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Thymbra spicata Extracts Against Soilborne Fungi: Linking Chemical Composition, Antifungal Activity, and Molecular Docking Insights

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In this study, the antifungal activities of essential oil, supercritical carbon dioxide (SC-CO_2) extract, methanol extract, and SC-CO_2+ethanol extract obtained from *Thymbra spicata* L. were evaluated *in vitro* against the soilborne fungal pathogens *Sclerotinia sclerotiorum*, *Macrophomina* sp., *Rhizoctonia solani*, and *Fusarium oxysporum,* which cause substantial economic losses in cultivated plants. The chemical profiles of the extracts were determined using GC–MS. Carvacrol and p-cymene were identified as the major constituents of the SC-CO_2 and SC-CO_2+ethanol extracts, accounting for 86.79% and 4.70%, respectively, of the SC-CO_2 extract and 72.07% and 4.6%, respectively, of the SC-CO_2+ethanol extract. Among the essential oils, carvacrol (89.61%) and trans-β-caryophyllene (1.86%) were identified as the predominant constituents, whereas the methanol extract was characterized by a greater proportion of non-volatile constituents, mainly carvacrol (33.93%) and linoleic acid (21.19%).

Antifungal bioassays were conducted using the contact effect method at concentrations of 0.25, 0.50, 1.00, 2.00, and 4.00 µl/ml. *T. spicata* essential oil (1.00, 2.00, and 4.00 µl/ml) and the SC-CO_2 extract (2.00 and 4.00 µl/ml) completely inhibited (100%) the mycelial growth of *S. sclerotiorum* in a dose-dependent manner, indicating fungicidal activity. Similarly, complete inhibition of *Macrophomina* sp. was observed with essential oil at 2.00 and 4.00 µl/ml and with SC-CO_2 and SC-CO_2+ethanol extracts at 4.00 µl/ml. The essential oil and SC-CO_2 extracts (1.00, 2.00, and 4.00 µl/ml) completely inhibited the mycelial growth of *R. solani* and *F. oxysporum*, demonstrating fungicidal activity. These differences in inhibition rates were statistically significant ($p \leq 0.05$). In contrast, the methanol extract had no inhibitory effect on any of the tested concentrations.

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ADMET analyses were performed to assess the acute toxicity profiles of carvacrol and p-cymene. Molecular docking against the 6CR2 target protein was conducted to assess ligand–protein interactions. Among the essential oil constituents evaluated, carvacrol and p-cymene exhibited the most favorable binding energies, supporting their potential as lead antifungal compounds.

Keywords: Soilborne fungal pathogens, carvacrol, supercritical CO_2, essential oil

INTRODUCTION

Plants are continuously affected by a range of biotic (fungi, bacteria, and viruses) and abiotic (temperature, wind, humidity, light, precipitation, and soil) stress factors that influence their physiological and pathological status (Scholthof, 2007). Fungi constitute the most important group of biotic agents because of the number and diversity of the diseases they cause (Sempere-Ferre et al. 2021). Soilborne fungal pathogens pose serious socioeconomic and ecological threats to agricultural systems (Mehta et al. 2014; Katan 2017), causing economic losses of 50–75% under both greenhouse and field conditions (Mihajlović et al. 2017; Panth et al. 2020). These diseases usually cause similar symptoms, such as root rot, wilting, yellowing, stunted growth, damping off, and dieback, in many cultivated crops (Mihajlović et al. 2017). Soilborne fungi such as *Fusarium*, *Macrophomina*, *Rhizoctonia*, and *Sclerotinia* represent major threats to crops (Dutta et al. 2023; Alizadeh et al. 2025). Pathogens can survive in the soil for several years without a host by forming resistant structures such as microsclerotia, sclerotia, chlamydospores, and oospores (Panth et al., 2020; Duff and Firrell, 2021; Parajuli et al., 2022), making them difficult to detect and manage.

Various strategies are being explored to improve global and regional agricultural systems in the future and to control diseases (Mihajlović et al. 2017; Genç Kesimci 2022). Chemical control is among the most commonly used methods for plant protection because it works quickly and effectively. The use of pesticides began with DDT (dichlorodiphenyltrichloroethane), which has continued to grow around the world, with more than 1,000 active substances now in use despite their known harmful effects (Choudhury and Saha, 2020). Although chemical fungicides are widely used, their restricted efficacy, harmful

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environmental effects, and possible food safety concerns have driven interest in natural plant-derived substances (van den Bosch et al. 2014). In nature, essential oils (EOs) are widely used because of their properties and play important roles in plant protection because of their antibacterial, antiviral, antifungal, insecticidal and repellent properties. Approximately 3000 EOs are currently known, 300 of which are of commercial importance for the pharmaceutical, agricultural, food, cosmetic and perfume industries (Bakkali et al. 2008). EOs are volatile and chemically complex extracts obtained from diverse botanical sources and are distinguished by the presence of a wide spectrum of constituents that are responsible for multiple biological functions (Hou et al. 2022).

The majority of the investigated plant species belong to the Lamiaceae family and include the genera *Origanum*, *Salvia*, *Thymus*, and *Mentha*. The bioactive constituents of these plants are consistently associated with potent antimicrobial properties (Greff et al. 2023). *Thymbra spicata* L. (Lamiaceae) is a perennial dwarf shrub that generally grows in arid and semiarid warm mountainous areas. *T. spicata* is distributed in the East Aegean Islands, Greece, Iran, Iraq, Lebanon, Syria, Israel and Türkiye. The species distribution in Türkiye is in western, southern and southeastern Anatolia. There are 4 taxa of *Thymbra* that are naturally found in the flora of Türkiye: *Thymbra spicata* var. *spicata* and *intricata* and *Thymbra sintenisii* var. *sintenisii* and *isaurica*.

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Thymbra spicata is also known as Zattar, Zhater or Karabas kekik in the Turkish and Arabic languages. The most notable visual difference between *T. sintenisii* and *T. spicata* is their corolla color: *T. sintenisii* has a white corolla, whereas *T. spicata* has a pink corolla. In some regions (Şanlıurfa, Gaziantep and Diyarbakır), both species can be found together in nature (Kızıl et al., 2011). On the other hand, in Hatay, only *T. spicata* occurs naturally.

