



Determination of chemical compositions of rosemary and sweet marjoram essential oils and their blends and their antifungal potential against potato rubbery rot disease agent *Geotrichum candidum*

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Abstract

In this study, the chemical compositions of rosemary (*Rosmarinus officinalis* Spenn.) and sweet marjoram (*Origanum majorana* L.) essential oils, both individually and in various blend ratios, were investigated by using Gas Chromatography-Mass Spectrometry. Their antifungal activities were also determined against the potato rubbery rot disease agent, *Geotrichum candidum*, under *in vitro* and *in vivo* conditions. Camphor (20.69%) and terpinen-4-ol (35.13%) were determined as primary constituents in rosemary and sweet marjoram essential oils, respectively. Sweet marjoram and rosemary essential oils completely inhibited mycelial growth of *G. candidum* at 55.0 and 65.0 µL/Petri concentrations, respectively. Essential oils at these concentrations were found as fungicidal. Notably, a synergistic fungicidal antifungal effects were also observed in essential oil blends. Blend of sweet marjoram and rosemary essential oil in a 3:1 ratio (O₇₅R₂₅) significantly enhanced antifungal activity at the relatively lower concentration (45.0 µL/Petri). Microscopic observations revealed structural deformations in exposed hyphae, including cytoplasmic coagulation and vacuolization. Essential oil blend (O₇₅R₂₅), sweet marjoram and rosemary essential oils completely inhibited the disease lesion caused by *G. candidum* on potato tubers at concentrations of 100.0, 125.0, and 150.0 µL/L air, respectively. These findings emphasized that essential oil blends of rosemary and sweet marjoram have synergistic antifungal potential and can be used as effective biofungicides against plant fungal diseases.

Keywords Antifungal activity · Essential oil blends · *Geotrichum candidum* · Gas chromatography-mass spectrometry

Introduction

The world population is estimated to reach about 10 billion people by 2050. To provide sufficient food for the growing world population, the amount of product that can be obtained from arable land per unit area must be increased (FAO 2017). Potato (*Solanum tuberosum* L.) is an important vegetable crop grown in more than 100 countries worldwide (Hussain 2016). Potato, the most consumed vegetable in the UK, is the 4th most consumed agricultural product in the world with a production of 374,777,763 tonnes after corn, rice and wheat (Zhang et al. 2017; Anonymous 2023).

Turkey is also among the world's leading countries for potato production with a production of 5,200,000 tonnes on 132,072 ha of cultivated land (Anonymous 2023). The production, yield and quality of potato is seriously threatened by soil-borne fungal diseases of various species (Koike et al. 2007). Fungal diseases such as *Fusarium sambucinum*, *Geotrichum candidum*, *Fusarium solani*, *Phytophthora cryptogea*, *Rhizoctonia solani*, *Sclerotinia sclerotiorum*, *Synchytrium endobioticum*, *Pythium ultimum* and *Verticillium dahliae* are the most important soil-borne fungal disease agents limiting potato production both in field and in warehouses worldwide (Ingram and Johnson 2010; Wang et al. 2011; Gore 2017; Adolf et al. 2020; Meno et al. 2021; Soylu et al. 2021).

Geotrichum candidum, which causes rubbery rot disease of potato tubers, produces water-soaked, soft rot lesions on the surface of potato tubers that spread an acidic, sour odour to the internal tissues, and symptoms covered with fluffy

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white mycelium on the surface of these lesions (Agrios 2005). The disease agent shows its typical symptoms in the tubers kept in the warehouses after harvest rather than in the field conditions. The disease agent has recently been reported in the world's leading potato growing countries (Kim et al. 2011; Song et al. 2020; Soylu et al. 2021; Duellman et al. 2021; Willbur et al. 2023). Disease caused by *G. candidum* is difficult to control in several crops including tomatoes, lemons, carrots, peaches, grapes, and sweet potatoes (Song et al. 2008, 2020; Cai et al. 2009; Kim et al. 2011; Yaghmour et al. 2012). Effective chemical control are not currently available to control rubbery rot disease of potato. One of the alternative methods to chemical control of diseases is the use of plant extract and essential oil (EO) with antimicrobial activity (Šernaitė et al. 2020; El-Tarabily et al. 2021; Kaur et al. 2021; Dassanayake et al. 2021; Abd El-Hack et al. 2022). The antimicrobial activities of essential oils from different plant species can vary (Burt 2004).

The antifungal properties of rosemary (*Rosmarinus officinalis*) EO were already reported for *Alternaria alternata*, *Aspergillus niger*, *Aspergillus parasiticus*, *Botrytis cinerea*, *Colletotrichum gloeosporioides*, *Colletotrichum nymphaeae*, *Fusarium culmorum*, *Fusarium oxysporum*, *Monilinia laxa*, *Penicillium expansum*, *Penicillium digitatum*, *Penicillium italicum* and *Phytophthora infestans* (Soylu et al. 2006, 2010; Ozcan and Chalchat 2008; Saglam et al. 2009; Lopez-Reyes et al. 2013; Yilmaz et al. 2016; Bakhtiarizade and Souri 2019; Ben Kaab et al. 2019; Hosseini et al. 2020; Šernaitė et al. 2020; Samara et al. 2021). Sweet marjoram (*Origanum majorana*) EO also showed an inhibitory activity against *Albifimbria verrucaria*, *Alternaria alternata*, *Aspergillus flavus*, *Aspergillus fumigatus*, *Botrytis cinerea*, *Cladosporium herbarum*, *Colletotrichum musae*, *Cryptococcus neoformans*, *Curvularia spicifera*, *Fusarium fujikuroi*, *Fusarium oxysporum* f. sp. *melonis*, *Fusarium solani*, *Macrophomina phaseolina*, *Microsporium canis*, *Microsporium gypseum*, *Monilinia laxa*, *Paramyothecium roridum*, *Penicillium expansum*, *Pestalotiopsis mangiferae*, *Rhizopus stolonifer*, *Stagonosporopsis cucurbitacearum*, *Trichophyton mentagrophytes* and *Trichophyton rubrum* (Lopez-Reyes et al. 2010, 2013; Souza et al. 2010; Dhauadi et al. 2018; Moumni et al. 2021a, b; Rizwana et al. 2022). Although the antimicrobial activity of rosemary and sweet marjoram essential oils against numerous fungal and bacterial disease agents has been described in previous studies (Lopez-Reyes et al. 2013; Ojaghian et al. 2014; Šernaitė et al. 2020; Bellumori et al. 2021), no study has investigated the antifungal activity of plant EO blends against the fungal disease agent *Geotrichum candidum*.

