Anti-*Candida* Activity of Essential Oils

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Abstract: Anti-*Candida* activity of essential oils has been widely studied and as a consequence they are being investigated as possible alternatives or complementary therapeutic agents for candidosis. We reviewed the most studied essential oils concerning chemical composition and *in vitro/in vivo* studies under the perspective of their possible clinical use.

Key Words: Essential oils, chemical composition, anti-*Candida* activity, candidosis, mechanism of action, therapeutic association, *in vitro* studies, *in vivo* studies.

INTRODUCTION

In recent years *Candida* infections, both mucocutaneous and systemic, have increased significantly [1, 2]. *C. albicans* and *C. glabrata* are pointed as major causes of nosocomial bloodstream infections in the United States of America and in Portugal and vulvovaginal candidosis (VVC) is one of the most common clinical manifestations by *Candida spp*, affecting 70-75% of women at least once in their lifetime [1, 3, 4].

Medicinal properties of plants are known since ancient times and their use has also increased in industrialized countries during the last decades [3].

Essential oils (EO) are natural complex mixtures of volatile compounds of terpenoid or non-terpenoid origin. They have been traditionally used by their bioactivity and considered to be valuable alternative therapies to treat women suffering from vaginitis and vaginosis [5]. The antifungal activity of EO on *Candida* species has been the subject of several published studies that confirmed their traditional use value and described their mode of action. Progress in isolation techniques and chemical characterization of EO has promoted recent research trying to correlate their chemical structure with the intensity of anti-*Candida* activity.

This article reviews the state of knowledge of *in vitro* activity of EO and and their major components against *Candida* spp, as well as studies in animal models and the impact in activity of recent therapeutic formulations.

CANDIDA SPP: CLINICAL ASPECTS

Candida spp are human commensal microorganisms of oral cavity, gastrointestinal tract and vagina [4, 6, 7].

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In recent years *Candida* infections, have increased significantly in consequence of increasing number of cases of AIDS, chemotherapy and immunesuppression therapy, gastrointestinal surgery, extensive burns, but also of intensive therapeutic efforts such as the frequent use of intravascular catheters and the administration of broad spectrum antibiotics [1, 8].

C. albicans is the most common fungal agent isolated from clinical microbiology specimens [1, 2].

During the last decade, *Candida* spp moved from the eighth up to the fourth position in the ranking of agents of invasive infection in Europe, *C. albicans, C. glabrata* and *C. parapsilosis* being considered the main agents of nosocomial fungal bloodstream infections [1-3].

In a prospective observational study conducted along 12 months at the biggest Portuguese hospital, the incidence of fungaemia and nosocomial fungaemia was 2.7 and 2 per 1,000 hospital admissions, respectively. The mortality rate associated was 39.3% and seventy-five percent of the fungaemia episodes were nosocomial, with 48% mortality [1].

Concerning mucocutaneous infections VVC is one of the most common clinical manifestations by *Candida spp*, affecting 70-75% of women at least once in their lifetime while 40-50% of them will experience a recurrence. It has been estimated that by 25 years old, about half of women already had, at least, one episode of VVC [4, 9]. The real significance of the presence of *Candida* spp in the vaginal millieu is unclear. *C. albicans* can colonize the vulva/vagina in up to 30% of all women. A prevalence of 10.1% was found in asymptomatic Portuguese women of reproductive age [6]. In spite of the existence of several putative epithelial defensive substances, the presence of yeast cells in the vagina may represent not true a commensalism state but a quiescent subclinical infection [10].

Vaginal irritation, vulvar burning, pruritus and vaginal discharge are complaints usually associated with VVC. Since

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the symptoms of VVC are not specific, diagnosis must be performed by microscopic examination of vaginal secretions, involving the detection of yeast cells and mycelia (usually considered a pathogenic phenotype) and vaginal samples from symptomatic women with negative microscopy should be cultured. The vaginal pH value in such cases is invariably normal (4.0-4.5) [9].

Nevertheless the recovery of *Candida* spp from symptomatic women being often unsuccessful, both VVC and recurrent vulvovaginal candidosis (RVVC) are frequently caused by *C. albicans*, followed by *C. glabrata*, *C. tropicalis* and *C. krusei*. *C. glabrata* is considered to be frequently involved with recurrent cases [4, 9, 11, 12].

Proposed risk factors for VVC include pregnancy, diabetes mellitus, oral contraceptive use, antibiotics, tight-fitting clothing, synthetic underwear, various dietary excesses or deficiencies, intense sexual activity, and the use of feminine hygiene and menstrual products [9, 13].

RVVC, for unknown reasons, has increased its prevalence during the last decades. A small percentage of women with acute *Candida* infection suffer from recurrent, even monthly, episodes of VVC. Its pathogenesis remains yet incompletely understood. Most recurrences may represent vaginal relapses by some persistent yeasts strains even after prolonged antifungal treatment, rather than exogenous reinfections [9, 14].

The pathogenic mechanisms involved in *Candida* spp infections are not yet well defined. Yeasts are opportunistic pathogens and possess multiple virulence attributes which in combination are able to overcome the host defenses.

Germ tube formation is considered to play a relevant role in tissue invasion. In animal models, germ tube and hyphae formation has been associated with pathogenicity, representing the phenotypic form found in symptomatic vaginosis. Conversely, yeast blastoconidia are responsible for asymptomatic colonisation of the vagina. Factors that enhance or promote germ tube formation tend to precipitate symptomatic vaginosis, whereas inhibition of germ tube formation may prevent VVC in asymptomatic carriers [9, 15-17].

Candida spp biofilms, which represent another relevant pathogenic mechanism, are characterized by yeasts and hyphae arranged in an organized structure that confer resistance to antifungal agents probably by restricting penetration of the therapeutic agents, as a result of phenotypic changes within the fungal cells and promoting the expression of resistance genes [18, 19]. This cellular organization is attracting scientist attention due to its relevance for the control and eradication of infection.

Therapeutic options for *Candida* infections include a small number of antifungal drugs. Amphotericin B, fluconazole and itraconazole are the most widely used. Other molecules, like voriconazole, posaconazole, ravuconazole, caspofungin and micafungin are also possible alternatives [20].

