

Article

Ovicidal and adulticidal effects of synthetic menthol, thymol and their mixtures against *Tetranychus urticae* (Acari: Tetranychidae)

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Abstract

The two-spotted spider mite, *Tetranychus urticae* Koch, is a polyphagous pest that causes considerable damage to field, horticultural and greenhouse crops. Currently, many plant essential oils and their active compounds have received attention because of their lethal effect against arthropod pests such as herbivorous mites. In this study, contact toxicity of three formulated compositions based on synthetic thymol and menthol including menthol 5%, thymol 5%, and a mixture of menthol 5% + thymol 5% tested against eggs and female adults of *T. urticae*. Adulticidal results showed that menthol 5% + thymol 5% had the lowest LC₅₀ value (656.77 µl/l) 24 hours after the treatment. Likewise, the mixture was highly effective against *T. urticae* eggs and had the lowest LC₅₀ value (967.24 µl/l). Although, the LC₅₀ value of menthol 5% and menthol 5% + thymol 5% were not significantly different from each other but both of them had a significant difference with the thymol 5% in manner of adulticide and ovicidal. Results also showed that a combination of thymol and menthol increased lethal effects compared to these two compounds.

Key words: Thymol, menthol, LC₅₀, bioassay, synergistic effect.

Introduction

The two spotted spider mite, *Tetranychus urticae* Koch (Acari: Tetranychidae) is an important pest and infests many crops causing reduction in plant productivity or even kills the host plants (Nachman & Zemek 2002; Zhang 2003). This species is a serious and destructive pest of crops, fruits and ornamental plants worldwide. Two-spotted spider mites damage plants both directly and indirectly. In their direct damage, they suck the chloroplasts on the upper surfaces of leaves and leave behind pale whitish yellowish spots. If the damage intensifies, the damaged leaves dry up and drop, and the plants may be totally covered by web and die before the reproductive stage (Zhang 2003). In their indirect damage, photosynthesis and transpiration levels drop, gas exchange through stomata is disrupted and eventually, the host plant dies (Janssen *et al.* 1997). Temperature, host plants, age, host leaf surface structure, and moisture are among factors influencing mite growth and the damage inflicted on crops (Wermelinger

et al. 1997). Chemical control is the most common manner for managing spider mites (Pontes *et al.* 2007). Chemical pesticides cause serious problems like pesticide resistance, secondary pest outbreak, pest resurgence and toxic residues in the environment (Isman 1999). Under greenhouse conditions, short life cycle and high reproductive potential of spider mites, combined with frequent pesticide applications, result in even more quick resistance to numerous miticides (Ambikadevi & Samarjit 1997). Spider mites have splayed resistance to more than 93 acaricides, and resistance has been reported from more than 105 countries (Whalon *et al.* 2012). Resistance and toxicity problems of the synthetic insecticides have resulted in the exigency of finding more effective and healthier alternatives. Hence, the alternative method for replacing the using of synthetic insecticides is needed. Among existing methods, essential oils have been suggested as alternative sources for control. Essential oils derived from many plants are known to possess biological activity against prokaryotic (Deans & Ritchie 1987) and eukaryotic organisms (Konstantopoulou *et al.* 1992). Many plants, including garlic (*Allium sativum* L.), rosemary (*Rosmarinus officinalis* L.), cinnamon (*Cinnamomum verum* J. Presl), and cedar (*Cedrus* spp.), have been used to control a variety of insects (Isman 2004). Many of plant compounds are selective to pests because they do not have side effects on the environment and non-target organisms or their effect is slight (Isman 2000). Thus, much attention has been focused on them as potential sources of commercial acaricide largely because certain plant essential oil preparations and their constituents meet the scales of minimum risk pesticides (USEPA 1996, 2009). Therewith, essential oils have a broad spectrum of insect and mite activity due to the presence of several modes of action including inhibition of molting, repellent and antifeedant activities and reduction in fecundity and growth (Saxena 1989; Arnason *et al.* 1993; Isman 2000; Enan 2001; Akhtar & Isman 2004). Well-documented records show that before 1850, 20 plant species belonging to 16 different families were used for control of agricultural and horticultural pests in Western Europe and China (Smith & Secoy 1981; Needham 1986). The rich knowledge of plants with pesticide properties was not lost in China as evidenced by a recent report stating that in China different parts or extracts of 276 plant species are used as pesticides (Yang & Tang 1988). New probe shows the capability of Lamiaceae essential oils to control *T. urticae* (EL-Zemity *et al.* 2009). Thymol and menthol are the important compounds of essential oils that extracted from Lamiaceae family plants (Buchanan & Shepherd 1981). Numerous studies have distinguished the antimicrobial effects of thymol, ranging from inducing antibiotic susceptibility in drug-resistant pathogens to strong antioxidant properties (Ündeğer *et al.* 2009). Thymol has been used to successfully control varroa mites and prevent zymosis and the growth of mold in bee colonies (Ward 2006). Also, menthol is an organic compound made synthetically or obtained from cornmint, peppermint or other mint oils. The potential of menthol to control of some arthropod pest have been reported (Badawy *et al.* 2010; Cavalcanti *et al.* 2010).

