Toxicities of active constituent isolated from *Thymus vulgaris* flowers and its structural derivatives against *Tribolium castaneum* (Herbst)

Hee-Kwon Lee1,2 · Hoi-Seon Lee1

Received: 29 September 2016 / Accepted: 7 October 2016 / Published online: 17 October 2016
© The Korean Society for Applied Biological Chemistry 2016

**Abstract** Insecticidal activity of 2-isopropyl-5-methylphenol isolated from *Thymus vulgaris* oil and its derivatives against *Tribolium castaneum* adults and larvae were evaluated using contact and fumigant bioassays. To elucidate the structure–activity relationship, 2-isopropyl-5-methylphenol and its derivatives were determined. Based on the LD50 values, 2-isopropylphenol was most toxic to *T. castaneum* adults and larvae, followed by 4-isopropylphenol, 2-methylphenol, 3-methylphenol, and phenol in contact and fumigant bioassays. These results indicate that the introduction of a functional group such as methyl and isopropyl group into the phenol skeleton has an important influence on contact and fumigant toxicities. Furthermore, 2-isopropyl-5-methylphenol and its derivatives could be used as an alternative to control stored-grain insects.

**Keywords** 2-Isopropyl-5-methylphenol · Stored-grain insects · *Thymus vulgaris* · *Tribolium castaneum*

**Introduction**

*Tribolium castaneum* is the destructive primary stored-grain insect of stored grains and cause serious damage to quantitative and qualitative losses of grain products (Padin et al. 2002). Insect control in stored grains is dependent in large measure on the use of synthetic insecticide. However, the use of synthetic fumigants and insecticides for decades to control stored-grain insects have led to a several undesirable effects, including health and environmental problems, insect toxicity and resistance to non-target organisms (Donahay et al. 1992; Sousa et al. 2009). Therefore, there is an urgent need to develop new types of safe, environmental, convenient and low-cost alternatives. For this reason, various studies have been concentrating their efforts on the search for plant materials as an alternative to synthetic fumigants and insecticides to control stored-grain insects (Huang et al. 1997).

Major constituents and essential oils derived from plants are significant natural sources of insect growth regulators, acute fumigants, fungicides, and insecticides against diverse insect species and they are developed as ecologically potential pesticides. To the best of our knowledge, nothing has been reported in journals about the insecticidal toxicities of the essential oils derived from *Thymus vulgaris* flowers against *T. castaneum* adults and larvae. The present study describes the insecticidal toxicities of active constituent isolated from *T. vulgaris* flowers and its derivatives using the contact and fumigant bioassays against *T. castaneum* adults and larvae.

**Materials and methods**

**Chemicals and material preparation**

The fumigant and contact toxicity of the following chemical and synthetic insecticide were tested: phenol (PubChem CID: 996), 2-methylphenol (PubChem CID: 335),
3-methylphenol (PubChem CID: 342), 2-isopropylphenol (PubChem CID: 6943), 4-isopropylphenol (PubChem CID: 7465), 2-isopropyl-5-methylphenol (PubChem CID: 6989), and dichlorvos (PubChem CID: 3039) were obtained from Aldrich (Milwaukee, WI, USA). The flowers of *T. vulgaris* (1 kg) were supplied from a local store in Jecheon, South Korea. The *T. vulgaris* flowers were powdered using a blender and extracted with steam distillation extraction. Essential oil (yield, 0.561%) was stored in a sealed glass vial at 3 °C prior to isolation.