The most prescribed therapeutic regimen for VVC involves the use of azoles, which represent the first choice, unless a confirmed or suspected azole-resistant *Candida* strain is involved. Efficacy of both oral and topical therapy has been shown to be equivalent [21].

The emerging incidence of fungal infections and the development of resistance to classical antifungal agents represent a serious clinical challenge. Resistance to azoles, especially among *C. tropicalis* isolates has been described [1]. In addition, to the limited number of antifungals available, the restrictions to its use (insufficient bioavailability, drug-related toxicity) stress the need for development and study of new molecules exhibiting distinct mechanisms of action and/or evasion of resistance. EO may represent a valuable therapeutic alternative for *Candida* infections, particularly in the case of mucocutaneous infections as is the case with VVC/RVVC [22].

ESSENTIAL OILS: DEFINITION AND CHARACTERIZATION

EO, also known as essences, volatile oils or aetheroleum, are complex mixtures of volatile compounds that result from secondary metabolism of plants. EO can be extracted from different parts of plants (flower, buds, seed, leaves, fruits) and are produced and stored either or both in external and internal secretory structures. Almost all volatile oils consist of chemical mixtures that are often quite complex. It is not uncommon for an oil to contain over 200 components, and frequently the trace constituents are also important for the overall activity of the oil. EO shows a number of physical properties in common. They are liquid, volatile, limpid (rarely coloured), soluble in organic solvents and insoluble in water. They have characteristic odours and high refractive indices, are optically active, being their specific rotation used as an identifying property [5, 23-25].

In industry several methodologies are used to obtain EO and other extracts from plants. The International Standard Organization on Essential Oils [26] considers as EO products exclusively obtained by water, steam or dry distillation from plant material. For the particular case of *Citrus* species a mechanical process is used for extraction.

Solvent extracts, like concretes, absolutes and oleoresins, as well as carbon dioxide extractives, are not considered to fall into this category, as they contain other products in addition to the EO. Standardization of the extraction method ensures that natural fluctuations in the level of active ingredients are balanced and the amount of active ingredients is the same in all samples [23, 27, 28].

Volatile compounds of terpenoid or non-terpenoid origin are frequently found on EO composition. All of them are hydrocarbons and their oxygenated derivatives. Some may also contain nitrogen or sulphur compounds. Monoterpenes, sesquiterpenes and even diterpenes constitute the main constituents of many EO. In addition, phenylpropanoids, fatty acids and their esters, or their decomposition products are also volatiles components [27].

Aromatic plants and their EO can vary greatly as a result of climate, soil quality and other external factors as well as genetic factors. Plants from the same taxon but with specific genetic characteristics can produce chemically distinct EO, so called chemotypes. Being so, not only the plant species

but also the chemical varieties (types) must be described. For instance, different samples of *Rosmarinus officinalis* EO studied by different authors revealed distinct chemical composition. Their antifungal activity was also diverse, as a consequence of the fact that those rich in α-pinene and 1,8 cineole appear to have only mild activity against *C. albicans* [29, 30]. On the contrary, EO obtained from the same species but rich in camphor, limonene and α-pinene presented notable fungistatic and fungicidal activity [31]. Thus, full characterization of EO composition must be made previously to their use.

Several techniques and criteria are used to assess the quality of EO, such as, sensory evaluations, physical and chemical tests and instrumental techniques. Physicochemical tests are required in EO monographs published in standards, pharmacopoeias and codices. Chromatrospectral techniques are modern methods used to assess their quality. The most important technique for EO analysis is gas chromatography (GC). Several detectors may be used in combination with GC. A flame ionisation detector is necessary for quantitative analysis of their constituents. A quadrupole mass detector or an ion-trap detector is indispensable for the identification of EO constituents and the technique is called mass spectrometry (MS). This combination is called GC/MS [27].

IN VITRO EVALUATION OF THE ESSENTIAL OILS ANTIFUNGAL ACTIVITY

In order to determine the antimicrobial activity of EO, several procedures are employed. Some of the methods are currently used in the evaluation of the activity of classical antifungal drugs and are proposed by Clinical Laboratory Standard Institution (CLSI). Their direct application in the study of EO brings some difficulties that need to be faced and solved. Some of those methodologies are described below.

Agar Diffusion Test

Diffusion methods are frequently used to study the effect of several EO against one microorganism. This methodology is dependent of a good diffusion coefficient and it was proposed to study polar compounds of small or medium molecular size [32].

It can be executed fulfilling disk papers or wells (cut into agar) with the EO. As there is loss of EO due to absorption by plastic material it is recommended that only glass Petri dishes shall be used. Volatilization, disk size, amount of compound in the disk, adsorption by the disk, agar type and agar-agar content are factors that may influence results. Frequently differences in diffusion properties of lipophilic EO components result in irregular inhibition halos lacking reproducibility and constitute a difficulty for the evaluation of the activity of the oil [3, 33-35].

Compounds with low antimicrobial activity but presenting a high diffusion coefficient may penetrate the agar rapidly and give large inhibition zones comparable to those of active compounds with less penetration [36]. This test is useful in achieving qualitative results and it is proposed as a screening test to check for primary antimicrobial activity of EO and to select those appearing to be the most active [33, 37].

Agar Dilution Test

This method allows the determination of antimicrobial activity of one sample of EO against several microorganisms. It was recommended for studying complex extracts, polar and non-polar compounds [32]. Agar Dilution Tests allow for minimal inhibitory concentrations (MIC) to be determined. As disadvantages these tests require high amounts of testing compound, and the addition of a solvent or detergent to the agar medium for homogenisation, while volatilization of test material may also occur influencing the results obtained [3].

Broth Dilution Test

This method is more sensitive than diffusion ones and is the best for establishing the potency of compounds [36]. However, solubilization of the EO is required. Organic solvents are often used but their efficiency in stabilizing the emulsion is questionable [3, 34, 35, 38].

Dilution tests seem to be more reproducible and allow for the determination of MIC and minimal lethal concentration (MLC) values, correlated to fungicidal and fungistatic effect of the EO, respectively [3, 34]. To determine those values, visual evaluation of growth, optical density (turbidity) measurement and enumeration of colonies by viable count are used.