T. spicata is cultivated in Eastern and Southeastern Anatolia for spice usage; however, hand-picking fresh shoots is difficult and costly (Figure 1). Since the harvest must be carried out before the plants pass the flowering stage by collecting fresh branches, it is necessary to operate under strict time constraints. Even if the essential oil content is very low, these plants need to be used in other areas.

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Figure 1. Cultivated *Thymbra spicata* field and manual harvesting process in Koz Kalesi, Hatay, Türkiye

Fresh and dried *T. spicata* leaves were used in breakfast side dishes, salads and herbal teas. In addition, *T. spicata* essential oils and different extracts have antioxidant, antimicrobial, antiproliferative and antiseptic activities (Kızıl et al., 2015; Eliuz and Bahadirli, 2023).

The aim of this study was to identify the most suitable extraction method by comparing different techniques in terms of yield, composition, and effectiveness against soilborne diseases. In this way, plants harvested by mechanical means can be directly extracted and subsequently applied to treat targeted diseases.

RESULTS AND DISCUSSION

GC–MS results

In the present study, *T. spicata* EO, SC-CO_2, SC-CO_2+Ethanol and methanol extracts were analyzed via GC/MS, and the results are presented in Table 1. Extracts obtained from 90–0 bars were not used in further analysis because of their low carvacrol content. In the methanol extracts, 12 compounds were determined to constitute 81.6% of the total detected compounds. The main compounds in the methanol extracts were carvacrol (33.93%), **γ**-terpinene (9.12%) and linoleic acid (21.19%). The SC-CO_2+Ethanol extract resulted in 28 compounds, which accounted for 85.04% of the total composition. The main

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components of SC-CO_2+Ethanol were 72.07% carvacrol and 4.60% p-cymene. The SC-CO_2 extract contained 14 compounds, which accounted for 97.58% of the total composition. The main components of SC-CO_2 were 86.79% carvacrol and 4.70% p-cymene. EO analysis revealed 9 components, which accounted for 95.07% of the total composition. The main components of the EO were 89.61% carvacrol and 1.86% trans-β-caryophyllene.

Table 1. Chemical profiling of *T. spicata* essential oil and SC-CO_2, methanol, and SC-CO2+ethanol extracts (%)

RI	**LRI**	**Compound**	**Essential Oil**	**SC-CO2**	**Methanol**	**SC-CO2+Ethanol**
947	948	α-Thujene			0.32	0.03
943	939	α-pinene	0.07	0.12	0.3	0.07
985	979	β-pinene				0.02
1004	1004	7-octen-4-ol				0.1
1011	1011	δ-3-carene				0.05
1014	1014	β-myrcene		0.09	0.58	0.13
1025	1025	α-phellandrene				0.05
1038	1036	α-terninene		0.1	0.83	0.21
1047	1046	p-cymene	0.93	4.7	5.58	4.6
1050	1050	β-phellandrene		0.05		0.06
1052	1052	1.8 cineole				0.03
1066	1061	γ-terpinene	0.06	0.32	9.12	0.48

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1105	1098	linalool		0.09		0.19
1110	1109	2-carene				0.04
1201	1200	terpinen-4-ol	0.59	0.47	0.12	0.66
1221	1221	β-fenchyl alcohol	0.14			0.08
1226	1226	carvone				0.03
1250	1244	thymoquinone		0.26		0.3
1293	1295	thymol	1.74	1.92	0.41	1.97
1304	1300	carvacrol	89.61	86.79	33.93	72.07
1402	1400	tetradecane				0.03
1437	1432	coumarin				0.03
1445	1418	trans-β-caryophyllene	1.86	2.39	1.48	1.53
1460	1454	α-humulene	0.07	0.09		0.18
1474	1470	γ-muurolene				0.01
1514	1513	ledene				0.08
1587	1582	spathulenol				0.22
1980	1977	n-hexadecanoic acid		0.19	7.74	1.79
2151	2154	linoleic acid			21.19	

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Total	**95.07**	**97.58**	**81.6**	**85.04**

RI: Retention indices calculated against *n*-alkanes, LRI: Retention indices reported in the literature, n.d.: not detected

In the present study, *T. spicata* extracts obtained by different extraction techniques exhibited remarkable variation in both total yield and relative chemical profile. Among the four tested methods, the SC-CO_2 extract had the highest relative proportion of volatile and semi-volatile constituents (97.58%), which was dominated by carvacrol (86.79%), followed by trans-β-caryophyllene (2.39%) and thymol (1.92%). These findings are consistent with those of Sonsuzer et al. (2004), who optimized SC-CO_2 parameters for *T. spicata* and reported carvacrol as the major compound under high-pressure, moderate-temperature conditions. Similarly, Barak et al. (2025) reported that *T. spicata* subsp. *spicata* oils rich in carvacrol exhibited strong antimicrobial, antibiofilm, and antiquorum-sensing activities, highlighting the biological relevance of this phenolic monoterpenoid. In addition, previous studies on SC-CO_2 extraction from oregano (*O. vulgare* L.) have shown that operational parameters significantly influence both the extraction yield and the relative abundance of phenolic monoterpenes, such as carvacrol and thymol, underscoring the importance of process optimization in shaping chemical profiles (Lin et al., 2021).

In contrast, the methanolic extract yielded a broader but less volatile profile (81.6%), with substantial amounts of fatty acids such as linoleic (21.19%) and n-hexadecanoic acid (7.74%), suggesting that polar solvents favor the extraction of high-molecular-weight non-volatiles. The SC-CO_2+Ethanol cosolvent system enhanced the recovery of moderately polar oxygenated monoterpenes (e.g., terpinen-4-ol, thymoquinone, and linalool) at relatively low proportions, reflecting the role of ethanol as a polarity modifier. Similar solvent-dependent compositional shifts have been reported for *Origanum vulgare* and *Satureja thymbra*extracted under SC-CO_2 conditions (Process Optimization, 2022; Isolation, 2002).

Compared with hydrodistilled essential oil (95.07%), the SC-CO_2 extract showed comparable selectivity toward carvacrol while minimizing the loss of thermolabile monoterpenes such as γ-terpinene and p-cymene. The

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predominance of carvacrol and thymol across all fractions supports the findings of previous studies linking these compounds to the antioxidant and antimicrobial potential of *T. spicata* (Unlu et al., 2009; Eruygur et al., 2021). Collectively, the data confirm that SC-CO_2 extraction not only improves yield and purity but also preserves the functional integrity of key terpenoids, making it a superior green alternative for obtaining high-quality *T. spicata* EO suitable for pharmaceutical and nutraceutical applications.