When EOs or their main components are used alone, they show low antimicrobial activity against the microorganisms for which they are tested against. However, their efficacy

can be very different when used as a blend with other EOs or components (Hyltdgaard et al. 2012; Ambrosio et al. 2017). It is known that this observed change in antimicrobial activity is usually due to the synergistic interaction of the component(s) with low antifungal activity with another component(s). There is a very limited number of studies that have looked at possible changes in antimicrobial activity when EOs are used as a blend. Due to the synergistic interactions of the compounds obtained by blending EOs, a limited number of studies have reported that they prevent pathogen resistance to fungicides, reduce costs and/or toxic side effects, and increase antifungal activity and spectrum of activity against various microbial species (Brochot et al. 2017; Purkait et al. 2020; Turkmen and Kaya 2021; Kara et al. 2022).

This study explored the chemical compositions of rosemary (*Rosmarinus officinalis* Spenn) and sweet marjoram (*Origanum majorana* L.) essential oils (EOs), as well as their blends, investigating their *in vitro* and *in vivo* antifungal activities against *Geotrichum candidum*. Additionally, morphological and cytostructural alterations induced by these EOs in the fungal mycelium were characterized using light microscopy.

Materials and methods

Plant materials

Rosemary and sweet marjoram leaves were collected from naturally growing plants in Antakya district, Hatay, Turkey (36°18'56"N, 35°22'48"E) in June 2021. The identification of the plant species was confirmed by Prof. Dr. İlhan ÜREMİS (Hatay Mustafa Kemal University, Hatay, Turkey). The voucher specimens were deposited at the BISAK Herbarium Centre of Hatay Mustafa Kemal University (MKUBK-R35 and MKUBK-O51).

Isolation of essential oils

Essential oils were extracted from air-dried leaves of each plant species using a water distillation process lasting 3–4 h with a Neo-Clevenger type apparatus (İldam, Ankara, Turkey). Two hundred grams of leaves per plant species were individually placed in 2000 ml round-bottomed distillation flasks with 500 ml of distilled water for each batch. Oil yields, calculated as a percentage of the dry weight of plant materials, were obtained and stored in amber vials at +4°C after dehydration using anhydrous sodium sulfate (Turkmen 2021).

Preparation of EO blends

Blends of essential oils (EOs) were prepared in Eppendorf tubes using varying ratios of 75:25%, 50:50%, and 25:75% (Turkmen and Kaya 2021; Kara et al. 2022). These ratios corresponded to different compositions of rosemary and sweet marjoram EOs as investigated in prior studies.

Determination of chemical components of EOs and their blends

The chemical constituents in the essential oils (EOs) and their blends were identified through Gas Chromatography-Mass Spectrometry (GC-MS) analysis using a Thermo Scientific Focus instrument with specific parameters. Compound concentrations, expressed as percentages, were determined by integrating chromatographic peak areas via Xcalibur software (Maral et al. 2018). GC-MS analysis utilized a TR-5MS column (5% phenylpolysilphenylenesiloxane, 60 m x 0.25 mm inner diameter, 0.25 layer thickness) and helium (99.9%) as the carrier gas, with an ionization energy of 70 eV and a mass range of m/z 50–650 amu. Sample injection volume was 1 μ L, and the oven temperature was initially set at 50 °C, then gradually increased from 50 to 220 °C at a rate of 3 °C/min. Relative retention indices were computed using a C8-C40 n-alkane series for calibration, while compound identification relied on retention indices and spectral comparisons with known compounds (Babushok et al. 2011; Adams 2007).

Isolation and purification of fungal pathogen

Geotrichum candidum, the fungal disease agent utilized in this study, was previously isolated from infected potato tubers grown in Hatay province (Soylu et al. 2021). A single spore culture of the fungal isolate (GCP19MKU) was used for the experiments. Total genomic DNA was isolated from mycelia (100 mg) obtained from single spore cultures of fungal isolate GCP19MKU grown on Potato Sucrose Agar (PDA), which were preliminarily identified using morphological and MALDI-TOF (Soylu et al. 2023), using DNeasy Plant Mini Kit (Qiagen, GeneMark Technology Co, Valencia, CA, Catalogue No. 69,104) by following the protocols recommended by the manufacturer. PCR amplification and DNA sequencing of the internal transcribed spacers (ITS) locus was performed using primer pairs ITS1 (5'-TC CGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGC TATTGATATATGC-3') (White et al. 1990). The consensus sequences of ITS locus sequence for GCP19MKU isolate was submitted to the NCBI GenBank database (MN621396).

Antifungal effects of EOs and their blends on mycelial growth of *Geotrichum candidum*

The antifungal effect of rosemary and sweet marjoram EOs and their blends on mycelial growth of *G. candidum* at the vapour phase was investigated *in vitro* (Kara et al. 2022). A single spore culture of the pathogen was cultivated on PDA medium at 25 °C for 3–5 days. Sterile glass Petri dishes (90 mm) were filled with PDA medium (20 μ L/Petri), and various concentrations of EO and their blends (ranging from 0.0 to 65.0 μ L/Petri) were applied to the lids of the Petri dishes using a micropipette. Mycelial discs (5 mm) from a 5-day culture of the pathogen were placed in the centre of PDA medium. Petri dishes containing only PDA (without EO) were used as controls. All Petri dishes were sealed with parafilm and incubated at 25 °C in an incubator. The mycelial growth in Petri dishes with varying concentrations of each EO and their blends was monitored daily, and the mycelial growth diameters were measured 4–5 days after the application. The inhibition rate of the different concentrations of EOs and blends was calculated using the %Abbott formula; Inhibition (%) = $[(MG_c - MG_t) / MG_c] \times 100$, MG_c = diameter of mycelial growth in control Petri plates (mm), MG_t = diameter of mycelial growth in treated Petri plates (mm).

To determine the fungicidal or fungistatic effect of minimum inhibition concentrations (MIC) of EOs that completely inhibit mycelial growth, fungal discs were transferred from Petri plates where no growth was observed to fresh PDA medium without PA. The discs were then re-incubated at 25 °C for 3–4 days. When the transferred mycelial discs failed to regrow on the PDA medium, the antifungal effect of PA at this concentration was fungicidal. When the mycelial growth resumed, the antifungal effect was considered fungistatic (Atay and Soylu 2023).

The effects of EOs and their blends on hyphal morphology

The effect of the most effective concentrations of EOs and their blends on hyphal morphology was also assessed using light microscopy. Fungal culture was allowed to grow in Petri dishes for 2–3 days. Minimum inhibition concentrations of EOs and their blends were precisely applied to the Petri dish lids using a micropipette, sealed with parafilm and incubated at 25 °C. After the incubation period, morphological alterations in the mycelium were examined using a light microscope equipped with a Nomarski/DIC attachment (Olympus BX-51, Tokyo, Japan) as described by Kara et al. (2022).

