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# Oral Toxicity of Thymol, α-Pinene, Diallyl Disulfide and Trans-Anethole, and Their Binary Mixtures against *Tribolium castaneum* Herbst Larvae (Coleoptera: Tenebrionidae)

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**Abstract**

Oral toxicity of thymol, α-pinene, diallyl disulfide and trans-anethole, as well as their binary combinations, was studied on the fourth larval instars of *Tribolium castaneum* Herbst. Serial dilutions of chemicals were prepared from the stock solution to obtain the concentrations between 2.5-200 µl/ml prior mixing with 500 mg of the diet. $LC_{50}$ values were determined as 6.79, 12.85, 8.52 and 1.03 µl/ml, for thymol, α-pinene, trans-anethole and diallyl disulfide after 24 hours and 3.74, 8.39, 6.48 and 0.68 µl/ml after 48 hours of exposure, respectively. $LC_{50}$ values confirmed that diallyl disulfide and α-pinene were the highest and lowest toxic chemicals. The results show that combined treatment of thymol synergized α-pinene activity at 24 and 48 hours post treatments. In addition, "diallyl disulfide and thymol", "thymol and trans-anethole" had additive effects on *T. castaneum* larvae. Our results suggest that the combination of thymol and α-pinene compounds were significantly more effective than each compounds alone on *T. castaneum* larvae.

**Keywords:** Acute toxicity, Essential oil components, Botanical insecticides, Synergy, *Tribolium castaneum*

## 1. Introduction

Essential oils, as botanical insecticides, are the complex mixture of volatile compounds, whose bioactivities depend on chemical composition, synergistic or antagonistic effects (Isman, 2006). Essential oils are lipophilic and dissolvable in the lipid medium classified into monoterpenes, sesquiterpenes (both including hydrocarbons and oxygenated derivatives) and aliphatic compounds (acids, alcohols, aldehydes, alkanes, alkenes and ketones) (Tripathi et al., 2009; Ebadollahi, 2013). These compounds are considered as main bioactive chemicals, which are distributed in plant families, such as Asteraceae, Apiaceae, Zingiberaceae, Myrtaceae, Lamiaceae, Piperaceae, Poaceae, Rutaceae, Lauraceae, Cupressaceae, Graminaceae, Pedaliaceae etc. (Ebadollahi, 2013). Recently, researchers have shown an increased interest in bioactive effects of essential oils (including larvicidal, insecticidal, repellency, antifeedant, deterrency, delay development, adult emergence and fertility) and their derivatives on insects (reviewed in Bakkali et al., 2008; Tripathi et al., 2009; Gonzalez-Coloma et al., 2010; Qin et al., 2010; Abbasipour et al., 2011; De Almeida et al., 2011). They are insect neurotoxicants that inhibit either GABA receptor or acetylcholinesterase (AChE) (Bakkali et al., 2008). Regarding their biological properties, some studies showed that complex essential oil compositions were more effective than pure compounds, which can be due to their synergistic effects. However, their modes of action are still obscure (Gonzalez-Coloma et al., 2010). Fumigant toxicity of essential oils have focused on stored beetles, such as *Sitophilus oryzae* L. (Col.: Curculionidae), *Sitophilus zeamais* M. (Col.: Curculionidae), *Tribolium castaneum* H. (Col.: Tenebrionidae) and *Rhyzopertha dominica* F. (Col.: Bostrichidae) (Rajendran and Sriranjini, 2008).

Despite that the red flour beetle *T. castaneum* is known as a key coleopteran insect model for genomic studies (Richards et al., 2008); it is a destructive pest of stored products feeding on different grain and products in stores (Garcia et al., 2005).

Thymol is a phenolic monoterpene with strong insecticidal and antimicrobial activities (Palaniappan and Holley, 2010; Kumrungsee et al., 2014). It disrupts GABA synapses function by binding to GABA receptors on membrane of postsynaptic neurons (Priestley et al., 2003).

