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Evaluating the antifungal potential of six essential oils against fungi causing wilt and dieback on olive trees

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Abstract

Essential oils, known for their antimicrobial properties, are being investigated as natural alternatives to synthetic fungicides in agriculture. This study aimed to assess the chemical composition of six commercial essential oils (clove, tea tree, rosemary, thyme, oregano, and garlic) and to evaluate their fungistatic and/or fungicidal activity against six phytopathogenic fungi that cause significant damage to olive trees in Tunisia. For this purpose, the essential oils' qualitative and quantitative chemical compositions were analyzed using gas chromatography-mass spectrometry. The antifungal activity was assessed using the poisoned substrate method at different concentrations (250, 500, 1000, and 4000 ppm). Results showed that the chemical composition analysis revealed that monoterpenoids were the dominant fraction in all oils except clove and garlic, which were primarily composed of eugenol (96.28%) and trisulfide (31.97%), respectively. The antifungal activity results showed that lower concentrations (250, 500, 1000 ppm) of tea tree, rosemary, thyme, and oregano oils had limited inhibitory effects on the tested fungi. However, *Biscogniauxia mediterranea* was highly sensitive to clove, garlic, and rosemary oils at 4000 ppm. *Fusarium oxysporum*, *Fusarium solani*, *Verticillium dahliae*, and *Lasiodiplodia theobromae* were mainly inhibited by clove oil at concentrations ranging from 500 to 4000 ppm, while Rhizoctonia bataticola was inhibited by clove and garlic oils at high concentrations. In conclusion, among the tested essential oils, clove oil demonstrated the highest antifungal efficacy, making it a promising natural alternative to synthetic fungicides for controlling olive tree phytopathogenic fungi.

Keywords: Essential oils, Phytopathogenic fungi, Gas chromatography–mass spectrometry (GC–MS), Agricultural applications, Fungistatic activity

1. Introduction

Olive groves are a very important cultivated crop in Tunisia with a total area of 2.3 million ha, and 102 million olive trees distributed in 30 % in the North, 38 % in the middle and 32 % in the South [1]. The annual production was recently estimated to be 220,000 tonnes of olives in 2023/2024. Tunisia is ranked as the fourth producer of olive oil worldwide. However, olive trees are constantly at risk of attack by fungi, which can cause severe damage to olive groves. Many pathogens constantly threaten olive cultivation. The dieback and wilting diseases induced by fungi have caused considerable economic losses in olive orchards in Tunisia [2-6]. Verticillium wilt is caused by the fungus *Verticillium dahliae,* which is one of the most important diseases occurring in olive-growing Mediterranean countries. The disease of olive was first reported in the Mahres region of Sfax, Tunisia by Triki et al, 2006 [6]. Soil-borne fungi including *Fusarium oxysporum, Fusarium solani*, *Phytium* sp., *Neonectria radicicola*, *Acremonium* sp., and *Rhizoctonia bataticola* were pathogenic causing dieback. Symptomatic trees exhibited several dead twigs and wilted leaves followed by death of the entire tree [7,8]. Other fungal species, such as *Neonectria radicicola* [3], [9], *Neofusicoccum australe* [10], *Nigrospora sp*. [9], and *Biscogniauxia mediterranea* [11] have been identified in new olive plantations and proved to be responsible of branch dieback. Previous studies in other Mediterranean countries reported that several fungi such as *Cylindrocarpon destructans, Phytophtora megasperma, Phytophtora palmivora, Phytophtora irregulare, Athelia rolfsii, Phaeoacremonium parasiticum, Phaeoacremonium rubrigenum, Phaeoacremonium aleophilum, Rhizoctonia solani, Macrophomina phaseolina, Armillaria mellea*, and *F solani*, are implicated in olive dieback disease [12-14]. Twig and branch dieback was observed in Italy and Spain [15-17]. Recently, a new fungus was identified in Crotia as *Cytos-*

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pora pruinosa [18].

In managing Fungi causing wilt and dieback on olive trees, several strategies and approaches were employed among them the use of synthetic fungicides which are the most common way to prevent fungal infections [1,19]. Several chemical molecules have long been employed to manage olive tree diseases and enhance crop yield through reducing losses [20]. They have been designed to inhibit fungal pathogens by targeting various components or mechanisms within the fungal cell, such as respiration, nucleic acid metabolism, cell membrane integrity, protein synthesis, signal transduction, and cell mitosis [21]. However, the use of these synthetic pesticides negatively impacted soil fertility, reduced the activity of soil microorganisms and affected immunity and reproduction in animals and humans [19]. Moreover, the frequent use of synthetic fungicides against plant pathogens led to microbial resistance that reduced their efficacy [21].

In response to mounting concerns about the negative impact of chemical synthetic fungicides, agriculture is now emphasizing sustainable and environmentally friendly alternatives. This shift encourages the adoption of biofertilizers, organic farming practices, and environmentally conscious agrochemical substitutes [19]. In this regard, as an alternative to conventional pesticides, biological control of plant pathogens has been suggested. Biocontrol products can be categorized into four main groups: macroorganisms, microorganisms, semiochemical products, and natural substances derived from plants, algae or animals. Essential oils (EOs), which occur naturally in various plant-based substances, are considered potential biocontrol products due to their inhibitory activity on pathogen growth either as main components or as antimicrobial adjuvants [22].