Effects of the EOs and their blends on disease development *in vivo* conditions

The antifungal activities of different concentrations of EOs and the most effective EO blend ($O_{75}R_{25}$) were evaluated on potato tubers under *in vivo* conditions. All experiments were arranged in a completely randomized split-plot design with two replicates of four potato tuber per treatment. Different concentrations of EOs and their blend $O_{75}R_{25}$ were prepared by dissolving the requisite amounts in sterile Tween 20 (0.1%, v/v) solution. Potato tubers were first washed with sterilized water and subsequently air dried for 1 h. Three wounds were made in the equatorial region of the air-dried potato tubers, using a sterilized cork borer (2–4 mm depth) and drop-inoculated with 50 μ l of a conidial suspension (10^5 conidia/ml) into the wounds. After the inoculation process, all tubers were dried for 1–2 h for fungal penetration. Inoculated potato tubers ($n=8$) were then placed in transparent storage boxes (having 6 L air capacities), and different concentrations of EOs and their blend (50, 75, 100, 125 and 150 mg/L air) solution were applied to the lids of the storage box using a micropipette. The control tubers were kept separately in boxes treated with 50 μ l of sterile Tween 20 (0.1%, v/v) without EOs. Storage boxes were sealed with parafilm and incubated at 22 °C with 90.0% humidity level for two weeks. The average lesion diameters (mm) at inoculation sites were measured at 15 days of incubation. Efficacy of EOs and the blend was calculated according to Abbott Formula [%effectiveness = $(C - T) / C \times 100$, where C = mean disease lesion in the control, and T = mean disease lesion in the relevant treatment]. Positive controls fungal-inoculated potato tubers; negative controls were healthy tubers treated with sterile Tween 20 (0.1%, v/v) without EOs and blend. All experiments were repeated at least twice.

Statistical analysis

The antifungal activities of individual essential oils (EOs) and their blend were evaluated *in vitro* and *in vivo*. Each study was replicated twice. Statistical analysis employed one-way analysis of variance (ANOVA) in SPSS statistical software (version 17.0, SPSS Inc., Chicago, IL, USA). Comparisons of differences among treatment and concentration were assessed using the Duncan Multiple Range Test ($P \leq 0.05$). Probit analysis was utilized to estimate the effective concentrations (EC_{50}), representing the concentrations inhibiting mycelial growth by 50%, of EOs and blends.

Results

Chemical constituents of EOs and their blends

The chemical constituents of the EOs and their blends were determined through GC-MS, and the results are presented in Table 1. A total of 27 constituents were determined in rosemary EO. The major components were camphor (20.69%), eucalyptol (14.13%), α -pinene (13.76%), verbenone (12.17%), borneol (8.98%) and linalool (5.21%). Sweet marjoram EO contained 30 constituents, with terpinen-4-ol (35.13%), γ -terpinene (12.58%), trans-sabinene hydrate (9.17%), o-cymene (7.60%), sabinene (6.15%), and α -terpinene (5.91%) as primary components.

The chemical components of the EO blends in different ratios ($O_{75}R_{25}$, $O_{50}R_{50}$, $R_{75}O_{25}$) were also determined by GC-MS, revealing 32–34 different chemical constituents (Table 1). Notably, in these blends, the proportions of terpinen-4-ol and camphor, the principal constituents of sweet marjoram and rosemary EOs respectively, varied: 25.10% and 6.71% for $O_{75}R_{25}$; 16.21% and 13.87% for $O_{50}R_{50}$; 13.14% and 14.13% for $R_{75}O_{25}$ (Table 1).

Isolation of fungal disease agent

The fungal disease agent *G. candidum* was isolated from water-soaked soft rot lesions of potato tubers (Fig. 1). The fungal species was identified based on the basis of morphological characteristics (Duellman et al. 2021; Soyly et al. 2021). The genomic DNA gene sequences of the isolate obtained were found to match the DNA sequences of *G. candidum* isolates obtained from different host plants in different parts of the world with 99% similarity, and the genomic DNA sequence of the potato isolate (GCP19MKU) used in the analysis was registered in GenBank [GenBank accession No. MN621396].

Antifungal effects of EOs and their blends on mycelial growth of *G. candidum*

The *in vitro* antifungal efficacies of rosemary and sweet marjoram EOs and their blends in vapor phase were determined against *Geotrichum candidum* mycelial growth (Fig. 2; Table 2). Minimum inhibitory concentrations (MICs) for sweet marjoram and rosemary EOs, completely inhibiting mycelial growth, were found to be 55 μ L/Petri and 65 μ L/Petri, respectively. The antifungal activities of EOs were found to be fungicidal at doses that completely inhibited mycelial growth (MIC).

The EO blends displayed varied inhibitory effects on mycelial growth, dependent on terpinen-4-ol to camphor ratio and blend concentration. Although rosemary EO

Table 1 Chemical compounds (%) determined in essential oils of *R. officinalis* and *O. majorana* and their blends