The aim of this study was to evaluate the lethal effect of thymol and menthol as well as the mixture of both compounds as formulated compositions against adult and egg stages of two spotted spider mite, *T. urticae*.

Materials & Methods

Culture colony

Spider mites were reared on bean plants *Vigna unguiculata* (L.) in the Research Institute of Biotechnology, University of Zanjan, without exposure to any pesticide.

Formulated compositions

Synthetic thymol, menthol and their mixture with a ratio of 1:1 were obtained from Barij Essence Medicinal Plants Research Centre as emulsifiable liquid.

Bioassays

A modified leaf-dipping method described by Sokeli & Karaka (2005) was used to assess the lethal effect of these formulated compositions on *T. urticae*. Preliminary tests (bracketing tests) were conducted to determine five doses causing 10 to 90 percent mortalities. Each treatment was replicated four times with the five doses. Each experimental unit was kept in Petri dishes (9 cm) and consisted of a bean leaf disk, placed on cotton pads that had been soaked in water. A number of 15 adult females of the same age (17 ± 1 days) were placed on the surface of each bean leaf disk. The mites were allowed to acclimate for three hours. Subsequently, the bean leaf disks including mites were dipped for five seconds in prepared concentrations that had obtained from bracketing tests (Roh *et al.* 2011). Also, a Control treatment with distilled water was included. After drying at room temperature for 10 min., the leaf disks were transferred into growth chamber at 25 ± 1 °C and $70 \pm 5\%$ RH under a 16:8 LD photoperiod. Mortality was determined 24h after treatment. The mites were considered dead if no movement was apparent after probing with a fine brush (Miresmailli *et al.* 2006). In the case of ovicidal effect, seven mature female mites were released on each leaf disk. The mites were given 24 hours to lay eggs then a fine brush was used to remove mites and only eggs were left on leaf surfaces. Therefore, one-day old eggs were used to conduct the experiments. Ovicidal test accomplished by dipping the eggs into suspension of formulated composition same as adulticidal assay. The number of unhatched eggs counted after four days.

Data Analysis

The LC₅₀ values were determined using POLO-PC software (LeOra Software 1987). The lethal dose ratios were calculated according to Robertson *et al.* (2007). The Sigma Plot 12.3 software was used to graph the probits versus the log of the concentrations. The Chou-Talalay's method (1984) was used to investigate synergistic effects and classifying the interactions. The combination index was calculated by the following formula:

$$CI_x = \frac{LC_x^{\text{Thymol (m)}}}{LC_x^{\text{Thymol}}} + \frac{LC_x^{\text{Menthol (m)}}}{LC_x^{\text{Menthol}}}$$

Where LC_x^{Thymol} and LC_x^{Menthol} indicate doses of thymol and menthol needed to cause x mortality when used alone and $LC_x^{\text{Thymol (m)}}$ and $LC_x^{\text{Menthol (m)}}$ indicate doses of thymol and menthol needed to cause the same mortality when used in combination.

Dose-Reduction Index (DRI) was calculated using the following formula to measure how much the dose of thymol and menthol in the combination might be reduced at a given effect level:

$$DRI_{\text{Thymol}} = \frac{LC_x^{\text{Thymol}}}{LC_x^{\text{Thymol (m)}}} \text{ and } DRI_{\text{Menthol}} = \frac{LC_x^{\text{Menthol}}}{LC_x^{\text{Menthol (m)}}$$

Results

Bioassays on adults

The effects of three formulated compositions against the two-spotted spider mites were evaluated by comparison of LC_{50} values. Our results indicated that the tested formulations were toxic against the female adult of two-spotted spider mites and caused more than 80% mortality in high doses (Table 1). Mortality for control (water-treated) was less than 10%.