Vapor Phase Activity Test

The importance of volatile compounds on the biological activity of EO can be study by this method. An agar medium inoculated with the yeast is kept inverted, with the EO sample deposited on the cover. The effect of volatile compounds is analyzed by inhibition of the yeast growth on agar. Reports of vapor phase activities have been published [34, 39].

This technique is important to differentiate direct from indirect effects. For example, eugenol, a constituent of cinnamon oil, is known to be active in direct contact but showed only scarse activity against *C. albicans* in vapor phase studies indicating that vapor phase activity of this EO shall be attributed to other compound [39]. By contrast, lemongrass oil presented greater antifungal activity in volatile method than in direct contact [40]. The effect of sealing on the antifungal activity of some EO and its main constituents has been studied. The half-life under sealed conditions is longer. In spite of the loss of EO and its components being significant, growth of *C. albicans* was not affected by sealing [34].

The lack of methods standardization for the evaluation of antifungal activity of EO makes the comparison of results difficult. The variables are: the method and the plant material used to extract the EO, the inoculum size, the growth phase of the microorganism chosen to be tested, the culture medium and its pH value, the incubation time and temperature [3, 34, 35].

The biological activity of a plant product is related to its composition and can be distinct for different types of extracts. Their so, the exact nature of the plant extract used must be indicated on published studies. The effects of impurities interference must also be taken in account [3, 35, 41-43].

Different isolates of the same species of the microorganism, as well as both collection strains and clinical strains must be tested to cover differences in behavior [3, 35].

The influence of the medium on EO activity has also been described. MIC values of the same EO determined in two different media revealed to be distinct, being higher with agar media than those obtained with broth media [42]. Also, as commented above, the test chosen may influence results. For example, smaller and non-phenolic volatile compounds, such as citral and limonene, are more active when air phase is included and phenolic compounds, such as thymol and eugenol, have better performance when applied directly in the medium. Again, thymol being smaller and more volatile than eugenol, explains why results obtained with thyme oil are more intense than those of clove or cinnamon when using the volatile method [40].

As there is a need to dissolve oils in the medium, organic solvents are used. The possible interference of solvents with the substance to be studied may be synergistic or antagonistic in affecting yeast growth. High concentrations of Tween are considered to induce false-positives results due to modifications in membrane permeability. And a reduction of the oil bioactivity is attributed to Tween, due to micelle formation that prevents EO to directly contact microorganisms. For physical reasons this reduction in activity of EO is more evident in liquid medium than in agar medium [33-35]. Similar antagonistic effect is seen with dimethylsulfoxide (DMSO) a solubilizer agent largely used. It is believed that DMSO reduces oil and cell membranes contact by partitioning the EO into the aqueous phase and the solvent [44].

To skip the use of emulsifiers it has been proposed to perform MIC studies in liquid medium supplemented with 0.15% of bacteriological agar [35].

The adoption of distinct definitions of MIC and MLC values may also become a problem. MIC has been described as the lowest concentration needed to obtain a 90% reduction in yeast growth measured by spectrometry, or required to inhibit the visible growth and even as the value needed to maintain or reduce the inocula. Moreover, in broth microdilution methods, some studies present the results in EO volume units while others use weight [42, 44-50]. MIC values published under these conditions are not directly comparable.

ESSENTIAL OILS' ANTI-*CANDIDA* ACTIVITY

In vitro and *in vivo* studies have been published confirming the effect of EO and their major components on *Candida* spp [3, 29-31, 33, 41, 42, 45-63]. Usually, from the chemical composition of the EO it is possible to presume their activity. The anti-*Candida* activity of the most cited EO will be described below and resumed on Table **1**.

EO considered to be potent antifungals have usually in common high contents of phenols. *Origanum* spp and *Thymus* spp oils are the more cited as being rich in those compounds. Their activity has been largely evaluated [47-49, 52].

Origanum oil and one of its major compounds, carvacrol, were shown to be able to inhibit *in vitro* both the germination and the mycelial formation of *C. albicans*, in a dose-dependent manner [52]. Other studies confirmed that high amounts of carvacrol present in samples of *O. virens* explain its great antifungal activity against *Candida* spp, with clear inhibition of germ tube formation [29, 49]. Moreover, it is known that *O. vulgare* EO, as many other plant extracts, show great variability in its composition due to the existence of various chemotypes and also as a consequence of different environmental and climatic conditions. One extract obtained from leaves of *Origanum vulgare* spp *vulgare* from Turkey presented a different chemical composition from the others described above: caryophyllene and spathulenol were the major compounds. Apparently these findings display a new oil chemotype with good activity against *C. albicans* [43].

Thymus spp are also currently accepted as being active against *Candida* spp. Chemotypes, with higher phenols content, do have greater activity [29, 60]. Samples of different species and from chemotypes rich in carvacrol and thymol, namely *T. pulegioides, T. vulgaris* and *T. zygis*, exhibited a potent anti-Candida activity, classified as fungicidal. On the other hand, oils without phenolic compounds and rich in 1,8-cineole, like *T. mastichina* and *T. capitellatus,* showed to be less potent [47, 48, 64].

In the same way samples of *Satureja montana* and *O. vulgare* rich in carvacrol and thymol, showed to have greater activity than some *T. vulgaris* oils from distinct chemotypes (linalool, borneol and geraniol chemotypes) [29, 30].

Thymbra capitata is another species rich in carvacrol, which is recognized as being highly antiseptic and valuable for cutaneous infections. Sub-inhibitory concentrations of the EO were able to inhibit germ-tube formation and both the oil and its major compound presented a good fungicidal effect against *Candida* spp [50].

Melaleuca spp, is widely available in Australia and its EO, Tea Tree Oil (TTO), is considered to be a relevant anti-*Candida* agent [33, 45, 54, 56, 65-68]. Due to its importance, the composition of this EO is regulated by an international standard [69]. However, as the species of *Melaleuca* spp from which the oil must be sourced are not stipulated, the majority of the published data concerns *M. alternifolia* species. Nevertheless, plant materials from other species are reported as TTO source and those oils also respect the requirements of the standard [45, 56, 65, 67, 70].