EOs, naturally produced by aromatic plants and typically extracted through hydrodistillation or steam distillation, are synthesized by all parts of these plants, including flowers, leaves, seeds, fruits, roots, rhizomes or wood [2,22]. They are complex mixtures of volatile secondary metabolites and often contain a variety of compounds, including 20 to 60 active substances categorized into two major components, which are present at relatively high concentrations compared to other trace compounds: terpene hydrocarbons, (monoterpenes and sesquiterpenes) and oxygenated compounds (alcohols, phenols, aldehydes and esters) [22]. EOs composition varies due to several factors, including the organ from which they are extracted, growth conditions, climate, soil composition, and harvesting time. Additionally, chemical differences exist between plant species of the same genus and even among varieties of the same species [22]. Some major compounds characterize some EOs, for example, carvacrol (65 %) and thymol (15 %) in *Origanum heracleoticum* EO, linalool (68 %) in *Coriander sativum* EO [22], Eugenol (90.4 %) in *Sysygium aromaticum* EO [23] and diallyl disulfide (30-40 %) in *Allium sativum* L. EO [24]. Numerous studies have investigated the link between the major components of EOs and their antimicrobial properties, particularly their antifungal effects [25]. EOs derived from thyme (rich in thymol and carvacrol), tea tree (abundant in terpenes), and peppermint or clove have undergone extensive research for their antifungal properties. Moreover, various other EOs have also shown efficacy against fungal infections [23]. Recently, Eos and their predominant components were tested for antifungal activity against 14 phytopathogenic fungi isolated from olive trees. Chinese cinnamon and oregano EOs, along with their active compounds, completely inhibited all tested fungi, highlighting their potential as biological control agents. In contrast, lemon and peppermint EOs, as well as limonene, menthol, and thymol, were the least effective, while *Dothiorella iberica* and *Nigrospora gorlenkoana* were the most sensitive fungi [26]. The antifungal efficacy of lemon EO was tested *in vitro* against three pathogenic fungi attacking grapevine wood, including *Botryosphaeria dothidea*. Growth inhibition of *Botryosphaeria dothidea* was observed at 0.25%, with all tested concentrations proving lethal under the conditions [27]. The essential oil of *Ammoides verticillata*, rich in carvacrol, limonene, and p-cymene, inhibited *Alternaria alternata* and *Fusarium solani*, causing olive rot, by 89%. It also showed in vivo antifungal activity against *Aspergillus niger* and *Penicillium crustosum* and 100% fumigation toxicity against *Bactrocera oleae* [28].

In general, EOs exhibit three primary mechanisms of action against fungi: cytotoxic effects, anti-biofilm effects, and impacts on mycotoxins. Several researchers have identified various key elements in the mechanism of the EOs antifungal activity that appear dependent on the concentration used. A loss of membrane integrity and a decrease in the amount of ergosterol (major lipid of the fungal biomembrane) as well as an inhibition in cell wall formation have been reported. The disruption of fungal membrane integrity, leads to leakage of cellular contents, thereby impairing fungal cell function. A significant decrease in ergosterol content has been observed, weakening the membrane and increasing its permeability. EOs also interfere with the formation of the fungal cell wall, an essential structure for cell shape and protection, contributing to cell lysis. Moreover, EOs target mitochondria and the endoplasmic reticulum to inhibit membrane ATPases and cytokine interactions by disrupting the energy production process and leading to cellular dysfunction [23]. Furthermore, EOs can disrupt endoplasmic reticulum functions, impairing protein folding and transport, which can trigger stress responses that further inhibit fungal growth. These combined mechanisms ultimately lead to fungal cell death. Additionally, EOs have been shown to inhibit the formation of biofilms, which are protective layers formed by fungi to shield themselves from environmental stresses, thus enhancing their antifungal activity [29]. D'agostino et al. [23] summarized the main EO compounds antifungal mechanism against several strains. The same compound can affect in different ways the growth or the viability of fungi. Indeed, thymol was described to inhibit telomerase activity resulting in cell death, and arrest of the cellular cycle of *Saccharomyces cerevisiae*. While, against *Fusarium graminearum*, it induces electrolyte leakage and lipid peroxidation [23]. Carvacrol targets transmembrane protein and causes disruption and depolarization of the plasma membrane of *Candida tropicalis* while it causes endoplasmic reticulum disruption with unfold protein response against *Candida albicans* [23].

In this study, we investigated the chemical composition of six selected commercial EOs and their effectiveness against phytopathogens causing wilt and dieback on olive trees. Our focus was on assessing their potential as natural fungicides, considering their antimicrobial effect in reducing the risk of pathogen resistance, and their en-

vironmentally friendly properties such as biodegradability and lack of bioaccumulation.

2. Materials and methods

2.1 Essential oils

Six natural and pure commercial EOs were utilized: clove (*Syzygium aromaticum*), tea tree (*Melaleuca alternifolia L.*), rosemary (*Rosmarinus officinalis L.*), thyme (*Thymus vulgaris L.*), garlic (*Allium sativum L.*), and oregano (*Origanum vulgare L.*). Oils were produced by the Phytochimie Company, Sfax, Tunisia a manufacturer specializing in the production and standardization of plant-derived extracts and EOs. Each EO was obtained through steam distillation or cold pressing, ensuring purity and preservation of bioactive compounds. The oils were stored in amber glass bottles at 4°C to protect them from light and oxidation until use. These EOs were chosen based on their well-documented antimicrobial activity and potential applications in plant disease management.

2.2 Strains of filamentous fungi and growth conditions

The phytopathogenic fungi used in this study were sourced from the phytopathology team's collection at the Olive Institute. These fungi were isolated from the roots and stems of olive-infected trees in Tunisia. The identified species include *Fusarium oxysporum (F. oxysporum) , F. solani* [7], *Rhizoctonia bataticola* (*R. bataticola*) [30], *Verticillium dahliae (V. dahlia)* [4], *Lasiodiplodia theobromae* (Cheffi et al. 2024 submitted), and *Biscogniauxia mediterranea (B. mediterranea)* [11] (Table1). These fungi were cultured on Potato Dextrose Agar (PDA) for 5-7 days at 26 °C under ambient daylight conditions. The cultures were subsequently preserved at 4 °C and subcultured monthly.