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α-Pinene is an alkene terpene found in Apiaceae family, that possess insecticidal activity on pests, such as *Sitophilus granaries* L. (Col.: Curculionidae), *T. castaneum*, (Ebadollahi and Mahboubi, 2011), *Spodoptera litura* W. (Lep.: Noctuidae) and *Achaea janata* L. (Lep.: Noctuidae) (Rani et al., 2014). Despite playing a role as insecticide, antifeedant, repellency and development inhibition (Huang et al., 1998; Kim et al., 2010; Yang et al., 2014), α-Pinene is associated with attracting the ladybeetles *Chilocorus kuwanae* S. (Col.: Coccinellidae) (Zhang et al., 2009).

Diallyl disulfide is a major constituent of the essential oil of Alliaceae family (Block, 2010) preventing oviposition and exhibiting behavioral deterrence against insect adults like *S. zeamais*, *T. castaneum* (Huang et al., 2000), *Culex pipien*s L. (Dip.: Culicidae) (Ramakrishnan et al., 1989), *Sitotroga cerealella* O. (Lep.: Gelechiidae) (Yang et al., 2012) and *T. confusum* (Saglam and Ozder, 2013). Trans-anethole is an active terpene derived of phenylpropanoid which is the main compound in essential oils of anise and fennel plants (Ozcan and Chalchat, 2006; Heydarzade and Moravvej, 2012; Kim et al., 2013). It induces lethal effects during the larval development (Sousa et al., 2015) and inhibits acetylcholinesterase and butyrylcholinesterase activity in insects (Menichini et al., 2009).

It is hypothesized that the mixtures of essential oils from the different plant could improve the efficiency against insect pests (Hummelbrunner and Isman, 2001). In addition, plant essential oils are highly volatile components which extrinsic parameters, such as temperature, light and oxygen, show a crucial impact on their stability. Since essential oils process significant fumigant toxicity against insect pests, the application of alternative methods seems to be efficient at least to minimize degradation and improve stability of essential oils. The objectives of the present study are to determine the oral toxicity of thymol, α-pinene, diallyl disulfide and trans-anethole, individually and in binary mixtures to find the most effective compound on fourth larval instars of *T. castaneum*.

## 2. Materials and Method

### *2.1. Compounds*

The chemicals including thymol (99%), α-pinene (98%), diallyl disulfide (80%) and trans-anethole (99.5%) were purchased from Sigma-Aldrich (Spain). Acetone (AR grade, Merck Germany) was used as the solvent in all experiments.

### *2.2. Insect Rearing*

The method described by Mikhaiel (2011) was used for insect rearing. Adults of *T. castaneum* were collected from infested stores in Zabol, Iran then stock colonies were established. Insects were fed on wheat flour and yeast (10:1 w:w) under controlled conditions (30 ±2 ºC, 70±2% R.H, 16:8 (L:D)) at Department of Plant Protection, University of Zabol, Zabol, Iran.

### *2.3. Bioassay*

The experiments were carried out on the fourth larval instars of *T. castaneum* under laboratory conditions. Concentrations of 2, 4, 8, 16 and 20 µl/ml of thymol, α-pinene, trans-anethole and 0.25, 0.5, 1, 2 and 4 µl/ml of diallyl disulfide, were determined based on preliminary experiments. Then, 500 µl of each concentration was added to 500 mg of larval diet. Diets were well mixed with chemicals, and then were allowed to evaporate their solvent for 15 min at room temperature. Controls were treated with aceton alone. The experiments were carried out in three replications with 10 larvae in each replication on plastic Petri dishes (diameter 6 cm). Larval mortality (lack of mobility was a criterion of larval mortality) was recorded 24 and 48 hours post treatments and $LC_{50}$ values were calculated. Probit analysis was used to calculate $LC_{50}$ and the corresponding 95 % CI values were obtained using Polo-Plus Software.