Antifungal Results

In this study, the suppressive potential of *T. spicata* extracts produced through various extraction techniques against soilborne fungal pathogens was evaluated (Table 2-3). The extracts of *T. spicata* had fungicidal or fungistatic effects at different ratios depending on the pathogen. The results of the *in vitro* study demonstrated that the inhibition rates of pathogens differed significantly depending on both the type and concentration of EO, SC-CO_2, methanol and SC-CO2+ethanol applied (Figure 2).

Extracting SC-CO_2, EO, or SC-CO_2+ethanol at concentrations of 1.00, 2.00, or 4.00 µl/ml, respectively, from *T. spicata* completely inhibited the mycelial growth of *S. sclerotiorum*. In addition, 2.00 and 4.00 µl/ml SC-CO_2 extract and EO, respectively, completely inhibited *Macrophomina* sp., *R. solani*, and *F. oxysporum.* SC-CO_2+Ethanol and EO at a dose of 1 µl/mL, as well as SC-CO_2 at a dose of 0.5 µl/mL, inhibited the pathogen's mycelial growth by 100%. The inhibitory effect of the SC-CO_2 dose of 0.5 µl/mL on the mycelial growth of S. *sclerotiorum* was greater than that on the mycelial growth of *Macrophomiana* sp., *R. solani* and *F. oxysporum.* Additionally, a statistically significant difference was not detected between the measurements on day 3 and day 5, except at the SC-CO_2 dose of 0.25 µl/mL. All the doses of methanol applied did not significantly inhibit the mycelial growth of the tested pathogens, and the methanol treatment was equivalent to the control treatment for suppressing the pathogen's mycelial growth. The results of this study are similar to the findings reported in the literature. Lehtijärvi (2006) reported that the EO of *T. spicata* inhibited the growth of different *R. solani* isolates by 89–99%. Bayan et al. (2017) reported that at a concentration of 7 µL per Petri dish, the essential oil of *T. spicata* completely (100%) inhibited the mycelial growth of *A. solani* and

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F. oxysporum f. sp. *lycopersici*. In contrast, the mycelial growth of *R. solani* and *V. dahliae* was suppressed by 68.61% and 79.18%, respectively.

Table 2. Effects of different methods for extracting *Thymbra spicata* on the mycelial growth of the pathogens *Sclerotinia sclerotiorum, Macrophomina* sp., *Rhizoctonia solani* and *Fusarium oxysporum*

			Mycelial growth (cm)*							
Pathogens	**Extractions**	**Days**	C1**	C2	C3	0,25 (µl/mL)	0,5 (µl/mL)	1 (µl/mL)	2 (µl/mL)	4 (µl/mL)
Sclerotinia sclerotiorum	Essential oil	3	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d	0.0 a	0.0 a	0.0 a
		5	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d	**0.0 a**	**0.0 a**	**0.0 a**
	$SC\text{-}CO_2$	3	8.5 d	8.5 d	8.5 d	6.5 b	**0.0 a**	**0.0 a**	**0.0 a**	**0.0 a**
		5	8.5 d	8.5 d	8.5 d	8.5 d	**0.0 a**	**0.0 a**	**0.0 a**	**0.0 a**
	Methanol	3	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d	7.5 cd
		5	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d
	SC-CO2+ Ethanol	3	8.5 d	8.5 d	8.5 d	8.5 d	6.9 bc	**0.0 a**	**0.0 a**	**0.0 a**
		5	8.5 d	8.5 d	8.5 d	8.5 d	8.5 d	**0.0 a**	**0.0 a**	**0.0 a**
***Macrophomina* sp.**	Essential oil	3	8.2 i-k	8.4 k	8.2 i-k	6.0 g	4.1 d	0.0 a	0.0 a	0.0 a
		5	8.5 e	8.5 e	8.5 e	8.5 e	8.5 e	2.0 b	**0.0 a**	**0.0 a**
	$SC\text{-}CO_2$	3	8.2 i-k	8.4 k	8.2 i-k	6.6 h	4.6 e	1.5 b	**0.0 a**	**0.0 a**
		5	8.5 e	8.5 e	8.5 e	8.5 e	8.5 e	5.4 d	**0.0 a**	**0.0 a**
	Methanol	3	8.2 i-k	8.4 k	8.2 i-k	8.5 k	8.5 k	8.0 i	8.1 i	8.0 i
		5	8.5 e	8.5 e	8.5 e	8.5 e	8.5 e	8.5 e	8.5 e	8.5 e
	$SC\text{-}CO_2$+ Ethanol	3	8.2 i-k	8.4 k	8.2 i-k	7.5 ı	5.1 f	3.0 c	**0.0 a**	**0.0 a**
		5	8.5 e	8.5 e	8.5 e	8.5 e	8.5 e	8.5 e	3.1 c	**0.0 a**
Rhizoctonia solani	Essential oil	3	3.0 e	8.3 e	5.4 d	4.6 c	1.8 b	**0.0 a**	**0.0 a**	**0.0 a**
		5	8.5 f	8.5 f	8.3 f	7.2 e	3.8 d	**0.0 a**	**0.0 a**	**0.0 a**
	$SC\text{-}CO_2$	3	8.0 e	8.3 e	5.4 d	4.9 cd	1.3 b	**0.0 a**	**0.0 a**	**0.0 a**
		5	8.5 f	8.5 f	8.3 f	8.5 f	2.8 c	1.7 b	**0.0 a**	**0.0 a**
	Methanol	3	8.0 e	8.3 e	5.4 d	8.5 e	8.5 e	8.5 e	8.5 e	8.5 e
		5	8.5 f	8.5 f	8.3 f	8.5 f	8.5 f	8.5 f	8.5 f	8.5 f
	$SC\text{-}CO_2$+ Ethanol	3	8.0 e	8.3 e	5.4 d	8.5 e	4.9 cd	1.8 b	**0.0 a**	**0.0 a**
		5	8.5 f	8.5 f	8.3 f	8.5 f	8.3 f	3.1 cd	**0.0 a**	**0.0 a**
Fusarium oxysporum	Essential oil	3	2.9 f	2.6 e-f	2.6 e-f	2.7 e-f	1.5 c	**0.0 a**	**0.0 a**	**0.0 a**
		5	4.8 ı	4.7 h-ı	4.6 g-ı	4.5 g-h	3.0 d	1.0 b	**0.0 a**	**0.0 a**
	$SC\text{-}CO_2$	3	2.9 f	2.6 e-f	2.6 e-f	2.1 d	1.1 b	**0.0 a**	**0.0 a**	**0.0 a**
		5	4.8 ı	4.7 h-ı	4.6 g-ı	3.8 e	2.2 c	**0.0 a**	**0.0 a**	**0.0 a**
	Methanol	3	2.9 f	2.6 e-f	2.6 e-f	2.9 f	2.8 f	2.9 f	2.8 f	2.9 f
		5	4.8 ı	4.7 h-ı	4.6 g-ı	4.6 g-ı	4.7 h-ı	4.7 h-ı	4.7 h-ı	4.8 ı
	$SC\text{-}CO_2$+ Ethanol	3	2.9 f	2.6 e-f	2.6 e-f	2.4 d-e	2.4 d-e	1.5 c	**0.0 a**	**0.0 a**
		5	4.8 ı	4.7 h-ı	4.6 g-ı	4.4 f-ı	4.1 f	3.1 d	**0.0 a**	**0.0 a**