The two most reported TTO oils are terpinen-4-ol and 1,8-cineole chemotypes. Their activity seems to be distinct, being terpinen-4-ol chemotypes, the most active against several strains of *Candida* spp. The activity of the oil and its major component was similar and revealed to be more potent than amphotericin B and 5-fluorocytosine [45, 56, 65]. Although reports attribute the activity of this oil mainly to terpinen-4-ol, some results indicate that linalool and α-terpineol also contribute to the general activity of the oil [33].

EO of some other *Melaleuca* species were studied: *M. ericifolia* (methyl eugenol), *M. leucadendron* (1,8 cineole; α-terpineol) and *M. armillaris* (1,8 cineole; terpinen-4-ol). Regarding anti-*Candida* activity, *M. armillaris* oil had a marked effect compared to the other samples [65]. This re-

Table 1. Essential Oils Origin, Major Compounds and Activity on *Candida* spp

Essential Oil	Plant origin	Major Compounds	Activity on *Candida* spp	References
Origanum oil	*O. virens* *O. vulgare*	Carvacrol	Inhibit *C. albicans* germination and mycelial elongation; fungicidal effect	[29, 49, 52]
	O. vulgare spp *vulgare*	Caryophyllene Spathulenol	Good activity against *C. albicans*	[43]
Thymus oil	*T. pulegioides* *T. vulgaris* *T. zygis*	Thymol Carvacrol	Potent effect; Fungicidal activity	[47, 48]
	T. mastichina *T. capitellatus*	1,8-Cineole	Low activity	[48, 64]
Thymbra oil	*T. capitata*	Carvacrol	Sub-MIC values inhibit germ tube formation; fungicidal effect	[50]
Tea Tree Oil	*M. alternifolia*	Terpinen-4-ol	Good activity	[45, 56]
	M. alternifolia	1,8-Cineole	Low activity	[65]
	M. armillaris	Terpinen-4-ol 1,8-Cineole	Marked activity	[65]
Mint Oil	*M* x *piperita*	Menthol Menthone 1,8-Cineole	Potent activity	[30]
	M x *piperita*	Linalool Carvone	Moderate activity	[41, 44]
	M. cervina	Pulegone Isomenthone	Samples with differences in chemical content had similar activities	[57]
Cinnamon Oil	*C. camphora*	Linalool	Moderate activity	[30]
	C. verum	Eugenol	Potent activity	[30]
Clove Oil	*S. aromaticum*	Eugenol	Potent activity	[29, 55, 72]

sult might be due to the presence of terpinen-4-ol, reported as the active compound of *M. alternifolia* [66].

Concerning mint, *Mentha* spp, their EO are known since antiquity as having antifungal properties [41, 57, 71]. Species from *M* x *piperita*, *M. spicata* and *M. cervina* showed to have good to potent effect against *Candida* spp. Samples from *M* x *piperita* rich in linalool and carvone were considered to have moderate activity and samples with high content in menthol, menthone and 1,8 cineole demonstrated an activity similar to other oils consider to be potent antifungals (*T. vulgaris* and *M. alternifolia*) [30, 41, 42, 44, 45, 68]. In the specific case of *M. cervina* differences in chemical composition were observed, particularly in the content of pulegone and isomenthone. However, this factor, apparently, was not determinant for the anti-*Candida* activity [57].

Another important EO is cinnamon oil, characterized by the presence of aromatic compounds (phenylpropane pathway), less frequent in EO but with an interesting activity against yeasts. Two samples, one of *Cinnamomum camphora*, rich in linalool, and one of *Cinnamomum verum*, rich in eugenol, were studied. The *C. verum* sample presented greater activity, probably due to the presence of eugenol [30]. This result is in accordance with those obtained with *Syzygium aromaticum* EO, which contains important amounts of eugenol and is classified as a good anti-*Candida* agent. Its activity was considered to be directly dependent with the concentration of eugenol [29, 55, 72].

Among EO rich in aldehydes, *Cymbopogon citrate*, commonly known as Lemongrass oil, showed a potent antifungal activity against *Candida* spp. The effect of the oil and its major compound, citral, was similar [53, 73].

There is no consensus in relation to antifungal effectiveness of *Santalum album* EO. Studies demonstrated the absence of activity even at high concentration [30, 74], while others presented better results with this oil than with *T. vulgaris* and *O. vulgare* ones, using agar dilution technique [42]. It seems that the methodology explains the divergence in effects.

Concerning *Salvia officinalis* its EO showed a moderate activity against *Candida* species. Those EO are characterized by high contents of thujones, known as hepatotoxic, a limitation factor on its possible clinical use [31, 42, 45, 75].

CHEMICAL COMPOUNDS AND ANTI-*CANDIDA* ACTIVITY

As it has been stated, the efficacy of the EO is intimately related to its chemical composition. EO are frequently mixtures of hydrocarbons and their oxygenated derivates are the responsible for EO odour and taste [24, 76, 77]. Generally, EO constituents do have low molecular weight and may have two original structures terpene and phenylpropane. Terpenes are biosynthesized from isoprene units and are classified as monoterpenes (C10), the most representative molecules, and sesquiterpenes (C15). Those compounds show a great variety of structures [23]. In addition, some monoterpenes and other components of EO do also occur in plants under a glycoside form. Glucosides of monoterpenols and of 2-phenylethanol are translocated from leaves to flowers as aroma precursors [77].

Phenylpropane compounds derive from shiquimat and present aromatic structure. They are less frequent and with lower structural diversity than terpenes. Eugenol is one of the most studied molecules included in this group. Aliphatic, sulfur and nitrogen substituted compounds are not regularly found [3, 23].

Phenylpropanoids

Eugenol (Fig. **1**) is considered to have a potent anti-*Candida* activity [72]. While recent studies concluded that eugenol is more active with a free phenolic OH than with both OH methylated [30], it has been previously stated that the activity with or without methylation is comparable [78]. Eugenol proved to be significantly less cytotoxic to human red blood cells than amphotericin B [58].

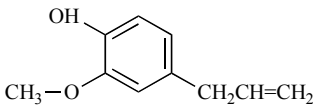

Fig. (1). Eugenol.