No.	Rt	Compound name	RI	RI lit	<i>R. officinalis</i>	<i>O. majorana</i>	Blend O ₇₅ R ₂₅	Blend O ₅₀ R ₅₀	Blend R ₇₅ O ₂₅
1	6.15	Thujene	927	927	nd	1.76	nd	nd	nd
2	6.30	α -Pinene	936	936	13.76	0.11	5.49	7.74	10.35
3	6.79	Camphene	965	965	2.92	nd	0.92	1.53	2.18
4	7.08	Sabinene	981	981	nd	6.15	4.70	2.27	1.77
5	7.35	Myrcene	995	995	1.49	0.96	1.14	0.98	1.35
6	11.9	cis-Ocimene	1101	1102	nd	nd	nd	0.57	0.82
7	12.86	3-Carene	1122	1122	0.64	nd	0.36	nd	nd
8	16.32	α -Phellandrene	1186	1186	0.10	0.18	0.13	nd	0.13
9	16.92	α -Terpinene	1195	1195	nd	5.91	3.41	0.38	1.68
10	17.08	Limonene	1198	1198	3.51	0.73	1.49	2.20	2.54
11	18.35	γ -Terpinene	1222	1222	0.23	12.58	7.66	2.29	3.38
12	18.89	Eucalyptol	1232	1231	14.13	nd	nd	9.31	10.86
13	19.85	o-Cymene	1248	1248	nd	7.60	nd	nd	nd
14	20.18	Linalyl acetate	1254	1254	nd	1.34	0.84	0.44	0.34
15	20.52	Bornyl acetate	1259	1259	2.43	0.07	nd	1.71	1.74
16	20.61	α -Terpinolene	1261	1261	nd	3.42	nd	nd	nd
17	21.19	p-Cymene	1270	1270	2.46	nd	8.04	7.47	6.50
18	21.81	3-Octanone	1280	1280	0.23	nd	0.07	0.13	0.16
19	23.29	Terpinen-4-ol acetate	1302	1302	nd	2.68	1.77	1.01	0.61
20	24.68	Terpinen-4-ol	1328	1328	1.24	35.13	25.10	16.21	13.14
21	30.38	trans-Caryophyllene	1420	1420	0.25	nd	nd	nd	nd
22	33.10	cis Sabinene hydrate	1460	1460	nd	nd	6.69	0.78	0.32
23	35.62	Camphor	1495	1495	20.69	0.18	6.71	13.87	14.13
24	36.09	β -Guaiene	1503	1503	nd	0.70	0.29	0.21	0.25
25	36.10	Isopinocampnone	1504	1504	2.30	0.14	0.79	1.46	1.56
26	36.19	Linalool	1507	1507	5.21	2.09	2.99	4.21	3.98
27	37.29	trans Sabinene hydrate	1548	1548	0.12	9.17	6.31	2.93	0.93
28	38.38	Caryophyllene oxide	1587	1587	0.13	0.27	0.24	0.37	0.25
29	38.75	Dihydrocarvone	1600	1600	nd	0.43	0.20	nd	nd
30	40.58	Verbenol	1647	1647	0.29	nd	nd	nd	nd
31	41.06	α -Terpineol	1659	1659	2.96	5.19	4.70	4.21	3.71
32	41.73	Isoborneol	1675	1675	nd	0.13	0.85	nd	nd
33	42.72	Borneol	1699	1699	8.98	nd	2.82	5.89	5.96
34	43.02	Neodihydrocarveol	1707	1707	0.93	nd	0.26	0.59	0.69
35	44.07	Verbenone	1735	1735	12.17	0.08	3.56	8.31	7.94
36	44.26	Neryl acetate	1740	1740	nd	0.20	0.11	nd	nd
37	44.63	cis-Piperitol	1750	1750	nd	1.02	0.70	0.32	0.17
38	45.92	Thymol	1783	1783	0.08	0.08	0.09	0.09	0.10
39	46.25	Myrtenol	1792	1790	1.04	nd	0.24	0.64	0.53
40	47.15	p-Cymen-8-ol	1813	1813	0.83	0.10	0.32	0.60	0.60
41	48.02	Nerol	1832	1832	nd	0.57	0.42	0.62	0.28
42	50.25	Carvacrol	1880	1880	0.13	0.30	0.33	0.30	0.30
		Monoterpene hydrocarbons			39.7	46.82	51.59	39.23	41.62
		Oxygenated monoterpenes			59.17	48.06	47.62	59.83	57.13
		Sesquiterpene hydrocarbons			0.25	4.12	0.29	0.21	0.25
		Oxygenated sesquiterpenes			0.13	0.27	0.24	0.37	0.25
		Total identified components (%)			99.25	99.27	99.74	99.64	99.25

Rt: Retention time; RI: Retention indices relative to C₈-C₄₀ on the TR-5MS capillary column; RI (lit): Retention indices according to Babushok et al. (2011). nd: not determined

Monoterpene hydrocarbons (No.1–12, 14, 18, 23, 26, 28, 30, 36, 38)

Oxygenated monoterpenes (No.13, 15, 16, 19–21, 24, 27, 31–35, 37, 39–42)

Sesquiterpene hydrocarbons (No.17, 22, 25)

Oxygenated sesquiterpenes (No.29)

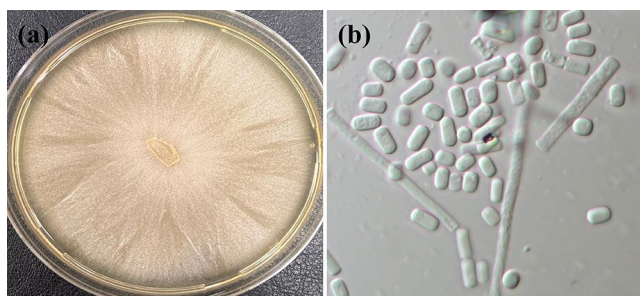


Fig. 1 Five-day mycelial growth (a) and typical conidia (b) (arrow) of the fungal disease agent *G. candidum* on PDA medium

completely inhibited mycelial growth at 65 $\mu\text{L}/\text{Petri}$, $\text{O}_{75}\text{R}_{25}$ blend completely at a lower concentration of 45 $\mu\text{L}/\text{Petri}$. Similarly, while sweet marjoram EO completely inhibited growth at 55 $\mu\text{L}/\text{Petri}$, $\text{O}_{75}\text{R}_{25}$ blend showed inhibition at 45 $\mu\text{L}/\text{Petri}$ (Table 2). The antifungal activity of EO blends was found to be fungicidal at the concentrations that completely inhibited mycelial growth (MIC).

Probit analysis determined the lowest effective concentration (EC_{50}) inhibiting fungal growth by 50%. The EC_{50} values for sweet marjoram and rosemary EOs were higher

compared to their blends ($\text{O}_{75}\text{R}_{25}$ and $\text{R}_{75}\text{O}_{25}$). Specifically, sweet marjoram and rosemary EO alone exhibited EC_{50} values of 14.645 and 17.433, respectively, whereas their blends ($\text{O}_{75}\text{R}_{25}$ and $\text{R}_{75}\text{O}_{25}$) demonstrated lower EC_{50} values of 12.132 and 15.017 (Table 2).

The effects of EOs and their blends on hyphal morphology

Microscopic examination of fungal hyphae treated with EO blends in Petri dishes at inhibitory concentrations of 100% revealed substantial morphological alterations. The volatile compound(s) within the EO blends elicited damage to the fungal plasma membrane, leading to observed deformities, including cytoplasmic coagulation, hyphal diameter constriction, and fragmentation of the hyphal wall (Fig. 3).

Antifungal effects of the EOs and their blends on disease development *in vivo* conditions

The results of the antifungal effectiveness of essential oils (EOs) and their blends on disease development on artificially

