percentages: 48.9%, 45.3%, and 26.19%, respectively, while Jaafari et al. in 2007 [38] found that these same constituents are the main components in thyme EO from Morocco and can hit the following percentages: 85%, 42%, and 23%, respectively. These variations in the composition of the EO could be due to factors such as plant age, plant part, development stage, the geographical localization, harvesting period, temperature, and environmental factors prevailing in the Mediterranean regions and principally by chemotype since they influence the plant biosynthetic pathways and consequently, the relative proportion of the main characteristic compounds [39].

3.2. Antioxidant Activity. Two complementary colorimetric methods, namely the DPPH and ABTS assays are compared to the reference standards butylated hydroxyl toluene (BHT) and ascorbic acid (AA), and the results are presented in Figure 1. The DPPH and the ABTS radicals are the two most widely used and stable chromogen compounds to measure the antioxidant activity of biological material [40]. In addition, the model of the DPPH radical-scavenging and ABTS radical cation decolorization assay can be used to evaluate the antioxidant activities in a relatively short time compared with other methods [41, 42]. In the present study, the capacity of the EO to scavenge the free radicals $DPPH^{\bullet}$ and $ABTS^{+\bullet}$ and their reducing power was determined on the basis of their concentration providing 50% inhibition (IC_{50}) and the lower IC_{50} value reflects high radical-scavenging activity [43].

3.2.1. DPPH Free Radical-Scavenging Activity. The effect of antioxidant on DPPH radical-scavenging was conceived to their hydrogen-donating ability [44]. DPPH is a stable free radical that accepts on electron or hydrogen radical to become a stable diamagnetic molecule [43].

From the analysis of Figure 1(a), we can conclude that the radical-scavenging activity of the EO and positive controls increased with increasing concentration (Spearman correlations $r = 0.856$ with P values < 0.0001). Furthermore, the results obtained in this study indicated that the *T. capitata* EO exhibited a high DPPH radical-scavenging activity and its percentage inhibition reached 85.44 ± 1.06% at a concentration of 200 μg/mL. The graph (Figure 1(a)) showed that the radical-scavenging activity of *T. capitata* EO was 44.16 ± 0.809 μg/mL, which appeared lower than of synthetic antioxidants BHT and ascorbic acid, with values of IC_{50} = 39.97 ± 1.64 μg/mL and 1.136 ± 0.305 μg/mL, respectively.

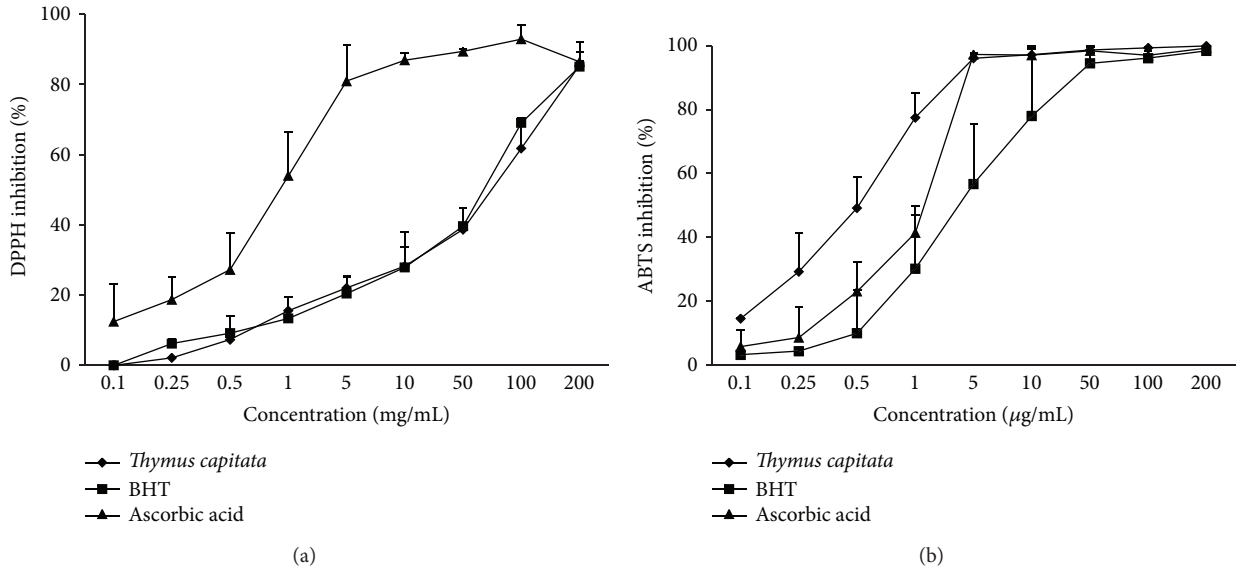

Figure 1: The antioxidant activities of *Thymus capitata* essential oil as determined by DPPH (a) and ABTS (b) free radical-scavenging activity. The absorbance values were converted to scavenging effects (%) and data plotted as the means of replicate scavenging effect (%) values. (Results are expressed as means ± standard deviation of three measurements.)

3.2.2. ABTS Free Radical-Scavenging Activity. Similar to DPPH, the decolorization of ABTS radical reflects the capacity of an antioxidant species to donate electron or hydrogen atoms to inactivate this radical cation [45]. The ABTS results were in good agreement with DPPH method that the scavenging activity of the EO was increased with the increasing concentration (Spearman correlations $r = 0.89$ with P values < 0.0001). From the analysis of Figure 1(b), we can conclude that the *T. capitata* EO exhibited higher ABTS radical-scavenging activity (99.98 ± 0.01%), which was comparable to that of BHT (98.46 ± 0.95%) and ascorbic acid (99.33 ± 0.59%) for the same concentration 200 μg/mL. These findings were confirmed by calculating the IC_{50} values for the *T. capitata* EO ($IC_{50} = 0.463 \pm 0.122\,\mu$g/mL), which was found to be significantly ($P < 0,05$) better than that of BHT ($IC_{50} = 3.204 \pm 3.541\,\mu$g/mL) and ascorbic acid ($IC_{50} = 1.126 \pm 0.19\,\mu$g/mL). These results are in agreement with previous studies [46, 47], which showed that greater antioxidant potential of several *Thymus* species EOs could be related to the nature of phenolic compounds and their hydrogen ability. Besides, it could be ascribed to the oxygenated types of compounds, such as carvacrol and thymol [26, 48]. Moreover, the activities of EOs of *Thymus* species depend on several structural features of the molecules and are primarily attributed to the high reactivity of hydroxyl group substituent [49].

Scavenging the ABTS radical by the *T. capitata* EO was found to be much higher than that of DPPH radical. These differences can be explained by the mechanism of the involved reaction. The ABTS radical reactions involve electron transfer and take place at a much faster rate compared to DPPH radicals [50]. Furthermore, various factors like stereoselectivity of the radicals or the solubility of the tested sample in different testing systems and functional groups present in the bioactive compounds have been reported to affect the capacity of the sample to react and quench different radicals [51]. Wang et al. in 1998 [52] showed that some compounds which have $ABTS^+$ scavenging activity may not show DPPH scavenging activity.

3.3. Antimicrobial Activity. In the present study, the *in vitro* antimicrobial activities of *T. capitata* EO against the studied microorganisms were qualitatively and quantitatively assessed by the presence or absence of inhibition zones and MIC values, respectively (Table 3). The results obtained from the disc- diffusion method indicated that EO exerted a strong antibacterial activity against all tested strains. Results were comparable to those of the antibiotics (chloramphenicol and streptomycin), used as positive controls. The size of the inhibition zone of *T. capitata* EO varied from 15 to 80 mm, while the inhibition zones of the chloramphenicol and streptomycin ranged from 18–27 mm to 12–22 mm, respectively.

Referring to the large inhibition zones observed with disk-diffusion method for *T. capitata* EO, the MIC values were determined by the microdilution broth assay (Table 3). The results of the MIC values against tested Gram-positive and Gram-negative bacteria varied from 0.32 to 5 mg/mL and from 0.63 to 20 mg/mL, respectively. We found that the antibacterial activity of the EO depends on its concentration and the tested bacteria strain. Interestingly, we have found that *Staphylococcus aureus* ATCC 6538 is the most sensitive tested microorganism, with the lowest MIC value (0.32 mg/mL), and it was closely followed by *Bacillus cereus* ATCC 11768. This antimicrobial spectrum obtained with the EO of *T. capitata* is comparable in most cases to the one reported by Bounatirou et al. in 2007 [9]. In addition, *Vibrio cholerae* (clinical isolate) is the most sensitive Gram-negative

Table 3: Antibacterial activity of essential oil from *Thymus capitata*, using paper disc-diffusion method and microdilution test.

Strains	Disc-diffusion method (DD)			MIC
	Thymbra capitata (L.)	Antibiotics		*Thymbra capitata* (L.)
		a	b	
Pseudomonas aeruginosa ATCC 27853	23	21	12	10
Pseudomonas aeruginosa ATCC 9027	15	19	13	20
Pseudomonas aeruginosa (clinical isolate)	17	22	16	20
Escherichia coli ATCC 25922	70	NA	12	2.5
*Enterococcus faecalis*ATCC 11700	60	20	14	2.5
Enterobacter cloacae ATCC 13097	80	18	13	5
Salmonella typhimurium ATCC 14028	50	22	15	2.5
Salmonella enteritidis ATCC 502	80	21	13	5
Salmonella salamae ATCC 6633	75	22	15	5
Salmonella anatum (food isolate)	80	20	18	2.5
Shigella flexneri ATCC 29903	80	18	15	2.5
Shigella sonnei (clinical isolate)	80	20	14	1.25
Staphylococcus aureus ATCC 2592	20	20	22	5
Staphylococcus aureus ATCC 6538	75	23	NT	0.32
Streptococcus pyogenes ATCC 12344	75	21	NT	2.5
Listeria monocytogenes ATCC 19118	70	23	16	5
Morganella morganii (clinical isolate)	75	NT	NT	1.25
Klebsiella oxytoca (clinical isolate)	70	21	15	2.5
Vibrio cholerae (clinical isolate)	80	NT	NT	0.63
Yersinia enterocolitica ATCC 23715	80	NT	NT	10
Proteus mirabilis ATCC 29906	45	NT	NT	5
Bacillus cereus ATCC 11768	50	20	16	0.63
Bacillus cereus (food isolate)	80	NA	NA	1.25
Bacillus subtilis (food isolate)	70	27	15	5

(3) Disc-diffusion method. Inhibition zone in diameter around the discs impregnated with 12 μL of essential oil. The diameter (9 mm) of the disc is included. MIC: minimal inhibitory concentration; values given as mg/mL for the essential oils.
a: Chloramphenicol (30 μg/μL); b: streptomycin B (10 μg/μL); NT: not tested; NA: not active.

bacteria with the lowest MIC value (0.63 mg/mL). Our results confirmed that Gram-positive bacteria were more susceptible to the antimicrobial properties of EO than Gram-negative ones. These differences could be attributed in part to the great complexity of the double membrane-containing cell envelope in Gram-negative bacteria compared to the single membrane structure of the positive ones [53, 54]. These differences may be attributed also to the presence of the lipopolysaccharides in the outer membrane of the Gram-negative bacteria, which make them inherently resistant to external agents, such as hydrophilic dyes, antibiotics, detergents, and lipophilic compounds [14, 55]. However, the ability of EOs to disrupt the permeability barrier of cell membrane structures and the accompanying loss of chemiosmotic control is the most likely reason for its lethal action [56]. The EOs can coagulate the cytoplasm and damage lipids and proteins [3]. Their mechanism of action would be similar to other phenolics, that is, the disturbance of the proton motive force, electron flow, active transport, and coagulation of cell contents. Instead, enzymes such as ATPases are known to be located in the cytoplasmic membrane and to be bordered by lipid molecules [3, 13].

Generally, antimicrobial activities of the EOs are difficult to correlate with a specific compound due to their complexity and variability; nevertheless, some investigators reported that there is a relationship between the chemical composition of the most abundant components in the EO and the antimicrobial activity [57, 58]. In the present study, carvacrol was the main component of *T. capitata* EO. It has been reported to be biocidal, resulting in bacterial membrane perturbations that lead to leakage of intracellular ATP and potassium ions and ultimately cell death [59, 60]. Previous studies [61] mentioned that carvacrol at concentrations of 0.5% and 1% shows antibacterial activity against *Shigella sonnei* and *Shigella flexneri*. Besides, it has been reported that carvacrol causes perturbation in the bacterial membrane and thus potentially can exert antibacterial activity also at intracellular sites [60, 62]. These results are in accordance

with the earlier findings [12, 54] that showed that *Thymus* species' essential oils rich in carvacrol were demonstrated to be potent antimicrobial *in vitro*.

However, other constituents, such as terpinene and *p*-cymene have been shown to display relatively good activity due to their potential synergistic or antagonistic effects [10, 63].