Table 1. Adulticidal effects of menthol, thymol and their mixture on *T. urticae*

Compounds	N	$LC_{50}(\mu\text{l/l})$	99% Confidence Limits ($\mu\text{l/l}$)		Chi-square (df=2)	Slope \pm SE
			lower	upper		
Menthol 5%	353	744.57	356.15	1306.95	2.69	0.96 \pm 0.14
Thymol 5%	360	1410.65	731.85	2320.72	2.45	1.09 \pm 0.14
Menthol 5% + Thymol 5%	367	656.77	344.39	1092.50	0.53	1.07 \pm 0.14

Furthermore, results show that menthol 5% + thymol 5% had the lowest LC_{50} value (656.77 $\mu\text{l/l}$) and thymol 5% had the highest LC_{50} value (1410.65 $\mu\text{l/l}$) 24 hours after the treatment. Although, the LC_{50} values of menthol 5% and menthol 5% + thymol 5% were not significantly different but both of them had a significant difference with the thymol 5% (Table 2). Both the equality and the parallelism hypotheses for the probit lines were rejected in adulticidal bioassays (Table 3).

Table 2. Lethal dose ratio of the formulated compositions based on essential oils tested on adults of *T. urticae*, 24h after treatment

	Menthol 5% + Thymol 5%	Thymol 5%
Menthol 5%	ratio = 0.882 lower limit = 0.472 upper limit = 1.647	ratio = 1.894* lower limit = 1.015 upper limit = 3.536
Thymol 5%	ratio = 0.465* lower limit = 0.257 upper limit = 0.841	

* There is no significant difference between LC_{50} values, if the lower and upper limits include 1.

Table 3. Equality and the parallelism hypothesis for the probit lines ($\alpha = 0.05$)

	Equality	Parallelism
Chi-square	10.9257	0.5080
Degrees of freedom	4	2
Tail probability	0.027	0.776

If the probability level is greater than 0.05, the hypothesis is accepted; otherwise, it is rejected.

Bioassays on eggs

The effects of three formulated compositions based on essential oils against the eggs of two-spotted spider mites were evaluated by comparison of LC_{50} value. The 24h ovicidal results of the tested compounds are presented in Table 4.

Like the adulticidal bioassays, results showed that the Menthol 5% + Thymol 5% had the lowest LC_{50} value (967.24 $\mu\text{l/l}$) and thymol 5% had the highest LC_{50} value (1410.65 $\mu\text{l/l}$) on eggs of *T. urticae*. The LC_{50} values of Menthol 5% and Thymol 5% were not significantly different but both of them had a significant difference with Menthol 5% + Thymol 5% (Table 5).

Table 4. Ovicidal effects of menthol, thymol and their mixture on *T. urticae*

Compounds	N	$LC_{50}(\mu\text{l/l})$	99% Confidence		Chi-square	Slope \pm SE
			Limits ($\mu\text{l/l}$)			
			lower	upper		
Menthol 5%	352	4303.47	2713.13	6137.09	2.25	1.51 \pm 0.20
Thymol 5%	523	6078.94	4548.89	7686.01	0.36	1.78 \pm 0.19
Menthol 5% + Thymol 5%	415	967.24	598.89	1475.72	0.91	1.07 \pm 0.12

Both the equality and the parallelism hypotheses for the probit lines were rejected in ovicidal bioassays (Table 6). Results also show that the combined use of thymol and menthol increased the lethal potentialities of these two compounds in adulticidal and ovicidal bioassays. In other words, the combined use of these two compounds had greater effects compared to the individual use of either of them. In cases where the combination index is higher than 1 has the antagonistic effect; if it is less than 1, the combined use of the two compounds has synergistic effects; however, if the combination index is equal to one, the combined use of the compounds has additive effects. Results showed that the synergistic interaction of the two components in the mixture of 1:1 ratio on eggs is stronger than on adults. The synergistic effect of using thymol and menthol in combination is shown in Table 7.

Table 5. Lethal dose ratio of the formulated compositions based on thymol, menthol and their mixture tested on eggs of *T. urticae*.

	Menthol 5% + Thymol 5%	Thymol 5%
Menthol 5%	ratio= 0.244* lower limit = 0.144 upper limit = 0.350	ratio= 1.412 lower limit = 0.991 upper limit = 2.012
Thymol 5%	ratio= 0.159* lower limit = 0.108 upper limit = 0.234	

* There is no significant difference between LC_{50} values, if the lower and upper limits include 1.

Figure 1 show the dose-reduction index plot at 0.25, 0.5 and 0.75 effect levels for egg and adult of the two spotted spider mite. Combined use of menthol and thymol led to favourable dose reduction of the both components especially in ovicidal bioassays.

