

Acaricidal Activity of *Thymus vulgaris* Oil and Its Main Components against *Tyrophagus putrescentiae*, a Stored Food Mite

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ABSTRACT

The acaricidal activities of compounds derived from *Thymus vulgaris* (thyme) oil against *Tyrophagus putrescentiae* were assessed using an impregnated fabric disk bioassay, and were compared with those of the synthetic acaricides, benzyl benzoate and *N,N*-diethyl-*m*-toluamide. The observed responses differed according to dosage and chemical components. The 50% lethal dose (LD₅₀) value of the *T. vulgaris* oil against *T. putrescentiae* was 10.2 µg/cm². Biologically active constituents derived from *T. vulgaris* oil were purified by using silica gel chromatography and high-performance liquid chromatography. The structures of acaricidal components were analyzed by gas chromatography–mass spectrometry, ¹H nuclear magnetic resonance (NMR), ¹³C NMR, ¹H-¹³C COSY-NMR, and DEPT-NMR spectra, and were subsequently identified as carvacrol and thymol. Carvacrol was the most toxic compound with LD₅₀ values (4.5 µg/cm²) significantly different from thymol (11.1 µg/cm²), benzyl benzoate (11.3 µg/cm²), and *N,N*-diethyl-*m*-toluamide (13.9 µg/cm²). Linalool was as toxic as was *N,N*-diethyl-*m*-toluamide. The lower LD₅₀ of carvacrol indicates that it may be the major contributor of the toxicity of *T. vulgaris* oil against the stored food mite, although it only constitutes 14.2% of the oil. From this point of view, carvacrol and thymol can be very useful as potential control agents against stored food mite.

Tyrophagus putrescentiae is one of the most important stored food mites among more than 60 species of mites that contaminate stored foods (10). *T. putrescentiae* causes serious economic losses (28), and reduces nutrient content and germination abilities (13). Additionally, stored food mites were responsible for allergic diseases among farmers and workers who handled heavily infested stored products, causing acute enteritis (10) and systemic anaphylaxis (17) upon the ingestion of contaminated food. Control of this mite depends on chemical methods such as fumigation, spraying with organophosphorus compounds, or treatment with commercial benzyl benzoate and *N,N*-diethyl-*m*-toluamide (2, 5, 7, 12). However, the repeated use of these chemicals has resulted in the widespread development of resistance, has undesirable effects on nontarget organisms, and fosters environmental and human health concerns (9, 12, 25). These problems have highlighted the need for the development of new strategies for selective control of stored food mites.

The plant kingdom is a good source of natural preparations containing effective bioactive compounds that can be used for different applications—in this context, for their acaricidal activities against *T. putrescentiae* in stored foods (11). The *Thymus* genus, as well as many other aromatic plants, biosynthesize several volatile compounds, termed essential oils (11). Therefore, chemical classification of such plants is based on the main essential oil components

(16). *Thymus vulgaris* also possesses various beneficial effects, e.g., antiseptic, carminative, antimicrobial, and antioxidative properties (3, 15). However, relatively little work has been done with respect to the management of stored food mites, although extracts and essential oils of *T. vulgaris* are insecticidal agents. Therefore, a study of the *T. vulgaris* extract and isolated compounds was performed to determine the mite control activities of agents against *T. putrescentiae*, a stored food mite.

MATERIALS AND METHODS

Chemicals. Benzyl benzoate, camphene, *N,N*-diethyl-*m*-toluamide, linalool, and α-pinene were purchased from Sigma-Aldrich (St. Louis, Mo.). D-Limonene, γ-terpinene, and *trans*-caryophyllene were supplied by Sigma-Aldrich. All other chemicals were of reagent grade. The chloroform used for the high-performance liquid chromatography (HPLC) mobile phase was of HPLC grade, and those used for other purposes were of analytical grade.