*: There is a significant difference between the numbers shown with different letters ($p \leq 0.05$).

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**: Control 1: sterile distilled water, Control 2: 70% ethanol, and Control 3: 70% methanol.

Table 3. Inhibitory effects of different methods for isolating *Thymbra spicata* from *Sclerotinia sclerotiorum, Macrophomina* sp., *Rhizoctonia solani,* and *Fusarium oxysporum*

Treatment	Dose (µl/mL)	Mycelial growth inhibition (%)*			
		Sclerotinia sclerotiorum	***Macrophomina* sp.**	***Rhizoctonia solani***	***Fusarium oxysporum***
Essential oil	0.25	0.0 b	0.0 e	15.3 e	6.3 h
	0.5	0.0 b	0.0 e	55.3 d	37.5 d
	1	100 a	76.9 b	100 a	79.2 b
	2	100 a	100 a	100 a	100 a
	4	100 a	100 a	100 a	100 a
SC-CO_2	0.25	0.0 b	0.0 e	0.0 f	20.8 e
	0.5	100 a	0.0 e	67.1 c	54.2 c
	1	100 a	36.5 d	80.0 b	100 a
	2	100 a	100 a	100 a	100 a
	4	100 a	100 a	100 a	100 a
Methanol	0.25	0.0 b	0.0 e	0.0 f	4.2 h
	0.5	0.0 b	0.0 e	0.0 f	2.1 h
	1	0.0 b	0.0 e	0.0 f	2.1 h
	2	0.0 b	0.0 e	0.0 f	2.1 h
	4	0.0 b	0.0 e	0.0 f	0.0 h
SC-CO_2+ Ethanol	0.25	0.0 b	0.0 e	0.0 f	8.3 g
	0.5	0.0 b	0.0 e	2.4 f	14.6 f
	1	100 a	0.0 e	63.5 c	35.4 d
	2	100 a	63.5 c	100 a	100 a
	4	100 a	100 a	100 a	100 a
Control 1	Sterile water	0.0 b	0.0 e	0.0 f	0.0 h
Control 2	Ethanol 70%	0.0 b	0.0 e	0.0 f	0.0 h
Control 3	Methanol 70%	0.0 b	0.0 e	0.0 f	0.0 h

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*: There is a significant difference between the numbers shown with different letters (p≤0.05).

Fungal diseases induce physiological changes in plants, interfere with their normal function, suppress growth, and can cause cellular mortality (Gheorghe et al. 2022). Globally, various management strategies, including the use of resistant cultivars, fungicides, biological control agents and natural compounds, have been employed to suppress fungal diseases (Mihajlović et al. 2017; Ma et al., 2023). In numerous studies, extracts and EOs from medicinal and aromatic plants have been reported to have antifungal effects against various plant pathogens. In particular, EOs exhibit strong antifungal effects through the suppression of mycelial development and/or spore germination in several fungal pathogens, such as *Fusarium*, *Aspergillus*, *Penicillium*, *R. solani*, *Verticillium dahliae*, and *S. sclerotiorum* (Greef et al., 2023).