### *2.4. Insecticidal Activity of Binary Mixture of Chemicals*

Acute effects of binary mixtures of different sublethal concentrations of compounds used in the survey were according to the described method above. Then the actual and expected mortalities of the treated larvae were compared using the following formula as were described by Trisyono and Whalon (1999): Em= Oa+ Ob (1- Oa), where O and E are the observed and expected mortalities using first (Oa) and second chemicals (Ob) in the binary mixtures, respectively. Following Em formula, using X2' formula (X2'= (Om- Em)2 / Em), where Om and Em are the observed and expected mortalities in the binary mixture. Additive, antagonistic or synergistic effects were determined. The values of X2' were compared to the values of X2 value in chi-square distribution table (X2df=1,α= 0.05 =3.84). As described by Kumrungsee et al. (2014), when X2' values > 3.84 and < 3.84, synergistic and additive effects were represented, respectively. If the observed mortality were less than the expected one, it would be interpreted as antagonistic effect of the mixtures.

## 3. Results

### *3.1. Toxicity of Pure Compounds*

Thymol, α-Pinene, trans-anethole and diallyl disulfide showed the oral toxicities against larvae of *T. castaneum* at different concentrations and all exposure times. $LC_{50}$ values were found to be 6.79, 12.85, 8.52 and 1.03 µl/ml, for thymol, α-pinene, trans-anethole and diallyl disulfide 24 hours post-treatment, respectively (Table 1). Although after 48 hours post-treatment, $LC_{50}$ values of thymol, α-pinene, trans-anethole and diallyl disulfide were 3.74, 8.39, 6.48 and 0.68 µl/ml, respectively (Table 1). $LC_{50}$ values confirmed that diallyl disulfide was the most toxic compounds. All chemicals showed a significant positive correlation between increasing chemical concentrations, exposure times and increased larval mortality (Figure 1).

**Table 1.** Toxicity of different compounds (µl/ml) to early 4th instar larvae of *T. castaneum* at 24 and 48 hours post-treatment

| Compound | N | Time (h) | $LC_{50}$(95% CL) | $X^2$(df) | Slope±SE | P-Value |
|---|---|---|---|---|---|---|
| Thymol | 150 | 24 | 6.79 (4.86-9.25) | 1.22 (3) | 1.66±0.31 | 0.747 |
| | 150 | 48 | 3.74 (2.51-4.96) | 1.82 (3) | 1.93±0.33 | 0.610 |
| α-pinene | 150 | 24 | 12.85 (8.86-23.58) | 1.13 (3) | 1.32±0.30 | 0.769 |
| | 150 | 48 | 8.39 (5.60-13.29) | 0.67 (3) | 1.26±0.29 | 0.881 |
| Trans-anethol | 150 | 24 | 8.52 (6.07-12.42) | 1.02 (3) | 1.5±0.30 | 0.795 |
| | 150 | 48 | 6.48 (4.34-9.23) | 1.79 (3) | 1.43±0.30 | 0.616 |
| Diallyl disulfide | 150 | 24 | 1.03 (0.72-1.49) | 0.41 (3) | 1.45±0.27 | 0.939 |
| | 150 | 48 | 0.68 (0.47-0.92) | 0.84 (3) | 1.68±0.29 | 0.839 |