Estragole has been recognized as one of the most potent antifungal compounds and *trans*-cinnamaldehyde is another phenylpropanoid that revealed a high anti-Candida activity [30].

Terpenoids

Hydrocarbons

This chemical group is the most heterogenous one. γ-Terpinene and α-terpinene are considered to have some fungistatic effect. ρ-Cymene, a thymol precursor, is generally defined as inactive although different results have been published [30]. Among the pinene variants, α isomer is considered as inactive while β-pinene (Fig. **2**) was found to have a good activity against *Candida* sp. β-fellandrene (Fig. **3**) demonstrated a great activity against yeasts in contrast with its similar compound limonene that is indicated as inactive, even at maximum concentrations. While their main chemical difference is the different position of the double ties, this

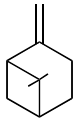

Fig. (2). β pinene.

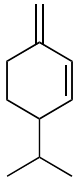

Fig. (3). β phellandrene.

seems to be insufficient to explain the distinct biological activity. On the other hand, limonene is considered to have some activity when vapor phase methodology is applied [30, 33, 40, 60, 79].

Moreover, in general, hydrocarbon monoterpenes are significantly less active than oxygenated monoterpenes [33].

Aldehydes and Ketones

Citral (a mixture of two isomers: geranial (Fig. **4**) - citral A and neral (Fig. **5**) - citral B), an aldehyde, has been recognized as active against yeasts being able to inhibit mycelial growth form of *C. albicans* [3, 30, 34, 53]. This compound seems to be able to form a charge transfer complex with an electron donor of fungal cell, acting as a fungicidal agent [78].

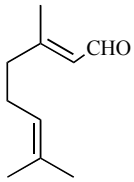

Fig. (4). Geranial.

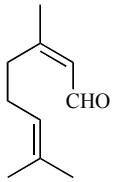

Fig. (5). Neral.

On the other hand, pulegone and ***cis*-**thujone are important ketones known to be hepatotoxic, justifying the need to control their level within EO composition [57, 75]. Their presence does not seem to influence the oil activity against *Candida* spp.

Alcohols

Alcohols do show in general antimicrobial activity. Ciclyc monoterpenic alcohols are considered to have scarce antifungal activity in contrast to aciclyc ones (geraniol, *β*-

citronellol, nerol and linalool) [30]. Primary alcohols (geraniol, citronellol) are more potent than secondary (borneol) and tertiary (linalool) alcohols [78].

The activity of linalool in literature is not consensual. Some papers classify this compound as inactive [60] while others consider it to have antifungal activity with a fungicidal effect [30, 33]. Geraniol, β-citronellol, nerol are considered to be good anti-*Candida* agents [30]. α-Terpineol and terpinen-4-ol (Fig. **6**) are recognized as potent compounds with a fungicidal effect [33, 60, 66, 67] . Farnesol, a sesquiterpenic alcohol, was described as inactive against *C. albicans* even at its maximum concentration [30]. Previously, this compound was identified as an inhibitor of yeast-to-mycelium germ tube formation, a pathway witch is independent of *C. albicans* viability and replication mechanism [80].

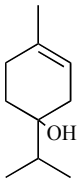

Fig. (6). terpinen- 4 –ol.

Ether Compounds

1,8-Cineole (Fig. **7**) is the most studied molecule of this group. It showed some anti-*Candida* activity with a fungicidal effect [33, 56, 66]. However, it has been reported to cause skin irritation [56], an effect not seen by others consensual [67].

Fig. (7). 1,8cineole.

The activity of several benzylic ether derivatives of 1,8-cineole with different substituents in the aromatic ring has also been investigated. Results suggested that the type of substituent introduced in the aromatic ring influenced the intensity of action. Compounds containing a methyl group show a wider activity than compounds with methoxyl group, probably due to its lipophilic nature. Contrasting with the position of the methoxyl group in the aromatic ring, the position of the methyl group does not seem to interfere with the activity of the tested compounds against *C. albicans* [81].

Phenols

Phenolic compounds appear to be the most powerful antifungal molecules found in EO composition [29, 60].

When comparing the activity profiles of molecules of this group, it is apparent that the addition of alkyl group to benzene ring of phenol enhanced the antifungal activity: the larger the size the higher the activity [78]. In this way, thymol is more potent than phenol concerning to antifungal activity. Thymol Fig. (**8**) demonstrated a potent antifungal activity on *Candida* spp including the ones highly resistant to classical antifungals. Carvacrol Fig. (**9**) and thymol as well as estragole (phenylpropanoid compound) are the most active among phenols [29, 30, 48, 49].

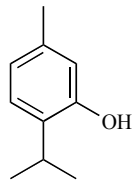

Fig. (8). Thymol.

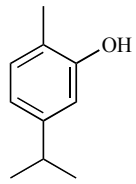

Fig. (9). Carvacrol.

MECHANISM OF ACTION OF ESSENTIAL OILS AND THEIR CONSTITUENTS

Overall it is believed that due to their highly complex composition, EO antimicrobial activity is not related with one single mechanism of action but result from the effect of different compounds on several cell targets. Similar effects are produced by EO against yeast and bacteria and mechanisms involved are thought to be non specific [79]. MIC and MLC values are usually equivalent indicating that they are mainly fungicidal [33, 60].

EO activity is directly dependent on their partition characteristics. They are able to pass through the cell wall and locate between fatty acid chains of lipid bilayers, altering membrane fluidity, causing degradation of cell wall, disruption of cytoplasm membrane and damage of membrane proteins. The leakage of cell contents, coagulation of cytoplasm, depletion of proton and lysis are consequences of this inducted changes in membrane structure and function. Oxygenated terpenic compounds, such as thymol and carvacrol, are frequently considered as those major responsible for this effect due to their ability to modify membrane permeability by chemical reaction with amino and hydroxilamine groups of membrane proteins. Other oxygenated monoterpenes (linalool, geraniol, citral and camphor) are also important although they are less active [3, 23, 61, 82].

