



Determination of In-Vitro Antifungal Activities of Essential Oils Against Fungal Pathogens

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ABSTRACT

This study was carried out to determine the in-vitro antifungal activities of essential oils obtained from thyme (*Thymus vulgaris* L.), Turkish pickling herb (*Echinophora tenuifolia* subsp. *sibthorpiana*), clove (*Syzygium aromaticum* L.), cinnamon (*Cinnamomum zeylanicum* Blume), eucalyptus (*Eucalyptus globulus* Labill), dill (*Anethum graveolens* L.), juniper (*Juniperus communis* L.), and ginger (*Zingiber officinale* Rosc.) against *Rhizoctonia solani* and *Fusarium solani* infections. Essential oils were added to autoclaved potato dextrose agar (PDA) medium at 0, 500, 1000 and 2000 ppm doses. Mycelium disks of both fungi with a diameter of 5 mm were transferred to the center of petri dishes and incubated at 24±2 °C for 7 days. The biocontrol efficiencies of the essential oils were calculated by measuring the mycelial development diameters. Essential oil applications showed significant antifungal activity against *R. solani* pathogen and cinnamon, thyme and clove essential oils at 1000 ppm, and dill essential oil at 2000 ppm doses completely inhibited mycelial development. The biocontrol efficiencies of juniper (2.4-12.6%), eucalyptus (2.8-26.6%) and ginger (18.2-37.3%) essential oils against *R. solani* were found to be low. While clove and thyme essential oils completely inhibited *F. solani* mycelial development at 2000 ppm dose, the biocontrol efficiencies of ginger (1.6-3.7%), eucalyptus (1.2-7.4%) and dill (2.9-9.8%) essential oils were low. It was concluded that especially clove, thyme, cinnamon and Turkish pickling herb essential oils showed high in-vitro antifungal activity against both phytopathogens and may have the potential to be used as an alternative to synthetic fungicide active substances.

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Introduction

The use of pesticides is considered the most effective method for disease and pest control; however, pesticides have several disadvantages, such as negative effects on the environment and human health, toxicity to non-target organisms, the development of widespread resistance to various fungal species, and high levels of toxic residues in the product. In addition to the negative effects of pesticides, increasing public awareness and growing demand for safer and healthier agricultural products make it necessary to develop alternative pest control and plant protection methods instead of relying on pesticide use. In today's context, where sustainable farming practices are gaining prominence, research on alternative compounds or active ingredients that can be used instead of synthetic chemicals has gained momentum. One of the most emphasized topics in this field is the synthesis of secondary metabolites by certain plants (such as alkaloids, glycosides, and essential oils). The eco-friendly nature of essential oils

and their components, along with their ability to possess a wide range of different mechanisms of action in plants, offers an alternative approach for their use as biostimulants and antifungal agents. The antifungal activities of many essential oils and active compounds against certain fungal pathogens that are problematic for agricultural production have been demonstrated, particularly through in vitro and in vivo studies (Sivrikaya et al., 2021). Secondary metabolites are organic molecules synthesized by plants, particularly under stress conditions, and they play a crucial role in plant defense mechanisms (Mazid et al., 2011). Monoterpenes and sesquiterpenes are known as volatile compounds in plants and constitute a significant portion of essential oils. It is well known that many derivatives of monoterpenes are important tools in plant defense against phytopathogens (Marei & Abdelgaleil, 2018; Zhang et al., 2018). It is known that sesquiterpenes create a defense mechanism in plants against abiotic stress factors by

scavenging reactive oxygen species (ROS) formed under stress conditions, and that they act as phytoalexins in direct defense mechanisms against diseases and pests, either by functioning like phytoalexins or by stimulating phytoalexin synthesis (Phillips & Croteau, 1999). Another group of volatile compounds, phenylpropanoids, generally possess high antioxidant and antimicrobial activities and play an important role in resistance to biotic and abiotic stress factors (Ramaroson et al., 2022). Essential oils, beyond their medicinal and aromatic uses, offer a "natural" alternative approach in agricultural fields for defense against stress factors because of their antimicrobial, antiviral, antifungal, nematocidal, insecticidal, and antioxidant activities (Dorman & Deans, 2000; Cavanagh, 2007; Ntalli et al., 2010; Lang & Buchbauer, 2012).

In this study, the antifungal activities of *E. tenuifolia* subsp. *sibthorpiana*, *A. graveolens* L., *C. zeylanicum* Blume, *S. aromaticum* L., *Z. officinale* Rosc., *T. vulgaris* L., *E. globulus* Labill, and *J. communis* L. essential oils against stem rot (*R. solani*) and dry rot (*F. solani*) were determined under in vitro conditions, the potential of these essential oils to be used as an alternative to pesticides has been investigated.