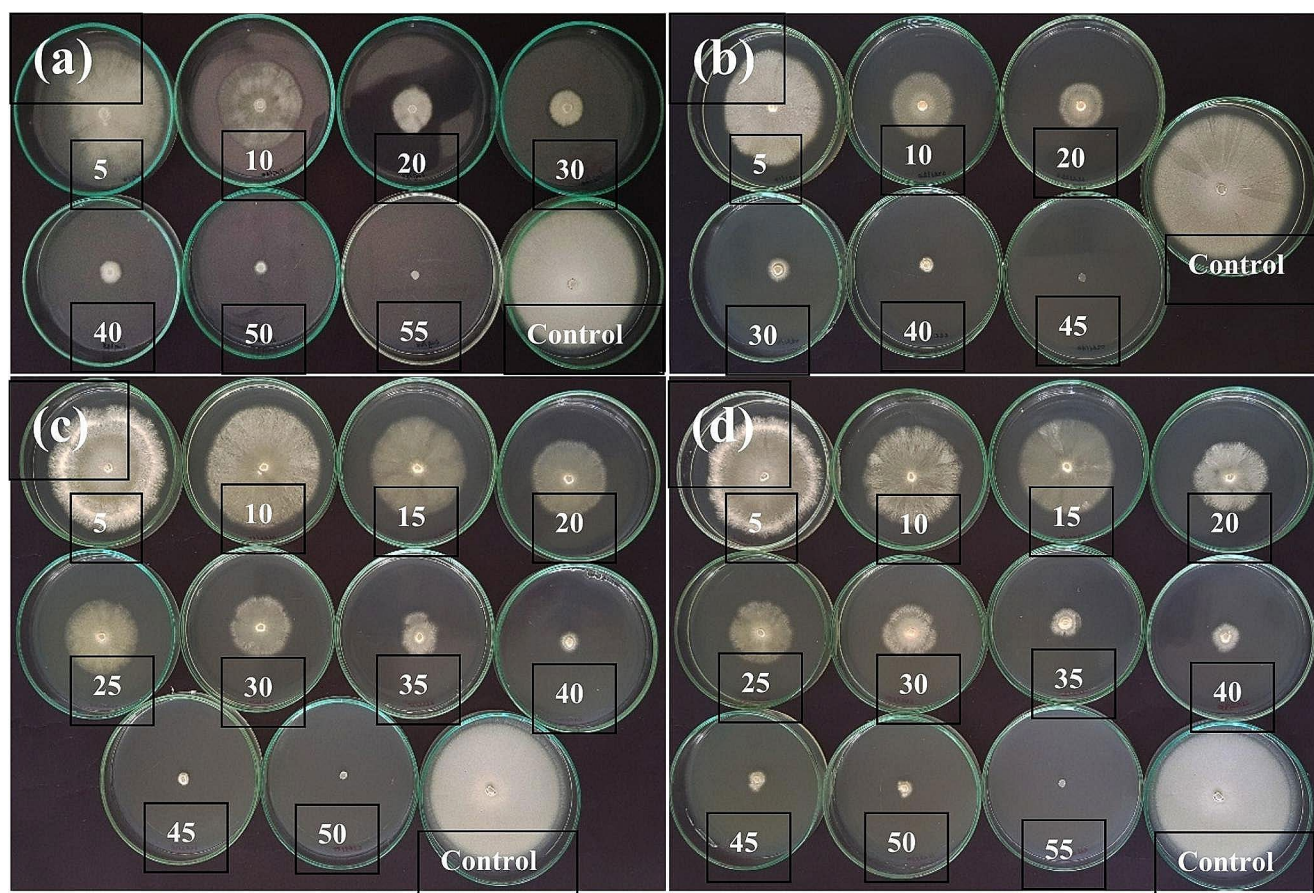


Fig. 2 Inhibition of mycelial growth by different concentrations of *R. officinalis* and *O. majorana* essential oils (a) and their blends $\text{O}_{75}\text{R}_{25}$ (b), $\text{O}_{50}\text{R}_{50}$ (c) and $\text{R}_{75}\text{O}_{25}$ (d)

Table 2 Antifungal effects of various concentrations of the volatile phase of essential oils and their blends on the mycelial growth (mm)^a and inhibition (%) of *Geotrichum candidum*

Mycelial Growth (MG) and Mycelial Growth Inhibition (MGI)										
Essential oils					Essential oil blends					
<i>R. officinalis</i>		MGI (%)	<i>O. majorana</i>		O ₇₅ R ₂₅	MGI (%)	O ₅₀ R ₅₀		R ₇₅ O ₂₅	MGI (%)
Conc. (μl/Petri)	MG (mm)		MG (mm)	MGI (%)			MG (mm)	MGI (%)		
0.00	90.00 ^{aA}	0.00	90.00 ^{aA}	0.00	90.00 ^{aA}	0.00	90.00 ^{aA}	0.00	90.00 ^{aA}	0.00
5.00	86.33 ^{bA}	4.07	77.67 ^{bB}	13.70	65.33 ^{bD}	27.41	79.67 ^{bB}	11.48	75.00 ^{bC}	16.67
10.00	60.00 ^{cAB}	33.33	58.00 ^{cBC}	35.56	54.00 ^{cC}	40.00	63.33 ^{cA}	29.63	58.33 ^{cB}	35.19
15.00	47.00 ^{dB}	47.78	43.33 ^{dC}	51.85	42.67 ^{dC}	52.59	54.33 ^{dA}	39.63	45.33 ^{dBC}	49.63
20.00	39.67 ^{eAB}	55.93	38.67 ^{eB}	57.04	35.00 ^{eC}	61.11	41.33 ^{eA}	54.07	38.33 ^{eB}	57.41
25.00	31.00 ^{fA}	65.56	27.33 ^{fB}	69.63	24.33 ^{fC}	72.96	29.33 ^{fAB}	67.41	30.00 ^{fA}	66.67
30.00	25.00 ^{gA}	72.22	23.33 ^{gAB}	74.07	19.67 ^{gB}	78.15	22.00 ^{gAB}	75.56	24.67 ^{gA}	72.59
35.00	23.33 ^{gA}	74.07	16.33 ^{hC}	81.85	10.67 ^{hD}	88.15	17.00 ^{hC}	81.11	21.00 ^{hB}	76.67
40.00	19.33 ^{hA}	78.52	10.00 ^{iB}	88.89	8.33 ^{iC}	90.74	10.33 ^{iB}	88.52	18.67 ^{iA}	79.26
45.00	15.33 ^{iA}	82.96	7.33 ^{jC}	91.85	0.00 ^{iD}	100.00	7.33 ^{jC}	91.85	11.33 ^{jB}	87.41
50.00	11.33 ^{jA}	87.41	6.33 ^{jC}	92.96	0.00 ^{iD}	100.00	0.00 ^{kD}	100.00	8.33 ^{kB}	90.74
55.00	9.33 ^{jA}	89.63	0.00 ^{kB}	100.00	0.00 ^{iB}	100.00	0.00 ^{kB}	100.00	0.00 ^{iB}	100.00
60.00	6.33 ^{kA}	92.96	0.00 ^{kB}	100.00	0.00 ^{iB}	100.00	0.00 ^{kB}	100.00	0.00 ^{iB}	100.00
65.00	0.00 ^{lA}	100.00	0.00 ^{kA}	100.00	0.00 ^{iA}	100.00	0.00 ^{kA}	100.00	0.00 ^{lA}	100.00
EC₅₀^b	17.433		14.645		12.132		16.077		15.017	

^a The mean mycelial growth of the fungal agent determined is based on measurements of 3 replicate plates recorded 7 days after inoculation. Means within a column or row followed by different lower and upper case letters are significantly different according to the Duncan Multiple Range Test ($P \leq 0.05$)

^b Efficient concentration (EC₅₀) values (ml/Petri) for each essential oil and their blends were estimated using Probit analysis

inoculated potato tubers (*in vivo*) at different concentrations are presented in Table 3. A dose-dependent increase in antifungal activity was evident with rising concentrations of essential oils and blends. In line with the *in vitro* findings, lesions on tubers treated with oregano and rosemary essential oils were completely inhibited at concentrations of 125 μl/L air and 150 μl/L air, respectively (Fig. 4). In contrast, the blend exhibited complete lesion inhibition at a relatively lower concentration of 100 μl/L air. Statistically significant differences in antifungal activities among the concentrations used were observed (Table 3).