**Isolation and identification**

The isolation of active constituent from *T. vulgaris* (5 g) oil was carried out by the method of Jeon et al. (2015). The essential oil was chromatographed on a silica gel column chromatography (Merck 70–230 mesh, 60 mm inside diameter × 800 mm in length; Rahway, NJ, USA) and eluted using a mixture of solvents [hexane/ethyl acetate (10:0, 9:1, 8:2, 7:3, 5:5, v/v)]. Each fraction (250 mL) was collected and concentrated at 30 ± 1 °C, and similar fractions detected by TLC profiles were combined to yield four fractions. Fractions (H 1–4) that bioassayed using contact and fumigant toxicity against *T. castaneum* adults and larvae were at 0.56 and 0.84 mg/cm², respectively. The H3 showed fumigant and contact toxicities against *T. castaneum* adults and larvae and was rechromatographed on a silica gel column using a mixture of solvent [hexane–ethyl acetate (8:1, v/v)] to give four fractions (H 31–34). Thus, the active H33 was purified by prep. HPLC (LC-908, Japan Analytical Industry Co., Ltd, Tokyo, Japan) using a Jai gel GS series column (GS310 500 + GS310 300 mm) with mixture of solvents [hexane/ethyl acetate (9:1, v/v)] at 5.0 mL/min to give three fractions (H 331–333). Furthermore, the active H 331 was separated into two further fractions (H 3311–3312) using a Jai gel-W253 column (2 cm i.d. × 50 cm L., JAI Co., Tokyo, Japan) with a chloroform (100 %) system at 5.0 mL/min. Finally, the active R 3311 was isolated as a single peak. The structure of H 3311 was elucidated based on JNM EX-600 spectrometer (JEOL Co., Tokyo, Japan) with CD3OD, 13C-NMR (150 MHz), 1H-NMR (600 MHz), and DEPT NMR (100 MHz). Furthermore, the UV–visible absorption spectrum was obtained using a UV spectrometer (DR-4000 spectrophotometer, H ACH, Loveland, Co, USA) and EI-MS spectra were determined on a JEOL GSX 400 mass spectrometer.

**Stored grain insect and bioassay**

*Tribolium castaneum* adults and larvae were obtained from National Academy of Agricultural Science, RDA (Korea). They were reared wheat flour in plastic boxes (W × L × H, 30 × 30 × 20 cm). The laboratory cultures maintained in a 15-h light/9-h dark photoperiod at 71 ± 5 % relative humidity (RH) and 28 ± 1 °C. For the fumigant and contact bioassays, about 2–3 weeks old adults and 12- to 16-day-old larvae were tested in this study. To investigate the insecticidal activities of *T. vulgaris* oil, the active compound, and its structurally related derivatives against *T. castaneum* adults and larvae, the fumigant and contact methods modified by Jeon et al. (2015) were used. To evaluate fumigant toxicity against *T. castaneum* adults and larvae, filter paper (5 cm diameter) was impregnated with acetone solutions (100 µL) at various concentrations (0.60–0.010 mg/cm³) of each sample. To evaluate contact toxicity at various concentrations (1.0–0.01 mg/cm²) for the contact toxicity were liquefied in acetone (100 µL) and applied to filter paper. Each piece of filter paper for the fumigant bioassay was attached to the lid of Petri dish (5.5 × 1.5 cm) after being dried in air. The larvae and adults were added to the Petri dish, and lid was closed. The treatment and control Petri dishes were placed in incubators (27 ± 2 °C; 60 ± 5 % RH; photoperiod of 12 h light/12 h dark) for 48 h. All treatments were replicated five times. After 48 h of treatment, the mortality and lethal dose (LD50) values of adults and larvae were determined.

**Statistical analysis**

Mortality was corrected for negative control mortality using Abbott's formula. The LD50 values of the test sample were calculated by probit analysis and transformed to arcsine square-root values for analysis of variance (Finney 1971).

**Results and discussion**

To study the contact and fumigant toxicities of the essential oil extracted from *T. vulgaris* flowers, the dosage mortality responses to *T. castaneum* adults and larvae were determined (Table 1). Significant differences were observed in contact and fumigant toxicities against *T. castaneum* adults and larvae. The LD50 value of *T. vulgaris* oil against *T. castaneum* adults and larvae were 0.141 and 0.117 mg/cm³ in the fumigant bioassay, respectively. In the contact bioassay, the LD50 value of *T. vulgaris* oil against *T. castaneum* adults and larvae was 0.236 and 0.158 mg/cm², respectively. *Tribolium castaneum* adults and larvae were about 1.7 and 1.4 times more susceptible to the fumigant
Table 1  Fumigant and contact toxicity of *Thymus vulgaris* oil against *T. castaneum*