3.4. The Effects of the EOs on L. monocytogenes Inoculated in Minced Beef Meat. In this part of our work, we studied *in vivo* the anti-*Listeria* activity of different concentration of *T. capitata* EO when inoculated in minced beef meat, as well as the effect of EO on the extension of shelf life and the preservation of the freshness of meats. It is well known that not all microbiologists demonstrated that decontamination of meat is required or even desirable. It has been argued by Jay in 1996 [64] that high levels of indigenous nonpathogenic microorganisms may have a protective effect on meat and its products, by out-competing the pathogens. Despite this fact, our samples were decontaminated in order to reduce the number of factors involved in the microorganisms' growth in such food model and to avoid interferences of colonies on plating agar. The bacteria count, which is related to survival time of *L. monocytogenes* in our processed food model following treatment with various concentrations (0.01; 0.05; 0.25, and 1.25% (v/w)) of *T. capitata* EO, was presented in Figure 2. Results showed that the initially recorded population of *Listeria monocytogenes* in untreated samples (control) increased approximately from 5log CFU/g to 7.13 log CFU/g during 15 days of storage. However, data from each of the four preparations showed a gradual decrease in the bacteria count with the increasing EO concentration. It appears that the used concentrations are higher than those applied for the *in vitro* tests. This cannot be misleading, because it is well established that intrinsic factors such as composition (e.g., proteins, fat) as well as extrinsic factors (temperature, oxygen limitation) of the food affect the behavior of bacteria in food ecosystems and may act synergistically with preservatives such as antimicrobial agents [32]. Indeed, food components, such as proteins and fat, are known to bind and/or solubilized phenolic compounds, reducing their availability for antimicrobial activity. Furthermore, it has been reported by many authors that antimicrobial activity of spice is lower in food systems than in microbiological media [65].

Indeed, a reduction of 4×log/g in the level of *L. monocytogenes* was recorded in 3 days of storage with a concentration of 0.25 or 1.25% (v/w) of *T. capitata* EO, compared to the control (not treated), and those treated either with a concentration of 0.01 or 0.05% (v/w) of *T. capitata* EO. The differences in the values were statistically significant (P values < 0.001). Thus, at the end of experimentation (15 days of storage), bacteria count in minced beef treated with a concentration of 0.25 and 1.25% (v/w) of *T. capitata* EO decreased and reached 1.45 and 1.13×log CFU/g, respectively. However, we did not notice immediate lethal (bactericidal) effects on *L. monocytogenes* when *T. capitata* EO was applied as described by other studies [66, 67], but we observed a strong inhibitory activity against *L. monocytogenes*. These discrepancies between our results

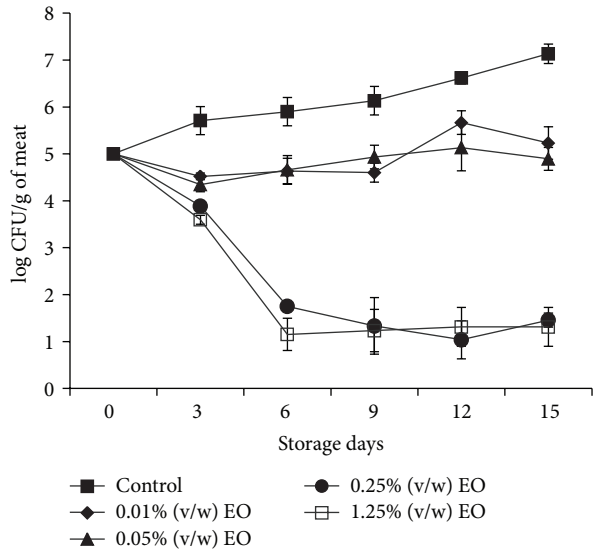

Figure 2: Time-related survival of *Listeria monocytogenes* at 7°C following treatment with increasing concentrations of *Thymus capitata* essential oil. Bacteria were supplemented in minced beef meat samples at 10^5 CFU/g of meat. Values are the average of three individual replicates.

and others that found full lethal effects can be explained by the fact that the activity depends on the type, composition and the concentration of the EO, the strain, and the dose of target microorganism inoculated in the meat. In this study, we used high inoculum (10^5 CFU/g) before treating mince beef compared to low inoculum (10^3 CFU/g) used by Hsouna et al. in 2011 [67]. Taken together, these results demonstrated that EOs derived from *T. capitata* have a great potential in terms of activity against the tested strains of *L. monocytogenes*. Thus, the dose-related inhibitory activity suggests the possibility of using this product as meat preservative. In agreement with our findings, Djenane et al. in 2011 [14] showed a high decrease of bacteria load when minced beef is treated with *Pistacia lentiscus* and *Satureja montana* EOs against *Listeria monocytogenes* CECT 935.

These results are in accordance with previous studies reveling that thyme EO significantly reduced viable counts of *Listeria monocytogenes* in Russian-type salad during one-week storage at 10°C when combined with Enterocin AS-48 (30–60 μg/g) [68] and exhibited a reduction about 0.25% of initial populations of *L. monocytogenes* in minced pork by 2 and 2.3×log CFU/g after 8 days of storage at 4°C and 8°C [69]. In fact, the potent antimicrobial activities of *T. capitata* EO observed in this study can be attributed to the presence of high concentration of carvacrol, which has a well-documented antibacterial potential [14].

4. Conclusion

In conclusion, this study focused on the correlation between the chemical concentration and the effectiveness of *T. capitata* EO as an antioxidant and antimicrobial. The results of this work show that *T. capitata* EO can exhibit strong antioxidant

and antimicrobial activity, probably due to its particular chemical composition, mainly the high amounts of carvacrol. In the second part, *L. monocytogenes* populations in minced beef treated with essential oil were significantly lower than those in control samples throughout the storage period. The application of 0.25 or 1% EOs (v/w) of *T. capitata* EO to minced beef coupled with low temperature storage can reduce the potential of *L. monocytogenes* contamination. So, this EO can be used for the preservation of meats against *L. monocytogenes* and for increasing their shelf life. All results obtained herein suggest that the *T. capitata* EO exhibited a bioprotector effect and therefore it could be used in many biotechnological fields as a natural preservative ingredient of food and/or pharmaceutical industries.

Abbreviations

EOs: Essential oils
T. capitata: *Thymus capitata*
GC-MS: gas chromatography, mass-spectrometry.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Essential Oils for Complementary Treatment of Surgical Patients: State of the Art

Susanna Stea,[1] Alina Beraudi,[1,2] and Dalila De Pasquale[1,2]

[1] *Medical Technology Laboratory, Istituto Ortopedico Rizzoli, Via di Barbiano 1/10, 40136 Bologna, Italy*
[2] *Prometeo Laboratory, Istituto Ortopedico Rizzoli, Via di Barbiano 1/10, 40136 Bologna, Italy*

Correspondence should be addressed to Susanna Stea; stea@tecno.ior.it

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Aromatherapy is the controlled use of plant essences for therapeutic purposes. Its applications are numerous (i.e., wellbeing, labour, infections, dementia, and anxiety treatment) but often they have not been scientifically validated. The aim of the present study is to review the available literature to determine if there is evidence for effectiveness of aromatherapy in surgical patients to treat anxiety and insomnia, to control pain and nausea, and to dress wound. Efficacy studies of lavender or orange and peppermint essential oils, to treat anxiety and nausea, respectively, have shown positive results. For other aspects, such as pain control, essential oils therapy has shown uncertain results. Finally, there are encouraging data for the treatment of infections, especially for tea tree oil, although current results are still inconclusive. It should also be considered that although they are, allergic reactions and toxicity can occur after oral ingestion. Therefore, while rigorous studies are being carried out, it is important that the therapeutic use of essential oils be performed in compliance with clinical safety standards.

1. Introduction

Patients undergoing surgery can benefit from complementary medicine treatments, such as acupuncture, relaxation techniques, massage, and soft manipulation, without putting a burden on the therapeutic plan but, on the contrary, relieving it.

Amongst complementary medicine treatments a particular attention is to be given to essential oils (EOs) treatments that, for their pleasantness and inexpensiveness, can result to be quite useful. Aromatherapy is often associated with other treatments, such as massage; therefore it is difficult to isolate their effect when applied topically. Nevertheless, there is some clinical scientific evidence in favour of EO use in various phases of pre- and postoperative treatment. It should be remarked that this approach is successful also in economically disadvantaged countries where the medium/low cost of this therapy can be supported by the national health system [1].

The mechanism of action of inhaled aromatherapy starts with the absorption of volatile molecules through the nasal mucosa. Odor molecules are then transformed into chemical signals, which move towards the olfactory bulb, and possibly other parts of the limbic system, interacting with the neuropsychological framework to produce characteristic physiological and psychological effects.

The aim of the present work is to illustrate the applications of aromatherapy to surgical patients, on the basis of scientific evidence.

2. Preoperative Anxiety

Preoperative anxiety is a common problem that patients undergoing surgery are facing. Surgical procedures, regardless of the difficulty of the intervention, can cause considerable apprehension, mainly reported as the fear of being unconscious, the operation itself, and pain when recovering from anesthesia.

Many anxious patients receive medications such as sedatives that may be associated with adverse side effects and reduce their capacity to actively and positively participate in

post-op care. The most appropriate oils to treat anxiety are lavender and orange and there is a variety of literature on them.

Evidence of efficacy of EOs in randomized clinical trials (RCTs) is not definitive. Two meta-analyses performed in 2011 [2] and in 2012 [3] on RCTs, both analyzing papers published up to 2010, came to slightly different conclusions: the first analysis concluded that there were insufficient clinical trials examining the effects of aromatherapy in general among people with anxiety disorders as primary condition, but that aromatherapy can be considered as a safe and pleasant intervention in case of secondary anxiety.

The second analysis verified more specifically the effect of lavender EO and concluded that evidence for oral administration of lavender is promising but remains inconclusive.

One possible reason for doubtful results may be linked to essential oil source; there are indeed various types of lavender species used as an EO in clinical aromatherapy practice. Lavender oils include true lavender (*Lavandula angustifolia*), *lavender stoechas* (*Lavandula stoechas*), spike lavender (*Lavandula latifolia*), and lavandin (*Lavandula* × *intermedia*) and this should be considered when efficacy is evaluated, as their chemical composition may differ considerably.

One of the major components of lavender EO is linalool, which has been demonstrated to act postsynaptically, possibly via the modulation of the activity of cyclic adenosine monophosphate (cAMP) [4]. In animal models, linalool has been found to inhibit GABA(A) binding receptor in the central nervous system inducing a relaxed state [5, 6]. Until recently, this activity had not been proven in human studies.

More recently, Schuwald gave further evidence of lavender EO mechanism of action using experimental models of low oral doses corresponding to dosages given in humans (80 mg/d) and demonstrating an inhibition of voltage-dependent calcium channels VOCCs [7].

Clinical evidence of the relaxing efficacy of lavender EO was obtained by Braden et al. [8] who enrolled 150 adult patients undergoing different types of surgery and then randomly assigned to either control (standard care), experimental (standard care plus EO lavandin, *Lavandula hybrida*), or sham (standard care plus jojoba oil) groups. Oils were sniffed and applied on the skin before surgery. Visual analog scales were used to assess anxiety on admission to preoperative suite and operating room transfer. It resulted in that the lavandin group showed significantly lower anxiety during operating room transfer.

The evidence of the efficacy of lavender essential oil was also confirmed by Kim et al. [9] who assessed stress and pain level of needle insertion in the preoperative area after inhalation of *Lavandula augustifolia* from a swab put in the oxygen mask.

Similar results were obtained by Lehrner et al. [10], in the waiting time for dental procedures; even if this cannot be considered as properly surgery, it represents an extremely "strong" emotional situation. 200 patients were either stimulated with ambient odor of orange (*Citrus sinensis*) or ambient odor of lavender. These conditions were compared to a music condition and a control condition (no odor and no music). It resulted in that both ambient odors of orange and lavender reduced anxiety and improved mood in patients waiting for dental treatment.

Orange essential oil diffused in the ambient demonstrated the capacity to reduce stress, measured as salivary cortisol and cardiac pulse, also in a different group of pediatric patients, during dental treatment [11].

The efficacy of *Citrus sinensis* has been proved in stressful conditions different from surgery but in very controlled and rigorous conditions (such as comparison to other smells or absence of other olfactive stimuli) also by Goes et al. who showed an acute anxiolytic activity of sweet orange aroma [12].

Evidence of the efficacy of other essential oils in anxiety control is modest with the exception of an interesting finding, by Hongratanaworakit, who observed a relaxing effect of *Rosa damascena* EO administered to volunteers by transcutaneous absorption, excluding olfactory stimulation [13]. Blood pressure, breathing rate, and oxygen saturation measurements indicate a decrease of autonomic arousal.

Also neroli EO (that is extracted from flowers of *Citrus* × *aurantium*) demonstrated the capacity to reduce systolic pressure in patients undergoing colonoscopy, even if anxiety was not affected [14].

Finally, it has been demonstrated that also a blend of essential oils, lavender (*Lavandula officinalis*), roman chamomile (*Anthemis nobilis*), and neroli at a ratio of 6 : 2 : 0.5 can reduce anxiety, increase sleep, and stabilize the blood pressure of patients undergoing cardiac stent insertion [15].

3. Pain

Perioperative pain is actually well controlled by drugs whose adverse effects are well known. In the control of pain, psychological support techniques can achieve good results by distraction, muscle relaxation, and imagination, thus decreasing the requirements for traditional analgesics and hence reducing the incidence of adverse effects.