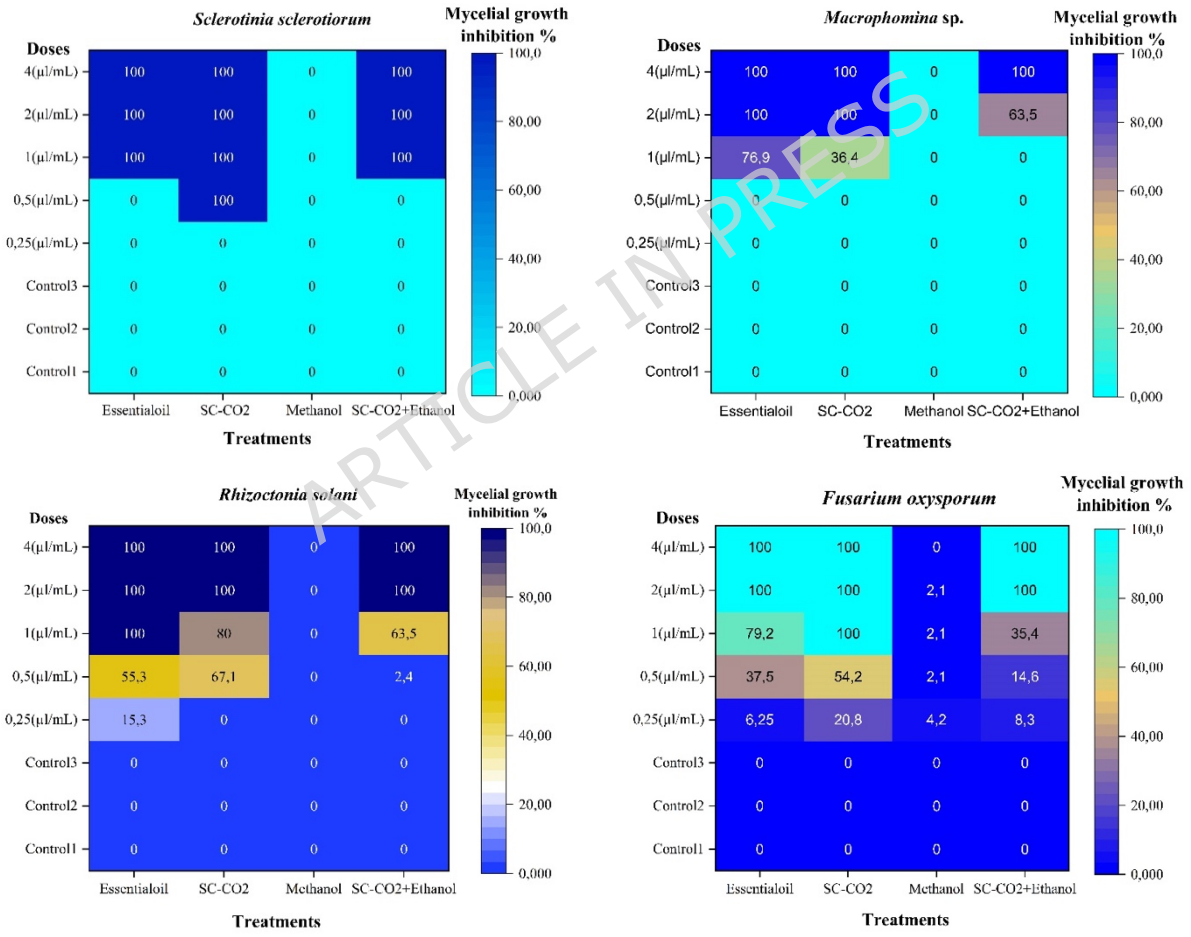


Figure 2. Inhibitory effects of different methods of *Thymbra spicata extraction* on the mycelial growth of *Sclerotinia sclerotiorum, Macrophomina* sp., *Rhizoctonia solani*, and *Fusarium oxysporum* (Control 1: sterile distilled water; Control 2: 70% ethanol; and Control 3: 70% methanol).

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Numerous studies have demonstrated that plant-derived EOs exhibit varying levels of efficacy against a wide range of fungal diseases. This variability largely reflects differences in the chemical composition of volatile oils and the physiological characteristics of the target fungi (Schmitt, 1994; Romagnoli et al., 2005). The sensitivity of fungal pathogens is influenced by factors such as hyphal morphology, resistance mechanisms, and biochemical pathways targeted by the EO. In addition, variations in inhibition rates may arise from experimental conditions, including exposure time and concentration (Moghaddam et al., 2013).

EOs display antifungal effects through multiple mechanisms, including disruption of energy metabolism, inhibition of cell wall and membrane synthesis, loss of structural integrity, suppression of hyphal development, and inhibition of mycotoxin biosynthesis. These combined effects enable EOs to effectively hinder fungal growth, supporting their potential as eco-friendly antifungal agents (Li et al., 2025). For example, linalool treatment has been shown to disrupt membrane integrity, induce oxidative stress by increasing reactive oxygen species levels, reducing glutathione levels, and inhibiting antioxidant enzyme activity in *Fusarium oxysporum* f. sp. *radicis-lycopersici*. Transcriptomic and proteomic analyses have indicated that linalool downregulates several metabolic and pathogenicity-related pathways, including those involved in cell wall degradation and G-protein signaling, while activating salicylic acid- and jasmonic acid-mediated plant defenses (Li et al., 2022). Similarly, essential oils from *Thymus pallescens, Artemisia herba-alba*, *Laurus nobilis*, and *Cymbopogon citratus* exhibit strong antifungal activity by suppressing the mycelial growth, sporulation, and spore germination of *F. oxysporum* f. sp. *ciceris* (Moutassem et al., 2019).

The high carvacrol concentration in the *T. spicata* strain used in our study may be attributed to its strong antifungal properties. The main components of T. spicata were carvacrol from methanol (33.93%), SCO_2-Eth (72.07%), SC-CO_2 (86.79%), and EO (89.61%). Sempere-Ferre et al. (2021) reported that carvacrol is a monoterpenoid phenol with strong antifungal activity that is fungicidal or fungistatic and is isolated from essential oils at varying concentrations. Wang et al. (2019) investigated the *in vitro* antifungal activity of carvacrol against *Phytophthora capsici*, *P. nicotianae*, *Alternaria solani*, *Botrytis cinerea*, *F. oxysporum*, *Pyricularia grisea*, and *R. solani*. Their results revealed that

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carvacrol exhibited broad-spectrum antifungal activity. Mueller-Riebau et al. (1995) reported an inhibitory effect of carvacrol against *Fusarium moniliforme*, *R. solani*, *S. sclerotiorum*, and *Phytophthora capsici*. Reports in the literature indicate that carvacrol affects the fungal cell membrane by altering its structure and function (Papantzikos et al. 2025). Zhao et al. (2023) reported that carvacrol interferes with membrane-associated polycarbohydrates, including chitinase and β-1,3-glucanase. These findings confirm our findings regarding the antifungal effects of these compounds. In this study, the SC-CO_2 extraction method resulted in notably high antifungal activity. These findings suggest that this approach has the potential to be at least as effective as EOs in the management of soil-borne diseases.

In silico molecular docking analysis

Molecular docking of carvacrol and p-cymene against the target protein 6CR2 was performed to assess their binding affinities and interaction modes. As shown in Table 4, compared with p-cymene, carvacrol exhibited stronger binding affinity (-4.8 kcal/mol) compared to p-cymene (-4.0 kcal/mol), suggesting a more stable interaction. The detailed data for the alternative binding modes (Modes 2–9) are provided in Supplementary Table 1. The best binding mode for both ligands was identified as Mode 1. The RMSD values are reported as 0 Å because only the top-ranked binding pose was considered for each ligand, and no reference ligand conformation was used.

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Table 4. Binding affinity, RMSD binding mode and interpretation of carvacrol and p-cymene binding to the 6CR2 protein

Ligand	Protein	Best Binding Affinity (kcal/mol)	RMSD (Å)	Binding Mode	Interpretation
Carvacrol	6CR2	**–4.8**	0.00	Mode 1	Stronger binding compared to p-Cymene
p-Cymene	6CR2	**–4.0**	0.00	Mode 1	Moderate binding affinity

The RMSD values are reported as 0 Å since only the best binding pose was considered for each ligand.