| Insect   | Bioassay  | Stage | Dose (mg/cm³) | % mortality (mean ± SE) | LD⁵₀ (mg/cm³) | 95 % CI  | Slope (±SE) | χ² (df, p) |
|----------|-----------|-------|---------------|-------------------------|--------------|----------|-------------|-----------|
| *T. castaneum* | Fumigant | Larvae | 1.0           | 100.0 ± 0.0ᵃ           | 0.117        | 0.109 ± 0.139 | 2.45 ± 0.55 | 3.439 (4, 0.329) |
|          |          |       | 0.5           | 100.0 ± 0.0ᵃ           |              |           |             |           |
|          |          |       | 0.25          | 100.0 ± 0.0ᵃ           |              |           |             |           |
|          |          | Adults| 1.0           | 100.0 ± 0.0ᵃ           | 0.141        | 0.129 ± 0.157 | 2.96 ± 0.59 | 7.168 (4, 0.128) |
|          |          |       | 0.5           | 100.0 ± 0.0ᵃ           |              |           |             |           |
|          |          |       | 0.25          | 89.3 ± 2.4ᵇ           |              |           |             |           |
|          | Contact  | Larvae| 1.0           | 100.0 ± 0.0ᵃ           | 0.158        | 0.121 ± 0.183 | 1.80 ± 0.33 | 4.105 (4, 0.392) |
|          |          |       | 0.5           | 100.0 ± 0.0ᵃ           |              |           |             |           |
|          |          |       | 0.25          | 82.2 ± 1.9ᵇ           |              |           |             |           |
|          |          | Adults| 1.0           | 100.0 ± 0.0ᵃ           | 0.236        | 0.208 ± 0.275 | 2.33 ± 0.44 | 2.126 (3, 0.547) |
|          |          |       | 0.5           | 93.6 ± 2.1ᵃ           |              |           |             |           |
|          |          |       | 0.25          | 54.7 ± 1.7ᵃ           |              |           |             |           |
| Control  | Fumigant | Larvae| 1.0           | 0ᵃ                     | –           | –        | –           | –         |
|          |          | Adults| 1.0           | 0ᵃ                     |              |           |             |           |
|          | Contact  | Larvae| 1.0           | 0ᵃ                     | –           | –        | –           | –         |
|          |          | Adults| 1.0           | 0ᵃ                     |              |           |             |           |

ᵃ Exposed for 48 h
ᵇ Negative control

Fig. 1 Structures of 2-isopropyl-5-methylphenol and its analogs: (1) phenol; (2) 2-isopropyl-5-methylphenol; (3) 2-methylphenol; (4) 3-methylphenol; (5) 2-isopropylphenol; (6) 4-isopropylphenol
action of *T. vulgaris* oil than contact action. *Tribolium castaneum* adults were more tolerant of *T. vulgaris* oil than the larvae to the fumigant and contact action. Due to the contact and fumigant toxicities observed from *T. vulgaris* oil, the active constituent was identified by $^{13}$C-NMR, $^1$H-NMR, DEPT NMR, $^1$H–$^1$H COSY, HMQC, and EI-MS analyses. The bioactive constituent was characterized as 2-isopropyl-5-methylphenol (C$_{10}$H$_{14}$O); UV (MeOH): $\lambda_{\text{max}}$ = 285 nm; $R_f$ = 0.54. EI-MS (70 eV), m/z (relative intensity) M$^+$ 150 (45), 135 (100, base peak), 107 (15), 91 (28), 58 (31); $^1$H NMR (CDCl$_3$, 600 MHz, $\delta$ ppm) $\delta$ 6.89–6.99 (1H, d, $J$ = 7.9 Hz), 6.69–6.72 (1H, d, $J$ = 7.9 Hz), 6.56 (1H, s), 3.13–3.17 (1H, t, $J$ = 6.9 Hz), 1.23–1.24 (3H, d, $J$ = 6.9 Hz), 1.23–1.24 (3H, d, $J$ = 6.9 Hz), and 1.28 (3H, s); $^{13}$C NMR (CDCl$_3$, 150 MHz, $\delta$ ppm) $\delta$ 152.3 (C-1), 136.7 (C-5), 131.5 (C-2), 126.3 (CH, C-3), 121.0 (CH, C-4), 116.3 (CH, C-6), 26.8 (CH, C-1'), 22.9 (CH3, C-2', C-3'), and 21.1 ppm (CH3, C-5). The spectroscopic data of the active compound matched those of previous studies (Jeon et al. 2015).