In the "distraction hypothesis" any perceived sensory environmental stimulus is sufficient to reduce the pain experienced because the stimulus itself reduces the cognitive resources focusing on pain.

Aromatherapy is one of the potential methods of reducing perioperative pain, but its evidence remains poor.

Preliminary studies have been carried out on the different effects of pleasant and unpleasant smells on pain perception [16]; these studies found unexpected results: both pleasantand unpleasant odors lead to the perception of a greater degree of pain compared to unexposed subjects. This has then been confirmed clinically by Kim et al. [17], who did not find lavender EO effective in reducing pain in patients undergoing breast biopsy. Lavender oil was given as a swab in the oxygen face mask.

Likewise, the efficacy of mandarin EO (*Citrus reticulata*) associated with massage was not proven to be effective to reduce discomfort of babies (3–36 months old) after major maxillofacial surgery [18]. Conversely, a randomized clinical trial on pediatric patients evaluated the effect of lavender EO on pain related to tonsillectomy and found that periodic

inhalation of the essence decreased the amount of analgesic required [19].

Good results were obtained also to control pain after laparoscopic gastric banding [20] and cesarean section [21] in a small cohort of patients. The authors concluded that inhaled Lavender essence may be used as a part of the multidisciplinary treatment of pain, but it is not recommended as the sole pain management.

In nonsurgery-related situations, a rigorous study demonstrated that some EOs have analgesic activity (ginger and orange) for a limited period of time; in fact the statistically significant effect observed immediately after application soon wore off. Ginger is one of the most popular herbal remedies and is recommended for rheumatic conditions in Chinese medicine [22].

A recent research added some doubts on the real efficacy of EO in reducing the perception of pain. Masaoka et al. [23], demonstrated that information given to the patients on the lavender effect, the lavender odor itself, and slower breathing contributed to the reduced perception of pain in a controlled study, suggesting a placebo effect. In this apparently well-controlled study, there is one missing information, which is the quality and source of lavender "odor". It is possible that the lack of efficacy beyond the placebo effect is in fact related to the choice of the EO.

4. Postoperative Nausea and Vomiting

Postoperative nausea and vomiting occur as a common side effect of general anesthesia. About one-third of all people undergoing surgery suffer from these conditions at various degrees of intensity. Current therapy has sedation as side effect.

The indication for EOs is mainly limited to ginger (*Zingiber officinale*), spearmint (*M. spicata*), and peppermint (*M.* × *piperita*). Peppermint oil is one of the oldest European herbs used for medicinal purposes. It is a hybrid species of spearmint and water mint (*Mentha aquatica*). The EOs are derived by steam distillation of the fresh aerial parts of the flowering plant. The active ingredients are menthol (35–45%) and menthone (10–30%). Peppermint oil is recommended for its antiemetic and antispasmodic effects on the gastric lining and colon. One possible mechanism of action of peppermint oil in the gastrointestinal system is the inhibition of muscular contractions induced by serotonin and substance P.

Several past studies have shown the efficacy of peppermint in reducing postoperative nausea and vomiting as reported by Tate [24] and Lane et al. [25], in post-cesarean section, where current therapies can interfere with breast feeding. Conversely, a recent Cochrane review [26] concluded that there is currently no reliable evidence for the use of peppermint oil. Similar conclusions were reached by Lua and Zakaria [27] in a review on nausea and vomiting associated with various conditions, not necessarily related to surgery.

After the publication of the review, few other papers were issued. Ferruggiari et al. [28] evaluated the efficacy of inhaled peppermint oil in treating the post-op nausea in a small group of women; their results indicated a good effect of the aroma in reducing the nausea, also compared to standard pharmacological intervention, but statistical significance was not reached due to the small sample of patients.

Recently, Hunt et al. [29] conducted an accurate randomized trial and ascertained that both ginger essential oil and a blend of essential oils of ginger, spearmint, peppermint, and cardamom are effective in reducing nausea and the requirement for antiemetic medications when inhaled following ambulatory surgery.

The use of mint EO has demonstrated applications and advantages as antiperistaltic agent during endoscopy [30] and colonoscopy [31]. In the first study gastric peristalsis was quantified using video-recorded endoscopic imaging and the results confirmed the efficacy of the substance in a dose-dependent manner.

In the second study, premedication with oral administration of capsules of mint EO was beneficial in terms of the time required for cecal intubation and total procedure time, reducing colonic spasm, increasing endoscopist satisfaction, and decreasing pain in patients during colonoscopy.

5. Disinfection

The cytotoxic activity of essential oils, mostly due to the presence of phenols, aldehydes, and alcohols, is successfully exploited against prokaryotic cells. Bacteria exposed *in vitro* to different EO show permeabilization of membranes, loss of ions, leakage of macromolecules, and lysis. [32].

The EOs most commonly used for their antibacterial and antifungal properties are the tea tree oil, steam distilled from the leaves, and terminal branchlets of *Melaleuca alternifolia*. In particular the tea tree oil has been shown to be effective *in vitro* on several strains of *Staphylococcus aureus* isolated from wounds (even surgical wounds) and on methicillin-resistant and -sensitive bacteria (MRSA and MSSA) [33].

Its components have shown both bacteriostatic and bactericidal activity *in vitro*. Tea tree oil has also been shown to increase monocytic differentiation *in vitro* and reduce inflammation, therefore assisting the healing of chronic wounds. The main component of tea tree oil, terpinen-4-ol, has been shown to suppress inflammatory mediator production by activated monocytes *in vitro* [33].

Few studies have been published, and some of them, even if encouraging, do not reach sufficient strength, for example, Chin and Cordell [34], who observed a reduction of infected wound healing time when the dressing was treated with tea tree essential oil, or Edmondson et al. [35], who observed a favorable effect of tea tree on wound healing, without negativization of the antibiogram.

In view of this, the general toxicology profile of *M. alternifolia* essential oil suggests that severe reactions would be extremely rare in the absence of ingestion; rare cases of sensitization can be observed probably due to alpha-terpinene, a major constituent in tea tree oil.

The ability of tea tree oil to reduce dermal colonization by methicillin-resistant *Staphylococcus aureus* (MRSA) in critical patients was not proved in a randomized study [36].

Table 1: Summary of the evidences for the use of EO in surgical patients.

Condition	Essential oil	Number of reference
Anxiety	Lavender	[3, 8–10]
	Citrus sinensis	[10–12]
	Rosa damascene	[13]
	Neroli	[14]
	Lavandula officinalis + *Anthemis nobilis* + *Neroli*	[15]
Pain	*Citrus reticulata*	[18]
	Lavender	[19–21]
Nausea	*Menta* × *piperita*	[24–31]
	Zingiber officinale + *Mentha spicata* + *Menta* × *piperita*	[29]
Infection	*Melaleuca alternifolia*	[33–36]
	Menta spicata + *Thymus vulgaris* + *Eucalyptus globulus*	[37, 38]

Other essential oils have demonstrated *in vitro* activity, but the step from laboratory experimentation to clinical use is not so straightforward.

Promising studies in these files were conducted by Edwards-Jones et al. [39] who demonstrated the *in vitro* potential of essential oils and of their vapors (*Patchouli*, tea tree, geranium, lavender EOs, and commercial mixture grapefruit seed extract) as antibacterial agents for the treatment of MRSA infection. Muthaiyan et al. [40] tested terpeneless cold pressed Valencia orange oil (CPV) for topical therapy against MRSA using an *in vitro* dressing model and skin keratinocyte cell culture model. Warnke et al. [41] tested the *in vitro* efficacy of many EOs (Eucalyptus, tea tree, thyme white, lavender, lemon, lemongrass, cinnamon, grapefruit, clove bud, sandalwood, peppermint, kunzea, and sage oil) with the agar diffusion test, against strains of several common and hospital-acquired bacterial and isolated yeasts and found that thyme white, lemon, lemongrass, and cinnamon oils were effective. The other oils also showed considerable efficacy [42].

Oregano essential oil, which is attributed to antiseptic proprieties by the traditional medicine, has not been tested in clinical trials.

It must be also pointed out that researches currently taking place demonstrate promising *in vitro* results on the synergic activity of EO compared to antibiotics. The *in vitro* inhibitory activity of some antibiotics not only demonstrates additive activity compared to essential oils but also a superior bactericidalcapacity compared to the sum of the activity of the individual substances alone.

Currently, the major applications as antiseptics have been found for infections prophylaxis during small oral surgery, using EOs included in mouthwash, mainly menthol, thyme, and eucalyptol [37, 38].

Data presented are summarized in Table 1, where only "*in vivo*" studies are presented.

6. Miscellanea

Essential oils can be successfully applied in few other situations related to surgery.

For example antimicrobial activity of tea tree oil can be exploited also for hand washing. The use of antiseptics is critical in healthcare settings for the prevention of transmission of infections. Messager suggests that tea tree oil-containing hand wash formulations may help reduce the skin carriage of potentially pathogenic organisms also in the surgical environment [42].

Topical application of black pepper may be a viable and effective way to enhance vein visibility and palpability prior to intravenous catheter insertion in patients who have limited vein accessibility. Black pepper essential oil may improve vein access and reduce the need for repeated insertion attempts, thereby reducing patient discomfort and improving patient care [43].

Other promising applications have been proposed for *Helichrysum italicum* as antispastic [44], rose geranium as anti-inflammatory [45], *Origanum majorana* as antimutagenic [46], and many others, but clinical validation is not ready at the moment.

7. Final Considerations

Essential oil rigorous studies are still at the beginning and there is some space for new research improving traditional medicine, even from nonoccidental cultures, and transposing it in a modern system where efficacy evidence is the main focus of physicians. Researchers can give their contribution in understanding EO mechanism of actions, as recently proposed by Zhang et al. who demonstrated that the metabolomics approach can capture the subtle metabolicchanges resulting from exposure to EOs [47].

So far, efficacy evidence is contrasting and some literature reviews give very negative opinions like Lee et al. who performed a systematic review stating that "due to a number of caveats, the evidence is not sufficiently convincing that aromatherapy is an effective therapy for any condition" [48].

It should not be forgotten that essential oils can be contact sensitizers [49] and due to their very complex composition characterized by two or three major components at a relatively high concentration and different substances present in trace, their activity is not completely understood; therefore possible interactions with drugs or peculiar metabolic conditions of surgical patient must be considered [32].

A complete review of the available literature has collected 71 cases of patients who experienced adverse effects of aromatherapy. Adverse effects ranged from mild to severe and included one fatality. The most common adverse effect was dermatitis. Lavender, peppermint, tea tree oil, and ylang-ylang were the most common essential oils responsible for adverse effects, possibly because they are the most commonly used [50]. A case report of seizure related to rosemary EO, possibly secondary to loss of tissue sodium/potassium gradient leading to increased cellular hyperexcitability, must be taken into particular account [51] as well as a case of

coma induced by oral long-term abuse and intoxication from methol contained in cough droplets [52].

Finally, it should also be taken into account that there is a trend to use uncommon EOs, often derived from wild plants which have a tendency to produce numerous cultivars with different chemical compositions. Often the different chemotypes have not been tested toxicologically, and possible further problems could derive from this in an uncontrolled market [53].

To conclude, we confirm the need of rigorous clinical trials to disprove the false belief of essential oils as a panacea, and we believe it is necessary that these substances are used at therapeutic level with the same degree of precautions normally followed by the use of pharmacologically active substances.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Exploring the Anti-*Burkholderia cepacia* Complex Activity of Essential Oils: A Preliminary Analysis

Isabel Maida,[1] Antonella Lo Nostro,[2] Giovanna Pesavento,[2] Martina Barnabei,[2] Carmela Calonico,[2] Elena Perrin,[1] Carolina Chiellini,[1] Marco Fondi,[1] Alessio Mengoni,[1] Valentina Maggini,[3] Alfredo Vannacci,[3] Eugenia Gallo,[3] Anna Rita Bilia,[4] Guido Flamini,[5] Luigi Gori,[3] Fabio Firenzuoli,[3] and Renato Fani[1,6]

[1] *Department of Biology, University of Florence, Via Madonna del Piano 6, Sesto Fiorentino, 50019 Florence, Italy*
[2] *Department of Health Sciences, University of Florence, Viale G. B. Morgagni 48, 50134 Florence, Italy*
[3] *Center for Integrative Medicine, Careggi University Hospital, University of Florence, 50139 Florence, Italy*
[4] *Department of Chemistry Ugo Schiff, University of Florence, Via della Lastruccia 3-13, Sesto Fiorentino, 50019 Florence, Italy*
[5] *Department of Pharmacy, University of Pisa, Via Bonanno 33, 56126 Pisa, Italy*
[6] *Laboratory of Microbial and Molecular Evolution, Department of Biology, University of Florence, Via Madonna del Piano 6, Sesto Fiorentino, 50019 Florence, Italy*

Correspondence should be addressed to Renato Fani; renato.fani@unifi.it

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In this work we have checked the ability of the essential oils extracted from six different medicinal plants (*Eugenia caryophyllata*, *Origanum vulgare*, *Rosmarinus officinalis*, *Lavandula officinalis*, *Melaleuca alternifolia*, and *Thymus vulgaris*) to inhibit the growth of 18 bacterial type strains belonging to the 18 known species of the *Burkholderia cepacia* complex (Bcc). These bacteria are opportunistic human pathogens that can cause severe infection in immunocompromised patients, especially those affected by cystic fibrosis (CF), and are often resistant to multiple antibiotics. The analysis of the aromatograms produced by the six oils revealed that, in spite of their different chemical composition, all of them were able to contrast the growth of Bcc members. However, three of them (i.e., *Eugenia caryophyllata*, *Origanum vulgare*, and *Thymus vulgaris*) were particularly active versus the Bcc strains, including those exhibiting a high degree or resistance to ciprofloxacin, one of the most used antibiotics to treat Bcc infections. These three oils are also active toward both environmental and clinical strains (isolated from CF patients), suggesting that they might be used in the future to fight *B. cepacia* complex infections.