For some oil, the effect on the integrity of yeast cell was investigated using a fluorescent marker - propidium iodide (IP) - and flow citometry. *Thymus* spp, *Origanum virens, Syzygium aromaticum* and their major compounds (carvacrol, thymol, eugenol) acted by inducing primary lesion of the cytoplasmic membrane [48, 49, 72].

Analysing the chemical structure of thymol, a potent antifungal compound, an amphypatic and/or hydrophobic behav-

iour is inferred. This characteristic confers to thymol the ability to migrate from the aqueous phase to membranes, affecting their integrity and altering surface electrostatics. Membrane permeability and its proteins activity may be modified [72, 83]. The interference of thymol with formation and viability of *Candida* spp hyphae has been related to its ability to affect fungal cell-wall synthesizing enzymes [59]. A growth phase dependent morphological damage of the membrane caused by thymol and eugenol has been observed [84]. Thymol presented a more pronounced effect than eugenol.

Changes in permeability and in membrane fluidity occurred when yeasts cells were pre-treated with *M. alternifolia* EO and/or its components (terpinen-4-ol, 1,8-cineole, α-terpineol, γ-terpinene and α-terpinene). Those effects were probably related to different terpene compounds positioning within membrane lipid bilayer, thought to be dependent on their hydrophobicity. The activity of this EO was also mediated by plasma membrane ATPase inhibition [54, 61].

Germ tube formation, a change in morphology that is important for pathogenicity, was significantly inhibited by *Thymus* oils, TTO, thymol and eugenol. Interestingly, *T. mastichina* and 1,8 cineole, that showed to have lower fungicidal activity, presented greater inhibition ability than others *Thymus* spp oils and their phenolic components [48, 72, 85].

As described above the activity of antifungal drugs against yeasts may be limited by their penetration and chemical reaction into biofilm matrices. Recent studies evaluated the interference of antifungal EO on the formation of biofilms by *Candida* strains [63, 68]. Published data confirm that thymol is able to inhibit both the initial formation and the stability of mature biofilms. The architecture of the mature biofilms was observed under fluorescence microscopy confirming the destruction of the 3D morphology, remaining only a few damage filamentous forms. Cell viability studies evidenced the fungicidal effect of this compound. This effect in addition to the ability to reduce the amount of metabolically active yeasts and the dimorphic switching from yeasts to filamentous forms have been proposed as involved mechanisms [59].

Similarly, eugenol proved to be active against pre-formed *C. albicans* biofilms, adhesion of cells and subsequent biofilm formation in concentrations similar to MIC values. It was also proved that eugenol-treated cells had a suppressed filamentous growth capacity [58].

The constituents of many volatile oils have been stated to interfere with respiration and electron transport in a variety of bacteria [77]. *M. alternifolia* oil produced the same effect on yeasts cells at MIC values [61].

Because EO seems to have no specific cellular targets, no particular resistance has been described [23].

NEW THERAPEUTIC APPROACH FOR *CANDIDA* SPP INFECTIONS: ESSENTIAL OILS AS AN ALTERNATIVE OR A THERAPEUTIC COMPLEMENT TO CLASSICAL ANTIFUNGAL AGENTS

New trends for antifungal drugs are being devised. Problems related with the low selectivity of classical antifungal molecules to defined targets, as well as, toxicity effects and fungistatic versus fungicidal action are urgent to overcome. Although the need for new drugs is claimed, investigation to obtain new molecules seems to be modest.

Natural compounds, especially EO, mainly during the last decades have been considered as possible alternatives or therapeutic complements to classical antifungals.

Amphotericin B, a classical antifungal widely used on *Candida* spp infections, is a polyene antifungal molecule with amphipatic characteristic allowing binding to the ergosterol of the fungal cell membrane creating pores across this structure. From this interaction results a modification of the permeability causing osmotic instability which results in a fungistatic and fungicidal effect. On the other hand, fluconazole is an azole derivative that inhibits the ergosterol biosynthesis pathway, compromising the fluidity, asymmetry and integrity of the fungal cell membrane, resulting in a fungistatic effect [20, 86]. These antifungal effects on yeast cell are similar to the ones previously described for EO, announcing a possible synergistic advantage on the therapeutic association of those compounds.

Reports considering EO more active than classical antifungals have been published [53, 56]. However, those results are not consensual [55, 87].

Recognizing the interest of EO as anti-*Candida* agents, the association of those compounds with classical antifungals has been evaluated, particularly considering the treatment of resistant cases. This has been the case for the association of *T. vulgaris* and *Cinnamomum cassia* EO with amphotericin B, resulting in a significant reduction of the MIC value of the later agent. Another synergistic effect was observed with the association of estragole and ketoconazole, resulting in a clear fungicidal activity in contrast to the fungistatic effect of ketoconazole alone. Interestingly the association of eugenol with amphotericin B showed to have antagonism or no effect [29, 51, 88, 89].

On the same way, the association of fluconazole with compounds with a phenolic nature depleted ergosterol in sensitive and resistant *Candida albicans* isolates enhancing the inhibition effect of ergosterol biosynthesis [90]. Those potentiating effects obtained *in vitro* may be promising for the development of less toxic and more effective therapies.

When analyzing the mode of action of EO it seems unlikely that resistance may occur, since multiple simultaneous mutations are required and numerous targets would have to adapt to overcome all the distinct antifungal actions of each and all of the components.

In fact, the association of EO with classical antifungals for *Candida* spp infections may be a future way to potentate their activities, as the final effect seems to be related to a greater accessibility of antifungal classical agents to their fixation sites inside yeasts cells, involving the formation of transmembrane pores with leakage of intracellular components, resulting in cell death. Some studies defend this concomitant use in order to reduce antibiotic concentration and secondary effects, stepping up to fungicidal activity of the usually fungistatic pharmacologic [29, 51, 88, 91].

It is also important to consider in possible associations, the role of EO showing complementary action, in addition to antimicrobial ones. It will be relevant, for example, to develop combinations with immunostimulant agents that will turn to reality a true prophylactic regimen

IN VIVO STUDIES, TOXICOLOGICAL RESULTS AND PHARMACEUTICAL FORMULATIONS

Despite the traditional use of EO through inhalation, oral or topical routes for different clinical purposes controlled clinical trials and scientific data supporting EO *in vivo* safety and effectiveness are far from sufficient [92]. Even so, some EO are included as active ingredients in many topical formulations with curative or prophylactic goals, including oral and genital hygiene products [67].