2.3 Gas chromatography analysis

The chemical composition of the EOs was determined both qualitatively and quantitatively using gas chromatography coupled with gas chromatography-mass spectrometry (GC-MS). The analysis was conducted on an HP6890 gas chromatograph coupled with an HP 5973A mass spectrometer (Agilent 19091S-433, Germany). A non-polar capillary column (HP-5MS) with dimensions of 30.0 m length, 0.25 mm internal diameter, and 0.25 µm film thickness was employed. Helium served as the carrier gas at a flow rate of 1 mL/min. The temperature range for analyses was 35-250 °C, with a heating rate of 10 °C/min ensuring optimal separation of volatile compounds. Solutions of the tested samples in dichloromethane (at a 1:50 v/v ratio) were introduced which facilitated efficient vaporization without altering the chemical composition. The solvent type did not impact the chemical composition. Separated fragments were identified by MS (Agilent 5973 inert MS) in Electron Impact (EI) mode. In the mass range of 50 to 700 m/z, ions were detected at 1 scan/s. Compound identification was achieved by comparing the obtained mass spectra with those available in the Wiley mass spectrometry database and retention indices from literature data.

2.4 Antifungal activity assay

The antifungal activity of the tested EOs was evaluated using the poisoned food technique, following the methodologies of Soliman and Badeaa [31]. To enhance solubility, the EOs were first dissolved in sterile distilled water containing 0.01% Tween 20 as a surfactant to obtain a stock solution of 10 mg/mL. These solutions were then incorporated into liquified Potato Dextrose Agar (PDA) to obtain final concentrations of 250, 500, 1000, and 4000 ppm. These concentrations were prepared by corresponding dilution in sterile distilled water containing 0.01% Tween 20. The final concentrations (250, 500, 1000, and 4000 ppm) were obtained by serial dilution using the following formula: C_1. V_1= C_2.V_2 were C_1: initial concentration of the EO stock solution (ppm or mg/mL), C_2: final desired concentration (ppm), V_2: final total volume of the PDA medium (mL). Control plates were prepared without EOs but contained the same amount of Tween 20 to ensure consistency. Once prepared, the Petri dishes were inoculated with the fungal cultures and incubated at 26°C in the dark. The incubation period ranged from 4 to 12 days, depending on the fungal growth rate, until the mycelial expansion in the control plates reached the edges. The diameter of fungal colonies was measured in two perpendicular directions to determine the extent of growth inhibition. Each treatment was conducted in triplicate to ensure reliability. The fungistatic effect was assessed based on the percentage of fungal colony growth inhibition, calculated using the following equation:

I=(K-C)/K*100 [32].

I: Inhibition coefficient-growth stimulation (%),

K: Diameter of the fungus colony on the control plate (mm),

C: Diameter of the fungus colony on the plate with the given oil (mm).

2.5 Statistical analysis

The results were reported as mean values ± standard deviation. Graphical representations were created using R Studio. A two-way hierarchical cluster analysis, based on Ward's method, identified clusters with similar patterns. Additionally, principal component analyses were performed to assess variable relationships.

3. Results

3.1 Essential oil chemical composition

The EOs of clove, tea tree, rosemary, thyme, garlic, and

Table 1. List of fungi used in this study.

Fungi	Isolation source	Symptom	Reference
Fusarium oxysporum	Roots	Dieback	[7]
Fusarium solani	Roots	Dieback	[7]
Rhizoctonia bataticola	Roots	Dieback	[30]
Verticillium dahliae	Roots	Wilt	[4]
Lasiodiplodia theobromae	Stems	Dieback	Cheffi et al. submitted
Biscogniauxia mediterranea	Stems	Dieback	[11]

oregano were subjected to GC-MS analysis. The details of these EOs regarding their compositions are shown in Figure 1 and in Table 2. These results showed a total of 74 different compounds representing 100 % of the total compounds of the clove, tea tree, rosemary and oregano EOs and 97% of thyme and garlic. Specific compounds were identified in each EO: 2 in clove, 18 in tea tree, 7 in thyme and garlic, 11 in oregano, and 12 in rosemary (Fig. 1, Table 2). Moreover, shared compounds were also detected. Rosemary, thyme, and oregano showed the highest shared compounds composed of alpha-pinene, gamma-terpinene, linalool, borneol, camphene, alpha-terpinene, alpha-phellandrene, beta-pinene and beta-myrcene (9 compounds); followed by rosemary and thyme sharing camphor, alpha-fenchyl acetate, alpha-terpinolene, and thujene (4 compounds).