Isolation and identification. The leaves of *T. vulgaris* (5 kg) were purchased from a local market in Chonju and identified by Prof. Sang-Hyun Lee (Forestry Department, Chonbuk National University, South Korea). The samples were washed twice with 1 liter of distilled water, dried in an oven at 40°C for 2 days, and then ground into a fine powder. The *T. vulgaris* oil was extracted by steam distillation as described previously (4).

The oil (10 g) was fractionated on a silica gel column (Merck 70-230 mesh; 600 g; inside diameter [i.d.], 10 by 50 cm) and successively eluted with a gradient step of hexane–ethyl acetate (8:1, 3:1, and 0:1, vol/vol), giving the four fractions (H1 to H4). The active H3 (7.5 g) fraction was rechromatographed on a silica

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gel column and successively eluted with hexane–ethyl acetate (8:1, vol/vol). The column fractions were analyzed by thin-layer chromatography (TLC) and fractions with similar TLC patterns were pooled. In this step, the four fractions (H31 to H34) were obtained and bioassayed at 80 $\mu\text{g}/\text{cm}^2$. The active H33 fraction (4.95 g) was purified by HPLC (Recycling Preparative HPLC, Japan Analytical Industry Co., Ltd., Tokyo, Japan) for separation of the biologically active constituents. The first column was a Jaigel GS Series Column (GS310 50 cm plus GS310 30 cm, 21.5-mm i.d., 800 mm long; Japan Analytical Industry Co., Ltd.), and chloroform (100%, vol/vol) was utilized at a flow rate of 5 ml/min and detection at 289 nm. In this step, the three fractions (H331 to H333) were obtained and bioassayed at 80 $\mu\text{g}/\text{cm}^2$. The active H333 fraction (3.5 g) was further chromatographed on a Jaigel W Series Column (W-253 50 cm plus W-252 50 cm, 20.0-mm i.d., 1,000 mm long; Japan Analytical Industry Co., Ltd.) under the same conditions. Finally, the potent active principles (H3311 and H3312, 1.92 g and 0.44 g, respectively) were isolated. The structures of the active isolates were determined by instrumental analysis. ^1H and ^{13}C NMR spectra were recorded in deuteriochloroform with a JNM-LA 400F7 spectrometer at 600 and 150 MHz (TMS as an internal standard), respectively, and chemical shifts (δ) are given in parts per million. Unambiguous ^1H and ^{13}C NMR chemical shifts were obtained using a ^1H – ^{13}H DEPT spectrum as well as a ^1H – ^{13}H COSY spectrum. UV spectra were obtained in chloroform with a Jasco V-550 spectrometer.

GC-MS. *T. vulgaris* oil was analyzed on a gas chromatograph (6890, Agilent)–mass spectrometer (5973IV, Agilent) (GC-MS). A DB-5 (0.25-mm film) fused silica capillary column (i.d., 30 m by 0.25 mm; J&W Scientific, Folsom, Calif.) was used as the GC column. The GC conditions were as follows: injector temperature, 210°C; column temperature, isothermal at 50°C for 15 min, then programmed to rise to 200°C at 2°C/min and held at this temperature for 15 min; and ion source temperature, 230°C. Helium was used as the carrier gas at a rate of 0.8 ml/min. The effluent of the GC column was introduced directly into the source of the mass spectrometer. Spectra were obtained in the electron ionization (EI) mode with 70-eV ionization energy. The sector mass analyzer was set to scan from 50 to 600 amu for 2 s. Compounds were identified by comparison with retention times and the mass spectra obtained with the authentic standards on the GC-MS system used for analysis. When an authentic sample was not available, identification for caryophyllene oxide was carried out by comparison of mass spectra obtained experimentally with those in the mass spectra library (The Wiley Registry of Mass Spectral Data, 6th ed.).

Stored food mite. Cultures of *T. putrescentiae* were maintained without exposure to any known acaricides. They were reared in plastic containers (15 by 12 by 6 cm) containing 30 g of sterilized diet (fry feed no. 1 and dried yeast, 1:1 by weight) at 25 $\pm$ 1°C and 75% relative humidity in the dark. The fry feed (Miropa) was purchased from Korea Special Feed Meal, Chonju, South Korea.