1. Introduction

Essential oils (EOs) consist of a complex blend of volatile and fragrant substances typically synthesized by all plant organs as secondary metabolites and extracted by water or steam distillation, solvent extraction, expression under pressure, supercritical fluid, and subcritical water extractions [1]. EOs include two biosynthetically related groups, mainly terpenes and terpenoids and, secondarily, aromatic and aliphatic constituents, all of them characterized by low molecular weight. Biological properties of EOs terpenoids are not well elucidated but a function of protecting plants against predators and microbial pathogens is postulated and they could be important in the interaction of plants with other organisms (e.g., attraction of pollinators). The same plant species can produce different EOs chemotypes (i.e., chemical components). For example, *Thymus vulgaris*, morphologically identical species with a stable karyotype, consist of seven different chemotypes depending on whether the dominant component of the essential oil is thymol, carvacrol, linalool, geraniol, sabinene hydrate, α-terpineol, or eucalyptol.

In recent years, the emergence of bacterial resistance against multiple antibiotics has accelerated dramatically. The quinolones/fluoroquinolones, azole, and polyene classes of

antimicrobials often are the last resort to treat infections; hence the chances of acquiring resistance against these antimicrobials are higher [2]. EOs and other plant extracts possess antibacterial, antifungal, and antiviral properties and have been screened worldwide as potential sources of novel antimicrobial compounds [3]. Thus EOs and their constituents can hopefully be considered in the future for more clinical evaluations and possible applications and as adjuvants to current medications [4]. The antimicrobial properties of EOs have been reported in several studies. High antimicrobial activity of *Thymus* and *Origanum* species has been attributed to their phenolic components such as thymol and carvacrol and those of *Eugenia caryophyllus*, *Syzygium aromaticum*, and *Ocimum basilicum* to eugenol [1]. In fact thyme and oregano EOs can inhibit some pathogenic bacterial strains such as *Escherichia coli*, *Salmonella enteritidis*, *Salmonella cholerasuis*, and *Salmonella typhimurium,* with the inhibition directly correlated to carvacrol and thymol [5]. The mechanisms by which essential oils can inhibit microorganisms involve different modes of action and in part may be due to their hydrophobicity. As a result, they get partitioned into the lipid bilayer of the cell membrane, rendering it more permeable, leading to leakage of vital cell contents [6]. There are fewer reports on the mechanisms of action of EOs combination or their purified components on microorganisms. They include the sequential inhibition of a common biochemical pathway, inhibition of protective enzymes, and use of cell wall active agents to enhance the uptake of other antimicrobials. The capacity of hydrocarbons to interact with cell membrane facilitates the penetration of carvacrol into the cell. In many cases the activity results from the complex interaction between the different classes of compounds such as phenols, aldehydes, ketones, alcohols, esters, ethers, or hydrocarbons found in EOs [1]. It is likely that it will be more difficult for bacteria to develop resistance to the multicomponent EOs than to common antibiotics that are often composed of only a single molecular entity [3]. For example the multicomponent nature of tea tree oil could reduce the potential for resistance to occur spontaneously, since multiple simultaneous mutations may be required to overcome all of the antimicrobial actions of each of the components. This means that numerous targets would have to adapt to overcome the effects of the oil [7].

Clinical studies with EOs are scarce. Topical use is the most promising strategy at the moment, for both skin and mucous membranes. Some hope exists for inhalation uses, but clinical evaluation is needed. There is little information regarding safety in relation to oral administration of EOs, so an increase in the knowledge about pharmacokinetics, pharmacodynamics, and the potential toxicity of EOs administered by this route is required [3].

Particularly interesting from this viewpoint is the possibility to treat infections of cystic fibrosis (CF) patients. One of the most important opportunistic CF pathogens is represented by bacteria belonging to the *Burkholderia cepacia* complex (Bcc) belonging to the very heterogeneous genus *Burkholderia*, which currently comprises more than seventy species, isolated from wide range of niches. Many members of the genus can cause infection in plants, animals, and humans, and most studies have thus focused on these pathogenic species due to their clinical importance [8]. However, recently, an increasing number of *Burkholderia* species associated with plants or with the environment and able to fix nitrogen, to nodulate legume or to promote plant growth, were described [8]. Among the pathogenic species, the Bcc bacteria, a group of genetically distinct but phenotypically similar bacteria that up to now comprises 18 closely related bacterial species [9, 10], have become known as opportunistic pathogens in humans. Although they are not considered important pathogens for the normal human population, some of them are considered serious threats for specific patient groups such as CF patients [11]. CF is the most fatal genetic disease of Caucasians [9], and the main cause of morbidity and mortality in patients is chronic lung infection involving different species of bacteria (mainly *Pseudomonas aeruginosa*), fungi, and viruses [12]. Regarding Bcc species, the prevalence (2009 and 2010) of chronic infection is reported to vary between 0 and 12% of the CF population attending various CF centres [13]. Although it is not high compared to other CF pathogens, Bcc infections correlate with poorer prognosis, longer hospital stays, and an increased risk of death [14].

One of the reasons for the high rate of mortality in infections caused by Bcc species is their high resistance to antibiotics: they are intrinsically resistant to many antibiotics and can develop *in vivo* resistance to essentially all classes of antimicrobial drugs [14, 15]. This high antibiotics resistance is the result of mechanisms specific for certain classes of antibiotics and of an intrinsic resistance, characteristic of all Gram-negative bacteria, due to the cooperation between the outer membrane barrier and the expression of efflux systems [14, 16]. Between multidrug efflux systems, the intrinsic drug resistance of Gram-negative bacteria is mainly attributable to RND (resistance-nodulation-cell division protein family) type drug exporters [17]. The presence and distribution of these kinds of proteins in some available *Burkholderia* genomes are known [18, 19], and some of these systems have also been experimentally characterized [20–23].

New antimicrobial agents are always needed to counteract the Bcc resistant mutants that continue to be selected by current therapeutic regimens. Bacterial resistance often results in treatment failure that causes severe aftermath especially in critically ill patients [24]. Inappropriate or unnecessary antibiotic prescriptions, the excessive use of antibiotics in the agricultural and livestock industries, and the lack of patient adherence to full antibiotic regimens, all of which select resistant bacteria, appear to be the key contributors to the emergence of antibiotic resistance. Resistant bacteria may also spread and become broader infection-control problems, not only within healthcare institutions but within communities as well. For this reason there is a pressing need to develop new antibacterial therapies not only against Bcc bacteria but also against other different human pathogens [25]. In this context one of the most important approaches is represented by the search of new natural drugs from "unusual" sources; particularly interesting might be the essential oils since they are multi-component and, in principle, the probability of bacteria to develop resistance to this

Table 1: List of bacterial strains used in this work and their sensitivity to the essential oils tested in this work.

Strain	Origin	Species	*Burkholderia cepacia* complex strains Sensitivity to						
			Eugenia caryophyllata	*Origanum vulgare*	*Rosmarinus officinalis*	*Lavandula hybrida*	*Melaleuca alternifolia*	*Thymus vulgaris*	Ciprofloxacin
LMG 13010	CF	*B. multivorans*	ES	ES	S	S	S	ES	VS
J2315	CF	*B. cenocepacia*	ES	ES	S	S	S	ES	S
LMG 14294	CF	*B. stabilis*	ES	ES	S	S	S	ES	NS
LMG 24064	CF	*B. latens*	ES	ES	ES	S	S	ES	ES
LMG 24065	CF	*B. diffusa*	ES	ES	VS	S	S	ES	VS
LMG 18943	CF	*B. dolosa*	ES	ES	VS	S	VS	ES	NS
LMG 24067	CF	*B. seminalis*	ES	ES	S	S	S	ES	VS
LMG 24068	CF	*B. metallica*	ES	ES	S	S	S	ES	ES
LMG 26883	CF	*B. pseudomultivorans*	ES	ES	VS	S	S	ES	VS
LMG 23361	AI	*B. contaminas*	ES	ES	VS	S	S	ES	ES
LMG 1222	Env	*B. cepacia*	VS	ES	S	S	S	ES	VS
LMG 10929	Env	*B. vietnamiensis*	ES	ES	ES	S	VS	ES	ES
LMG 19182	Env	*B. ambifaria*	ES	ES	NS	S	S	ES	ES
LMG 20980	Env	*B. anthina*	ES	ES	VS	S	ES	ES	ES
LMG 14191	Env	*B. pyrrocinia*	ES	ES	VS	S	ES	ES	ES
LMG 22485	Env	*B. lata*	ES	ES	S	S	S	ES	ES
LMG 24066	Env	*B. arboris*	ES	ES	VS	S	S	ES	ES
LMG 20358	Env	*B. ubonensis*	ES	ES	ES	S	VS	ES	ES

CF: strain isolated from cystic fibrosis patient; Env: environmental strain; AI: animal infection; NS, S, VS, and ES: not sensitive, sensitive, very sensitive, and extremely sensitive, respectively (according to Ponce et al., 2003) [26].

mixture of substances might be much lesser than to a single molecule.

Therefore, the aim of this work was to explore the antimicrobial activity of six different essential oils versus a panel of Bcc bacteria, some of which exhibiting multiresistance to different drugs and with either clinical or environmental source, in order to check the possibility of using essential oils to fight Bcc infections in CF patients.

2. Materials and Methods

2.1. Bacterial Strains and Growth Conditions. The bacterial strains used in this work are listed in Table 1. They were grown either on Tryptone Soya Agar (TSA, Oxoid S.p.A., Strada Rivoltana, 20090 Rodano, MI, Italy) medium at 37°C for two days or in liquid Tryptone Soya Broth (TSB, Oxoid S.p.A., Strada Rivoltana, 20090 Rodano, MI, Italy) medium at 37°C with shaking.

2.2. Aromatograms

2.2.1. Preparation of Microbial Suspensions and Media. Each bacterial strain was grown at 37°C in liquid medium (TSB) with shaking; the growth was checked at regular time intervals (as spectrophotometric reading at OD_{600}) until the end of the growth exponential phase was reached. Serial dilutions 1 : 10 to 10^{-5} of each bacterial suspension were plated on TSA Petri dishes in order to count the microorganisms and verify that the number of bacteria in the samples was appropriate to the performance of the tests.

TSA, used to perform the agar diffusion assays, was enriched with a suitable volume of Dimethylsulphoxide (DMSO, Carlo Erba Reagenti S.p.a., Strada Rivoltana km 6/7, 20090 Rodano, MI, Italy), sterilized by filtration through filters with a pore diameter of 0.22 μm (Sartorius Italy Srl, Viale A. Casati 4, 20835 Muggiò, MB, Italy), thus obtaining 0.5% (v/v) solutions identified by the abbreviations of DTSA. The addition of DMSO, an aprotic organic solvent belonging to the category of sulfoxides, had the purpose of facilitating the solubilisation of essential oils in the aqueous medium represented by the culture media.

2.2.2. Preparation of Dilutions of Essential Oils. The essential oils used in this study (*Eugenia caryophyllata*, *Origanum vulgare*, *Rosmarinus officinalis, Lavandula hybrida, Melaleuca alternifolia* and *Thymus vulgaris*) were all extracted by steam distillation method, and purchased from the same retailer (Prodotti Phitocosmetici Dott. Vannucci di Vannucci Daniela e C. Sas, Via la Cartaia Vecchia 3, 59021 Vaiano (PO), Italy). All EOs and EOs dilutions were stored at 4°C before use.

2.2.3. Agar Disk Diffusion Assay. Burkholderia cell suspensions were streaked on DTSA Petri dishes. Sterile filter paper disks (Oxoid SpA. Strada Rivoltana, 20090 Rodano, MI,

Italy) of 6 mm diameter were soaked with 10 μL of each not diluted EO, and placed on the surface of the dishes. In addition, positive and negative controls were applied to the surface of agar plates; they were, respectively, the antibiotic ciprofloxacin (3 μg/10 μL) (Oxoid S.p.A. Strada Rivoltana, 20090 Rodano, MI, Italy) and a solution of DMSO 0.5% in sterile deionised water. The plates were incubated at 37 ± 1°C for 48 h aerobically. After incubation, the diameter of the inhibition zones was measured in millimeters, including the diameter of disk. The sensitivity to the EOs was classified by the diameter of the inhibition zones as follows: *not sensitive* for total diameter smaller than 8 mm, *Sensitive* for total diameter 9–14 mm, *very sensitive* for total diameter 15–19 mm, and *extremely sensitive* for total diameter larger than 20 mm [26]. Each assay was performed in triplicate on three separate experimental runs.

