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Article**Chemical Composition and Antifungal Activities of the Essential Oils of *Thymus* Species (*Thymus pectinatus*, *Thymus convolutus*, *Thymus vulgaris*) Against Plant Pathogens****Hüseyin Aksit¹, Yusuf Bayar^{2*}, Samed Simsek³ and Yakup Ulutas⁴**¹ Erzincan Binali Yıldırım University, Faculty of Pharmacy, Department of Analytical Chemistry, 24100, Erzincan, Turkey² Kırşehir Ahi Evran University, Faculty of Agriculture, Department of Plant Protection, Kırşehir, 40100, Turkey³ Erzincan Binali Yıldırım University, Çayırılı Vocational School, Medical Services and Techniques Dept. 24500, Erzincan, Turkey⁴ Erzincan Binali Yıldırım University, Science and Art Faculty, Department of Chemistry, 24100 Erzincan, Turkey* Corresponding Author: yusuf.bayar@ahievrn.edu.tr (Yusuf Bayar)

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Abstract: The use of herbal extracts can be considered as an alternative method to minimize the effects of synthetic chemical pesticide used to control plant pathogens on human and environmental health. The essential oils (EOs) obtained from *Thymus pectinatus* (TP), *Thymus convolutus* (TC), and *Thymus vulgaris* (TV) by hydrodistillation were analyzed by GC/MS. The main components of TP, TC, and TV were thymol (52.2%), *p*-cymene (17.03%), eucalyptol (24.47%), camphor (16.84%), α -pinene (12.88%), and carvacrol (72.47%). The antifungal activity of EOs against plant pathogens including *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL), *Phytophthora infestans*, and *Rhizoctonia solani* was evaluated by using the agar dilution method. The EOs of TV and TP inhibited mycelium growth 100 % in FORL *P. infestans*, and *R. solani* at 4 μ L/petri dose. The EOs of TC did not affect mycelial growth of *R. solani* and *R. infestans* while the mycelial growth of FORL was inhibited by 36.11%. Our findings suggested the use of these essential oils can prevent fungal infections on plants as alternatives to synthetic ones.

Keywords: Antifungal activity, Essential oils, GC/MS analysis, *Thymus* spp.**Introduction**

Lamiaceae family is represented by 250 genus and 7133 species spread over all the world, especially in the Mediterranean region^{1,2}. This family includes herbs, shrubs, and trees that are well known for economic value. The *Lamiaceae* species including *Thymus*, *Salvia*, *Mentha*, *Origanum*, *Rosmarinus*, and *Satureja* were used for spice, flavoring agents, and folk medicine^{2,3}. Most of the family members are

important for the pharmacology, perfumery, and cosmetics industries because they contain essential oils. *Thymus* genus is also among the essential oil-containing plants and has many species, subspecies, and taxa. *Thymus* is one of the 8 largest genera of the *Lamiaceae* family in terms of the number of species⁴. *Thymus* species are distributed in Asia and Europe, especially in the Mediterranean region, and in North Africa⁵. *Thymus* species reported having some biological

effects such as antibacterial ⁶, antioxidant ⁷, and antifungal ⁸ activities.

Fungal diseases cause an average of 14% yield loss each year all over the world in vegetable production ^{8,9}. *FORL*, *Phytophthora infestans*, and *Rhizoctonia solani* are the plant pathogens that cause significant losses in Turkey as well as the world. Root rot and wilt disease caused by *Fusarium oxysporum* f. sp. *radicis-Iycopersici* is a fungal disease that is seen in the roots of tomato plants and the stem parts that close to the soil ¹⁰. The causative agent of the disease called soft rot in roots and tubers of potatoes, which causes the significant yield losses in potatoes by *R. solani* ¹¹. *Phytophthora infestans* appear as small, pale green or yellowish spots on potato leaves, and as the disease progresses, these spots turn brown and the middle parts lose their vitality. However, serious problems have emerged as a result of chemical control against plant diseases for many years. Studies to find alternative methods, especially in developed countries, have gained momentum. As a result of the use of intensive pesticides, the natural balance has been destroyed and it has become threatening to the environment and human health ¹². For this reason, it is a necessity to research and apply alternative methods to chemical control to sustain agriculture. One of these alternative methods is the determination of plant origin compounds and their use in the fight against plant diseases, pests, and weeds. In the studies, there are many results on the fungicidal effects of the compounds and EOs bearing plants and their biological activities ¹³⁻¹⁶.