Molecular docking analyses of carvacrol and p-cymene with 6CR2 revealed distinct binding patterns and interaction profiles (Figures 3 and 4). Carvacrol established multiple interactions within the active site, including a hydrogen bond with SER A mediated by its hydroxyl group, π–π stacking interactions with

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PHE A and TYR A, a π–cation interaction with ARG A, and hydrophobic contacts with LEU A (Figure 2A–D). In contrast, p-cymene displayed a simpler binding mode dominated by hydrophobic interactions with VAL A and PRO A, without the formation of hydrogen bonds or π-interactions (Figure 3A–C). The 2D interaction diagrams summarize all key contacts for each ligand, whereas 3D visualizations illustrate their overall orientation within the binding pocket. These results are consistent with the calculated binding affinities, indicating that compared with p-cymene, carvacrol engages in a broader spectrum of stabilizing interactions, which may explain its stronger docking score.

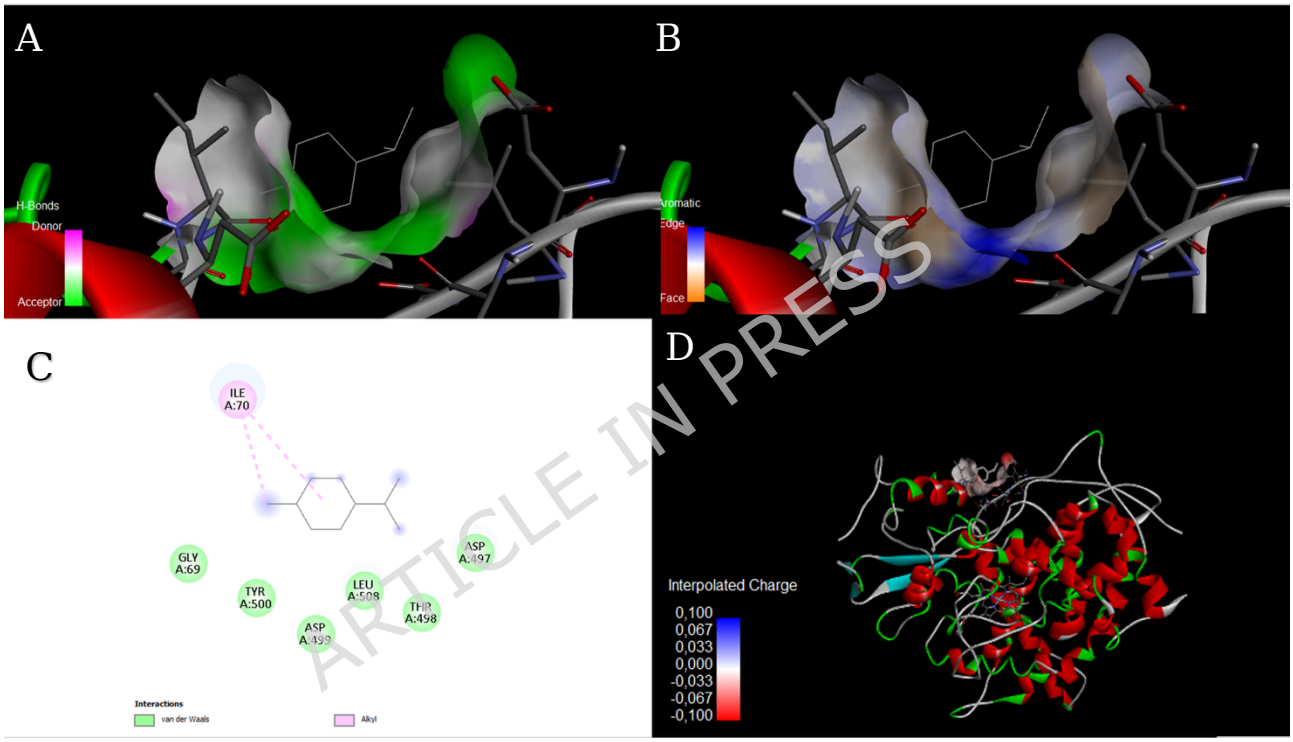


Figure 3. Molecular docking of carvacrol with 6CR2: binding interactions and structural representation. (A) Hydrogen bond interactions, showing donors and acceptors. (B) Aromatic interactions, including edge-to-face and face-to-face π–π interactions. (C) 2D interaction diagram of the ligand within the active site. (D) 3D visualization of the ligand binding site.

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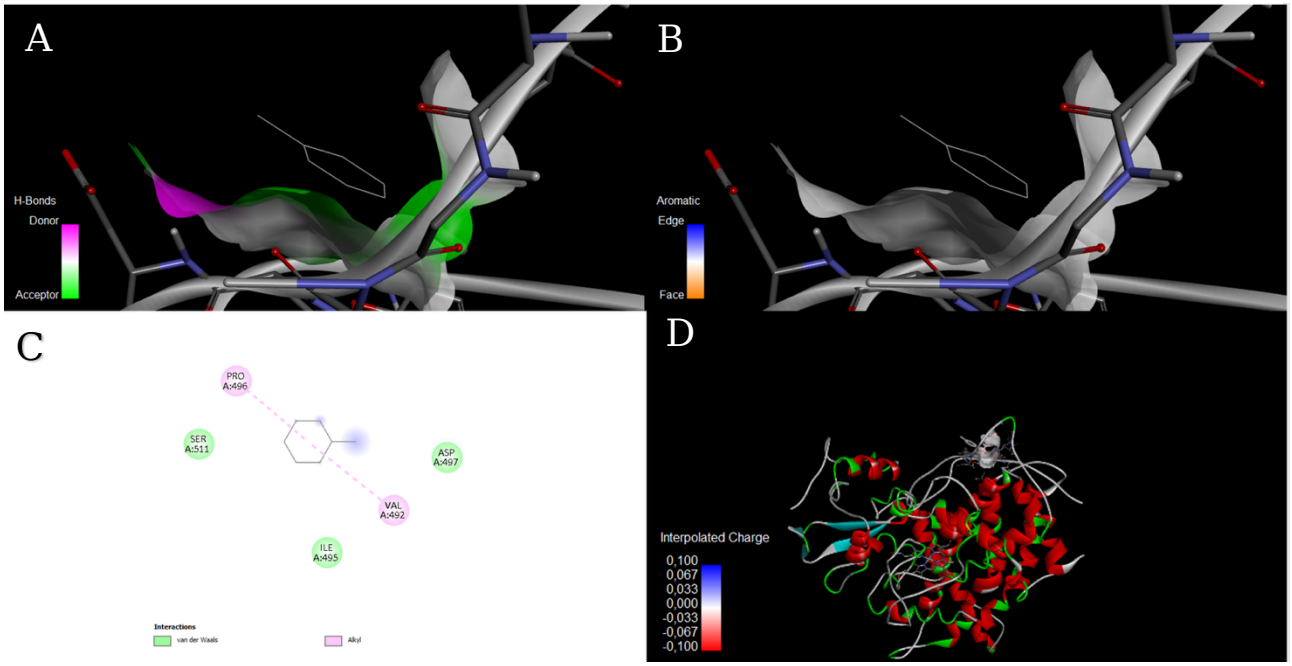