The contact and fumigant toxicities were determined for 2-isopropyl-5-methylphenol and its derivatives against *T. castaneum* adults and larvae and compared with famous insecticide, dichlorvos, which was the positive control (Fig. 1; Tables 2 and 3). The LD$_{50}$ values of 2-isopropyl-5-methylphenol in the fumigant toxicity were 0.088 and 0.110 mg/cm$^3$ against *T. castaneum* larvae and adults (Table 2). The LD$_{50}$ values of 2-isopropyl-5-methylphenol in the contact toxicity were 0.118 and 0.105 mg/cm$^2$. *Tri- bolium castaneum* larvae were about 1.3 times more susceptible to fumigant action of 2-isopropyl-5-methylphenol. However, there were no significant differences were observed between contact and fumigant toxicity against *T. castaneum* adults. The fumigant toxicities of five derivatives were compared with that of dichlorvos against *T. castaneum* adults and larvae (Table 2). Based on the LD$_{50}$ values, 2-isopropylphenol (0.073 and 0.104 mg/cm$^3$) was most toxic to *T. castaneum* larvae and adult, followed by 4-isopropylphenol (0.079 and 0.106 mg/cm$^3$), 2-methylphenol (0.101 and 0.117 mg/cm$^3$), 3-methylphenol (0.116 and

| Insect | Stage | LD$_{50}$ (µg/cm$^3$) | 95% CI | Slope (±SE) | $\chi^2$ (df, p) |
|--------|-------|----------------------|-------|------------|-----------------|
| Phenol | Larvae | >0.140 | – | – | – |
|        | Adults | >0.140 | – | – | – |
| 2-Methylphenol | Larvae | 0.101 | 0.0893 ± 0.1182 | 2.01 ± 0.45 | 3.737 (3, 0.291) |
|        | Adults | 0.117 | 0.1014 ± 0.1431 | 1.94 ± 0.44 | 2.516 (4, 0.472) |
| 3-Methylphenol | Larvae | 0.116 | 0.1031 ± 0.1382 | 2.27 ± 0.46 | 1.309 (4, 0.727) |
|        | Adults | 0.132 | 0.1156 ± 0.1594 | 2.33 ± 0.46 | 1.131 (3, 0.770) |
| 2-Isopropylphenol | Larvae | 0.073 | 0.0674 ± 0.0881 | 2.03 ± 0.42 | 1.421 (3, 0.701) |
|        | Adults | 0.104 | 0.0908 ± 0.1294 | 2.17 ± 0.45 | 2.347 (4, 0.504) |
| 4-Isopropylphenol | Larvae | 0.079 | 0.0686 ± 0.0883 | 1.46 ± 0.29 | 3.065 (4, 0.547) |
|        | Adults | 0.106 | 0.0893 ± 0.1284 | 2.49 ± 0.59 | 1.148 (4, 0.651) |
| 2-Isopropyl-5-methylphenol | Larvae | 0.088 | 0.0727 ± 0.0989 | 2.24 ± 0.33 | 2.292 (4, 0.682) |
|        | Adults | 0.110 | 0.0925 ± 0.1464 | 2.04 ± 0.31 | 1.838 (4, 0.651) |
| Dichlorvos$^a$ | Larvae | 0.009 | 0.0076 ± 0.0127 | 1.68 ± 0.27 | 1.638 (3, 0.651) |
|        | Adults | 0.020 | 0.0153 ± 0.0287 | 1.53 ± 0.28 | 1.838 (3, 0.607) |