2.3. Determination of Essential Oil Composition. Gas cromatographic (GC) analyses were accomplished with an HP-5890 series II instrument equipped with a HP-5 capillary column (30 μm × 0.25 mm, 0.25 μm film thickness), working with the following temperature program: 60°C for 10 min, ramp of 5°C/min to 220°C; injector and detector temperatures, 250°C; carrier gas, nitrogen (2 mL/min); detector, dual flame ionization detection (FID); split ratio, 1 : 30; injection, 0.5 μL. The identification of the components was performed, for both columns, by comparison of their retention times with those of pure authentic samples and by means of their linear retention indices (LRI) relative to the series of *n*-hydrocarbons. Gas chromatography-electron impact mass spectrometry (GC-EIMS) analyses were performed with a Varian CP 3800 gas chromatograph (Varian, Inc. Palo Alto, CA) equipped with a DB-5 capillary column (Agilent Technologies Hewlett-Packard, Waldbronn, Germany; 30 m × 0.25 mm, coating thickness 0.25 mm) and a Varian Saturn 2000 ion trap mass detector. Analytical conditions were as follows: injector and transfer line temperature at 250 and 240°C, respectively, oven temperature being programmed from 60 to 240°C at 3°C/min, carrier gas, helium at 1 mL/min, splitless injector. Identification of the constituents was based on comparison of the retention times with those of the authentic samples, comparing their LRI relative to the series of *n*-hydrocarbons and on computer matching against commercial and homemade library mass spectra built from pure substances and components of known samples and MS literature data [27–32]. Moreover, the molecular weights of all the identified substances were confirmed by gas chromatography-chemical ionization mass spectrometry (GC-CIMS), using methanol as chemical ionization gas.

2.4. Statistical Analyses. Inhibition zones in Bcc strains from the different EOs were analyzed by using principal component analysis as implemented in PAST software [33]. Kruskal-Wallis test with Bonferroni error protection was applied for comparing the overall inhibition zones from the different EOs by using the Analyse-it software (Analyse-it Software, Ltd.).

3. Results and Discussion

3.1. Composition of Essential Oils. Essential oils are very complex natural mixtures, which can contain about 20–60 components at quite different concentrations. They are characterized by two or three major components at fairly high concentrations (20–70%) compared to other components present in trace amounts. Terpenoids (mainly monoterpenoids and sesquiterpenoids) generally represent the principal constituents but some essential oils are characterised by the presence of aromatic (phenylpropanoids) and aliphatic constituents, all characterized by low molecular weight.

The tested essential oils were commercial samples and analysed by GC using as detector a dual FID and electron impact mass spectrometry. Constituents were identified by comparison of their retention times of both columns with those of pure authentic samples and by means of their linear retention indices (LRI) relative to the series of *n*-hydrocarbons and MS data from homemade library mass spectra and literature.

Almost 100% of the volatiles of oregano essential oil were identified, being 77.2% of oxygenated monoterpenes, principally represented by carvacrol representing 71.8% of the total essential oil; 19.2% of constituents were represented by monoterpene hydrocarbons, principally *p*-cymene; 2.9% were sesquiterpenes hydrocarbons, and 0.6% were oxygenated sesquiterpenes.

Also in the case of rosemary essential oil the identified volatiles were 99.9% and major constituents were represented by oxygenated monoterpenes (64.6%) being the main volatile 1,8-cineole (43.9%). Monoterpene hydrocarbons were 25.9%, principally α-pinene. Sesquiterpene hydrocarbons were 9.1% and oxygenated sesquiterpenes were only 0.3%.

Total identified constituents of thyme oil were 99.5%. These volatiles were characterized by 53.7% of monoterpene hydrocarbons being 47.9% *p*-cymene and oxygenated monoterpenes 45.6%, principally thymol (43.1%). Only 0.2% of the volatiles were sesquiterpenes hydrocarbons.

About 98% of constituents of clove oil were identified and the main metabolite was eugenol (85%), a typical phenylpropanoid, while 11.2% of the constituents were recognised as sesquiterpene hydrocarbons being β-caryophyllene the main molecule (9%).

Approximately all (99.1%) of the constituents of *M. alternifolia* were identified; principal compounds were oxygenated monoterpenes being 4-terpineol the principal one (39.9%). The rest of the oil was mainly represented by monoterpene hydrocarbons (41.4%) being γ-terpinene (14.4%) and α-terpinene (8.8%) the principal molecules.

3.2. Antimicrobial Activity of the Essential Oils against Burkholderia cepacia Complex (Bcc) Strains. The antimicrobial activity of the six different EOs (*E. caryophyllata* (Ec), *O. vulgare* (Ov), *R. officinalis* (Ro), *L. hybrida* (Lh), *M. alternifolia* (Ma), and *T. vulgaris* (Tv)) was checked versusthe 18 Bcc type strains listed in Table 1 and representative of the 18 known Bcc species; this panel comprises strains of either clinical or environmental origin.

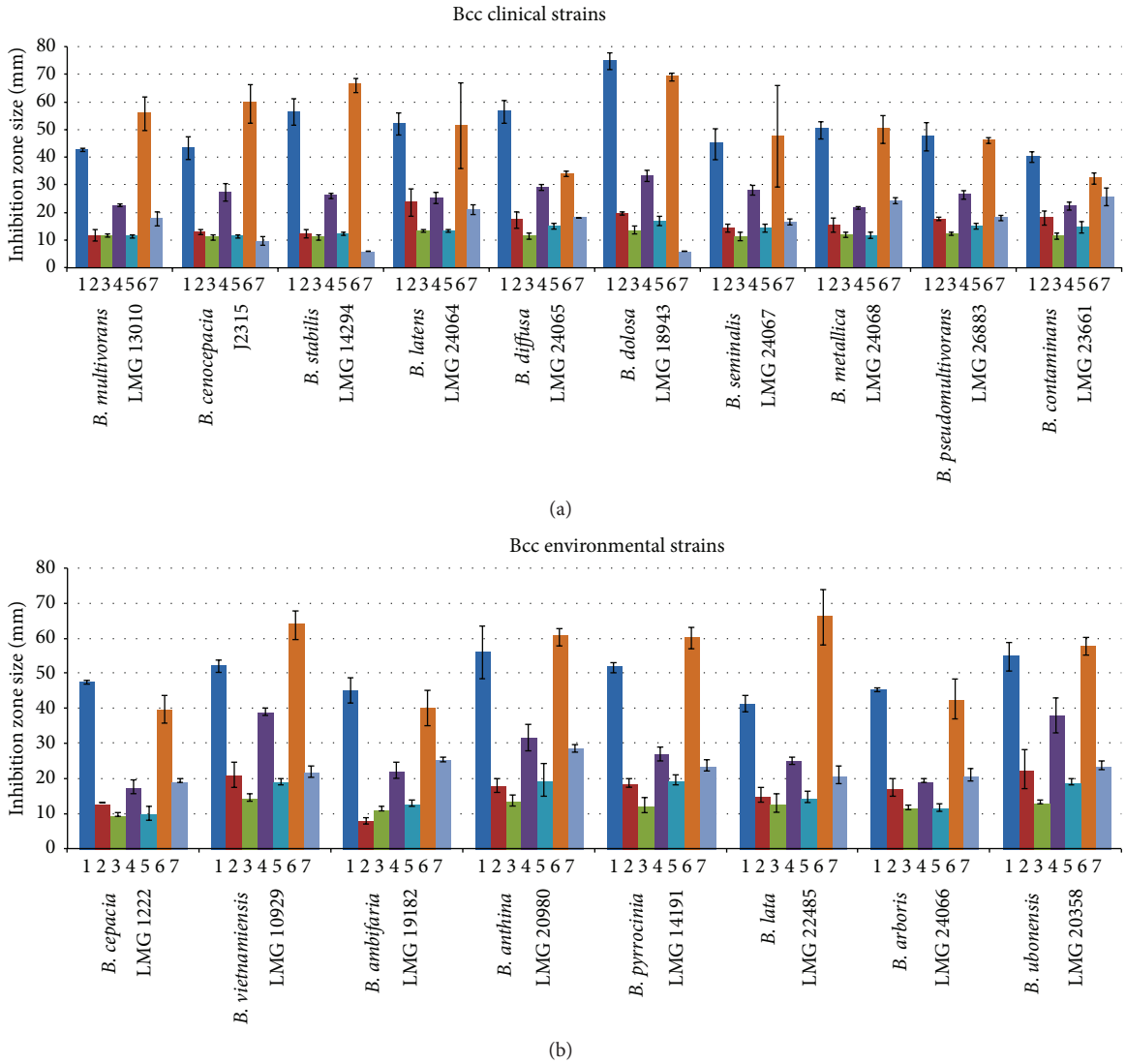


Figure 1: Inhibitory power of essential oils. Results for the agar diffusion assay performed on the 18 Bcc type strains are presented. Each bar of the histogram represents the mean of the inhibitory zone obtained for each of the EOs analyzed. In the graphics are reported the standard deviations for every arithmetic average obtained: (1) *Thymus vulgaris*, (2) *Rosmarinus officinalis*, (3) *Lavandula hybrida*, (4) *Eugenia caryophyllata*, (5) *Melaleuca alternifolia*, (6) *Origanum vulgare*, and (7) *Ciprofloxacin*.

Data obtained are reported in Figure 1 and showed the following.

(i) All the 18 bacterial strains, from both clinical and environmental origin, exhibited, although at a different extent, sensitivity to each of the six EOs tested.

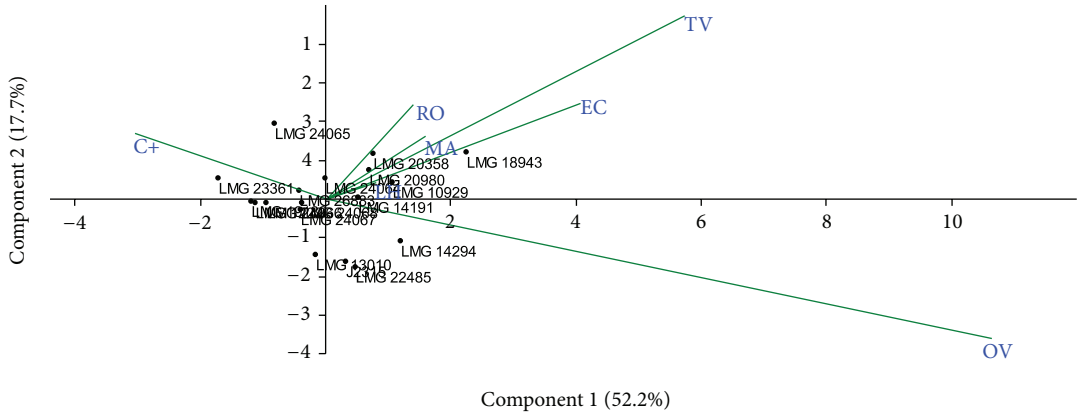
(ii) According to Ponce et al. [26], three essential oils, that is, Ec, Tv, and Ov, exhibited a very high inhibitory power *versus* all the Bcc strains tested. Indeed, all of them were *extremely sensitive* to these three EOs.

(iii) Quite interestingly, these three EOs gave an inhibitory halo much larger than that produced by ciprofloxacin, suggesting that they are more active than this antibiotic.

(iv) The other three EOs (Ro, Lh, and Ma) exhibited a degree of inhibition of Bcc growth lower than that exhibited by the three EOs mentioned above; however, the inhibitory halos they produced were similar and in many cases larger than those exhibited by ciprofloxacin.

(v) Apparently, clinical and environmental strains did not exhibit a different sensitivity to a given EO (or to a set of EOs), but they were differently sensitive to ciprofloxacin (Table 1). Two of them, that is, LMG 14294 (*B. stabilis*) and LMG 18943 (*B. dolosa*), were resistant to the antibiotic and *B. cenocepacia* J2315, representing the model system for the study of Bcc infection in CF patients, exhibited a low sensitivity to ciprofloxacin. These three strains have a clinical origin. In spite of this, the same three strains were extremely sensitive to the three most active EOs.

(vi) Environmental Bcc strains were much more sensitive to ciprofloxacin than their clinical counterparts.



P-values of pairwise comparison of inhibitory halo diameters

	TV	RO	LH	EC	MA	OV	C+
TV	0	***	***	***	***	n.s.	***
RO	***	0	**	***	n.s.	***	*
LH	***	**	0	***	*	***	***
EC	***	***	***	0	***	***	**
MA	***	n.s.	n.s.	***	0	***	*
OV	n.s.	***	***	***	***	0	***
C+	***	n.s.	*	**	n.s.	***	0

Figure 2: Differences in the patterns of inhibition of essential oils. Upper panel: principal component analysis biplot of inhibitory patterns 18 Bcc strains (centroids) treated with different EOs and ciprofloxacin (C+). The percentage of variance explained by the first two principal components is reported. Lower panel: *P* values of pairwise comparisons (Kruskal-Wallis test and Bonferroni error protection) between EOs and C+. n.s.: not significant; $^{*}P < 0.01$; $^{**}P < 0.001$; $^{***}P < 0.0001$.