Bioassay. An impregnated fabric disk bioassay was used to assess the acaricidal activities of test samples. Various amounts (2.5, 5.0, 10, 20, 40, 60, and 80 $\mu\text{g}/\text{cm}^2$) of each test material dissolved in 100 μl of acetone were applied to disks of black cotton fabric (0.5 g, 5-cm diameter: 700 mesh). Control fabric disks received only 20 μl of acetone. After the disks of black cotton fabric were dried in a fume hood (19°C) for 30 s, each piece was placed in the bottom of a petri dish (5-cm diameter by 1.2 cm). Then 30 individuals of *T. putrescentiae* (7 to 10 days

TABLE 1. *Acaricidal activity of constituents derived from T. vulgaris oil and synthetic acaricides against T. putrescentiae, using the impregnated fabric disk bioassay method^a*

Compound	LD ₅₀ ($\mu\text{g}/\text{cm}^2$)	95% confidence limit	RT ^b
Oil	10.2	9.70–11.09	0.7
α -Pinene	— ^c	—	—
Camphene	—	—	—
D-Limonene	—	—	—
γ -Terpinene	—	—	—
Linalool	14.6	14.17–14.92	0.8
Thymol	11.1	10.89–11.23	1
Carvacrol	4.5	4.10–5.03	2.5
<i>trans</i> -Caryophyllene	—	—	—
Benzyl benzoate	11.3	11.04–12.02	1
<i>N,N</i> -Diethyl- <i>m</i> -toluamide	13.9	13.12–14.73	0.8

^a Exposed for 24 h.

^b RT, relative toxicity; equals the LD₅₀ value of benzyl benzoate/LD₅₀ value of each chemical.

^c —, no activity.

old) were placed in each petri dish and covered with a lid. Treated and control mites were held at 25 $\pm$ 1°C and 75% relative humidity in the dark. Mortalities were determined 24 h after treatment under a binocular microscope ($\times 20$). Mites were considered dead if appendages did not move when prodded with a pin. All treatments were replicated three times. Fifty percent lethal dose (LD₅₀) values were calculated by probit analysis. Treatment means were compared and separated by Scheffe's test at $P = 0.05$ (SAS Institute) (21).

RESULTS AND DISCUSSION

Acaricidal effects of constituents derived from *T. vulgaris* oil against *T. putrescentiae* were examined using an impregnated fabric disk bioassay, and were then compared with those of the widely used benzyl benzoate and *N,N*-diethyl-*m*-toluamide, which served as positive controls for acaricidal activity (Table 1). The observed responses varied according to dosage and mite species based on the 24-h LD₅₀ value of the oil being 10.2 $\mu\text{g}/\text{cm}^2$ against *T. putrescentiae*. No mortality occurred in the untreated controls. Due to the potent activity of the oil derived from *T. vulgaris*, the isolation of the active components was pursued. The isolation procedure used to purify the acaricidal constituents from *T. vulgaris* oil is shown in Figure 1.

Bioassay-guided fractionation of the *T. vulgaris* oil afforded an active constituents identified by spectroscopic analyses, including HPLC, GC-MS, EI-MS, ^{13}C NMR, and ^1H NMR by the direct comparison with authentic reference compound. In this regard, the biologically active constituents were characterized as carvacrol and thymol. These compounds were identified based on the following evidence. (i) Carvacrol (5-isopropyl-2-methylphenol) ($\text{C}_{10}\text{H}_{14}\text{O}$, molecular weight [MW]: 150; EI-MS (70 eV) m/z (percent relative intensity) M^+ 150, 135, 131, 121, 115, 107, 103, 99, 95, 91, 87, 81, 77, 73, 66, 62, 58, 51, 41; ^1H NMR (CD_3OD , 600 MHz) δ 6.944–6.966 (1H, d), 6.633–6.647 (1H, d), 6.599 (1H, s), 2.705–2.571 (1H, m), 1.119–1.130 (3H, m), 2.126 (3H, s), 4.781 (1H, s); ^{13}C