The detected compounds were classified according to their chemical classes (Fig. 2). The two-way hierarchical cluster analysis showed clusters grouping oregano and rosemary and another grouping tea tree and thyme, whereas clove and garlic showed specific profiles (Fig. 2a). More specifically, oregano and rosemary showed high amounts of hydrocarbon monoterpene (33.47 and 28.74 %, respectively) and other oxygenated monoterpenes (61.88 and 48.67 %, respectively), whereas tea tree and thyme were composed mainly of monoterpenoid alcohol (39.59 and 64.97 %, respectively) and hydrocarbon monoterpene (44.27 and 28.23 %, respectively). The main chemical

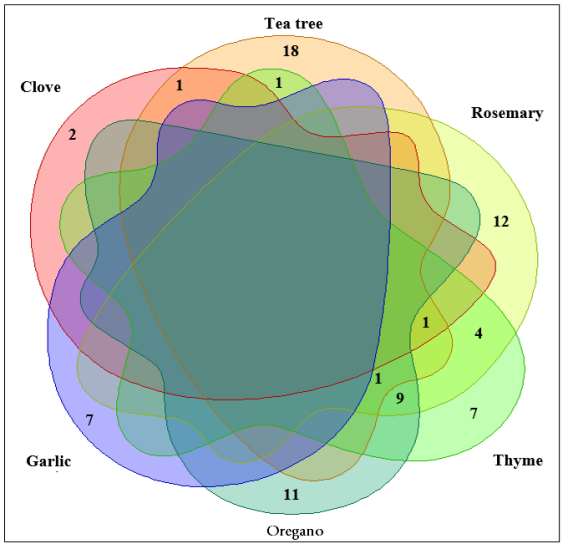

Fig. 1. Venn diagram comparing the chemical composition of the six essential oils. Area without specified number means the absence of shared compounds between the essential oils.

Table 2. List of specific and shared compounds of the six essential oils determined by GC-MS analysis

Essential oil(s)	N	Compounds
Clove	2	Acetyleugenol, Cyclohexanol
Tea tree	18	Thujene, Alpha-pinene, 2-Beta-pinene, Beta-myrcene, l-Phellandrene, (+)-4-Carene, P-cymene, Gamma-terpinene, Alpha-terpinolene, Alpha-terpineol, Alpha-gurjunene, (+)-Aromadendrene, Naphthalene, Azulene, Delta-cadinene, Cadina-1,4-diene, Globulol, Methanoazulene
Rosemary	12	Tricyclene, Eucalyptol, Alpha-terpineol, Ylangene, Copaene, Alloaromadendrene, Beta-selinene, Alpha-amorphene, Alpha-muurolene, Beta-bisabolene, Gamma-cadinene, Delta-cadinene
Thyme	7	Sabinene, O-Cymene, Beta-trans-ocimene, Cis-sabinenehydrate, Thymol methyl ether, Anthranilic acid, Beta-bisabolene
Oregano	11	Alpha-thujene, Delta3-carene, O-cymene, P-cymene, Terpinolene, Alpha-terpineol, Carvacrol methyl ether, Thymol, Carvacrol, Trans-caryophyllene, Alpha-humulene
Garlic	7	Diallyl sulfide, Diallyl disulphide, Disulfide, 3,4-Dihydro-3-vinyl-1,2-dithiin, 3-Vinyl-1,2-dithiocyclohex-5-ene, Trisulfide, Diallyl tetrasulphide
Clove and Tea tree	1	Eugenol
Tea tree and Thyme	1	Aromadendrene
Rosemary and Thyme	4	Camphor, Alpha-fenchyl acetate, Alpha-terpinolene, Thujene
Rosemary, Thyme and Oregano	9	Alpha-pinene, Gamma-terpinene, Linalool, Borneol, Camphene, Alpha-terpinene, Alpha Phellandrene, Beta-pinene, Beta-myrcene,
Rosemary, Tea tree, Thyme and Clove	1	Caryophyllene
Rosemary, Tea tree, Thyme and Oregano	1	Terpinene-4-ol

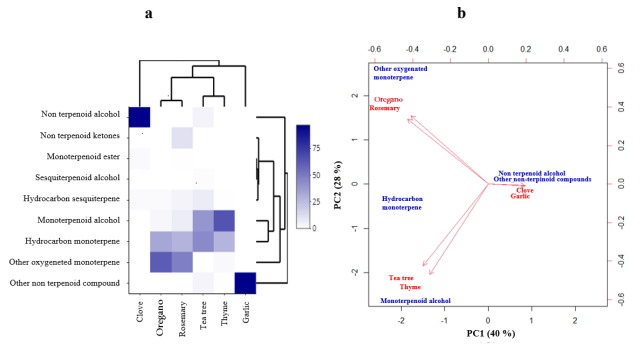

Fig. 2. Chemical class compositions of the six essential oils. **(a)** Two-way hierarchical clustering of the detected chemical classes and the six essential oils. The color scale from white to blue shows the gradually increasing intensity of the chemical class. **(b)** Principal component analysis profile of the detected chemical classes and the six essential oils. The percentage of variation explained by each axis is given in brackets. Only the main chemical classes are shown.

classes detected in clove and garlic were non-terpenoid alcohol (96.83 %) and other non-terpenoid compounds (96.99), respectively. Tea tree and rosemary comprise also classes present more than 3%. These include hydrocarbon sesquiterpene (6.83 %), non-terpenoid alcohol (4.6 %), and other non-terpenoid compounds (3.87 %) in tea tree and monoterpenoid alcohol (6.47 %), hydrocarbon sesquiterpene (4.62 %) and non-terpenoid ketones (11.12 %) in rosemary. The dominance of non-terpenoid alcohols in clove EO composition is primarily due to the high concentration of eugenol (96.28%), which belongs to this class. Eugenol is a phenolic compound biosynthesized through the phenylpropanoid pathway rather than the terpenoid biosynthetic pathway [33].

The principal component analysis performed using the above-mentioned traits also confirmed the results of the two-way hierarchical cluster analysis (Fig. 2b). Globally, the two dimensions explain 68 % of the variability with 40 % of the variation for the first axis and 28 % for the second.