Candida spp infections, particularly vulvovaginal candidosis, have been traditionally treated with EO topical remedies. The previously described *in vitro* studies on EO and their components may explain this traditional application but *in vivo* experiments are essential to conclude about their usefulness as dugs. Available data from animal model studies and small clinical trials, even scarce, support the *in vivo* efficacy of specific EO and isolated components against *Candida spp* highlighting their promising role in the future therapeutic approach of these infections.

Animal models of either topical or systemic candidosis have been used to study the *in vivo* effect of EO.

On a systemic candidosis model, 80% survival rate was achieved after a daily oral administration of either origanum oil or carvacrol for 30 days. The small daily oral dose was determined by toxicity studies and the EO was administered mixed with olive oil being well tolerated, with no apparent clinical abnormalities. Mice that were treated with the whole oil exhibited better clinical outcomes suggesting the importance of other components to the overall efficacy of the oil [52]. More research on the pharmacological properties of oregano EO or other EO with similar activities may bring new possibilities in the prophylaxis and treatment of systemic candidosis, particularly in immunosupressed patients.

Mucocutaneous candidosis was studied using animal oral and vaginal models. Carvacrol and eugenol activity was evaluated in immunosuppressed rats. The compounds were suspended in a viscous agar solution in order to promote good adhesion to the oral and vaginal tissue following topical application. Considerable reduction in the number of colonies was obtained. No acute toxicity was observed probably due to the low doses of carvacrol and eugenol used in these studies [93, 94].

Low doses of geranium oil and its major component geraniol were shown to exhibit both anti-*Candida* and anti-inflammatory activity when combined with vaginal washing, in the treatment of vaginal candidosis in similar models [95]. The observed anti-inflammatory activity is an important therapeutic issue since symptoms like itching and soreness are strongly associated with vulvovaginal candidosis. Simultaneous antifungal and anti-inflammatory activity should act as synergistic towards the desirable therapeutic outcome. Clove oil was also shown to exhibit antifungal *in vivo* activity on vaginal candidoses animal models [55].

The use of TTO and its main bioactive component, terpinen-4-ol, as main treatment for vaginal candidosis even when caused by azole-resistant strains has been supported by other *in vivo* animal studies [66, 96]. Indeed, traditional medicine practices have suggested the use of medicated tampons containing this EO as simple house-made treatment for this vaginal infection [92]. In 1985, Belaiche published a small clinical study on the success of TTO in the treatment of vaginal candidosis. The administration of this natural product was achieved by preparing vaginal suppositories (also referred as ovules or pessaries) made of gelatin with *M. alternifolia* EO. Good clinical results and low incidence of adverse reactions were described with daily administration for 90 days [97]. Further preclinical and larger clinical studies need to be performed.

Recognizing the importance of the vaginal lactobacilli protective flora in maintaining the physiological equilibrium and reducing possible recurrences, the effect of EO proposed as therapeutic agents for VVC on these microorganisms is urgent. TTO seems to have the advantage of not disturbing significantly *Lactobacillus* spp growth. The need to perform studies in order to clarify adverse reactions of its application has been highlighted [22]. In the same way, this EO was also considered to be useful in removing skin transient flora while suppressing but maintaining resident flora [98]. Indeed, studies evaluating the effect of EO with interest in anti-*Candida* activity on tissues commensal flora must be performed previously to clinical studies.

Cases of oral candidosis refractory to conventional therapy in immunocompromised patients, particularly AIDS patients, have been treated using TTO-containing mouthwashes. Water-based instead of alcohol-based formulations are proposed to reduce local reaction and discomfort. Although there have been good results regarding the efficacy of this alternative therapeutic approach, additional clinical trial data based on studies with larger numbers of participants and other patient groups are necessary [99, 100].

Toxicity and compliance must be better explored but these preliminary data are encouraging particularly because of success on azole-resistant strains. The use of these products has also been proposed as a potential preventive or therapeutic strategy in the management of oral candidosis in advanced cancer patients. This possibility is based on the good *in vitro* susceptibility patterns to TTO of isolated yeasts from these patients when compared with conventional therapies [62].

TTO has also been investigated for the treatment of Denture Stomatitis, an inflammatory reaction of the palatal and alveolar mucosa underlying removable dental protheses, in which *C. albicans* can be regularly isolated. 1mL TTO mixed with 4mL of a well tolerated tissue conditioner (Coe-Comfort, GC America Inc. Alsip, IL) was placed on the maxillary prosthesis in three sessions during a total 12 day period. Clinical remission of Denture Stomatitis was even faster with TTO-tissue conditioner than with Nystatin-tissue conditioner mixture, used as control. TTO mixed with the Coe-Comfort tissue conditioner could thus be used as an alternative therapy for denture stomatitis resistant to traditional therapies [101].

In most of the reviewed studies on the anti-*Candida* activity of EO there is a lack of data on formulation, pharmacokynetics and the influence of delivery systems on the efficacy of the drug. However, these are main issues regarding the therapeutic application of EO. The development of suitable delivery systems should make EO easy to administer, biocompatible and able to maintain their antifungal properties all over the storage period, with no toxicity. EO particular physical-chemical characteristics present certain difficulties for formulation and packaging. Their lipophilicity becomes a problem when solubility in water is required, while their volatility requires technological resources to assure stability and bioavailability.

A study comparing the *in vitro* activity of both non-formulated TTO and several intra-vaginal products containing the oil as active ingredient has been published. These products were shown to have MIC and MLC comparable to those of non-formulated TTO suggesting that formulated TTO retains the activity of the former [45].

Similar results were obtained in a study that compared *Thymus vulgaris* EO activity before and after incorporation in a polycarbophil-based gel [102]. Even so, *in vivo* studies and clinical trials are required to confirm these results and determine the real usefulness of these products.