In this study, chemical components of the essential oils of *T. pectinatus*, *T. convolutus*, and *T. vulgaris* and their antifungal activities against three plant pathogens were evaluated.

Material and method

Plant material and isolation of essential oils

T. pectinatus, *T. convolutus*, and *T. vulgaris* samples were collected each plant materials approximately 2 kg of fresh weight from Erzincan in 2018 and were authenticated by Prof. Dr. Ali Kandemir, Department of Biology, Faculty of Science and Art, Erzincan Binali Yıldırım University. The essential oils were obtained by hydrodistillation

using the Neo-Clevenger apparatus. To extraction of volatile compounds, the plant materials were weighed (100 g) then added deionized water (300 mL) and the distillation process was continued for 2 hours. The EOs were separated and dried with anhydrous sodium sulfate and stored in dark bottles at +4°C until used and analyzed.

GC/MS analysis

For the GC/MS analysis, a 1.33% w/v solution of the sample in acetone was prepared and 1 µL was injected with a split mode (60:1). Gas chromatographic (GC) analyses were performed using a Thermo Scientific Trace 1310 GC/MS system, equipped with a DB-5MS capillary column (30 m x 0.25 mm ID and 0.25 µm) according to our previous report ¹⁷. Helium (1.2 mL/min) was used as a carrier gas. The injection site temperature was set at 280°C. The column oven temperature was programmed as follows: the initial column oven temperature was 60°C, hold for 3 min, ramp to 200°C at a rate of 3°C/min and held for 0 min and, ramp to 240°C at a rate of 5°C/min and hold for 5 min. The total run time was 60 min. The mass spectrometer conditions were as follows: transfer line and ion source temperature were 280°C and the ionization energy was at 70 eV. The percentages of the essential oil components were calculated using peak areas without any correction factors. The retention indexes were calculated for all the components using the Van den Dool and Kratz equation based on homolog n-alkane series (C8-C20) retention times. The components were identified by comparing their mass spectral fragmentation patterns with NIST and Wiley mass spectral database and confirmed by comparison with RI's with previous reports on DB-5 column ¹⁸⁻²².

Fungi Culture

Plant pathogenic fungi were obtained from stock cultures in the Phytoclinical Laboratory, Kırşehir Ahievran University, Faculty of Agriculture, Department of Plant Protection. Fungus cultures were developed for 7 days at 25±2°C in 90 mm petri dishes containing 20 ml potato dextrose agar (PDA). These stock cultures were used (Table 1).

Table 1. List of plant pathogens

Plant pathogens	Origin
<i>Fusarium oxysporum</i> f.sp. <i>radicis-lycopersici</i> (FORL)	Tomato
<i>Phytophthora infestans</i>	Potato
<i>Rhizoctonia solani</i>	Potato

***In vitro* antifungal activity of Thymus essential oils**

The prepared PDAs were autoclaved and cooled to 40°C and transferred to 60 mm diameter petri dishes (to be 10 ml). A 5 mm of diameter sterile filter paper is affixed to the lids of the PDA transferred petri dishes. Mycelium from 7-days fungus cultures was transferred to petri dishes. Essential oils were dropped with a micropipette at a dose of 0 (Control), 0.5, 1, 2, and 4 µl/petri on filter papers adhered to the lids of the petri dishes. Fungus cultures were incubated for 7 days at 25±2°C. After inoculation, fungal development was recorded at the end of the period. Development inhibition was calculated according to the formula given below compared to the development in control ²².

$$I=100 \times (DC-DT)/DC$$

where, I: Percentage blocking compared to control (Mycelium development), DC: Mycelium development in control, DT: Mycelium development in EOs applications.

Statistical analysis

Data are expressed as means ± SEM of four independent experiments performed in triplicate. Statistical analysis was carried out by using SPSS Ver.15.0 software. Differences between the means were determined by the Duncan test and were considered statistically significant at $p < 0.05$ level.