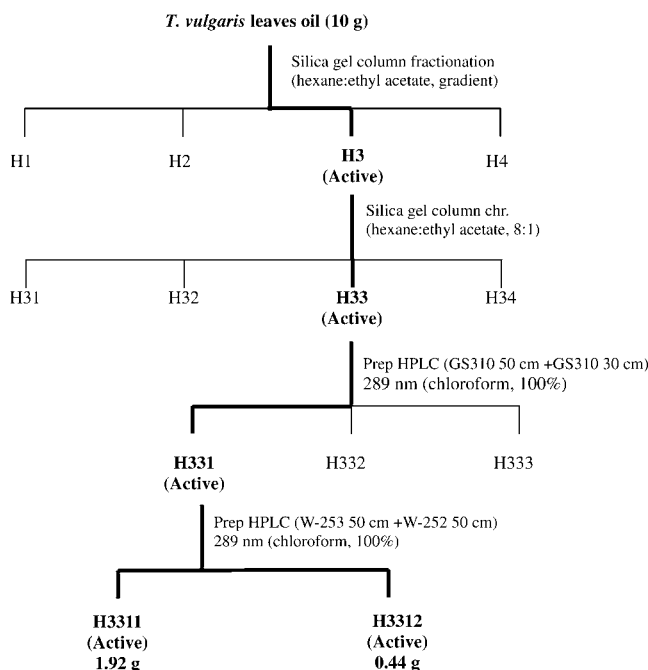


FIGURE 1. Isolation procedure of the acaricidal constituent of the oil from *T. vulgaris*.

NMR (CD_3OD , 150 MHz) δ 153.53, 148.39, 130.79, 120.85, 118.76, 112.99, 33.63, 23.95, 15.28. (ii) Thymol (2-isopropyl-5-methylphenol) ($\text{C}_{10}\text{H}_{14}\text{O}$, MW: 150): EI-MS (70 eV) m/z (percent relative intensity) M^+ 150, 135, 115, 103, 91, 77, 65, 51; ^1H NMR (CD_3OD , 600 MHz) δ 6.992~7.004 (1H, d), 6.641~6.654 (1H, d), 6.472 (1H, s), 3.080 (1H, s), 1.148~1.161 (3H, m), 2.178 (3H, s), 4.655 (1H, s); ^{13}C NMR (CD_3OD , 150 MHz) δ 152.45, 136.56, 131.33, 126.20, 121.66, 116.00, 26.67, 22.64, 20.82 (Fig. 2). The spectroscopic data of carvacrol and thymol were found to be the same as those for the carvacrol isolated from *Thujopsis dolabrata* var. *hondai* sawdust, and commercial thymol was purchased from Sigma (1, 6, 24).

The substances identified by GC-MS in the *T. vulgaris* oil are presented in Table 2. Analysis led to the identifica-

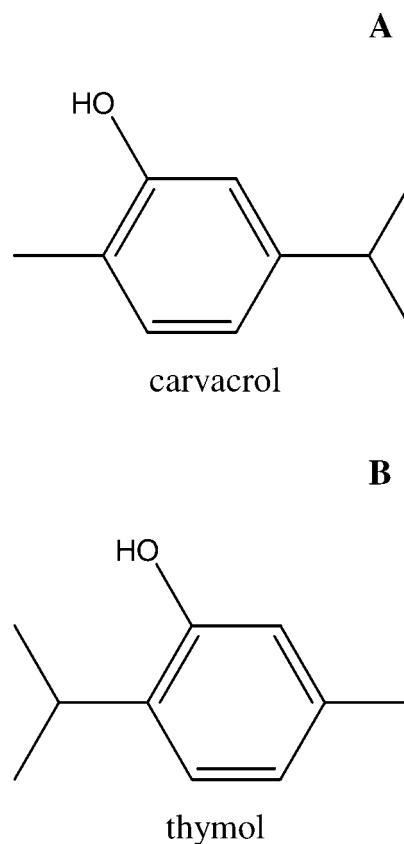


FIGURE 2. Structures of carvacrol and thymol isolated from *T. vulgaris* oil.