Materials and Methods

Origin of Essential Oil

The essential oils of *C. zeylanicum* Blume, *S. aromaticum* L., *Z. officinale* Rosc., *T. vulgaris* L., *E. globulus* Labill and *J. communis* L. used in this study were purchased. The essential oils of these species were selected based on the consideration that they meet the characteristics specified in their standards (GC-MS analysis results). For *E. tenuifolia* subsp. *sibthorpiana* and *A. graveolens* L., the essential oils were obtained by subjecting the plant parts containing active compounds to hydrodistillation for 3 hours using a Clevenger-type distillation apparatus (European Pharmacopoeia, 1980).

GC-FID/GC-MS Analysis

Gas Chromatography/Mass Spectrometry (GC-MS) analysis of the essential oils (50 µL of the oil was solubilized in 5 mL of n-hexane and injected into the split mode 1/100) were performed on Shimadzu 2010 Plus GC-MS equipped with a Quadrapole (QP-5050) detector. The analysis was employed under the following conditions: capillary column, CP-Wax 52 CB (50 m x 0.32 mm, film thickness 0.25 µm); injector and detector heats, 240 °C; stove heat program, from 60 °C (10 min. hold) to 90 °C rising at 4 °C/min., and increasing to 240 °C (11.5 min. hold) rising at 15 °C/min.; flow speed, 1 psi; detector: 70 eV; ionization type, EI; carrier gas, helium (20 ml/min.); sample injected 1 µl. Identification of constituents was carried out with the help of retention times of standard substances by composition of mass spectra with the data given in the Wiley, Nist, Tutor library (Rostad & Pereira, 1986; Adams, 2007). The quantitative analysis was conducted using Gas Chromatography/Flame Ionization Detector (GC-FID), Shimadzu Model Thermo Ultra Trace, operating at the same conditions of GC-MS.

Plant pathogens (*R. solani*, *F. solani*) were obtained from stock cultures at Department of Plant Protection, of Isparta University Applied Sciences. Fungi cultures were developed at 20 mL potato dextrose agar (PDA) on petri dish (90 mm) and kept at 22 ± 2°C for 7 days and these fungi were used for the experiment.

Fungi Cultures

In vitro antifungal activity and fungal growth inhibition of the EOs

Antifungal activity was determined using the agar plate method (Nwosu & Okafor, 1995). PDA (95 mL, w/v) was autoclaved and maintained at 40°C. The effect of essential oils (EOs) at concentrations of 500, 1000, 1500, and 2000 ppm were investigated on growth of the pathogens. The PDA medium treated with essential oils was prepared by adding the appropriate amount of essential oils to the melted medium, followed by the addition of Tween 80 (0.01%) to disperse the essential oils in the medium.

Table 1. Essential oils used in the study and major components

Family	Scientific Name	Common Name	Used Part	Major Components	Rate %
Lamiaceae	<i>T. vulgaris</i>	Thyme	Herba	Thymol	44.11
				Cymol	23.26
				Linalool	6.20
				δ-3-Carene	54.93
Apiaceae	<i>E. tenuifolia</i>	Turkish pickling herb	Herba	Methyleugenol	21.61
				Cymol	9.16
Myrtaceae	<i>S. aromaticum</i>	Clove	Bud	Eugenol	90.02
				trans-Caryophyllene	3.38
Lauraceae	<i>C. zeylanicum</i>	Cinnamon	Bark	Cinnamaldehyde	86.34
				δ-3-Carene	2.79
Myrtaceae	<i>E. globulus</i>	Eucalyptus	Leaf	Eucalyptol	68.19
				α - Pinene	17.70
Apiaceae	<i>A. graveolens</i>	Dill	Fruit	d-Carvone	57.87
				Limonene	33.22
Cupressaceae	<i>J. communis</i>	Juniper	Fruit	α - Pinene	88.47
				β- Myrcene	2.06
				Sesquithujene	28.94
Zingiberaceae	<i>Z. officinale</i>	Ginger	Rhizome	Camphene	10.42
				β- Sesquiphellandrene	10.37

Table 2. Results of analysis of variance for the inhibit growth of *R. solani* and *F. solani* pathogen by essential oils