Furthermore, Probit analysis revealed the lowest effective concentration (EC₅₀) inhibiting lesion development caused by the fungal pathogen by 50%. Individually, sweet marjoram (61.46 μl/L air) and rosemary EO (76.17 μl/L air) demonstrated higher EC₅₀ values compared to their blend (54.06 μl/L air) (Table 3).

Discussion

The limitations associated with fungicides, such as their decreasing effectiveness against resistant pathogens and potential adverse effects on beneficial microorganisms, underscore the urgency to explore alternative disease management strategies (Staub 1991). Plant extracts and essential oils with inherent antifungal properties offer promising

alternatives to chemical controls (Bakkali et al. 2008; Nazaro et al. 2017; El-Tarabily et al. 2021; Bhattacharya et al. 2021; Abd El-Hack et al. 2022). Current research predominantly focuses on studying the chemical components and antimicrobial efficacy of essential oils, which can vary significantly among plant species. While certain plants produce substantial essential oil quantities, their antimicrobial effectiveness may be modest, contrasting with plants possessing oils with potent antimicrobial properties despite lower oil yields. Recent studies emphasize exploring essential oil blends to combat plant fungal diseases (Hyldgaard et al. 2012; Ambrosio et al. 2017).

Rosemary and sweet marjoram essential oils, characterized by chemical constituents like borneol, p-cymene, camphor, 1,8-cineole, linalool, terpinene, thymol, β-pinene, α-pinene, and eucalyptol, align closely with prior research findings (Soylu et al. 2006, 2010; Ozcan and Chalchat 2008; Lopez-Reyes et al. 2010; Hosseini et al. 2020; Moumni et al. 2021b). Minor variations in their proportions were observed, yet overall, the results largely corroborate earlier studies. Discrepancies in chemical constituents and ratios within the essential oils could be reasons of diverse factors, including genetic variations in the source plants, geographical and climatic conditions, extraction techniques, harvesting times, and plant phenological stages (Turkmen 2021; Turkmen et al. 2021).

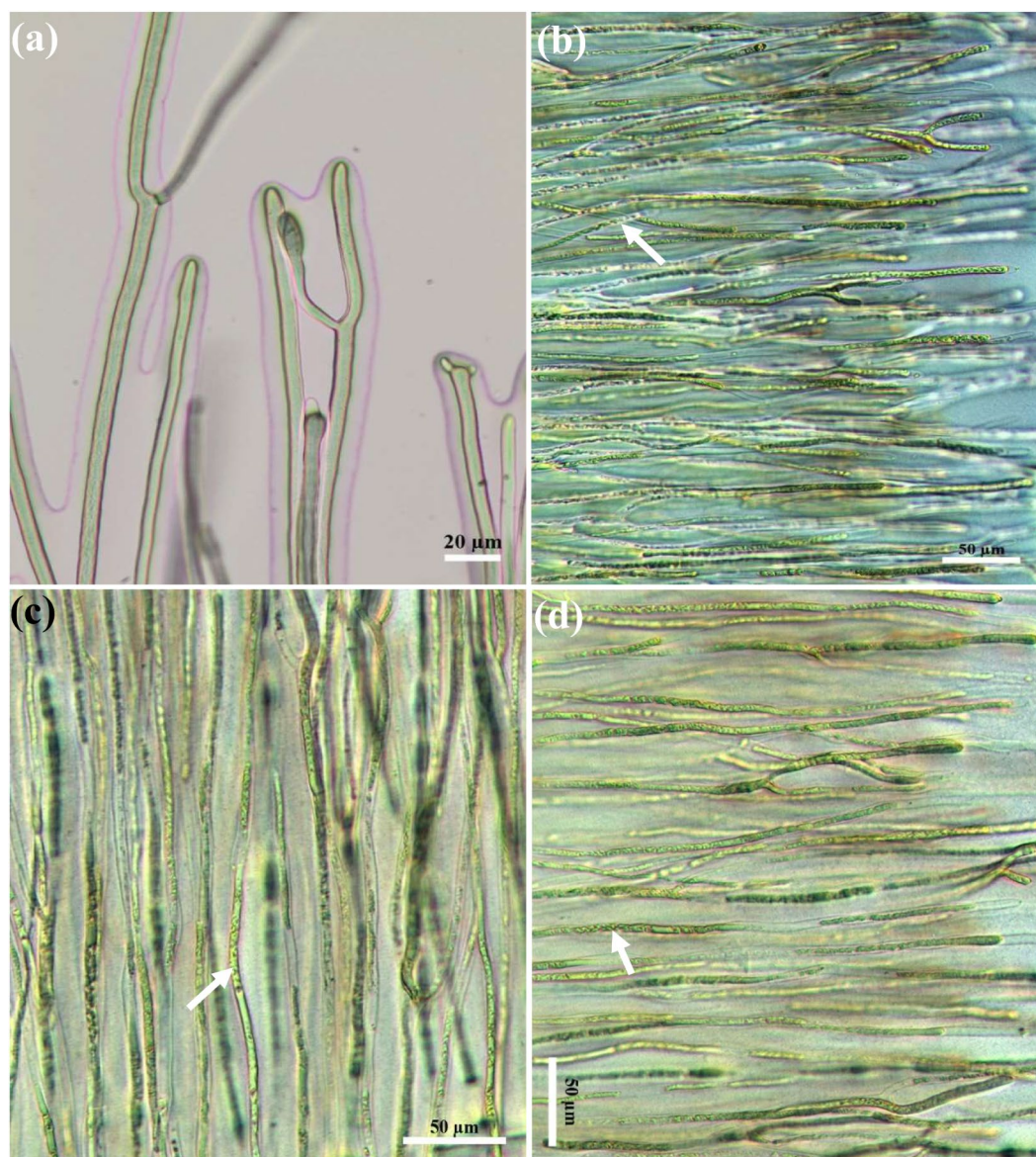


Fig. 3 Effects of essential oils blends on the morphological structures of fungal hyphae. (a) Fungal hyphae growing on the control plate. Morphological changes were observed as coagulation (arrow) in the

cytoplasm of hyphae growing on (b) $O_{75}R_{25}$, (c) $O_{50}R_{50}$ and (d) $O_{25}O_{75}$ essential oil blends added to PDA

Considering the chemical constituents and their respective proportions within the essential oil (EO) blends, a notable observation emerges: the blended EOs exhibit a higher count of constituents compared to their individual counterparts. These differences in constituent numbers and proportions within the blends may potentially evade detection using the Gas Chromatography-Mass Spectrometry (GC-MS) method. Such discrepancies could be due to the chemical interactions during the blending process, potentially leading to reduced quantities of certain minor components present in exceedingly minimal amounts ($<0.6\%$) within the blended ratios.