Exposed for 48 h
$^a$ Positive control
0.132 mg/cm³), and phenol (>0.140 mg/cm³). For five derivatives, the larvae of *T. castaneum* were about 1.1–1.4 times more susceptible to fumigant action compared to adults. The contact toxicities of five derivatives were compared with that of dichlovos against *T. castaneum* larvae and adults (Table 3). Based on the LD₅₀ values, 2-isopropylphenol (0.049 and 0.081 mg/cm²) was most toxic to *T. castaneum* larvae and adults, followed by 4-isopropyl phenol (0.109 and 0.96 mg/cm³), 2-methylphenol (0.113 and 107 mg/cm³), 3-methylphenol (0.131 and 0.115 mg/cm³), and phenol (>0.210 mg/cm³). For five derivatives except for 2-isopropylphenol, the adults of *T. castaneum* were more susceptible to contact action compared to larvae. The contact and fumigant toxicities of five derivatives and how toxicity varies with structure were investigated. Phenol, which is the skeleton of 2-isopropyl-5-methylphenol exhibited no contact and fumigant toxicities against *T. castaneum* adults and larvae. However, introduction of functional group, such as isopropyl and methyl, in phenol ring significantly increased their contact and fumigant toxicity against *T. castaneum* adults and larvae. 2-Isopropylphenol, conjugated with isopropyl group, exhibited the highest contact and fumigant toxicities against *T. castaneum* adults and larvae. In addition, 4-isopropylphenol (conjugated with isopropyl group) much more effective against *T. castaneum* adults in the contact and fumigant bioassay than 2-methylphenol (conjugated with methyl group) and 3-methylphenol (conjugated with methyl group). These results indicate that the contact and fumigant mode of action of these compounds may be largely attributable to isopropyl functional group in phenol ring. According to previous studies, the toxicity and efficacy of the monoterpenes against stored-grain insects influenced by the bioassay method (Prates et al. 1998; Park et al. 2003). For example, Abdelgaleil et al. (2009) reported that treatment of *T. castaneum* with 1,8-cineole and myrcene was the most toxic fumigant against *T. castaneum*, but the two compounds were not active as a contact toxicant. Lee et al. (1997) reported that the insecticidal susceptibilities were influenced

### Table 3 Contact toxicities of 2-isopropyl-5-methylphenol and its derivatives against *T. castaneum*

| Insect                        | Stage | LD₅₀ (µg/cm²) | 95% CI       | Slope ±SE (df, p) | χ² (df, p) |
|-------------------------------|-------|---------------|--------------|-------------------|------------|
| Phenol                        | Larvae| >0.210        | –            | –                 | –         |
|                              | Adults| >0.210        | –            | –                 | –         |
| 2-Methylphenol                | Larvae| 0.113         | 0.0931 ± 0.1382 | 2.19 ± 0.32     | 3.747 (4, 0.441) |
|                              | Adults| 0.107         | 0.0793 ± 0.1321 | 2.43 ± 0.34     | 4.307 (4, 0.366) |
| 3-Methylphenol                | Larvae| 0.131         | 0.1166 ± 0.01564 | 3.51 ± 0.48     | 6.575 (4, 0.160) |
|                              | Adults| 0.115         | 0.0871 ± 0.1476 | 1.84 ± 0.30     | 1.216 (4, 0.875) |
| 2-Isopropylphenol             | Larvae| 0.049         | 0.0384 ± 0.0592 | 2.03 ± 0.38     | 2.015 (3, 0.569) |
|                              | Adults| 0.081         | 0.0696 ± 0.1021 | 2.15 ± 0.32     | 6.005 (4, 0.199) |
| 4-Isopropylphenol             | Larvae| 0.109         | 0.0927 ± 0.1364 | 1.90 ± 0.30     | 5.302 (4, 0.258) |
|                              | Adults| 0.096         | 0.0748 ± 0.1225 | 2.77 ± 0.44     | 6.274 (4, 0.180) |
| 2-Isopropyl-5-methylphenol    | Larvae| 0.118         | 0.0914 ± 0.1437 | 2.09 ± 0.31     | 6.211 (4, 0.184) |
|                              | Adults| 0.105         | 0.0812 ± 0.1346 | 1.92 ± 0.31     | 4.497 (4, 0.343) |
| Dichlorvos*                   | Larvae| 0.005         | 0.0042 ± 0.0063 | 2.64 ± 0.50     | 1.106 (4, 0.776) |
|                              | Adults| 0.008         | 0.0057 ± 0.0098 | 1.79 ± 0.037    | 1.462 (4, 0.691) |