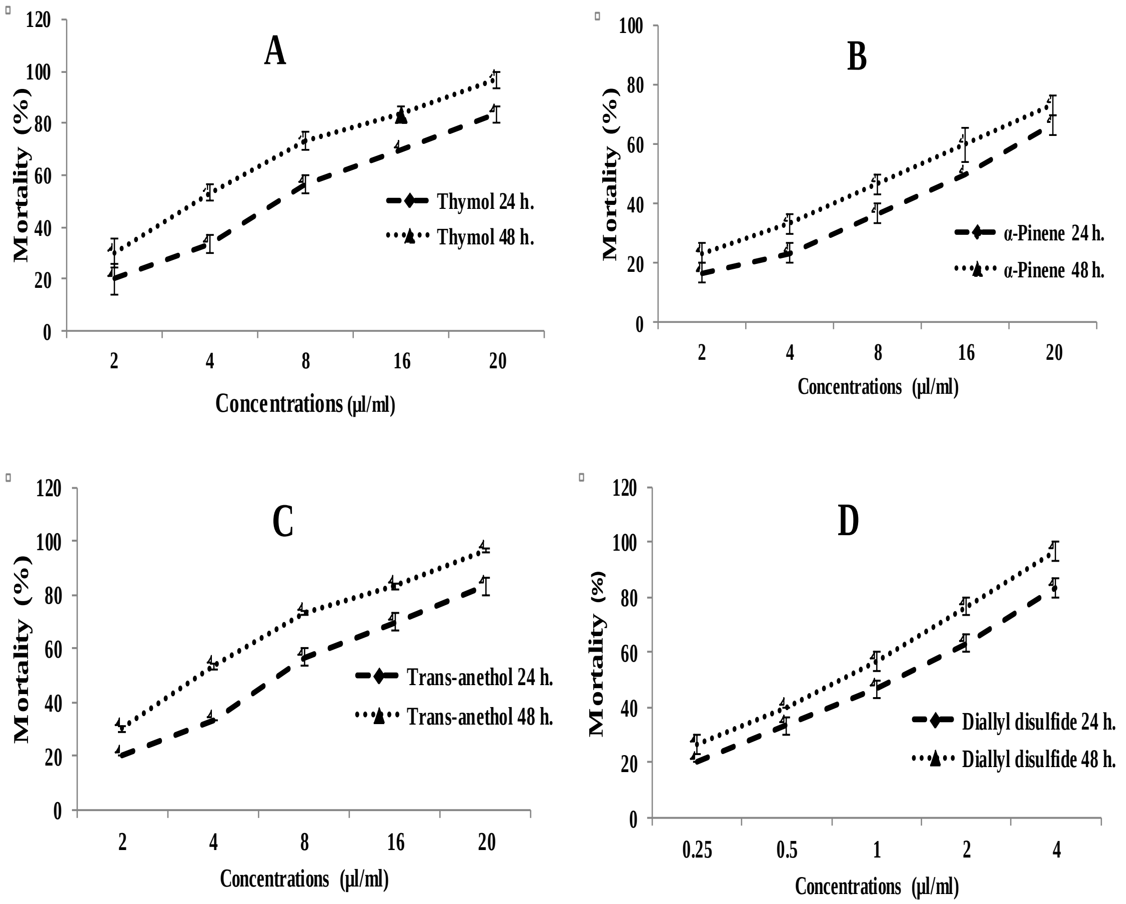


**Figure 1.** Relative toxicity of compounds to 4th instar larvae of *T. castaneum* at 24 and 48 hours post treatment. A) Thymol, B) α-pinene, C) Trans anethol, D) Diallyl disulfide

 

### *3.2. Combination of Pure Compounds*

In all concentrations, combination of thymol with diallyl disulfide or trans-anethole showed additive effects (Table 2). Binary mixtures of thymol and α-pinene synergized oral toxicity against *T. castaneum* (Table 2). In all concentrations, combinations of diallyl disulfide with trans-anethole or α-pinene and trans-anethole with α-pinene showed antagonistic effects 24 hours after exposure (Table 2).

**Table 2.** Relative toxicity of binominal mixtures of essential oil compounds to early 4th instar larvae of *T. castaneum* and measures of interactions at 24 hours post treatment