Sources of variation	Degrees of freedom	R. solani	F. solani
Application	7	850**	1246**
Dose	2	478**	1326**
Application * Dose	14	34.5**	138**
Error	48		
Total	71		
CV(%)		6.52	7.81

Table 3. Growth inhibition (%) of *R. solani* infection by essential oils applied at different doses

Application	Growth inhibition (%)			Mean
	Concentrations (ppm)			
	500 ppm	1000 ppm	2000 ppm	
<i>J. communis</i>	2.4 j	3.3 j	12.6 i	6.1 F
<i>S. aromaticum</i>	55.9 d	100.0 a	100.0 a	85.3 B
<i>A. graveolens</i>	21.7 gh	58.2 d	100.0 a	60.0 C
<i>E. tenuifolia</i>	37.4 e	69.4 c	82.0 b	63.0 C
<i>E. globulus</i>	2.8 j	18.2 hi	26.6 fg	15.8 E
<i>C. zeylanicum</i>	83.2 b	100.0 a	100.0 a	94.4 A
<i>T. vulgaris</i>	82.6 b	100.0 a	100.0 a	94.2 A
<i>Z. officinale</i>	18.2 hi	28.0 f	37.3 e	27.8 D
Mean	38.0 C	59.6 B	69.8 A	

The mycelium discs (10 mm in diameter) from 7-day-old fungal cultures were transferred to 60 mm petri dishes. Following incubation at 28°C for 72 hours, fungal growth was recorded daily (Onaran & Yılar, 2012). The positive control plates (without essential oil) were inoculated following the same procedure. The growth inhibition percentage was calculated using the formula described by Abd-El-Khair & El-Gamal Nadia (2011), as outlined below:

$$\text{Growth inhibition (\%)} = (C-T)/C \times 100$$

C= the diameter of mycelial growth in control plates

T= the diameter of mycelial growth in treated plate

Experimental Design and Data Analysis

The data obtained from the research were subjected to analysis of variance (ANOVA) using a completely randomized design. The significance test for comparing the differences among the means was performed using the Least Significant Difference (LSD) test ($p < 0.05$) with SAS (2009) statistical software.

Results and Discussion

Antifungal Activity

The differences between essential oil applications, doses and their interaction effects on growth inhibition of fungal pathogens were found to be statistically significant ($P < 0.01$) (Table 2).

The application of essential oils significantly affected the inhibition rate of *R. solani* infection. The highest inhibition rates were obtained from *C. zeylanicum* essential oil (94.4%) and *T. vulgaris* essential oil (94.2%), followed by *S. aromaticum* essential oil (85.3%). The lowest activity against *R. solani* was observed with *J. communis* essential oil (6.1%). As the essential oils doses increased, the inhibition rate of the infection also significantly increased. At a dose of 500 ppm, the average inhibition rate was 38.0%, while at 2000 ppm, it increased to 69.8%. The

inhibition rates of the applications varied depending on the dosages, with *C. zeylanicum*, *T. vulgaris*, and *S. aromaticum* essential oils achieving 100% inhibition at a dose of 1000 ppm, while for other essential oils, the inhibition rate was higher at a dose of 2000 ppm (Table 3).

Owing to the significant increase in the importance of fungal diseases, which are difficult to treat, it has become imperative to find new solutions beyond synthetic chemical fungicides. Plant essential oils are promising sources of antifungal compounds. Several studies on plant pathogenic fungi have demonstrated that some essential oils possess antifungal properties that inhibit fungal growth (Zaidi & Crow 2005). Essential oils exhibit antifungal activity in various ways, including the inhibition of biofilm formation in fungal pathogens, disruption of the environmental sensing system, effects on cell development and morphology, inhibition of fungal mycelial growth, disruption of the cell membrane/wall, and inhibition of ergosterol and fumonisin biosynthesis (Nazzaro et al., 2017). Many researchers have reported that various essential oils inhibit some fungal pathogens that cause economic damage in potatoes under in vitro conditions (Prabuseenivasan et al., 2006; Seema & Devaki, 2010; Galvão et al., 2012). Studies have shown that *Syzygium aromaticum*, *Salvia triloba*, *Thymus vulgaris*, *Laurus nobilis*, *Cuminum cyminum* L., *Juniperus communis* L., *Eucalyptus* sp., *Allium sativum*, and *Echinophora tenuifolia* species exhibit antifungal activity against various phytopathogens (Çakar et al., 2021), and their appropriate doses can be used as fungicides (Boyraz & Koçak, 2006; Arıcı et al., 2013; Erdoğan et al., 2014; Sharma et al., 2017; Er, 2018; Şanlı and Ok, 2023). Some researchers have also shown that the essential oils of cinnamon (*Cinnamomum verum*), thyme (*Thymus vulgaris*), sage (*Salvia fruticosa*), peppermint (*Mentha piperita*), clove (*Syzygium aromaticum*), and lemongrass (*Cymbopogon citratus*) exhibit strong antifungal activity against *R. solani* (Zambonelli et al., 2004; Vaillant et al., 2009; Amini et al., 2012; Khaledi et al., 2014).