The EO of clove revealed that eugenol belonging to the non-terpenoid alcohol class was the predominant compound (96.28 %) (Fig. 3a). The major compounds of rosemary oils were: Eucalyptol (48.67 %), Alpha pinene (14.02 %), and camphor (11.12 %). Other compounds were present but accounted for a lesser percentage. For garlic EO, the main detected compounds were trisulfide, diallyl disulfide, and diallyl sulfide with concentrations of 31.97, 27.79, and 12.43 %, respectively. Other compounds were also present such as disulfide (8.74 %). The major compounds of tea tree were terpinene-4-ol (35.17 %), gamma-terpinene (16.11 %), and p-cymene (9.34 %) whereas those of thyme were linalool (57.75 %) and O-cymene (8.64 %). Carvacrol showed the highest contribution (55.91 %) amongst oregano EOs followed by gamma-terpinene (15.46 %) and O-cymene (11.49 %). Figure 3b represents the principal component analysis indicating the major detected compound for each EO. The characteristics of the major compounds of the six EOs are shown in Table 3.

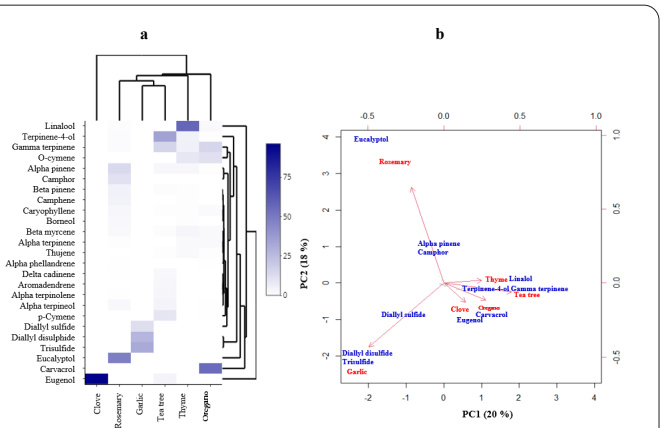

Fig. 3. Major compound composition of the six essential oils. **(a)** Two-way hierarchical clustering of the major compounds detected and the six essential oils. The color scale from white to blue shows the gradually increasing intensity of the major compounds. **(b)** Principal component analysis profile of the major compounds detected and the six essential oils. The percentage of variation explained by each axis is given in brackets. Only the main compounds are shown.

Table 3. Relative percentages of the major compounds of the six essential oils determined by GC-MS analysis.

Common name	Scientific name	Family	Main components	Retention time (min)	Percentages of the main constituents (%)
Clove	*Syzygium aromaticum*	Myrtaceae	Eugenol	21.644	96.28
Tea tree	*Melaleuca alternifolia*	Myrtaceae	Terpinene-4-ol	16.733	35.17
			Gamma-terpinene	12.410	16.11
			p-cymene	11.292	9.34
Rosemary	*Rosmarinus officinalis*	Lamiaceae	Eucalyptol	11.010	48.67
			Alpha-pinene	7.963	14.02
			Camphor	14.327	11.12
Thyme	*Thymus vulgaris*	Lamiaceae	Linalool	13.669	57.75
			O-cymene	10.716	8.64
Oregano	*Origanum vulgare*	Lamiaceae	Carvacrol	19.656	55.91
			Gamma-terpinene	11.692	15.46
			O-cymene	10.663	11.49
Garlic	*Allium sativum*	Alliaceae	Trisulfide	18.939	31.97
			Diallyl trisulfide	12.457	27.79

3.2 Antifungal activity of essential oils

The EOs were tested for their antifungal activity against the six fungi (Fig. 4 and Table 4). The antifungal activity was expressed as the percentage of radial growth inhibition. To evaluate the correlations between the antifungal activity and the EOs, the mean values of the percentage of radial growth inhibition were subjected to the two-way hierarchical cluster and the principal component analysis. The results are shown in Figure 4 and Table 4. According to the statistical analysis, the tested EOs showed different fungal growth inhibitory potency depending on the tested fungi. Two main clusters were defined through the two-way hierarchical cluster analysis. The first included the fungus *R. bataticola* and *B. mediterranea* which were inhibited by garlic at 500 ppm, 1000 ppm, 4000 ppm, but also by clove and rosemary at 4000 ppm. The second cluster included the other fungi. All of these latter were controlled by clove at a concentration higher than 500 ppm. *L. theobromae* was inhibited by clove at concentrations higher than 500 ppm, and rosemary at 4000 ppm. The other fungi; *F. oxysporum*, *F. solani*, *Verticillium dahliae*, and *B. mediterranea*; were also inhibited at varying extents by clove at 250 ppm, garlic at concentrations ranging from 250 to 4000 ppm, and rosemary at 4000 ppm. *F. solani* and *V. dahlia* were also inhibited by oregano at 1000 and 4000 ppm. Regarding the second dimension, two clusters were also defined. The first grouped the most active EOs being clove (250-4000 ppm), garlic (250-4000 ppm), and rosemary (4000 ppm). The second grouped the inactive and moderately active EOs including those of rosemary at low concentration, thyme, tea tree, and oregano (Fig. 4a).