Results and Discussion

The composition of Thymus sp. essential oils and their antifungal effects

The essential oil yields of TP, TV, and TC were 4.1%, 4.8%, 5.6%, respectively. Table 2 lists the chemical composition of essential oils of the three samples of Thyme species. The essential oils were characterized by a very high content

of oxygenated monoterpene hydrocarbons (58-65%) and monoterpene hydrocarbons (12-32%). Twenty-six constituents were present in the EOs of *T. pectinatus*, one of Turkey's endemic species, and is widespread over Sivas and Erzincan, mainly having the major components as 52.82% carvacrol, 17.03% *p*-cymene, and 8.87% γ -terpinene (Table 2). From the essential oil of *T. vulgaris*, 27 components were identified, 96.12% of the total amount. Carvacrol (72.47%), γ -terpinene (4.61%), and caryophyllene (4.21%) were the dominant components. From the essential oil of *T. convolutus* total of 21 components were identified, 95.37% of the total amount. Eucalyptol (24.47%), camphor (16.84%), α -pinene (12.88%) and camphene (9.6%) were the main components. *T. convolutus* was found poor in both thymol (<0.05%) and carvacrol (1.64%). These findings agree with those of previous reports ^{23,24}.

Phytochemical analysis of the essential oils showed that most of the *Thymus* genus contain phenolic compounds such as thymol and carvacrol predominantly. In contrast, it was reported the *T. convolutus* essential oil consists of *E*- β -ocimene (8.9%), camphor (16.6%), and eucalyptol (7.20%) ²⁴ as the major component. Our results support the previous report with the qualitative differences in the subclassing of the components. These differences can be explained by geographical, climatic, and collection stages of plant materials ²⁵. Previous reports showed that the *T. pectinatus* essential oil contains mainly thymol (35.0-49.8%), *p*-cymene (5.19-14.8%), γ -terpinene (3.54-16.1%), and borneol (2.7-17.7%) ^{26,27}, supporting our results. The composition of these main components is varied during maturation stages by from 1% to 12% ²⁶. Among the studied essential oil *T. vulgaris* was found to be the richest in carvacrol content.

Borugã *et al.* reported the main components of essential oil of *T. vulgaris* as *p*-cymene (8.41%), γ -terpinene (30.90%), and thymol (47.59%) ²⁸. Asllani and Toska reported that the 86 compounds were detected in the essential oils of *T. vulgaris*, from which more than 40 could be identified, and also, main components identified were: *p*-cymene: (7.76-43.75%), γ -terpinene (4.20-

Table 2. Chemical compositions of TP, TC, and TV essential oils

Compound number	RT*	RI**	RI***	Compound name	TP****	TC	TV
1	5.24	926	928	2-Carene	tr	0.36	tr
2	5.28	927	929	α -Phellandrene	0.1	0.15	tr
3	5.46	935	939	α -Pinene	0.3	12.88	0.29
4	5.86	951	954	Camphene	0.22	9.6	tr
5	6.60	977	975	Sabinene	tr	1.52	tr
6	6.65	979	979	β -Pinene	0.4	1.57	0.69
7	7.01	991	991	β -Myrcene	0.75	0.29	0.53
8	7.24	998	997	3-Octanol	0.11	1.3	0.14
9	7.85	1018	1017	α -Terpinene	1.15	0.58	0.58
10	8.14	1027	1025	<i>p</i> -Cymene	17.03	6.59	2.87
11	8.26	1031	1031	Limonene	0.45	tr	0.31
12	8.36	1034	1032	β -Phellandrene	2.89	0.71	2.36
13	8.45	1037	1038	Eucalyptol	nd	24.47	tr
14	9.32	1062	1060	γ -Terpinene	8.87	1.45	4.61
15	9.63	1070	1068	4-Thujanol	0.21	1.15	tr
16	10.90	1115	1116	Limonene oxide	nd	1.26	0.29
17	11.69	1124	1119	<i>cis</i> -sabinene hydrate	0.14	0.24	0.22
18	12.62	1149	1143	Camphor	0.24	16.84	0.18
19	13.44	1168	1167	Borneol	2.24	3.69	2.14
20	13.92	1180	1183	4-Terpineol	1.59	5.29	0.14
21	14.24	1187	1189	<i>p</i> -Cymen-8-ol	0.33	nd	0.11
22	14.48	1192	1189	α -Terpineol	0.5	tr	0.44
23	14.74	1198	1195	Bornyl acetate	0.14	tr	0.37
24	18.54	1242	1240	Carvone	tr	2.49	0.17
25	18.89	1297	1290	Thymol	52.82	tr	1.47
26	19.17	1304	1299	Carvacrol	5.96	1.64	72.47
27	19.37	1309	1305	Isopinocarveol	tr	0.28	0.11
28	21.28	1357	1355	<i>m</i> -Cymen-4-ol	0.87	nd	0.22
29	23.89	1420	1419	Caryophyllene	0.52	nd	4.21
30	27.47	1509	1505	β -Bisabolene	0.27	tr	1.08
31	28.03	1525	1523	β -Cadinene	0.16	1.02	tr
32	30.27	1583	1583	Caryophyllene oxide	0.33	tr	0.12
Total identified components (%)					98.59	95.37	96.12
Total unidentified components (%)					1.41	4.63	3.88
Monoterpen hydrocarbons (No: 1-7, 9-12, 14)					32.16	35.7	12.24
Oxygenated monoterpenes (No: 8, 13, 15-28)					65.15	58.65	78.47
Sesquiterpene hydrocarbons (No: 29, 30, 31)					0.95	1.02	5.29
Oxygenated sesquiterpenes (No: 32)					0.33	<0.05	0.12