Table 4. Inhibit growth (%) of *F. solani* infection by essential oils applied at different doses

Application	Growth inhibition (%)			Mean
	Concentrations (ppm)			
	500	1000	2000	
<i>J. communis</i>	7.8 ij	20.6 g	59.3 f	29.2 D
<i>S. aromaticum</i>	63.6 e	81.7 b	100.0 a	81.8 A
<i>A. graveolens</i>	2.9 k	7.8 ij	9.8 hi	6.8 F
<i>E. tenuifolia</i>	18.5 g	56.2 f	72.0 d	48.9 C
<i>E. globulus</i>	1.2 k	3.7 jk	7.4 ij	4.1 G
<i>C. zeylanicum</i>	1.2 k	3.7 jk	58.0 f	21.0 E
<i>T. vulgaris</i>	13.6 h	77.4 c	100.0 a	63.7 B
<i>Z. officinale</i>	1.6 k	2.9 k	3.7 jk	2.7 G
Mean	13.8 C	31.8 B	51.3 A	

Essential oil applications significantly affected the growth inhibition of *F. solani*. The highest average growth inhibition was obtained with *S. aromaticum* essential oil (81.8%), followed by *T. vulgaris* oil (63.7%) and *E. tenuifolia* oil (48.9%). As the concentration of essential oil increased, the growth inhibition also significantly increased. The mean inhibition growth rate, which was 13.8% at 500 ppm, rose to 51.3% at 2000 ppm. The inhibition of pathogen varied depending on the doses of the essential oils. For *J. communis*, *S. aromaticum*, *E. tenuifolia*, and *T. vulgaris* essential oils, the growth inhibition significantly increased with the application doses. However, for *A. graveolens* at 1000 and 2000 ppm, *E. globulus* and *C. zeylanicum* at 500 and 1000 ppm, and *Z. officinale* essential oil across the 500, 1000, and 2000 ppm doses, no significant differences were observed (Table 4).

The antimicrobial activities of essential oils are attributed to their ability to contain terpenes/terpenoids, due to their highly lipophilic nature and low molecular weight, disruption of cell membranes, induction of cell death, or inhibition of germination and sporulation of fungal spores (Taweechaisupapong et al., 2012; Nazzaro et al., 2017). The antifungal mechanisms of essential oils and their components are explained by the loss of fungal mitochondrial function, disruption of the cell membrane, alteration and inhibition of cell wall formation, inhibition of efflux pumps, and the production of Reactive Oxygen Species (ROS). In the in-vitro studies, *C. zeylanicum*, *T. vulgaris*, and *S. aromaticum* essential oils applied at 1000 ppm completely inhibited the growth of *R. solani*, whereas *E. tenuifolia* essential oil showed 82.0% inhibition at a concentration of 2000 ppm. *T. vulgaris* and *S. aromaticum* essential oils completely inhibited *F. solani* development at a concentration of 2000 ppm, whereas *C. zeylanicum* and *J. communis* essential oils exhibited a moderate inhibitory effects. Similarly, Şanlı and Ok (2023) reported that the essential oil of *E. tenuifolia* was effective in inhibiting or controlling *Fusarium oxysporum*, *Rhizoctonia solani*, and *Alternaria alternata*.

Conclusion

In vitro applications of essential oils against *F. solani* and *R. solani* infections, which are among the major diseases of potato plants, have showned varying degrees of effectiveness. In the current study, 1000 ppm concentration of *S. aromaticum*, *C. zeylanicum*, and *T. vulgaris* essential

oils, and the 2000 ppm dose of Turkish pickling herb essential oil, exhibited the highest antifungal activity against *R. solani*. For *F. solani*, *S. aromaticum* and *T. vulgaris* essential oils at a concentration of 2000 ppm were the most effective, completely inhibiting mycelial growth in both pathogens. It has been suggested that the main components of these oils have disease-reducing effects. In conclusion, it was been determined that to fully understand the mechanisms of action of essential oils, the main components of these oils and the most effective formulations based on these components should be re-examined.

Declarations

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