The results of the principal component analysis per-

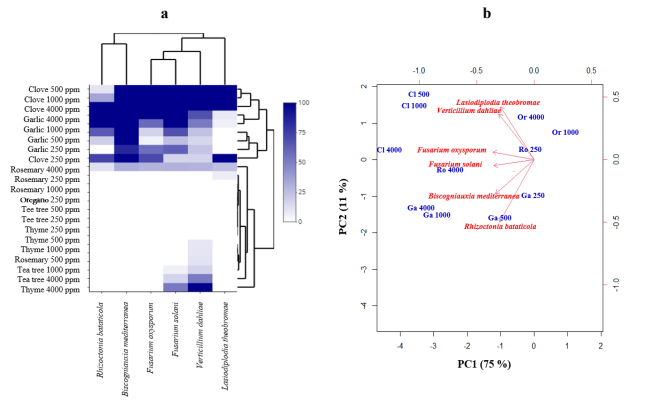

Fig. 4. Antifungal activities of the six essential oils. **(a)** Two-way hierarchical clustering of the antifungal activities and the six essential oils. The color scale from white to blue shows the gradually increasing intensity of the antifungal activities. **(b)** Principal component analysis profile of the antifungal activities and the six essential oils. The percentage of variation explained by each axis is given in brackets. Only the main type-essential oil concentrations are shown. Cl: clove, Ro: rosemary, Ga: garlic, and Or: oregano; 250, 500, 1000, and 4000 indicate the essential oil concentration used expressed as ppm.

Table 4. Antifungal activity of essential oils. The results were expressed as inhibition percentage (%) and presented as mean values ± standard deviation.

		Percentage of radial growth inhibition soil-borne and branch dieback fungi (%)					
Essential Oil	**Concentration (ppm)**	***Fusarium oxysporum***	***Fusarium solani***	***Verticillium dahliae***	***Biscognauxia mediterranea***	***Lasiodiplodia theobromae***	***Rhizoctonia bataticola***
Clove	250	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	*0.00±0.00*	0.00±0.00
	500	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	10.62±2.69
	1000	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	35.69±0.15
	4000	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00	100.00±0.00
Thea Tree	250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	500	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	1000	0.00±0.00	0.00±0.00	9.73±0.86	0.00±0.00	0.00±0.00	0.00±0.00
	4000	0.00±0.00	0.00±0.00	10.20±1.03	0.00±0.00	0.00±0.00	0.00±0.00
Thyme	250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	500	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	1000	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	4000	0.00±0.00	0.00±0.00	11.18±0.79	0.00±0.00	0.00±0.00	0.00±0.00
Rosmary	250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	500	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	1000	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	6.90±0.96	0.00±0.00
	4000	70.63±0.09	16.90±1.11	17.01±0.8	100.00±0.00	100.00±0.00	73.46±0.11
Oregano	250	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
	500	0.00±0.00	6.05±1.19	16.94±0.92	0.00±0.00	0.00±0.00	0.00±0.00
	1000	0.00±0.00	17.14±0.96	52.14±0.44	0.00±0.00	0.00±0.00	0.00±0.00
	4000	0.00±0.00	52.32±0.58	100.00±0.00	0.00±0.00	0.00±0.00	0.00±0.00
Garlic	250	5.28±0.17	24.76±0.84	15.76±0.76	100.00±0.00	0.00±0.00	19.31±0.34
	500	17.15±0.05	68.21±0.39	24.10±0.79	100.00±0.00	0.00±0.00	64.94±0.28
	1000	58.51±0.03	100.00±0.00	52.13±0.46	100.00±0.00	5.99±1.00	100.00±0.00
	4000	100.00±0.00	100.00±0.00	68.14±0.31	100.00±0.00	8.44±0.98	100.00±0.00

formed using the above-mentioned traits confirmed the results of the two-way hierarchical cluster analysis (Fig. 4b). Globally, the two dimensions explain 86 % of the variability with 75 % of the variation for the first axis and 11 % for the second. The plot showed that *R. bataticola* and *B. mediterranea* were mainly affected by garlic EO whereas *V. dahlia* and *L. theobromae* were mainly inhibed by clove EO and that *F. oxysporum*, *F. solani*, and *B. mediterranea* were affected by both. The lower antifungal effectiveness of thyme, tea tree, and oregano EOs at lower concentrations can be attributed to their specific chemical compositions and the threshold levels required for bioactive compounds to exert significant antifungal effects. These EOs primarily contain monoterpenes and monoterpenoid alcohols, which, while bioactive, may require higher concentrations to disrupt fungal cell membranes and metabolic processes effectively.

4. Discussion

The increasing cultivation of olive trees has intensified the demand for plant protection products, while resistance in phytopathogenic fungi remains a major challenge. Due to environmental concerns and regulatory restrictions, there is a growing shift toward sustainable alternatives, including natural compounds and Integrated Pest Management strategies [26]. EOs are among the naturally occurring substances showing negative effects on different filamentous fungi. Their broad availability and biodegradability have validated their growing use [32]. In this study, we investigated the chemical complexity of EOs of clove, tea tree, rosemary, thyme, garlic and oregano and their potential applications as bio-fungicides.

Analysis of the composition of clove EO revealed that eugenol (96.28 %), a non-terpenoid alcohol, is the major compound that was renowned for its antimicrobial and antioxidant properties [34]. The substantial eugenol content underscores clove EO unique profile and therapeutic uses, particularly in antimicrobial applications. Our result aligns with recent studies that identified eugenol as the dominant component in several clove EOs with high antimicrobial properties [35]. Indeed, it has been reported that clove EO contains along with minor components (beta-caryophyllene (21.24 %), alpha-copaene (1.16 %), and beta-caryophyllene oxide (0.45 %)) about 76.78 % eugenol [36]. Similarly, among 65 components identified in the EOs of clove from Algeria, eugenol was predominant (78.72 %), followed by beta-caryophyllene (8.82 %) and eugenyl acetate (8.74 %) [36]. This study demonstrated that clove EO has significant antioxidant properties and moderate antibacterial activity [37]. The differences in eugenol content across several studies could be attributed to the variations of extraction methods and environmental conditions that influence clove cultures and composition.