*Retention time (RT), **Retention index (RI), ***RI literature on DB-5MS column, *****T. pectinatus* (TP), *T. convolutus* (TC), *T. vulgaris* (TV)

27.62%), thymol (21.38-60.15%), carvacrol (1.15-3.04%) and β -caryophyllene (1.30-3.07%)²⁹. When compared with the literature, it is seen that although the main components of the

essential oils tested are parallel to our findings, there are some differences in terms of their proportions. In various studies, it was reported that the genotype, chemotype, geographic origin, environmental and soil conditions, and the time of collection of the plants are effective on the chemical composition and amounts of essential oils^{30,31}.

T. vulgaris, *T. pectinatus* var *pectinatus*, and *T. convolutus* plant essential oil *in vitro* fumigant activity against important plant pathogens *R.*

solani, *Phytophthora infestans*, and *FORL* was investigated. The results of our study are given in Table 3, Table 4, Table 5, and Table 6. According to the current results, *T. vulgaris* essential oil showed a high level of phytotoxic effect mycelium development on *R. solani*, *P. infestans*, and *FORL*. At 0.5 µl/Petri dose, *R. solani*, *P. infestans*, and *FORL* pathogens were inhibited at 51.15%, 57.66%, and 85.82% level, respectively, while 4 µl/Petri dose completely inhibited mycelium growth.

Table 3. Percent fumigant effect of thyme essential oils on *R. solani* mycelium growth

Doses (µl air ⁻¹)	Control	0.5	1	2	4
<i>T. vulgaris</i>	00.00±0.00d	51.15±7.17c	93.47±6.52b	100±0.00a	100±0.00a
<i>T. pectinatus</i>	00.00±0.00c	85.46±0.83b	100±0.00a	100±0.00a	100±0.00a
<i>T. convolutus</i>	00.00±0.00a	60.00±0.00a	60.00±0.00a	60.00±0.00a	60.00±0.00a

Table 4. Percent fumigant effect of thyme essential oils on *P. infestans* mycelium growth

Doses (µl/petri)	Control	0.5	1	2	4
<i>T. vulgaris</i>	00.00±0.00c	57.66±13.03b	100±6.52a	100±0.00a	100±0.00a
<i>T. pectinatus</i>	00.00±0.00d	58.32±07.41c	86.92±2.96b	100±0.00a	100±0.00a
<i>T. convolutus</i>	00.00±0.00a	60.00±00.00a	60.00±0.00a	60.00±0.00a	60.00±0.00a

Table 5. Percent fumigant effect of thyme essential oils on *FORL* mycelium growth

Doses (µl/Petri)	Control	0.5	1	2	4
<i>T. vulgaris</i>	00.00±0.00c	85.82±0.32b	100±0.00a	100±0.00a	100±0.00a
<i>T. pectinatus</i>	00.00±0.00c	86.72±0.38b	100±0.00a	100±0.00a	100±0.00a
<i>T. convolutus</i>	00.00±0.00c	00.00±0.00c	13.61±0.71b	27.54±3.32a	36.11±6.87a

Table 6. Dose-effect results of *Thymus* essential oils

Plants	LD Values	Test microorganisms		
		<i>R. solani</i>	<i>P. infestans</i>	<i>FORL</i>
<i>T. vulgaris</i>	LC ₅₀ (µL/air)	0.459	0.467	0.339
	Slope	4.95±0.70	7.097±1.40	6.33±2.83
	Heterojenite	0.35	0.961	0.49
<i>T. pectinatus</i>	LC ₅₀ (µL/air)	0.346	0.452	0.331
	Slope	6.43±2.79	3.63±0.536	6.22±2.87
	Heterojenite	0.154	1.81	0.145
<i>T. convolutus</i>	LC ₅₀ (µL/air)	-	-	5.32
	Slope	-	-	1.757±0.263
	Heterogeneity	-	-	8.127

The effect of essential oil of *T. pectinatus* on mycelium growth of pathogens increases depending on the dose was determined. 0.5 µl/Petri dose prevented the mycelium growth in the plant pathogens *P. infestans*, *R. solani*, and *FORL* about 58.32%, 85.46%, and 86.72%, respectively. At 1 µl/Petri dose, inhibited in the plant pathogens *P. infestans*, *R. solani*, and *FORL* about 86.92%, 100%, and 100%, respectively. It completely inhibited the mycelium growth of all pathogens at 2 and 4 µl/Petri doses.