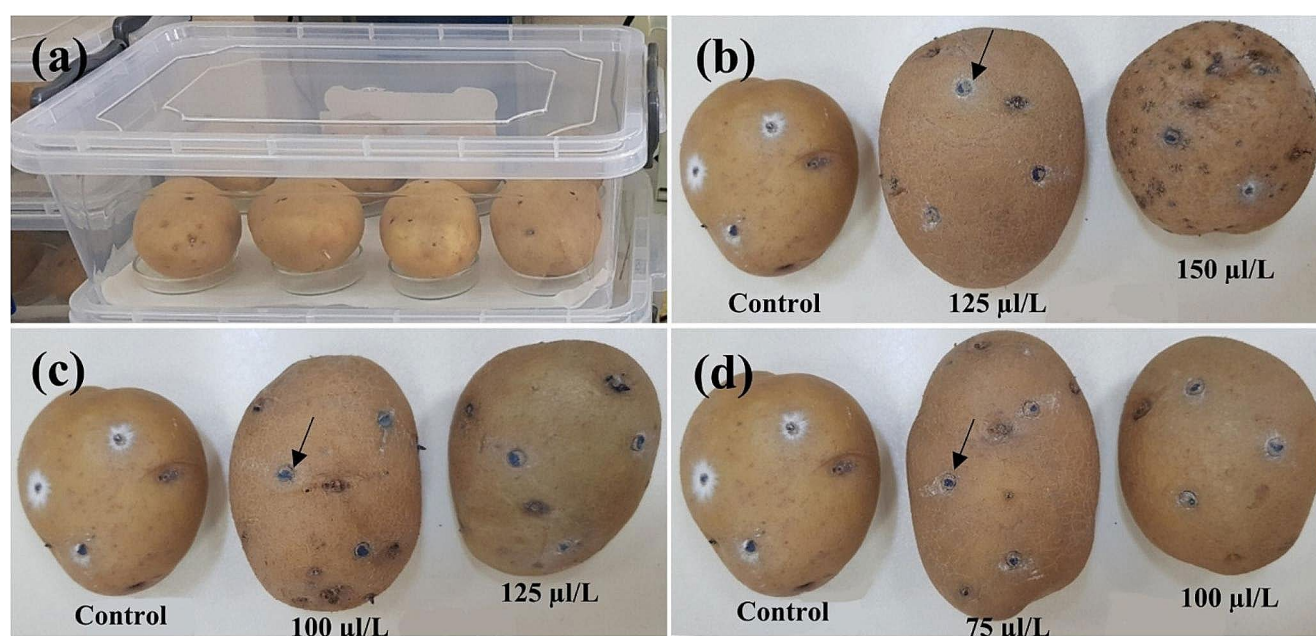
Previous literature lacks comprehensive investigations into the antifungal potential of plant essential oils (EOs) specifically against rubbery rot disease agent *G. candidum*. Promising antifungal efficacy in plant extracts obtained from *Cistus villosus*, *Pistacia atlantica*, *Halimium antiatlanticum*, *Inula viscosa*, *Ighermia pinifolia*, and *Hammada scoparia* from various regions in southern Morocco, exhibited antifungal activities against citrus sour rot disease agent *G. candidum* (Talibi et al. 2012). Additionally, the antifungal activity of chloroform extracts from plant species within the Lamiaceae family has been reported in the context of combating *G. candidum*, as well as the postharvest citrus green mold disease agent *Penicillium digitatum* (Balkan et

Table 3 Efficacy of different concentrations of essential oils and blend on disease lesion diameter (mm)^a and inhibition of disease lesion (%) caused by *G. candidum* in potato tubers under *in vivo* conditions

Concentrations ($\mu\text{L/L}$ air)	<i>R. officinalis</i>		<i>O. majorana</i>		<i>O</i> ₇₅ <i>R</i> ₂₅	
	DL (mm)	IDL (%)	DL (mm)	IDL (%)	DL (mm)	IDL (%)
0.0	16.5 ^f	0.00	16.5 ^e	0.00	16.5 ^d	0.00
50.0	12.38 ^{cC}	25.00	10.38 ^{dB}	37.12	9.38 ^{cA}	43.18
75.0	9.38 ^{dC}	43.18	6.88 ^{cB}	58.33	3.38 ^{bA}	79.55
100.0	6.13 ^{cC}	62.88	2.13 ^{bB}	87.12	0.00 ^{aA}	100.00
125.0	2.63 ^{bB}	84.09	0.00 ^{aA}	100.00	0.00 ^{aA}	100.00
150.0	0.00 ^a	100.00	0.00 ^a	100.00	0.00 ^a	100.00
EC₅₀		76.17		61.46		54.06

^a The mean disease lesion diameter caused by fungal agent determined is based on measurements of 8 replicate tubers recorded 15 days after inoculation. Means within a column or row followed by different lower and upper case letters are significantly different according to the Duncan Multiple Range Test ($P \leq 0.05$)

^b Efficient concentrations (EC₅₀ and EC₅₀) values ($\mu\text{L/ml}$ air) for each essential oil and their blend were estimated using Probit analysis

**Fig. 4** Efficacy of different concentrations of essential oils and blend (*O*₇₅*R*₂₅) on inhibition of disease lesion caused by *G. candidum* in potato tubers under *in vivo* conditions. (a) Inoculated potato tubers exposed to different concentrations of essential oils stored in a trans-

parent sealed storage box. (b) Inhibition of disease lesion (arrow) on potato tubers treated by different concentrations of *R. officinalis* (b), *O. majorana* (c) and essential oil blend (d)

al. 2019). In a recent study, antifungal activities of oregano and clove essential oils and their main components thymol, carvacrol and eugenol were demonstrated against citrus post harvest disease agents such as *Geotrichum candidum*, *Penicillium digitatum* and *Penicillium italicum* isolates which were resistant to the commercial fungicides imazalil or guazatin (Moussa et al. 2021). The results indicated that the essential oils and their main components were effective in inhibiting fungal growth, with thymol displaying the highest efficacy. Moreover, the chemical compositions and antifungal properties of EOs from *Thaumatococcus daniellii*, *Hyptis suaveolens*, and *Hildegardia barteri* against various tomato fruit rot disease agents, including *G. candidum*

(Dania and Olaleye 2022). Remarkably, the EO derived from *T. daniellii* exhibited significant efficacy against *G. candidum* at concentrations equal to or exceeding 0.25 $\mu\text{L/ml}$, surpassing the effectiveness of the fungicide mancozeb. Antifungal activity of ethanolic extract of clove (*Syzygium aromaticum*) buds was investigated against fungal isolates of *Geotrichum candidum*, *Alternaria alternata*, *Fusarium oxysporum* and *Mucor hiemalis* causing postharvest infections from potato and tomato. All fungal isolates were found to be inhibited by the extract (Suleiman et al. 2019).