Tea tree EO contains terpinen-4-ol (35.17 %), gamma-terpinene (16.11 %), and p-cymene (9.34 %) that are significant contributors of biological activities. Similarly, tea tree EO, was describedfor its high content of terpinen-4-ol, gamma-terpinene, and p-cymene which are most important for antimicrobial and anti-inflammatory activities [38]. A previous research attributed tea tree oil's efficacy against various pathogens to these key compounds [39].

Rosemary EO major compounds include eucalyptol (48.67 %), alpha-pinene (14.02 %), and camphor (11.12 %). These molecules are known to exhibit antioxidant, anti-inflammatory as well as antimicrobial properties [40]. Indeed, the investigation of the chemical composition and antimicrobial properties of rosemary EO using GC-MS analysis, identified 19 compounds, with a high content of 1,8-cineole and alpha-pinene [41]. The consistency across different studies highlights the stable chemical composition of rosemary EO [42].

Unlike thyme EO, which usually contains high levels of thymol, our analysis revealed significant amounts of linalool (57.75 %) and O-cymene (8.64 %) indicating that thyme oil possesses antimicrobial and anti-inflammatory properties as described by [43]. A comparison of chemical, antioxidant and antimicrobial studies of *Thymus vulgaris* EO using GC-MS, showed that thymol, p-cymene, and gamma-terpinene, with thymol, were the most abundant compounds [43]. This composition aligns with other findings that highlight linalool's content in thyme oil, contributing to its potent antibacterial and antifungal activities [23].

Current findings displayed that garlic EO contains high levels of sulfur-containing compounds including trisulfide (31.97 %), diallyl disulfide (27.79 %), and diallyl sulfide (12.43 %). Diallyl disulfide (30-40 %), diallyl trisulfide (20-30 %), and other sulfides were also highlighted as predominant in garlic EO that was attributed to the broad spectrum antimicrobial and cardiovascular protective effects [25], [44]. Indeed, Garlic EO's strong antifungal activity against *R. bataticola* and *B. mediterranea* at multiple concentrations can be attributed to its high content of sulfur-containing compounds, such as trisulfide, diallyl disulfide, and diallyl sulfide. These compounds are known for their potent antimicrobial and antifungal properties, as they can disrupt fungal cell membranes, inhibit essential enzyme activity, and interfere with fungal metabolism. Additionally, the presence of multiple bioactive sulfur compounds in garlic EO may contribute to a synergistic effect, enhancing its efficacy even at lower concentrations. The clustering results from the hierarchical and principal component analyses further confirm that *R. bataticola* and *B. mediterranea* are particularly susceptible to garlic EO, reinforcing its potential as a natural antifungal agent. The EO extracted from garlic bulbs cultivated in Spain, analyzed using GC-MS and gas chromatography-flame ionization detection (GC-FID), was rich in allyl polysulfides. These compounds ensure dual roles as flavoring agents in foods and as components of herbal medicine [45]. Moreover, EO derived from the white-skin garlic cultivar had lower concentrations of diallyl trisulfide (45.76 %) and diallyl disulfide (15.63 %), compared to the purple-skin cultivar (58.53 % and 22.38 %, respectively). These compounds showed a dose-dependent antimicrobial activity [46].

Significant amounts of carvacrol (55.91 %), gamma-terpinene (15.46 %), and O-cymene (11.49 %) were found in oregano EO. Carvacrol is well-known for its strong antimicrobial properties, which contribute to oregano oil's effectiveness in treating infections [47]. Martucci et al, 2015 [48] reported that carvacrol (65 %), p-cymene (10 %), and gamma-terpinene (15 %) as major compounds aligning closely with our current results, whereas Napoli et al, 2020 [49] showed that the major components of three different oregano plantations were thymol (36.91-60.14 %), gamma-terpinene (11.59-24.14 %), and p-cymene (2.56-9.38 %) with similar to the gamma-terpinene and o-

cymene composition as that found in our study.

Rosemary, thyme, and oregano EOs displayed several shared compounds such as alpha-pinene, gamma-terpinene, linalool, borneol, camphene, alpha-terpinene, alpha-phellandrene, beta-pinene, and beta-myrcene. These compounds contribute to similar biological effects, essentially antimicrobial and anti-inflammatory activities [50]. Additionally, rosemary and thyme shared common chemical profile with camphor, alpha-fenchyl acetate, alpha-terpinolene, and thujene. This result supports their grouping in hierarchical analysis and highlights their overlapping therapeutic properties.

The hierarchical cluster analysis and principal component analysis provided further insights into the relationships among all used EOs. Indeed, regarding their high levels of hydrocarbon monoterpenes and oxygenated monoterpenes, oregano and rosemary EOs were grouped. However, tea tree and thyme EOs are categorized together based on their significant monoterpenoid alcohol content. Clove and garlic EOs exhibited different profiles characterized by high amounts of non-terpenoid alcohol and sulfur-containing non-terpenoid, respectively. Interestingly, a recent study analyzing EOs from similar plants also found comparable clustering patterns. For example, EOs rich in monoterpenes and oxygenated monoterpenes, such as oregano and rosemary, exhibited similar clustering [51]. The clustering observed was related to the common major compounds, such as alpha-pinene and eucalyptol, found in all analyzed EOs. Moreover, a recent study reported that EOs of tea tree and thyme, which are rich in monoterpenoid alcohols like terpinene-4-ol and linalool, clustered together, supporting the results of our current analysis [44].