T. convolutus essential oil exhibited a weak fumigant activity on plant pathogens. It was observed that the essential oil of *T. convolutus* did not affect the mycelium development of *P. infestans* and *R. solani*. However, it was determined that *FORL* had a weak effect on mycelium development. The determined pathogen most sensitive to the essential oil of *T. convolutus* was *FORL*.

In the dose-effect study, LC₅₀ values of plant essential oils on pathogens were calculated (Table 2). The current LC₅₀ values of *T. vulgaris* essential oil on *R. solani*, *P. infestans* and *FORL* were calculated as 4.95 µL, 0.467 µL and 0.339 µL/air, respectively. For *T. pectinatus* var *pectinatus* LC₅₀ values on *R. solani*, *P. infestans* and *FORL* were 0.346 µL, 0.452 µL, and 0.331 µL, respectively. However, for *T. convolutus*, the LC₅₀ values for *R. solani*, and *Phytophthora infestans* could not be determined because the essential oil did not affect the mycelium growth of these fungi. However, the LC₅₀ value for *FORL* was calculated as 5.32 µL.

In terms of a lethal dose, it was determined that when three thyme species were compared, the LC₅₀ values of *T. vulgaris* and *T. pectinatus* essential oils were almost the same, while the LC₅₀ values of *T. convolutus* essential oils were higher.

Mycelium growth data showed that 3 thyme species used had a phytotoxic effect on the mycelium growth of plant pathogens. *T. vulgaris* and *T. pectinatus* essential oils are highly effective on the mycelium growth of plant pathogens. However, *T. convolutus*'s essential oil had a limited effect on the mycelium development of pathogens. The low activity of *T. convolutus* might be due to the absence of antimicrobial compounds such

as thymol and carvacrol. *T. vulgaris*'s essential oil contained 72% carvacrol, and *T. pectinatus*'s essential oil included 52.82% thymol. Comparing the previous data with the chemical composition of the essential oils, it becomes more evident that there was a relationship between the high activity of the *T. vulgaris* and *T. pectinatus* essential oils and the presence of phenol components, such as thymol and carvacrol. The high antifungal activity of these essential oils could be explained by the high percentage of phenol components³². Zambonelli *et al.* reported that thymol and carvacrol interfere with the cytoplasmic content of the fungus, causing the fungus hyphae to deteriorate. It has been reported that 7 commercially purchased *T. vulgaris* essential oils show antifungal activity on *Fusarium solani*, *Rhizoctonia solani*, and *Colletotrichum lindemuthianum*, and an increase in activity due to the increase in thymol content³³. Tullio *et al.* determined the antifungal effect of *T. vulgaris* essential oil against *Aspergillus niger*, *A. flavus*, *A. flavus* var *columnaris*, *A. fumigatus*, *Penicillium lanosum*, *P. frequentans*, *Alternaria alternata*, *Cladosporium cladosporioides*, *Mucor* spp and *Rhizopus* spp kary³⁴. Soto Mendivil *et al.* reported that a 1000 ppm dose of *T. vulgaris* essential oil prevented the mycelial growth of *Alternaria citri*³⁵.

Conclusions

In the current study, the chemical components and antifungal activities of the essential oils of three thyme species were determined *in vitro* conditions.

The main compounds of the essential oils of *T. pectinatus*, *T. vulgaris* and *T. convolutus* were thymol (52.82%), *p*-cymene (17.03%), γ -terpinene (8.87%); carvacrol (72.47%), γ -terpinene (4.61%), *p*-cymene (4.21%); (24.47%), camphor (16.84%), α -pinene (12.88%) and camphene (9.6%), respectively.

To conclude, the essential oils of *T. vulgaris* and *T. pectinatus* had higher level fumigant effects on the mycelial development of *R. solani*, *P. infestans* and *FORL*. These oils have the potential to control this disease. However, to determine this potential, more studies on this subject in field conditions will make the validity of the results more meaningful.

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