While extensive research has focused on assessing the individual antifungal properties of essential oils (EOs), there remains a relative scarcity in investigations concerning the

potential of EO blends (Brochot et al. 2017). The synergistic effect of active compounds found in essential oils, particularly those abundant in phenolic terpenoids, is a significant characteristic that makes EOs appropriate for use as natural antimicrobial agents (Teixeira et al. 2022). Several studies have shown that EOs and their blends derived from medicinal and aromatic plant species can increase the mortality rate of pests and decrease the MIC of commercially available pesticides, fungicides, and other bioactive molecules (Brochot et al. 2017; Dassanayake et al. 2021; Bhattacharya et al. 2021). Therefore, plant-derived essential oils are a valuable and innovative source of bioactive compounds that have potent synergistic effects in controlling crop-associated insect pests and phytopathogenic fungi. Notable instances in the literature include an inquiry into the antimicrobial efficacy of distinct EO combinations comprising lavender, petit-grain, clary sage, ylang-ylang, and jasmine against bacterial pathogens such as *Staphylococcus aureus*, *S. epidermidis*, *Escherichia coli*, alongside the fungal pathogen *Candida albicans* (Tadtong et al. 2012). Their results clearly revealed heightened antimicrobial effectiveness in the blended EOs compared to their singular constituents. Turkmen and Kaya (2021) explored the use of fennel and laurel EO blends to inhibit the mycelial growth of the white rot disease agent, *Sclerotinia sclerotiorum*. Furthermore, Hashem et al. (2019) investigated an EO blend comprising clove bud, lemon, cinnamon, rosemary, lavender, and cedarwood oils, displaying its ability to protect peach fruit from various post-harvest disease agents during prolonged cold storage, significantly extending shelf life. Additionally, a synergistic antifungal effect was observed in a blend of fennel and laurel EOs at a specific ratio (3:1), inhibiting mycelial growth and conidial germination of *Pestalotiopsis funerea* (Kara et al. 2022). These findings corroborate the present study's outcomes, collectively advocating for the enhanced efficacy of EO blends compared to their individual applications in combating a diverse spectrum of fungal disease agents. Zhang et al. (2023) evaluated the synergistic antifungal effects of natural compound against *Aspergillus flavus*. Different combinations of natural antifungal compound revealed that the cinnamaldehyde and nonanal blend displayed the strongest synergistic antifungal activities against *A. flavus* compared to when each agent was used separately.

The role of principal components within essential oils (EOs) in exhibiting antifungal activity is widely acknowledged. However, a significant gap exists in the current literature regarding the synergistic antifungal potential of terpinen-4-ol and camphor, major constituents prominent in sweet marjoram and rosemary EOs. Our study findings underscore the substantial impact of varying proportions of terpinen-4-ol and camphor on antifungal activity, with terpinen-4-ol determined as the primary influential contributor.

Prior investigations have highlighted terpinen-4-ol and camphor as pivotal constituents demonstrating significant antifungal properties (Morcia et al. 2012; Kong et al. 2022). Furthermore, the antifungal potential of other compounds such as β -citronellol, nerol, menthol, α -terpineol, carvone, borneol, and the commercial fungicide benomyl has been observed, highlighting potent inhibitory effects against a broad spectrum of fungal species, including *Alternaria alternata*, *A. solani*, *Botrytis* spp., *Drechslera* spp., *Fusarium* spp., *Nigrospora* spp., *Penicillium* spp., *Phoma* spp., *Pythium ultimum*, *Rhizoctonia solani*, *Sclerotinia* spp., *Verticillium* spp., and *V. tenerum* (Kordali et al. 2007). The antifungal activity of camphor was meticulously evaluated against four commonly encountered *Fusarium* spp., such as *Fusarium oxysporum*, *F. solani*, *F. verticillioides*, and *F. graminearum*. The minimum inhibitory concentrations of camphor against these fungi were recorded at 4.0 mg/ml, 4.0 mg/ml, 4.0 mg/ml, and 2.0 mg/ml, respectively (Kong et al. 2022).

The cell membrane represents a primary target of action for EOs (Shao et al. 2013; da Rocha Neto et al. 2019). Previous research has reported that EOs, particularly those derived from *Thymbra* spp., *Thymus* spp., *Origanum* spp., and *Foeniculum* spp., induced significant alterations in the morphological structures of both fungal and bacterial disease agents. This structural deterioration likely arises from the impact of the principal EO components on enzymatic reactions that regulate cell wall synthesis, consequently damaging the microorganism's cell membrane (Yong et al. 2015; Kachur and Suntres 2020; Turkmen et al. 2021; Kara et al. 2022). Notably, *Citrus reticulata* EO exhibits cytotoxic effects on *Penicillium italicum* and *Penicillium digitatum*, compromising the cell membrane integrity and leading to the release of cellular components (Tao et al. 2014). Tea tree EO demonstrates efficacy in inhibiting *Botrytis cinerea* by disrupting plasma membrane integrity and inducing hyphal morphological alterations (Shao et al. 2013). Moreover, tea tree EO prompts morphological and ultrastructural changes in *Monilinia fructicola* hyphae (Xu et al. 2021). Similarly, camphor disrupts cytomembranes, increasing permeability and triggering the release of intracellular macromolecules like nucleic acids and proteins (Morcia et al. 2012).

In previous studies, assessments of antifungal activities of essential oils have primarily conducted *in vitro* conditions. However, the absence of established antifungal efficacy concentrations from *in vivo* studies has limited their practical applicability in natural (*in vivo*) conditions (Soylu et al. 2006; Tegegne et al. 2008; Senouci et al. 2020). Consequently, the imperative arises to conduct *in vivo* investigations aimed at corroborating the observed antifungal activities witnessed *in vitro* (Soylu et al. 2010). Our results indicate that the antifungal activities of essential oils and

their blend demonstrated reduced potency compared to the recorded outcomes *in vitro* experiments. Specifically, the effective concentrations of essential oils and the blend required to suppress disease incidence were nearly double the concentrations observed *in vitro* conditions. These findings align with prior studies that explored the *in vivo* effectiveness of essential oils (Askarne et al. 2012; Hashem et al. 2019). This study contributes to the understanding of essential oils' antifungal activities, emphasizing the necessity of *in vivo* investigations to validate their potential in field conditions.

Conclusion

In conclusion, blends of rosemary and sweet marjoram essential oils exhibit enhanced antifungal activity at lower concentrations compared to their individual applications promises to play a pivotal role in the development of innovative, natural bio-fungicides. The outcomes of this study offer valuable insights to researchers in this field, highlighting the synergistic effects of EOs and their major constituents when utilized in combination. These findings suggest potential applications of EO blends, solely confined to crop protection but extending to potential uses within the food industry for preventing microbial contamination.

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Data availability The data that support the findings of this study are available from the corresponding author upon reasonable request.

Declarations

Ethical approval This article does not contain any studies with animals performed by the author.

Conflict of interest The corresponding author states that there is no conflict of interest.

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