tion of 11 components from the *T. vulgaris* oil. The main constituents were thymol (relative percent = 50.80%), benzene (20.08%), carvacrol (14.17%), linalool (2.17%), γ -terpinene (1.94%), *trans*-caryophyllene (1.03%), α -pinene (0.87%), D-limonene (0.65%), camphene (0.46%), caryophyllene oxide (0.29%), and camphor (0.15%). Together, benzene, carvacrol, and thymol made up 80.05% of the *T. vulgaris* oil. In a previous study, Venskutonis (27) reported on *Thymus vulgaris* L. leaves that were oven dried at 30°C

TABLE 2. Volatile compounds derived from the *T. vulgaris* oil identified by GC-MS

Peak no.	Compound	Mass spectral data ^a	Retention time (min)	Relative (%)
1	α -Pinene	27, 41, 77, 93, 105, 121, 136	3.88	0.87
2	Camphene	39, 41, 79, 93, 121, 126	4.15	0.46
3	Benzene	43, 51, 58, 65, 77, 91, 103, 107, 119, 128, 134	5.5	20.1
4	D-Limonene	39, 53, 68, 93, 107, 121, 136	5.55	0.65
5	γ -Terpinene	65, 77, 93, 105, 115, 121, 136	6.08	1.94
6	Linalool	15, 55, 71, 82, 93, 109, 121, 136, 154	6.77	2.17
7	Camphor	18, 27, 55, 59, 81, 95, 108, 119, 137, 152	7.6	0.15
8	Thymol	51, 65, 77, 91, 103, 115, 135, 150	9.9	50.8
9	Carvacrol	41, 51, 58, 62, 66, 73, 77, 81, 87, 91, 95, 99, 103, 107, 115, 121, 131, 135, 150	10.01	14.2
10	<i>trans</i> -Caryophyllene	37, 51, 53, 57, 65, 69, 74, 79, 83, 89, 93, 97, 105, 109, 115, 120, 124, 128, 133, 137, 141, 161, 175, 189, 204	11.67	1.03
11	Caryophyllene oxide	55, 63, 69, 79, 85, 93, 99, 107, 115, 121, 128, 131, 149, 161, 177, 187, 205, 220	13.73	0.29

^a Major fragmentation ions, base peak (listed first), and other ions in decreasing order of relative abundance.

after simultaneous distillation-extraction of oil from *T. vulgaris* leaves. The main constituents were thymol (42.27%), *p*-cymene (25.2%), γ -terpinene (6.37%), linalool (2.86%), carvacrol (2.81%), β -caryophyllene (2.39%), myrcene (1.93%), α -thujene (1.50%), 1-octen-3-ol (1.19%), α -pinene (1.16%), *trans*-sabinene hydrate (1.07%), α -terpinene (1.02%), borneol (0.98%), 1,8-cineol (0.96%), camphene (0.60%), terpinene-4-ol (0.52%), limonene (0.52%), and β -pinene (0.38%) (27). In this study, some differences in the concentration of volatile compounds between the *T. vulgaris* oil oven dried at 30 and 45°C were also noted. The amounts of compounds collected from the leaves of *T. vulgaris* that had been oven dried at 45°C compared with those collected from the leaves of *T. vulgaris* at 30°C can mainly be evidenced by the increased levels of carvacrol (12%) and thymol (8%), while the concentration of γ -terpinene was decreased by 5%. In addition, considering the solvent extraction technique, the distillation extract was richer in phenols containing three double bonds (carvacrol and thymol) than it was in the simultaneous distillation extract. Furthermore, it could cause the differences of essential oils concentrations in strains, types, and production year.