Clove oil has been formulated by means of an emulsion and oil-containing lipossomes to evaluate their potential as future drugs to treat vaginal candidosis in a murine model. The emulsion and oil-containing lipossomes were administered either subcutaneously or topically. It was found that topical treatment was more effective than subcutaneous treatment. On the other hand lipossomes showed to be more effective in the suppression of fungal burden highlighting the importance of pharmaceutical vehicles and formulation in the efficacy of vaginal drug delivery systems [55].

The use of liposomes and other techniques of encapsulating the oil can overcome topical undesirable reactions by avoiding direct contact of EO with skin or mucosal surfaces. Indeed, when administered topically EO were found to induce certain adverse reactions such as irritation and contact allergy. However these reactions seem to be strongly dependent on the EO composition, stability, dose and concentration. Extremely high doses of topically applied EO have been associated with systemic toxicity in animals and humans, due to transdermal absorption of the oil [55, 99, 100, 103].

In the way that some data suggest that skin reactions could be caused by oxidation products resultant from exposure to light and/or air, formulation factors may be crucial for the overcome of toxicity, while inhibiting the volatilization of the oil which avoids loss of the therapeutic effect [103]. On the other hand it is possible to allow the controlled release of this active ingredient by formulating adequate vehicles for the required application [104]. However, excipients may also have the opposite effect as has been shown by the diminished antimicrobial activity of TTO in the presence of some organic and surfactant excipients [42, 105]. The overall properties of the base into which the oil is incorporated were also shown to strongly influence its activity. It has been suggested that hydrophilic bases are more effective than lipophilic ones due to the greater affinity of EO components to lipophilic bases which impairs their release to the medium [106]. Additionally it is possible that interferences occur between the drug delivery system and some environmental products like secretions components or skin natural products that may affect the EO efficacy [107]. Concerning the EO release and skin permeation, *in vitro* studies on TTO topical formulations have shown that differences on the semisolid drug delivery system strongly influences the oil permeation through human skin [108]. In this case a semisolid oil-in-water emulsion and an ointment were shown to be superior to a cream.

Topical treatment of *Candida* spp superficial infections is strongly dependent on the ability of the administered formulation to be retained at the site of infection. In this way, the use of hydrophilic bioadhesive polymers can improve the contact of the active ingredient with the pathogen, once topically applied [109]. The development and evaluation of a polycarbophil mucoadhesive gel containing *Thymus vulgaris* essential oil revealed not only *in vitro* activity against *Candida* spp, but also adequate physical properties for vaginal administration [102]. Extensive *in vitro* and *in vivo* studies on the bioavailability and toxicology of drug delivery systems containing EO are needed. Studies on the absorption of the oil after topical administration are required to assure its safety. However, the search for new drug delivery systems with EO must not only be based on pharmacokinetic aspects but also on biologic tissue effects. Standardization of experimental procedures will probably be considered essential in the future in order to achieve quality assured results [110].

Overall EO represents a promising alternative, after incorporation in suitable delivery systems for the treatment of *Candida spp* infections. However there is a need for more extensive and appropriate evaluation of both pre-clinical and clinical investigations.

STUDY PERSPECTIVES AND CONCLUSIONS

A new attitude in relation to the treatment of infectious diseases is necessary in order to prevent antibiotics to become obsolete.

Medicinal plants are widely used as therapeutic agents since ancient times. However, the recognition of their importance by classical medicine has been delayed due to some difficulties in controlling their quality. In fact, herbs of different origins are often known by the same name although their chemical composition is distinct. These characteristics, which are determinant for their therapeutic use, are influenced by genetic factors, climatic conditions, soil composition and seasonal timing. Moreover, the plant parts used and the extraction process itself cause variation in potency and safety profile of the EO obtained.

In spite of those difficulties, EO have been studied looking for either their direct use as biological agents or as a basis for the development of new drugs with promising therapeutic application. To arrive to these objectives a multidisciplinary team working is needed in order to analyze the biological activity of the plants extracts, to understand which chemical compounds are responsible for that activity and to

search for possible side effects resulting from their use *in vivo*.

The *in vitro* antifungal activity against *Candida* spp of the most cited EO has been described. In view of the anecdotal nature of most of the reports on the anti-*Candida* activity of EO, more controlled research has been started, particularly using established standard methodology [47-50, 57, 64, 72]. In order to compare results of different studies, reference to plant species, plant material for EO isolation and extraction conditions should be perfectly described. Moreover, the EO composition must be determined and the antifungal activity of major compounds achieved. Indeed, results indicate that, at least for some major compounds, their antifungal activity is lower than the one of the EO, predicting the possibility of synergism within EO components [52, 79]. However opposite situations were reported describing in some cases a higher activity for the major compound when compared with the whole EO [66]. This effect appears to be related with a slower cidal activity of the oil against microorganisms [79].

Concerning to *in vitro* studies, methodology, growth medium and the organic solvent used to dissolve EO must be described. The microorganism species and strains used ought to be perfectly characterized. Clinical antifungal resistant strains and non-*C. albicans* species should be included.

EO use as an alternative or in association to antibiotics was discussed. However, extensive research is still needed to support their clinical use. Data concerning the EO use safety in complementary therapies on immunossupressed patients is emerging although in very limited surveys. There is a substantial lack of preclinical testing of formulated EO using suitable animal models of infection, a necessary prerequisite to large-scale clinical testing.

Toxicity against animal and human cells, elucidation on the mechanism of action, *in vivo* effects of their use and positive or negative interactions with common antibiotics are examples of topics still needing to be fully understood.

We feel, however, that in spite of the incomplete available data that requires more research on this field, EO are promising and valuable drugs as alternative or complementary therapies for *Candida* spp infections, especially mucocutaneous infections resistant to classical antifungals.

ABBREVIATIONS

EO	=	Essential Oil
VVC	=	Vulvovaginal Candidosis
RVVC	=	Recurrent Vulvovaginal Candidosis
TTO	=	Tea Tree Oil
GC	=	Gas Chromatography
MS	=	Mass Spectrometry
CLSI	=	Clinical Laboratory Standard Institution
MIC	=	Minimal Inhibitory Concentrations
MLC	=	Minimal Lethal Concentrations
DMSO	=	Dimethylsulfoxide
IP	=	Propidium Iodide

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