The differential sensitivity to EOs and ciprofloxacin was confirmed by a principal component analysis (Figure 2). As shown in the biplot the vectors accounting for EOs are differentially oriented than those of ciprofloxacin (C+). Moreover, the vectors for Ov and Tv greatly contributed in the differential pattern of sensitivity, thus confirming that the most active essential oils were *T. vulgaris* and *O. vulgare.* Finally a pairwise comparison (Kruskal-Wallis test) of the patterns of inhibition of EOs and ciprofloxacin (Figure 2) showed that large differences between inhibitory halos of different EOs and ciprofloxacin are present, highlighting the observed (Table 1, Figure 1) differences in the inhibitory power of the six EOs.

4. Conclusions

In this work we have performed a preliminary analysis of the ability of six different essential oils to inhibit the growth of strains belonging to the *B. cepacia* complex, whose members are dangerous for CF patients; indeed they can cause severe infections in immune-compromised patients, such as those affected by cystic fibrosis. This idea relies on previous findings demonstrating that essential oils are able to inhibit the growth of some human pathogens, such as *E. coli*, *S. enteritidis*, *S. choleraesuis*, and *S. typhimurium* [5]. However, to the best of our knowledge, nothing are known on the ability of these mixtures of chemical compounds to inhibit the growth of Bcc members.

For this reason we selected six different essential oils (*E. caryophyllata*, *O. vulgare*, *R. officinalis, L. officinalis, M. alternifolia*, and *T. vulgaris*) that were tested *versus* a panel embedding the type strains of the known 18 Bcc species.

The composition of the six EOs was quite different but, in spite of this, all of them exhibited an inhibitory activity *versus* all the 18 Bcc strains, suggesting that one compound or (more likely) more than one compound (see below) present in each essential oil might interfere with the Bcc cell growth. However, the six essential oils showed a different inhibitory activity and according to Ponce et al. [26] they might be split into two different clusters; the first one includes *T. vulgaris, O. vulgare* and *E. caryophyllata*, whereas the other one embeds *R. officinalis, M. alternifolia* and *L. officinalis* (Table 2). Indeed, Bcc strains were extremely sensitive to the EOs belonging to the first group and just sensitive to the other three.

However, all of them are able to inhibit the growth of Bcc strains; particularly interesting and intriguing is the finding that the inhibitory halos produced by most of EOs are (much more) larger than those produced by ciprofloxacin, one of the antibiotics used in CF infections therapy. We are completely aware that the sensitivity to a given drug or to a complex mixture of antimicrobial compounds may strongly vary also between strains belonging to the same bacterial species. However, in our opinion, the preliminary data reported in this work are particularly encouraging, since they demonstrate that the use of essential oils might represent an alternative way to fight Bcc growth. It is also quite interesting that, in spite of the high number of experiments performed in this work, no Bcc mutant resistant to any of the essential oils tested was isolated (data not shown). This represents a very important finding, which strongly suggests

Table 2: Composition (%) and principal classes (%) of the six essential oils used in this work.

Constituents	LRI	Essential oil					
		Lavandula hybrida	*Eugenia caryophyllata*	*Melaleuca alternifolia*	*Origanum vulgare*	*Rosmarinus officinalis*	*Thymus vulgaris*
Tricyclene	928					0.2	tr
α-Thujene	933			0.6		tr	
α-Pinene	941	0.4	0.2	3.8	1.7	11.5	4.3
Camphene	955	0.3		tr	0.4	4.1	0.1
Thuja-2.4(10)-diene	959					tr	
Sabinene	977	0.1	tr	0.6			
β-Pinene	982	0.6	0.1	2.1	0.4	3.8	1.2
Myrcene	993	0.5		0.6	1.3	1.3	
α-Phellandrene	1006			0.4	tr	0.2	
1-Hexyl acetate	1010	0.1					
δ-3-Carene	1013	tr	tr				tr
1.4-Cineole	1018						0.1
α-Terpinene	1020		tr	8.8	0.8	0.4	
p-Cymene	1027	0.3	tr	3.7	11.6	1.9	47.9
Limonene	1032	0.7	0.1	2.0	1.1	1.8	0.2
1.8-Cineole	1034	6.9	tr	2.9	0.6	43.9	0.2
(*Z*)-β-Ocimene	1042	0.3					
γ-Terpinene	1063		tr	14.4	1.7	0.4	
cis-Sabinene hydrate	1070	0.1		tr		tr	
cis-Linalool oxide (furanoid)	1077	0.3					
Terpinolene	1090			4.4	0.2	0.3	
trans-Linalool oxide (furanoid)	1090	0.2					
1-Pentyl butyrate	1094				tr		
trans-Sabinene hydrate	1099			0.3			
Linalool	1101	27.1			1.8	0.9	1.2
1-Octenyl acetate	1112	0.4					
exo-Fenchol	1118			tr		tr	tr
cis-*p*-Menth-2-en-1-ol	1123			0.4			
Terpinen-1-ol	1135			0.2			
trans-Pinocarveol	1141					tr	
trans-*p*-Menth-2-en-1-ol	1142			0.4			
Camphor	1145	8.4			tr	11.3	
1-Hexyl isobutyrate	1152	0.2					
Isoborneol	1158				0.2		
trans-Pinocamphone	1162					tr	
Pinocarvone	1164					tr	
Borneol	1168	3.2			0.4	4.2	
Lavandulol	1171	0.6					
cis-Pinocamphone	1175					tr	
4-Terpineol	1178	3.9	tr	39.9	0.2	0.8	
p-Cymen-8-ol	1185			tr			
α-Terpineol	1190	1.7		4.2	0.4	2.6	0.6
1-Hexyl butyrate	1193	0.6					
cis-Piperitol	1195			tr			

Table 2: Continued.

Constituents	LRI	Essential oil					
		Lavandula hybrida	*Eugenia caryophyllata*	*Melaleuca alternifolia*	*Origanum vulgare*	*Rosmarinus officinalis*	*Thymus vulgaris*
Verbenone	1206					0.2	
trans-Piperitol	1207			0.2			
Nerol	1230	0.2					
1-Hexyl 2-methylbutyrate	1235	0.1					
1-Hexyl 3-methylbutyrate	1244	0.3					
Chavicol	1252		tr				
Linalyl acetate	1259	30.4					
trans-Ascaridolglycol	1268			0.2			
Isobornyl acetate	1287				0.2	0.7	
Lavandulyl acetate	1291	3.3					
Thymol	1292				1.6		43.1
Carvacrol	1301				71.8		0.4
1-Hexyl tiglate	1333	0.2					
α-Cubebene	1352		tr			tr	
Eugenol	1358		85.0				
Neryl acetate	1365	0.4					
α-Ylangene	1373					0.2	
α-Copaene	1377		0.2	tr	tr	0.6	
Geranyl acetate	1383	1.0					
α-Gurjunene	1410			0.5			
β-Caryophyllene	1419	2.2	9.0	0.5	2.7	5.1	0.2
Lavandulyl isobutyrate	1424	0.1					
trans-α-Bergamotene	1437	0.2					tr
α-Guaiene	1440			1.4		0.2	
(Z)-β-Farnesene	1444	0.2					
α-Humulene	1455	tr	1.4	0.1	0.2	0.5	tr
(E)-β-Farnesene	1459	1.1					
Alloaromadendrene	1461			0.6			
γ-Muurolene	1478					0.6	
Germacrene D	1482	0.3					
Valencene	1493			0.3			
Viridiflorene	1494			1.3		0.2	
Bicyclogermacrene	1496			0.7			
α-Muurolene	1499			0.2		0.2	
β-Bisabolene	1509	0.2				0.2	
Lavandulyl 2-methylbutyrate	1513	0.4					
trans-γ-Cadinene	1514	0.5				0.4	
δ-Cadinene	1524		0.6	1.8		0.9	
trans-Cadina-1(2).4-diene	1534			0.2			
Spathulenol	1577			0.2			
Caryophyllene oxide	1582	0.6	0.5		0.6	0.3	tr
Globulol	1584			0.5			
Guaiol	1597			0.2			
1-*epi*-Cubenol	1629			0.3			

Table 2: Continued.

Constituents	LRI	Essential oil					
		Lavandula hybrida	*Eugenia caryophyllata*	*Melaleuca alternifolia*	*Origanum vulgare*	*Rosmarinus officinalis*	*Thymus vulgaris*
T-Cadinol	1640	0.2					
Cubenol	1643			0.2			
α-Bisabolol	1684	0.4					
Monoterpene hydrocarbons		3.2	0.4	41.4	19.2	25.9	53.7
Oxygenated monoterpenes		88.2	0.0	48.7	77.2	64.6	45.6
Sesquiterpene hydrocarbons		4.7	11.2	7.6	2.9	9.1	0.2
Oxygenated sesquiterpenes		1.2	0.5	1.4	0.6	0.3	tr
Phenylpropanoids		—	85.0	—	—	—	—
Other derivatives		1.9	—	—	tr	—	—
Total identified		99.2	97.1	99.1	99.9	99.9	99.5

LRI: linear retention indices relative to the series of *n*-hydrocarbons; tr: traces.

that the ability of essential oils to inhibit the growth of Bcc cells might be very likely due to the simultaneous presence in the oil of different molecules (whose mechanism of action is still unknown) that might work in a synergistic fashion to antagonize the Bcc growth. In addition to this, in our opinion, these combinations of compounds should not act on a single target, but on different molecular targets within the Bcc cell. If this is so, the simultaneous block of the activity of different molecular targets should strongly decrease the probability of the appearance of a mutant able to resist the essential oils. If this scenario is correct, these data might pave the way to the use of essential oils to fight Bcc infection in CF patients.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Hepatoprotective Effect of Pretreatment with *Thymus vulgaris* Essential Oil in Experimental Model of Acetaminophen-Induced Injury

Renata Grespan,[1] Rafael Pazinatto Aguiar,[1] Frederico Nunes Giubilei,[1] Rafael Rocco Fuso,[1] Marcio José Damião,[1] Expedito Leite Silva,[2] Jane Graton Mikcha,[3] Luzmarina Hernandes,[4] Ciomar Bersani Amado,[1] and Roberto Kenji Nakamura Cuman[1]

[1] *Department of Pharmacology and Therapeutics, State University of Maringá, 87020-900 Maringá, PR, Brazil*
[2] *Department of Chemistry, State University of Maringá, 87020-900 Maringá, PR, Brazil*
[3] *Department of Clinical Analysis, State University of Maringá, 87020-900 Maringá, PR, Brazil*
[4] *Department of Morphophysiology Sciences, State University of Maringá, 87020-900 Maringá, PR, Brazil*

Correspondence should be addressed to Renata Grespan; rgrespan2@uem.br
and Roberto Kenji Nakamura Cuman; rkncuman@uem.br

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Acute liver damage caused by acetaminophen overdose is a significant clinical problem and could benefit from new therapeutic strategies. *Objective*. This study investigated the hepatoprotective effect of *Thymus vulgaris* essential oil (TEO), which is used popularly for various beneficial effects, such as its antiseptic, carminative, and antimicrobial effects. The hepatoprotective activity of TEO was determined by assessing serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) in mice. Their livers were then used to determine myeloperoxidase (MPO) enzyme activity and subjected to histological analysis. In vitro antioxidant activity was evaluated by assessing the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH•)-scavenging effects of TEO and TEO-induced lipid peroxidation. TEO reduced the levels of the serum marker enzymes AST, ALT, and ALP and MPO activity. The histopathological analysis indicated that TEO prevented acetaminophen-induced necrosis. The essential oil also exhibited antioxidant activity, reflected by its DPPH radical-scavenging effects and in the lipid peroxidation assay. These results suggest that TEO has hepatoprotective effects on acetaminophen-induced hepatic damage in mice.

1. Introduction

Acetaminophen (APAP) at large doses causes serious liver injury that may develop into liver failure [1]. Hepatotoxicity induced by acetaminophen occurs through a biotransformation reaction that forms the reactive metabolite *N*-acetyl-p-benzoquinone imine (NAPQI) through the cytochrome P-450 mixed function of oxidase system. The metabolite is normally detoxified through a conjugation reaction with reduced glutathione (GSH). However, at large doses of acetaminophen, NAPQI levels increase, ultimately depleting GSH levels. Subsequently, sulfhydryl groups of hepatic proteins may react with the reactive metabolite, resulting in hepatic necrosis [2, 3]. Hepatocellular degeneration and necrosis are also associated with elevated enzyme markers, such as serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) that indicate hepatotoxicity [4]. Liver injury induced by acetaminophen in mice is a commonly used experimental model for screening substances with potential hepatoprotective activity [5]. Growing interest has been observed in the analysis of these natural entities for their potential benefits to human health. Accelerating research of plants used in folk medicine to treat liver diseases and boost liver function has been performed. In plants, essential oils are natural mixtures of terpenes, mainly monoterpenes and sesquiterpenes, which have been increasingly used in complementary therapies because essential oils are usually rich sources of phytochemical mixtures [6].