Figure 4. Molecular docking of p-cymene with 6CR2: binding interactions and structural representation. (A) Hydrogen bond interactions, showing donors and acceptors. (B) Aromatic interactions, including edge-to-face and face-to-face π–π interactions. (C) 2D interaction diagram of the ligand within the active site. (D) 3D visualization of the ligand binding site.

The detailed ligand–residue interactions of carvacrol (CAR) and p-cymene (PCM) with the 6CR2 protein are summarized in Table 5. Carvacrol formed a hydrogen bond with SER A (2.95 Å) through its hydroxyl group, while additional interactions included aromatic π–π stacking with PHE A (3.45 Å), a π–cation interaction with ARG A (3.20 Å), and hydrophobic contacts with TYR A (3.10 Å) and LEU A (3.50 Å). These interactions suggest the stable and multifaceted binding of carvacrol within the active site. In contrast, p-cymene exhibited only hydrophobic interactions with VAL A (3.55 Å) and PRO A (3.65 Å), reflecting a simpler binding mode dominated by aliphatic contacts. Overall, these results indicate that, compared with p-cymene, carvacrol engages in a broader spectrum of interactions, potentially contributing to its stronger binding affinity.

Table 5 Comparative protein–ligand interactions of carvacrol (CAR) and p-cymene (PCM) with the 6CR2 protein.

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Ligand	Amino Acid	Chain	Distance (Å)	Interaction Type	Notes
CAR	SER	A	2.95	Hydrogen bond	OH group forms H-bond
CAR	TYR	A	3.10	Hydrophobic	Aromatic ring interaction
CAR	PHE	A	3.45	Pi-pi stack	Aromatic ring stacking
CAR	ARG	A	3.20	Pi-cation	Aromatic + basic side chain
CAR	LEU	A	3.50	Hydrophobic	Aliphatic chain contact
PCM	VAL	A	3.55	Hydrophobic	Only aliphatic contact
PCM	PRO	A	3.65	Hydrophobic	Hydrophobic contact

The drug likeness and pharmacokinetic properties of p-cymene and carvacrol were predicted using the tools SwissADME and ADMET (Supplementary Table 2). Carvacrol exhibited a greater topological polar surface area (TPSA = 20.23 Å²) and possessed one hydrogen bond donor and acceptor, whereas p-cymene lacked a hydrogen bonding capacity (TPSA = 0.00 Å²). The molecular weights of both compounds were similar (134–136 g/mol), and the lipophilicity (consensus LogP) was greater for p-cymene (3.50) than for carvacrol (2.03). Carvacrol was predicted to have greater gastrointestinal absorption, while both compounds were capable of crossing the blood–brain barrier. Carvacrol fully satisfied Lipinski's rule of five, indicating good oral drug likeness, whereas p-cymene showed a single violation because of its slightly high lipophilicity. Both compounds had a moderate bioavailability score of 0.55, suggesting potential oral availability. Overall, these results indicate that compared with p-cymene, carvacrol has a slightly more favorable pharmacokinetic profile, supporting its consideration for further *in vitro* and *in vivo* studies.

Molecular docking analysis revealed that, compared with p-cymene, carvacrol exhibited stronger binding affinity for 6CR2, forming multiple stabilizing interactions, including hydrogen bonds with SER A, π–π stacking with PHE A

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and TYR A, π–cation interactions with ARG A, and hydrophobic contacts with LEU A. In contrast, p-cymene engaged mainly in hydrophobic interactions with VAL A and PRO A, reflecting a simpler binding mode. These results are consistent with previous in silico studies reporting that carvacrol can effectively interact with multiple protein targets, including HER2, PI3Kα, mTOR, and EGFR, mainly through hydrophobic interactions, π–π stacking, and hydrogen bonding, which have been associated with its reported anticancer and antimicrobial potential (Herrera-Calderon et al., 2020; Jianu et al., 2022). Similarly, in silico ADMET and docking approaches have been used to investigate the interactions and drug likeness profiles of essential oil components, and related studies have reported that monoterpenes such as carvacrol exhibit promising pharmacokinetic and biological activities against microbial targets, supporting the relevance of such computational analyses in natural product research (Sharifi-Rad et al., 2018; Azzouni et al., 2025). Taken together, these findings indicate that the structural features of carvacrol, particularly its hydroxyl group and aromatic ring, enable a broader spectrum of interactions within the active site, supporting its potential as a more effective bioactive compound than p-cymene. Moreover, these results highlight the importance of combining docking data with ADMET predictions and previous literature to rationalize ligand efficacy and guide future experimental validation.

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CONCLUSION

In the present study, *T. spicata* extracts obtained through different extraction techniques exhibited distinct chemical and biological profiles. The supercritical CO_2 (SC-CO_2) extract yielded the highest total volatile and semi-volatile content (97.58%) and was particularly rich in carvacrol (86.79%), followed by trans-β-caryophyllene. These results confirm that compared with conventional methods, SC-CO_2 extraction provides superior selectivity and preservation of thermolabile bioactive terpenoids. The results of antifungal assays demonstrated that both the SC-CO_2 and SC-CO_2–ethanol extracts had inhibitory effects comparable to those of the hydrodistilled essential oil, particularly against *S. sclerotiorum*, where complete growth inhibition (100%) was achieved even at low concentrations (0.5 µL/mL). Similarly, the inhibition rates of the SC-CO_2 extract and essential oil were nearly equivalent against *Macrophomina* sp.