| Compound A | Compound B | Concentrations (μl/ml) | N | Larval mortality (%) | | | | $X^2$ | Effect |
|---|---|---|---|---|---|---|---|---|---|
| | | | | Pure compound | | Binary mixtures | | | |
| | | | | $O_a$ | $O_b$ | $E_m$ | $O_m$ | | |
| Diallyl disulfide | Thymol | 0.25+2 | 30 | 20.00 | 20.00 | 36.00 | 46.67 | 3.16 | Additive |
| Diallyl disulfide | Thymol | 0.25+4 | 30 | 20.00 | 33.33 | 46.64 | 56.67 | 2.16 | Additive |
| Diallyl disulfide | Thymol | 0.5+2 | 30 | 33.33 | 20.00 | 46.64 | 50.00 | 0.24 | Additive |
| Diallyl disulfide | Thymol | 0.5+4 | 30 | 33.33 | 33.33 | 55.55 | 60.00 | 0.36 | Additive |
| Diallyl disulfide | Trans-anethol | 0.25+2 | 30 | 20.00 | 20.00 | 36.00 | 30.00 | - | Antagonist |
| Diallyl disulfide | Trans-anethol | 0.25+4 | 30 | 20.00 | 30.00 | 44.00 | 33.33 | - | Antagonist |
| Diallyl disulfide | Trans-anethol | 0.5+2 | 30 | 33.33 | 20.00 | 46.66 | 33.33 | - | Antagonist |
| Diallyl disulfide | Trans-anethol | 0.5+4 | 30 | 33.33 | 30.00 | 53.33 | 40.00 | - | Antagonist |
| Diallyl disulfide | α-pinene | 0.25+2 | 30 | 20.00 | 16.67 | 33.34 | 20.00 | - | Antagonist |
| Diallyl disulfide | α-pinene | 0.25+4 | 30 | 20.00 | 23.33 | 38.66 | 23.33 | - | Antagonist |
| Diallyl disulfide | α-pinene | 0.5+2 | 30 | 33.33 | 16.67 | 44.44 | 30.00 | - | Antagonist |
| Diallyl disulfide | α-pinene | 0.5+4 | 30 | 33.33 | 23.33 | 48.88 | 30.00 | - | Antagonist |
| Thymol | Trans-anethol | 2+2 | 30 | 20.00 | 20.00 | 36.00 | 43.33 | 1.49 | Additive |
| Thymol | Trans-anethol | 2+4 | 30 | 20.00 | 30.00 | 44.00 | 50.00 | 0.82 | Additive |
| Thymol | Trans-anethol | 4+2 | 30 | 33.33 | 20.00 | 46.66 | 53.33 | 0.95 | Additive |
| Thymol | Trans-anethol | 4+4 | 30 | 33.33 | 30.00 | 53.33 | 56.67 | 0.21 | Additive |
| Thymol | α-pinene | 2+2 | 30 | 20.00 | 16.67 | 33.34 | 56.67 | 16.32 | Synergy |
| Thymol | α-pinene | 2+4 | 30 | 20.00 | 23.33 | 38.66 | 60.00 | 11.78 | Synergy |
| Thymol | α-pinene | 4+2 | 30 | 33.33 | 16.67 | 44.44 | 66.67 | 11.12 | Synergy |
| Thymol | α-pinene | 4+4 | 30 | 33.33 | 23.33 | 48.88 | 73.33 | 12.23 | Synergy |
| Trans-anethol | α-pinene | 2+2 | 30 | 20.00 | 16.67 | 33.34 | 23.33 | - | Antagonist |
| Trans-anethol | α-pinene | 2+4 | 30 | 20.00 | 23.33 | 38.66 | 23.33 | - | Antagonist |
| Trans-anethol | α-pinene | 4+2 | 30 | 30.00 | 16.67 | 41.67 | 30.00 | - | Antagonist |
| Trans-anethol | α-pinene | 4+4 | 30 | 30.00 | 23.33 | 46.10 | 33.33 | - | Antagonist |

## 4. Discussion

In the present study, we demonstrated that the mixing of some secondary metabolites of plant essential oils can result in significant synergism to increase their toxicity. In general, fumigant and contact toxicity of essential oils have been successfully studied. Findings demonstrated that essential oils penetrate into insect respiratory system and cuticle (Kim *et al.*, 2010). Physical properties of essential oils, such as low vapor pressure, limit their application in stores. Although the application of traps baited with a mixture of diet and essential oil to prevent essential oil residues and food tainting in the products seems to be a useful technique to control the stored pests, there is a lack of data on the impact of essential oils on insect gustatory reaction and their antifeedent effects (Dubey, 2011; Hernández-Lambraño *et al*., 2014). Koul *et al.* (2013) suggested that the relation between insect gustatory reaction and toxic effect of plant secondary metabolites is species specific, which may vary among insects.

Some monoterpenoids, such as thymol, 1,8-cineole, pulegone, Fenchone, S-carvone, γ-terpinene, geraniol and etc., were recommended as alternatives to synthetic insecticides against pests (Tripathi *et al.*, 2009; Lopez *et al.*, 2008, 2010; Kumrungsee *et al.*, 2014). In the present study, diallyl disulfide was the most toxic compound on *T. castaneum* fourth larval instar, followed by thymol, trans-anethole and α-pinene.