The acaricidal activities of the isolated components (carvacrol and thymol) and six commercially available compounds (camphene, *trans*-caryophyllene, D-limonene, linalool, α -pinene, γ -terpinene) derived from the *T. vulgaris* oil against *T. putrescentiae* adults were examined by direct contact application (2, 12), and were compared with those of benzyl benzoate and *N,N*-diethyl-*m*-toluamide, which served as reference materials. On the basis of 24-h LD₅₀ values, the compound that was most toxic against *T. putrescentiae* was carvacrol (4.52 $\mu\text{g}/\text{cm}^2$), followed by thymol (11.09 $\mu\text{g}/\text{cm}^2$), benzyl benzoate (11.29 $\mu\text{g}/\text{cm}^2$), *N,N*-diethyl-*m*-toluamide (13.89 $\mu\text{g}/\text{cm}^2$), and linalool (14.63 $\mu\text{g}/\text{cm}^2$). The acaricidal activities of carvacrol and thymol were much more effective than benzyl benzoate and *N,N*-diethyl-*m*-toluamide were. However, no activity was observed for α -pinene, camphene, D-limonene, γ -terpinene, and *trans*-caryophyllene. These results suggest that the acaricidal activity of the *T. vulgaris* oil can be primarily attributed to carvacrol and thymol because carvacrol was approximately 2.5 and 3.1 times more toxic than were benzyl benzoate and *N,N*-diethyl-*m*-toluamide, respectively, and thymol was about 1 and 1.3 times more toxic than were benzyl benzoate and *N,N*-diethyl-*m*-toluamide. Furthermore, a previous study reported that the acaricidal activity of the oil of *Foeniculum vulgare* seeds could be attributed to thymol (14). In this study, monoterpenoid alcohols such as phenols containing three double bonds (carvacrol and thymol) merit further study as potential stored food mite control agents or as lead compounds.

Various compounds, including alkaloids, phenolics, and terpenoids, exist in plants, and jointly or independently contribute to the generation of a variety of biocidal compounds (26). In addition, plant-based chemical substances are potential sources for stored food mite control (8, 12, 19). In this vein, a number of studies have reported naturally occurring acaricidal compounds to be effective against stored food mites, including fenchone, linalyl acetate, men-

thol, and menthone from *Eucalyptus globules* Labill., *Lavandula angustifolia* Miller, *Lavandula stoechas* L., and *Mentha piperita* L. (19); cinnamaldehyde, benzaldehyde, 3-phenylpropionaldehyde, cinnamyl alcohol, salicylaldehyde, and 2-hydroxycinnamic acid from *Cinnamomum cassia* Blume barks (12); and terpinene and pulegone from natural monoterpenes (20). The *Thymus* genus comprises 215 species, with *T. vulgaris* being one of the most important and thoroughly investigated aromatic plants (16). In addition, the essential oils containing large amounts of carvacrol and thymol were reported to possess the highest antioxidant activity. These compounds also exhibit other bioactivities, e.g., thymol is antiseptic, and carvacrol possesses antifungal properties (18). In this study, the carvacrol and thymol isolated from the *T. vulgaris* oil exhibited acaricidal activities against *T. putrescentiae*, a stored food mite. In a previous study, the oral LD₅₀ values of carvacrol and thymol for rats were reported to be 810 and 980 mg/kg, respectively, suggesting a relatively low acute toxicity to mammals (22).

In conclusion, these results indicate that phenols containing three double bonds, carvacrol and thymol, which constituted 64.97% of all quantified volatiles, were found to be major contributors to the acaricidal activity of the volatile extract of *T. vulgaris*, and were comparable to commercial benzyl benzoate and *N,N*-diethyl-*m*-toluamide against *T. putrescentiae*, using the same method. More importantly, carvacrol and thymol can be very useful as potential agents against mites infesting stored food and lead compounds, and may act in the prevention of allergic disease. Further research should be performed to assess potential safety issues of these compounds related to human health, the acaricidal mode of action, and formulations to improve the acaricidal potency and stability.

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