Essential oils extracted from fresh leaves and flowers can be used as aroma additives in food, pharmaceuticals, and cosmetics [7]. The leaves of thyme (*Thymus vulgaris*) can be used fresh or dried as a spice. Thyme also possesses various beneficial effects, including antiseptic, carminative, antimicrobial, and antioxidative effects [8]. Recently, in our laboratory it was showed that constituents, thymol and carvacrol, of *Thymus vulgaris* L. essential oil present effects on the inflammatory response [9]. Besides, essential oils and phenolic compounds, such as thymol in thyme, have antioxidative properties and may have hepatoprotective properties [8, 10]. To our knowledge, scarce information is available about the effects of *Thymus vulgaris* essential oil (TEO) in experimental hepatotoxicity models. Therefore, the present study investigated the hepatoprotective effect of TEO on acetaminophen-induced hepatic damage in mice.

2. Methods

2.1. Extraction of Essential Oil. The fresh leaves of *Thymus vulgaris* L. were collected from the Proffessor Irenice Silva Medicinal Plant Garden on the campus of the State University of Maringá, Paraná, Brazil. The leaves were identified and authenticated by botanist Maria Aparecida Sert. A voucher specimen was deposited in the Herbarium of the Department of Botany, State University of Maringá (number 11329). The essential oil was obtained by hydrodistillation using a Clevenger-type apparatus. Approximately 556 g of the leaves was subjected to steam distillation for 2 h. The oil was dried over sodium sulfate and stored in an amber flask at 4°C. The TEO yield was 1.76% v/w.

2.2. Essential Oil Analysis

2.2.1. Gas Chromatography-Mass Spectrometry (GC-MS). Gas chromatographic (GC) analysis was performed with a Thermo Electron Corporation, Focus GC model, under the following conditions: DB-5 capillary column (30 m × 0.32 mm, 0.50 mm); column temperature, 60°C (1 min) to 180°C at 3°C/min; injector temperature 220°C; detector temperature 220°C; split ratio 1 : 10; carrier gas He; flow rate: 1.0 mL/min. The volume injected 1 μL diluted in chloroform (1 : 10). The GC/MS analysis was performed in a Quadrupole mass spectrometer (Thermo Electron Corporation, DSQ II model), operating at 70 V. Identification of the individual components was based on comparison of their GC retention indices (RI) on apolar columns and comparison with mass spectra of authentic standard purchased from Sigma-Aldrich literature data.

2.2.2. Nuclear Magnetic Resonance (NMR). ^{1}H (300.06 MHz) and ^{13}C NMR (75.45 MHz) spectra were recorded in deuterated chloroform ($CDCl_3$) solution in a Mercury-300BB spectrometer, with δ (ppm) and spectra referred to $CDCl_3$ (δ 7.27 for ^{1}H and 77.00 for ^{13}C) as internal standard.

2.3. Animals. Male Balb/c mice, weighing 24 ± 2 g, were provided by the Central Animal House of the State University of Maringá. The animals were housed at 22 ± 2°C under a 12/12 h light/dark cycle. Prior to the experiments, the animals fasted overnight, with water provided *ad libitum*. The experimental protocols were approved by the Ethical Committee in Animal Experimentation of the State University of Maringá (CEAE/UEM 126/2010).

2.4. Treatment of Animals. The experimental animals were divided into six groups of five animals each. Firstly, each group received orally during seven days the following treatment: Group I, the mice did not receive any treatment. In Group II, the mice received TEO vehicle (saline that contained 0.1% Tween 80). In Groups III–V, the mice were pretreated with TEO at doses of 125, 250, and 500 mg/kg, respectively. In Group VI, the mice were pretreated with the standard drug, silymarin (200 mg/kg). After this time, the animals fasted for 8 h and then received oral acetaminophen on the seventh day at a dose of 250 mg/kg in Groups II–VI. Group I orally received saline that contained 0.1% Tween 80 (APAP vehicle). After 12 h, the mice were anesthetized with halothane, and blood was collected for the determination of serum AST, ALT, and alkaline phosphatase (ALP). The livers were then used to determine myeloperoxidase (MPO) enzyme activity and for histological analysis.

2.5. Determination of Serum ALT, AST, and ALP Levels. Blood samples were collected and centrifuged at 3000 ×g for 15 min at 4°C. Serum ALT, AST, and ALP levels were then measured using the Analyze Gold enzymatic test kit.

2.6. Determination of MPO Activity. The livers were used to determine MPO enzyme activity in the homogenate supernatant of the liver sections, which were placed in potassium phosphate buffer that contained hexadecyltrimethylammonium bromide in a Potter homogenizer. The homogenate was stirred in a vortex and centrifuged. Ten microliters of the supernatant was added to each well in triplicate in a 96-well microplate. Two hundred microliters of the buffer solution that contained 16.7 mg *O*-dianisidine dihydrochloride (Sigma), 90 mL double-distilled water, 10 mL potassium phosphate buffer, and 50 μL of 1% H_2O_2 was added. The enzymatic reaction was stopped by the addition of sodium acetate. Enzyme activity was determined by absorbance measured at 460 nm using a Spectra Max Plus microplate spectrophotometer.

2.7. Histopathological Analysis. The livers were washed in 0.9% (w/v) sodium chloride solution and placed in 10% neutral buffered formalin for fixation. Subsequently, the livers were processed to paraffin embedded and sectioned in semiserial at a 6 μm thickness on a Leica rotary microtome (Leica Microsystems, Gladesville, New South Wales, Australia). The sections were stained with hematoxylin and eosin to evaluate tissue morphology using light microscopy (Olympus BX-41, Tokyo, Japan). The graded lesions were subjectively classified as absent, mild, moderate, or severe according to lesion area.

2.8. Lipid Peroxidation Assay. A lipid peroxidation assay was performed as previously reported with a minor modification [11]. Egg yolk homogenates were prepared as lipid-rich media. Briefly, 0.1 mL of TEO (5, 50, 500, 2500, and 5000 μg/mL) in methanol was thoroughly mixed with 0.5 mL of egg yolk homogenate (10%, v/v, diluted with pure water) and made up to 1 mL with pure water. Ferrous sulfate (50 μL, 70 mM) was added to induce lipid peroxidation, and the mixture was incubated for 30 min at 37.5°C. Afterward, 1.5 mL of 20% acetic acid (v/v, pH 3.5, diluted with pure water) and 1.5 mL of 0.8% (w/v) thiobarbituric acid in 1.1% sodium dodecyl sulfate (w/v, diluted with pure water) were added, and the resulting mixture was vortexed and heated at 95°C for 60 min. After cooling, 5 mL of 1-butanol was added to each tube and centrifuged at 5000 rotations per minute for 15 min. The organic upper layer was collected and measured spectrophotometrically at 532 nm using a Beckman DU-65 spectrophotometer. The essential oil was diluted in methanol (the solvent expressed no antioxidant activity). Ascorbic acid was used as a positive control. The inhibition of lipid peroxidation was calculated as follows: Inhibition (%) = $(1 - A_{\text{sample}}/A_{\text{control}}) \times 100$. A_{control} was considered the absorbance of the control (i.e., methanol, instead of the sample). The IC_{50} value, representing the concentration of the essential oil that caused 50% inhibition of lipid peroxidation in the Fe^{2+}/ascorbate system, was determined by linear regression analysis from the obtained inhibition (%) values.

2.9. DPPH Assay. Free radical-scavenging capacity (RSC) was evaluated by measuring the 2,2-diphenyl-1-picrylhydrazil (DPPH)-scavenging activity of TEO. The DPPH assay was performed as previously described [12], with minor modifications. The samples (60–2500 μg/mL) in methanol were mixed with 1 mL of a 25 mM DPPH• solution (Sigma, St. Louis, MO, USA), with the addition of 95% methanol to a final volume of 4 mL. The absorbance of the resulting solutions and blank (i.e., with the same chemicals, with the exception of the sample) were recorded against ascorbic acid (Chem Cruz; used as a positive control) after 30 min at room temperature. For each sample, four replicates were recorded. The disappearance of DPPH• was measured spectrophotometrically at 515 nm using a Beckman DU-65 spectrophotometer. The percentage of RSC was calculated using the following equation: $\text{RSC}(\%) = 100 \times (A_{\text{blank}} - A_{\text{sample}}/A_{\text{blank}})$. The IC_{50} value, representing the concentration of the essential oil that caused 50% RSC inhibition, was determined by linear regression analysis from the obtained RSC values.

3. Results

The thyme essential oil showed a predominance of carvacrol (45.54%), α-terpineol (22.96%), and endo-borneol (14.29%) as the major components (data not shown). In the acute toxicological study, TEO tested orally showed an LD50 value of 4.000 mg/kg. All doses used in the present study were lowest of LD50 values observed. Consequently, no apparent behavioural side effects were observed in the animals during our studies. The high LD50 values also suggest that the TEO was relatively safe and nontoxic to the animals.

We evaluated the effects of TEO on serum enzyme markers. As shown in Table 1, acetaminophen-induced hepatic damage markedly elevated serum ALT, AST, and ALP enzyme levels compared with the normal animals. Pretreatment with 250 and 500 mg/kg TEO but not 125 mg/kg TEO for 7 days prior to acetaminophen administration markedly reduced serum ALT, AST, and ALP levels compared with vehicle-treated controls. The effect of TEO was also comparable to silymarin, a standard hepatoprotective agent.

The activity of MPO in TEO-pretreated mice that received doses of 250 and 500 mg/kg was significantly decreased (0.073 ± 0.008 and 0.069 ± 0.008 IU/L, resp.) compared with the group that received acetaminophen only (0.251 ± 0.149 IU/L; Table 1).

The histopathological analysis of control group (APAP vehicle) did not show hepatocellular damage (Figure 1(a)). However, the acetaminophen-treated group showed severe injury characterized by hemorrhagic and necrotic areas, presence of inflammatory infiltrate and piknotic nucleus, (Figure 1(b)). Considering the sylimarin group (standard drug), although the hepatic parenchymal did not present homogenous, necrotic areas were not observed. Also, cellular nucleus was more basophilic than of that control mice and many cells showed cytoplasm vacuolization, showing minor injuries (Figure 1(c)). The group of animals treated with TEO 125 mg/Kg showed interspersed necrotic areas with non-necrotic areas, with cytoplasm vacuolization and hemorrhagic points, characteristics of moderate injuries (Figure 1(d)), differently to that observed after TEO 250 mg/kg treatment, where mild injuries were observed, characterized by basophilic nucleus almost piknotic with shrinkage level (Figure 1(e)). Besides, the group treated with 500 mg/kg of TEO showed the hepatic parenchyma with similar morphology to the control group (Figure 1(f)). Therefore, TEO appeared to provide significant protection against hepatocyte injury.

In the DPPH test, the ability of TEO to act as a donor for hydrogen atoms or electrons in the transformation of DPPH• in its reduced form (DPPH-H) was measured spectrophotometrically. The RSC of TEO at concentrations of 60–2500 μg/mL showed significant antioxidant activity in vitro (IC_{50} = 1377 ± 1.6970 μg/mL; Figure 2(a), Table 2). The IC_{50} value of ascorbic acid (i.e., the positive control) was 4.40 ± 0.07928 μg/mL in the DPPH assay (Figure 2(b) and Table 2).

Egg yolk lipids undergo rapid lipid peroxidation when incubated in the presence of ferrous sulfate. The effect of TEO on nonenzymatic peroxidation is shown in Figure 2(c) and Table 2. At concentrations of 5–5000 μg/mL, TEO significantly inhibited lipid peroxidation (IC_{50} = 8461 ± 7.778 μg/mL). The IC_{50} value of ascorbic acid (i.e., the positive control) was 63.00 ± 3.3870 μg/mL in the lipid peroxidation assay (Figure 2(d) and Table 2). Therefore, TEO was significantly correlated with total antioxidant activity ($R > 0.99$), demonstrating that TEO had antioxidant activity.

4. Discussion

In the present study, it was evaluated the hepatoprotective effect of TEO using the hepatotoxicity model induced by

Table 1: The effect of Thymus essential oil on biomarkers of hepatic damage.

Groups and designe of treatment	ALT	AST	ALP	MPO
		(IU L^{-1})		
Group I, control, Tween 80	48.88 ± 2.05	113.3 ± 15.39	138.40 ± 10.67	0.058 ± 0.009
Group II, APAP control (250 mg kg^{-1})	11130 ± 973.40[a]	6860 ± 140.00[a]	181.90 ± 24.75[a]	0.251 ± 0.149[a]
Group III, 125 mg kg^{-1} of TEO + APAP	3847 ± 3673	3201 ± 2731	110.40 ± 35.74	0.101 ± 0.008
Group IV, 250 mg kg^{-1} of TEO + APAP	261.10 ± 84.41[b]	176.0 ± 76.50[b]	71.37 ± 11.49[b]	0.073 ± 0.009[b]
Group V, 500 mg kg^{-1} of TEO + APAP	110.70 ± 35.79[b]	110.8 ± 22.53[b]	79.20 ± 1.591[b]	0.069 ± 0.009[b]
Group VI, 200 mg kg^{-1} of SLM + APAP	388.80 ± 148.10[b]	286.7 ± 150.40[b]	101.40 ± 40.01[b]	0.091 ± 0.003[b]

Values are mean ± SEM. 5 mice in each group ($n = 5$), $P < 0.05$ values are considered statistically significant. [a]$P < 0.05$ acetaminophen (APAP) treated group compared with animals in control groups. [b]$P < 0.05$ mice treated with the Thyme essential oil (TEO) or Silymarin (SLM) compared with acetaminophen group.