Exposed for 48 h

* Positive control
by structural characteristics of monoterpenoids such as degree of saturation, shape, and types of functional groups. Furthermore, Samarasekera et al. (2008) reported that the structural variation in the molecule is a key factor in enhancing the mosquitocidal activity. Similar studies have been reported by Lee et al. (2008), introduction of functional group, such as ally, benzyl, isopropyl, and vinyl cinnamates had potent insecticidal activities against stored-grain insect, *Sitophilus oryzae*. Our results confirm the importance of the isopropyl groups in the insecticidal mode of action of phenol skeleton, as reported by others (Jeon et al. 2015). These observations raise the possibility that the presence of isopropyl functional group augments toxicity. As to questions on possible toxicity of *T. vulgaris* oil, the fact that it is consumed by humans, as herb food flavoring is indicative of its non-toxicity to humans. Furthermore, Sigma-Aldrich acute toxicity database indicated that the mammalian toxicity of 2-isopropyl-5-methylphenol isolated from *Ruta graveolens* oil and its structural analogs are relatively low mammalian toxicity (Sigma-Aldrich 2010, USA). So it may be given preference on synthetic pesticides for managing stored-grain insects.

**Acknowledgments** This work was carried out with the support of “Development of crop pest management techniques using the functional materials derived from Coriandrum sativum and Valeriana fauriei, Project No. PJ011983022016“ Rural Development Administration.

**References**

Abdelgaleil SAM, Mohamed MIE, Badawy MEI, EI-arami SAA (2009) Fumigant and contact toxicities of monoterpenes to *Sitophilus oryzae* (L.) and *Tribolium castaneum* (Herbst) and their inhibitory effects on acetylcholinesterase activity. J Stored Prod Res 35:518–525

Donahay E, Zalich D, Rindner M (1992) Comparison of the sensitivity of the development stages of three strains of the red flour beetle (Coleoptera: Tenebrionidae) to modified atmospheres. J Econ Entomol 85:1450–1452

Finney DJ (1971) Probit analysis. Cambridge University Press, Cambridge, p 333

Huang Y, Tan JM, ML, Kini RM, Ho SH (1997) Toxic and antifeedant action of nutmeg oil against *Tribolium castaneum* (Herbst) and *Sitophilus zeamais* Motsch. J Stored Prod Res 33:289–298

Jeon JH, Lee SG, Lee HS (2015) Isolation of insecticidal constituent from *Ruta graveolens* and structure-activity relationship studies against stored-food pests (Coleoptera). J Food Prot 78:1536–1540

Lee S, Tsao R, Peterson C, Goats JR (1997) Insecticidal activity of monoterpenoids to western corn rootworm (Coleoptera: Chrysomelidae), two spotted spider mite (Acarii: Tetranychidae), and house fly (Diptera: Muscidae). J Econ Entomol 90:883–892

Lee EJ, Kim JR, Choi DR, Ahn YJ (2008) Toxicity of *Cassia* and cinnamon oil compounds and cinnamaldehyde-related compounds to *Sitophilus oryzae* (Coleoptera: Curculionidae). J Econ Entomol 101:1960–1966

Padin S, Dal Bello G, Fabrizio M (2002) Grain loss caused by *Tribolium castaneum*, *Sitophilus oryzae* and *Acanthoscelides obtectus* in stored durum wheat and bean treated with *Beauveria bassiana*. J Stored Prod Res 38:69–74

Park IK, Lee SG, Choi DH, Park JD, Ahn YJ (2003) Insecticidal activities of constituents identified in the essential oil from leaves of *Chamaecyparis obtusa* against *Callosobruchus chinensis* (L.) and *Sitophilus oryzae* (L.). J Stored Prod Res 39:375–384

Prates HT, Santos JP, Maquill TM, Fabris JD, Oliveira AB, Foster JE (1998) Insecticidal activity of monoterpenes against *Rhizopertha dominica* (F.) and *Tribolium castaneum* (Herbst). J Stored Prod Res 34:243–249

Samarasekera R, Weerasinghe IS, Hemalal KDP (2008) Insecticidal activity of menthol derivatives against mosquitoes. Pest Manag Sci 64:290–295

Sigma-Aldrich (2010) In Material Safety Data Sheet (MSDS): Toxicological Information, Section 11. Sigma-Aldrich, St Louis

Sousa AH, Faroni LRDA, Pimentel MAG, Guedes RNC (2009) Developmental and population growth rates of phosphine-resistant and -susceptible populations of stored-product insect pests. J Stored Prod Res 45:241–246