As natural alternatives to synthetic fungicides, EOs antifungal effects have gained significant attention. In this study, antifungal activity of six EOs was evaluated against six fungal strains infecting olive trees. In order to elucidate the relationships between the EO and their antifungal efficiency, the data were analyzed using two-way hierarchical cluster analysis and principal component analysis. Significant variations in the antifungal effects of EOs were observed. Clove, garlic and rosemary at high concentrations were the most active EOs. Whereas rosemary (at lower concentrations), thyme, tea tree, and oregano had moderate to no activity. *B. mediterranea* was the most sensitive fungi inhibited by clove, garlic, and rosemary at 4000 ppm. While *F. oxysporum, F. solani*, *V. dahliae,* and *L. theobromae* were inhibited mainly by clove at concentrations ranging from 500 to 4000 ppm. *R. bataticola* was inhibited by clove and garlic at high concentrations. Indeed, [52] showed that thyme EO effectively inhibited *Fusarium culmorum*, *Fusarium graminearum* and *Fusarium avenaceum* at 0.025 %, while garlic EO required a higher concentration of 0.500 % for similar effectiveness. Garlic oil inhibited *Fusarium graminearum* and *Fusarium avenaceum* at 1.000 %, whereas tea tree EO was described to be effective at 0.250 %. Against *F. oxysporum*, garlic EO demonstrated fungicidal effects at higher concentrations, whereas thyme EO at 0.125 % [52]. The research conducted by Krzyśko-Łupicka et al. [52] evaluated the oils' fungistatic activity at various concentrations (0.025 % to 2.0 %) using the disc plate method. Comparable to the commercial fungicide Funaben T which was effective at all tested concentrations, thyme EO exhibited the strongest fungicidal activity where *F. culmorum* was the most susceptible and *F. oxysporum* the most resistant [52]. Similarly, clove EO demonstrated significant antifungal activity against *Cladobotryum mycophilum*, and Rosemary EO was most effective against *Trichoderma aggressivum*. Clove EO exhibited the highest antifungal activity, achieving 100 % inhibition of *Phytophthora parasitica* and *Sclerotinia sclerotiorum* at concentrations of 15 % to 30 %. Schmidt et al, 2007 [53] investigated the antifungal properties of eugenol-containing EOs from four spices against 38 isolates of *Candida albicans*. Among the EOs tested, clove oil demonstrated the strongest antifungal activity against all *C. albicans* strains [53]. Rosemary EO showed antifungal effect against all tested fungi, except *Pythium aphanidermatum* [25]. Another study found that rosemary EO exhibits strong antifungal activity, particularly against *C. albicans*, *Trichophyton tonsurans*, and *Trichophyton rubrum* [54]. Özcan et al, 2000 [55] found that a 10 % oregano decoction completely inhibited mycelial growth in culture fungi including *F. oxysporum*, *Macrophomina phaseoli*, *Botrytis cinerea*, *Rhizoctonia solani*, *Alternaria solani*, and *Alternaria parasiticus*. Recently, Petrovic et al, 2025 showed that Oregano commercial EOs and their components completely inhibited all tested fungi, highlighting their potential for biocontrol in sustainable agriculture [26].

Several studies have reported the antifungal properties and possible mechanisms of EOs. Majors EOs compounds that demonstrated the highest efficiency against fungi were isoeugenol, cinnamaldehyde, carvacrol, eugenol, and thymol [56]. The antifungal activity of the tested EOs could not be attributed to a single component. However, it can result from the synergistic effects of all components. In fact, it was suggested that EOs were significantly more effective than their individual major components [25]. It is important to consider that the composition and concentration of compounds in EOs can be influenced by various factors, including the plant's genotype, geographic location, seasonal variations, agricultural practices, and the extraction method used [25]. The antifungal mechanisms of these EOs can involve spore germination inhibition, fungal cell membrane disruption, and blocking enzymes essential for fungal growth and development. Moreover, another mechanism was proposed based on the hydrophobic nature of EOs that increases membrane permeability resulting in disruption in all membrane-dependent vital functions ultimately causing fungi cell death [57]. Among EO compounds of oregano, carvacrol interacts with cell membranes by altering their permeability, particularly affecting sterols in fungal membranes. The complex chemical composition of oregano EO allows it to target various cell structures. Garlic EO can penetrate cellular membranes and even the membranes of organelles like mitochondria, causing damage and resulting in the death of *Candida albicans*. Additionally, it has been reported that garlic EO influenced the expression of several critical genes, including those involved in oxidation-reduction processes, and the cellular response to drugs and starvation [3,58]. Further studies are essential to investigate the EOs impact on pathogens and plants, as well as to establish an optimal dosage that ensures efficacy against pathogens while minimizing potential harm to plants and the environment. Moreover, the long-term efficacy of EOs, their precise mechanisms of action against pathogens, and their potential effects on plant health could be investigated, en-

suring their practical application in sustainable disease management.

5. Conclusion

The comparative analysis of EOs composition from clove, tea tree, rosemary, thyme, garlic, and oregano was performed in order to identify their major compounds. Our findings highlight that EOs, particularly garlic and clove, showcase noteworthy antifungal efficacy against various pathogenic fungi strains. Hierarchical cluster analysis and PCA offer valuable insights into the relationships between these EOs major compounds and their antifungal activity. This analysis is in line with recent investigations emphasizing the potential use of EOs as natural antifungal agents to replace chemical fungicides. Further investigations about their mechanisms of action could conduct to the application of more sustainable biocontrol strategy based on eco-friendly fungicidal compounds.

Statements and Declarations

Competing interests

The authors declare no competing interests.

Ethics approval

Not applicable.

Data availability

The datasets generated during the current study are available from the corresponding author upon reasonable request.

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