Many researchers have studied bactericidal and insecticidal activities of garlic, *Allium sativum* (Liliaceae) essential oil against pests. The authors found that diallyl disulfide, as a major compound of garlic essential oil was highly toxic against *T. confusum* and *S. oryzae* (Huang *et al.*, 2000; Yang *et al.*, 2010; Saglam and Ozder, 2013).

Trans-anethole exhibited fumigant toxicity (with $LC_{50}$= 5.02 mg/L air) on *S. oryzae* at 24 h. (Kim *et al*., 2013). Kumrungsee *et al*. (2014) reported that thymol was highly toxic to *P. xylostella* ($LD_{50}$ = 0.22 ug/larva). Similarly, Kim *et al.* (2010) showed that thymol, α-pinene, camphene, p-cymene, and γ-terpinene were highly toxic to *T. castaneum* adults. Thymol binds GABA-gated chloride channels on the membrane of postsynaptic neurons and disrupts the function of GABA synapse (Priestley *et al*., 2003). It was proposed that biological activities of monoterpenoids are associated with molecular configuration, position and nature of functional groups (Tripathi *et al.*, 2009). The minor structural diversity of monoterpenoids may be accounted for the significant differences in their mode of action of insecticidal activity.

Synergistic and additive effects of secondary metabolites are important in plant defenses against herbivores insects. The effects of minor constituents in a complex of plant secondary metabolites may be synergized and enhanced via different mechanisms. Some of the binary mixtures of essential oils and volatile compounds potentially exhibit strong feeding deterrence in pests (Hummelbrunner and Isman, 2001; Faraone *et al*., 2015). In the present study, combination of different concentrations of pure compounds including thymol and α-pinene caused synergistic effects on mortality of *T. castaneum*. In addition, combination of thymol with diallyl disulfide or trans-anethole resulted in additive mortality effect. Some studies have shown that combination of thymol and other compounds increased toxicity on insects, such as Hummelbrunner and Isman (2001) who demonstrated that thyme oil, containing thymol and carvacrol, showed greater toxicity effects than either of the pure compounds. In a similar study, the binary mixture of compounds “thymol and trans-anethole” were synergistic against *S. littura* larva (Hummelbrunner and Isman, 2001; Koul *et al*., 2013). Singh *et al*. (2009) found that a combined treatment of thymol with 1,8-cineole or linalool significantly caused higher mortality than either treatment alone against the third instar of *Chilo partellus* S. (Lep.: Crambidae). However, combinations of “diallyl disulfide and trans-anethole”, “diallyl disulfide and α-pinene” as well as “trans-anethole and α-pinene” were antagonistic in a mixture. Diallyl disulfide was the most toxic of all compounds. “Trans-anethole and α-pinene” caused reduction in the toxicity of diallyl disulfide, which suggests that both of these compounds may be competing for the same receptor site (Kumrungsee *et al*., 2014). The combination of thymol with 1,8-cineole or linalool as well as 1,8-cineole with linalool mixtures showed an antagonistic effect against *P. xylostella* larvae (Kumrungsee *et al*., 2014).

Synergistic power of pure compounds when they are mixed, may depend on compatibility of two constitutes as well as the used concentrations (or doses). Probably, the compounds that cause low mortality, their toxicity may increase in a suitable combination. In addition, Faraone *et al*. (2015) demonstrated that the use of essential oils, such as linalool and thymol, as synergistic agents with conventional pesticides (imidacloprid and spirotetramat) may lead to greater uptake of essential oils for crop protection. Our findings confirmed that α-pinene has low toxicity but combinations of “thymol and α-pinene” demonstrated the highest synergic effect on *T. castaneum*.

Unlike the synergism observed in some mixtures, in several cases the insecticidal activity of essential oil components was antagonized when mixed. We found that diallyl disulfide was most toxic alone but in binary mixture showed low effectiveness.

## 5. Conclusion

In conclusion, the individual monoterpenoids, and their mixtures showed a potential to be developed as possible natural insecticides for the control of *T. castaneum* but needs further evaluation to enhance their insecticidal and antifeedent activity and effects on insect digestive physiology when fed on contaminated diets in the stores.

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