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Molecular docking supported these results, showing that compared with p-cymene, carvacrol had stronger affinity (−4.8 kcal mol^{-1}) for the 6CR2 target (−4.0 kcal mol^{-1}), resulting in the formation of stabilizing hydrogen bonds and hydrophobic interactions. These in silico findings provide a molecular explanation for the strong antifungal and antimicrobial effects observed experimentally. Overall, these findings highlight SC-CO_2 extraction as an efficient, green, and selective approach for obtaining high-quality *T. spicata* essential oils rich in bioactive terpenoids with strong antifungal potential.

MATERIALS AND METHODS

Harvest and Extractions

The plant material of *Thymbra spicata* used in this study was cultivated in Koz Kalesi, Hatay Province, Turkiye, before the flowering period. The species was taxonomically identified by Dr. Nadire Pelin Bahadirli. A voucher sample (HPB: 097) was deposited in the HMKU-Medicinal and Aromatic Plants Herbarium of Hatay Mustafa Kemal University. The plant samples were air-dried and ground for further analysis. Hydrodistillation was used to obtain essential oils, and 50 g of dried sample was distilled with a neovenger apparatus for 3 hours. The obtained essential oils were kept at 4°C in dark bottles until further analysis. Ten grams of ground sample was kept in 100 mL of methanol for 6 hours, filtered and evaporated with a rotary evaporator. Supercritical CO_2 (SC-CO_2) extracts were prepared with and without ethanol, and ethanol (10%) was used to obtain polar, semipolar and apolar components. For SC-$CO_{2,\ the}$ temperature was 43°C, the pressure was 200 bar, the flow rate was 1500 g/min, the separator pressure was 0/90 bar, and the total analysis was continued for 2 hours. The pressure ranged from 200 bar until 0, and the extracts were collected separately to compare their compositions at 200–90 bar and 90–0 bar.

GC–MS analysis

Analysis and identification of the compounds in the extracts obtained from the collected plants were carried out with a gas chromatograph coupled with a mass spectrometer. The gas chromatograph was a model 7890A. coupled with a mass spectrometer (model 5975C; Agilent Company, USA). A capillary column (30 m in length) with a stationary phase of 5% methyl phenyl siloxane was used for HP-5 MS. An internal diameter of 0.25 mm was used. and a stationary layer thickness of 0.25 µm was utilized. The column temperature was increased from 50°C to 320°C at a speed of 5°C per minute. The temperature of the injection

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room was 280°C. The carrier gas was helium with a flow velocity of 1.89 mL/min, and the mass spectrometer was operated with an ionization energy of 70 eV. The identification of the compounds was carried out by the study of mass spectra and comparison with the mass spectra of standard compounds. using the information available in the libraries (Wiley7Nist05, Flavor2 and W9N11) (Yabalak et al., 2025). Retention indices (RIs) were calculated relative to a homologous series of n-alkanes (C7-C40) analyzed under identical chromatographic conditions, and calculated RI values were compared with literature retention indices (LRIs) reported for nonpolar columns obtained from NIST WebBook (2026) and Babushok et al. (2011).

Fungal isolates

The isolates of *F. oxysporum*, *R. solani*, *Macrophomina* sp., and *S. sclerotiorum* were obtained from the culture collection of Iğdır University, Faculty of Agriculture, Department of Plant Protection and Phytopathology Laboratory. The fungal cultures were kept and grown on potato dextrose agar (PDA) slants at 25°C for seven days.

Antifungal activity of the extracts

The antifungal effects of the EO, supercritical carbon dioxide (SC-CO_2) extract, methanol extract, and SC-CO_2+ethanol extract obtained from *T. spicata* were assessed *in vitro* using the contact assay method. Extracts at concentrations of 0.25, 0.5, 1.00, 2.00, and 4.00 µl/ml (dissolved in 70% ethanol at a ratio of 1:2) were added to the growth media. PDA was allowed to solidify at 23°C for one hour. Mycelial plugs (0.5 cm in diameter) were excised from the actively growing margins of 7–10-day-old fungal cultures and placed centrally on Petri dishes containing essential oil-amended PDA following the protocol described by Soliman and Badeaa (2002). The control treatments consisted of sterile distilled water, 70% ethanol, and 70% methanol. The Petri dishes were sealed with paraffin to prevent volatilization and incubated at 25°C. Each treatment was replicated three times. Fungal growth was evaluated by measuring the diameter of the colonies in two perpendicular directions using calipers on the 3rd and 5th days post-inoculation. The percentage of mycelial growth inhibition for each concentration was calculated on the 5th day using the following formula.

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Mycelial growth inhibition (%) = [(C − T)/C] × 100, where C is the mean colony diameter in the control group and T is the mean colony diameter in the treatment group.

Computational details

The major compounds identified in the extracts guided the selection of two key bioactive molecules for molecular docking simulations. Interactions between these compounds and sterol 14-alpha demethylase (CYP51B, PDB ID: 6CR2) were investigated using AutoDock Vina (Trott and Olson, 2010). CYP51B was selected because it is a key enzyme in fungal ergosterol biosynthesis and is a well-established molecular target for antifungal agents. The protein structure was chosen because of its high-resolution crystal data, which provide precise active site coordinates suitable for in silico docking studies. Ligands were retrieved from PubChem (Kim et al., 2021), preprocessed, and docked using optimized grid parameters to ensure accurate binding predictions. The docking data, including the binding energy, ligand efficiency, and inhibition constant (Ki), were analyzed with a BIOVIA Discovery Studio Visualizer (BIOVIA, 2021). The crystal structure of 6CR2 was originally described by Friggeri et al. (2018). Protein–ligand interactions were analyzed using PLIP 2021 (Adasme et al., 2021).

The pharmacokinetic, physicochemical, drug likeness, and toxicity parameters of carvacrol and p-cymene were evaluated using SwissADME and ADMET 3.0 (Daina et al., 2017; Gu et al., 2024). The outputs obtained from both platforms were combined into a single comparative table, allowing assessment of the absorption, metabolism, distribution, excretion, and toxicity characteristics of the two major compounds.

Statistical analysis

The results obtained from this study were analyzed using the SPSS program. The effects of the essential oils and concentrations on the colony diameter were tested by analysis of variance, and mycelial growth inhibition caused by the extracts and concentrations was analyzed by ANOVA ($P \leq 0.05$). Differences between applications were evaluated with Duncan's multiple comparison tests.

Data availability statement: The data are available as described in the manuscript.

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Conflict of interest. The author(s) declare that there are no competing interests.

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