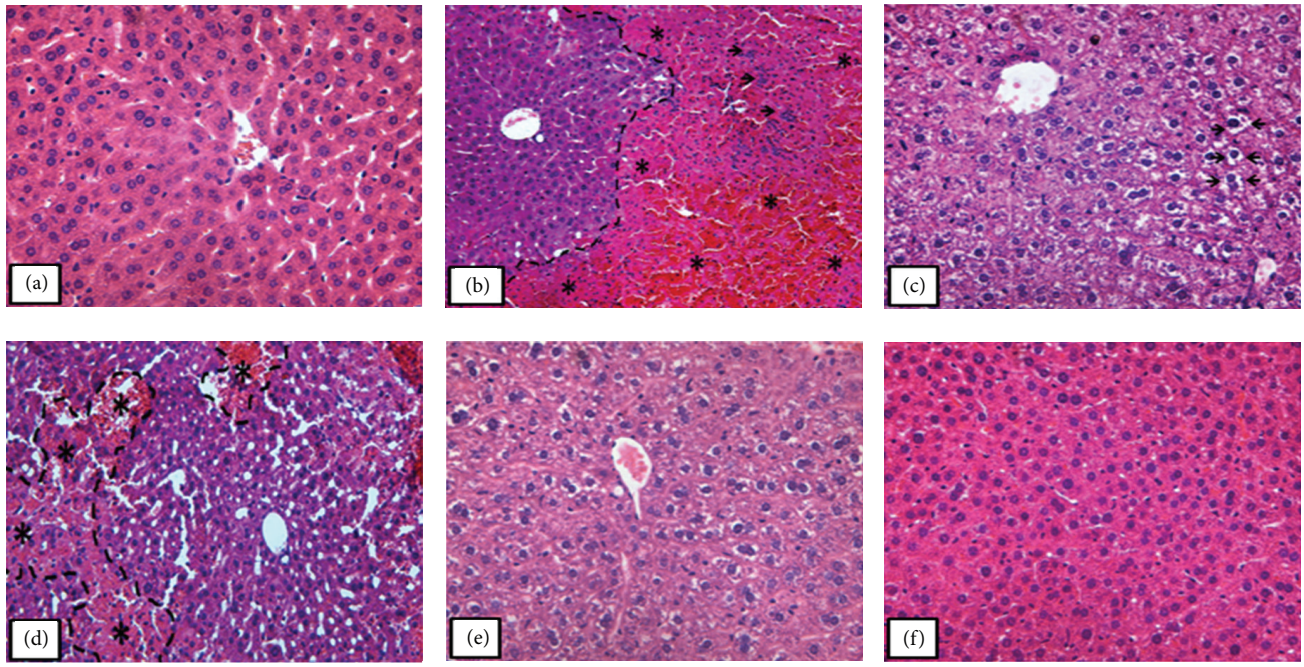

Figure 1: Photomicrograph of the liver in mice that received orally (a) saline, (b) acetaminophen on last day of treatment (250 mg/kg), (c) silymarin (200 mg/kg), and ((d)–(f)) acetaminophen, after being treated for 7 days with the essential oil of *Thymus vulgaris* (TEO), 125, 250, and 500 mg/kg, respectively. In (a) the liver showed normal morphology; (b) presence of necrosis and hemorrhagic points (*) in the defined area; ((c) and (e)) parenchyma stands out for having vacuolated hepatocytes (arrows); (d) observed necrotic areas (*); (f) hepatic parenchyma morphology similar to that observed in the control. Original magnification 40x in (a), (c), (e), and (f); original magnification 20x in (b) and (d). The sections stained with hematoxylin and eosin.

acetaminophen. This drug in an overdose (i.e., at doses that are different from analgesic doses that are safely and effectively used therapeutically) can induce severe hepatotoxicity in experimental animals and humans [13–15]. In our work, hepatotoxicity was reflected by a marked elevation of the levels of serum marker enzymes (AST, ALT, and ALP), increased MPO activity, and histopathologic alterations. These enzymes in serum are useful quantitative markers of the extent and type of hepatocellular damage. High levels of AST indicate a loss of the functional integrity of the liver, similar to the effects seen in viral hepatitis, cardiac infraction, and muscle injury. The ALT enzyme catalyzes the conversion of alanine to pyruvate and glutamate and is released in a similar manner. Therefore, ALT is more specific to the liver and thus is a better parameter for detecting liver injury [16–18].

Acetaminophen is converted to a toxic reactive intermediate called N-acetyl-p-benzoquinone imine (NAPQI) following metabolism by number of isozymes of cytochrome P-450 (CYPs), that is, CYP 2E1 [19], CYP 1A2 [20], CYP 2A6 [21], CYP 3A4, and CYP 2D6 [22]. NAPQI could be bound covalently to cellular proteins, including mitochondrial proteins [23] and that in turn leads to mitochondrial dysfunction. Mitochondrial respiration is inhibited, which results in the formation of reactive oxygen species (ROS) and peroxynitrite in the mitochondria [24, 25]. The massive production of reactive species (ROS) may lead to depletion of protective physiological moieties (glutathione and α-tocopherol), ensuing widespread propagation of the alkylation as well as peroxidation, causing damage to the macromolecules in vital biomembranes [26]. However, reduced

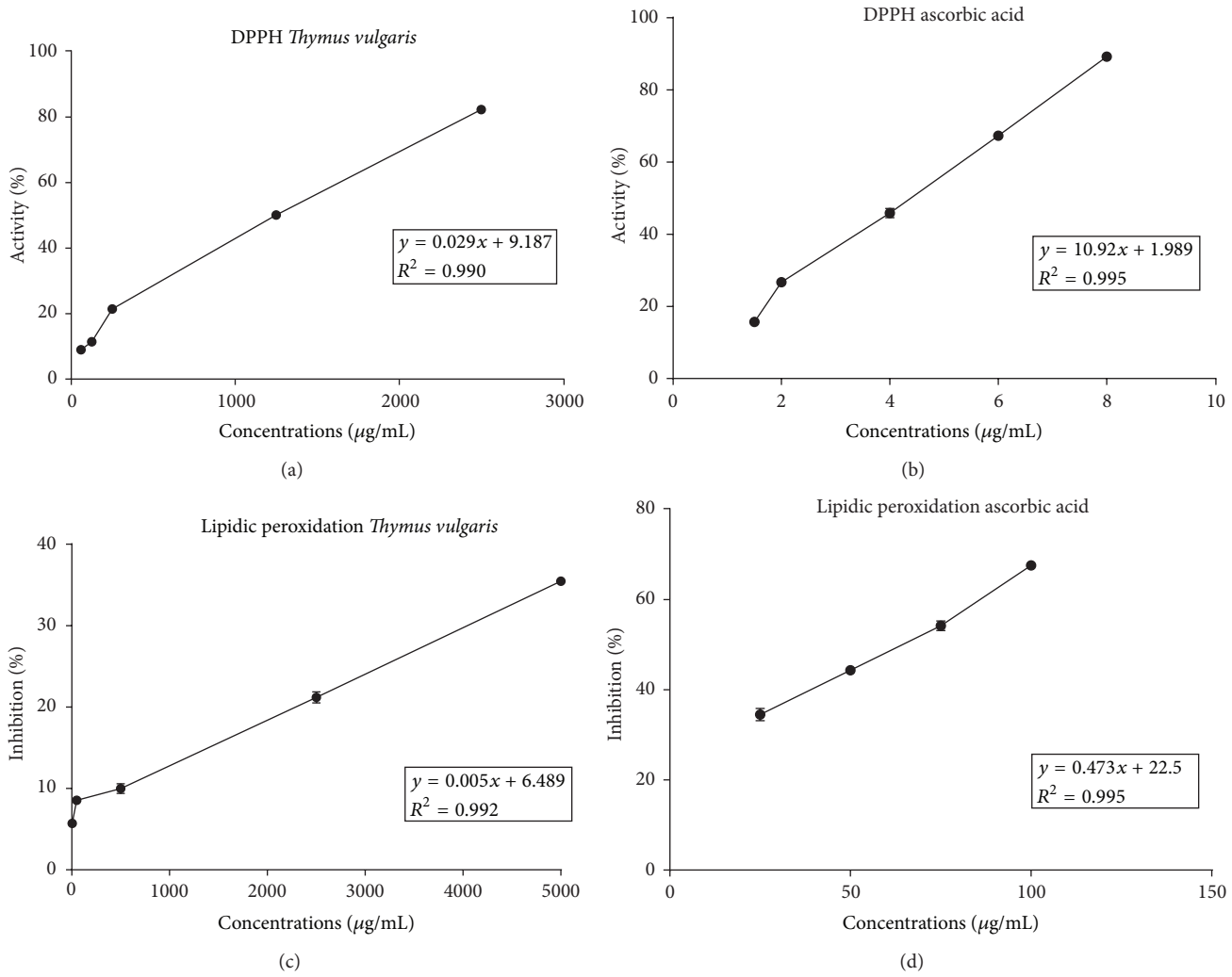

Figure 2: Antioxidant activity of the essential oil from *Thymus vulgaris*. The figure shows the percentage of neutralization of DPPH by (a) the essential oil of *T. vulgaris* and (b) ascorbic acid in the DPPH assay (μg/mL). The inhibition of lipid peroxidation (LP) in the Fe^{2+}/ascorbate system induced by (c) the essential oil of *T. vulgaris* and (d) ascorbic acid in the TBA assay is also shown.

glutathione is one of the main defense mechanisms against oxidative stress reducing peroxides and hydroperoxides [27]. In addition, the oxidative stress can induce a mitochondrial membrane permeability transition and adenosine-5′-triphosphate (ATP) depletion which results in membrane permeabilization, membrane rupture, and cell apoptosis [28–31].

We assessed the hepatoprotective effect of TEO in acetaminophen-induced hepatic damage in mice, and the results suggested that pretreatment with TEO could be protecting the functional integrity of hepatocytes and the cellular membrane from damage by toxic reactive metabolites produced by acetaminophen biotransformation [32, 33].

Inflammation also plays a central role during drug-induced acute hepatitis and products of arachidonic acid metabolism have been extensively involved in inflammatory processes [34]. The histopathological analysis of the livers obtained from the TEO-pretreated group showed mild sinusoidal congestion, less inflammatory cell infiltration, and well-preserved hepatocytes with less of an area of necrosis compared with the severe centrilobular necrosis observed in acetaminophen-treated mice. These results suggest that the anti-inflammatory properties of TEO are partially involved in the hepatoprotective effect of this essential oil. Similarly, previous studies have shown that extracts of plants protect the liver from acetaminophen overdose, suggesting that the hepatoprotective effect can be considered an expression of the functional improvement of hepatocytes that results from accelerated cellular regeneration [35, 36]. Thus, the cytoprotective effects of silymarin, a natural product, are also mainly attributable to its antioxidant and free radical-scavenging properties. Silymarin can interact directly with cell membrane components to prevent abnormalities in the content of the lipid fraction that is responsible for maintaining normal fluidity [37].

Furthermore, the free radical-initiated oxidation of cellular membrane lipids can lead to cellular necrosis and is now accepted to be important in various pathological

Table 2: Summary of IC_{50} values of thyme essential oil (TEO) and ascorbic acid.

	DPPH IC_{50} (μg/mL) ± SD	Lipid peroxidation IC_{50} (μg/mL) ± SD
TEO	1377 ± 1.6970	8461 ± 7.7781
Ascorbic acid	4.40 ± 0.07928	63.00 ± 3.3870

conditions. High acetaminophen doses significantly elevated reactive oxygen species levels, ultimately depleting the levels of superoxide dismutase (SOD) and GSH in liver tissue. This oxidative stress contributes to the initiation and progression of liver damage [33]. Apoptosis is a form of cell death and has deleterious consequences as observed in many diseases, including acquired immunodeficiency syndrome, cancer, and neurodegenerative disorders [38–40]. Apoptosis is induced by different stimuli such as oxidants, xenobiotics, glucocorticoids, and irradiation [41] which converge to trigger a common pathway of cell death, activating proteases as caspase-3 found only in cells undergoing apoptosis. Protease caspase-3 can cleave and inactivate a nuclear protein poly (ADP-ribose) polymerase (PARP), an enzyme used for DNA repair. Many bioactive substances exert their effect on apoptosis acting in cell cycle progression and/or triggering apoptotic cell death. However, the effects of essential oils inducing or inhibiting apoptosis via mitochondrial stress and caspase activation are controversial [42–45]. Thus, various essential oils have been shown to have antioxidant activity and have been used as antioxidant drugs in many diseases [46–48]. The ability of natural products to reduce acetaminophen-induced lipid peroxidation could be as a result of their antioxidant constituents [49]. The mechanisms of TEO in reduction of free radical species and their effects on cells and tissues damage should be elucidated.

5. Conclusion

TEO pretreatment improves the hepatotoxicity induced by acetaminophen in mice. The effects of TEO partially involve the antioxidative effect of this essential oil. However, further detailed studies are required to investigate the mechanism by which TEO exerts its effects and determine the specific constituents that are responsible for this action.

Conflict of Interests

The authors declare that there is no conflict of interest regarding the publication of this paper.

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