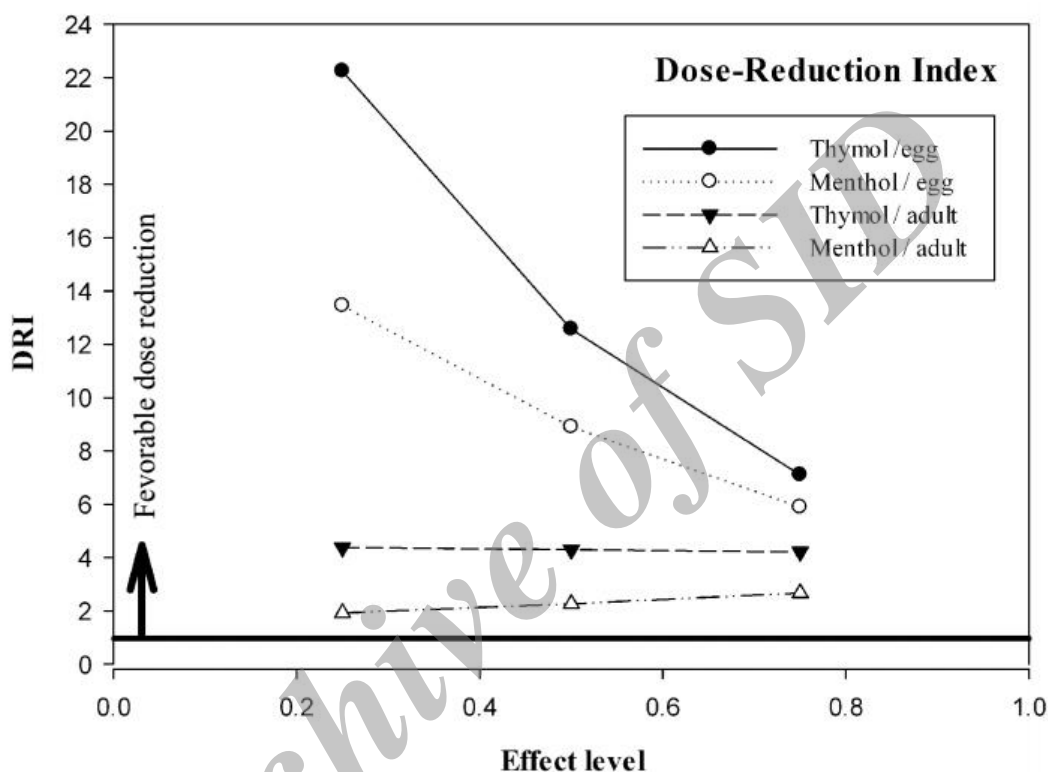
Table 6. Equality and the parallelism hypothesis for the probit lines ($\alpha = 0.05$)

	Equality	Parallelism
Chi-square	92.2026	11.1428
Degrees of freedom	4	2
Tail probability	0.000	0.004

If the probability level is greater than 0.05, the hypothesis is accepted; otherwise, it is rejected.

Table 7. Combination index (CI) of the thymol and menthol mixture (at 1:1 ratio) on the egg and adult stages of *T. urticae*

Lethal concentration level	CI value			
	Egg	Interaction	Adult	Interaction
LC ₂₅	0.119	Strong synergism	0.747	Moderate synergism
LC ₅₀	0.191	Strong synergism	0.673	Synergism
LC ₇₅	0.310	Synergism	0.611	Synergism

**Figure 1.** The dose-reduction index plot at 0.25, 0.5 and 0.75 effect levels.

Discussion

In this study, we found that menthol and thymol have about 5.8 and 4.3-fold less contact ovicidal vs. adulticidal potency, respectively. Similarly, Badawy *et al.* (2010) showed limited contact effect of these two monoterpenoid on hatching rate of *T. urticae* eggs. In fumigant toxicity tests, although Lim *et al.* (2011) found significant inhibition of egg hatching at 25 °C, overall they concluded that the adults are more sensitive than the eggs to essential oils and their constituents. The same conclusion has been affirmed by Tsolakis & Ragusa (2008) for a commercial product of vegetable, essential oils and fatty acid potassium salts. The toxicity is the resultant of toxicokinetics and toxicodynamics processes (Walker *et al.* 2012). Definite explanation of minor sensitivity of the egg stage to these compounds needs to know the exact mode of action of essential oils and their compounds as well as the mite's egg physiology and anatomy but very little information is available. Here we give some hypothetical reasons: Because of large surface-to-volume ratio of the mites' eggs, their chorion is waterproofed to prevent from desiccation.

This causes suppression of gas exchanges, too. However, a specialized device as an air-duct system is used as respiratory system (Dittrich & Streibert 1969). This organ is formed and connected to egg shell in *T. urticae* a few hours before egg hatching. It has been suggested that the organ is an important route of entry for some acaricides (Dittrich & Streibert 1969; Dittrich, 1971). So, it could be concluded that the tested compounds on early eggs could not penetrate well until the respiratory ducts are developed within the eggs. If so, it may be also assumed that the compounds undergo some degradation by the time of formation of their site of action. Increase in *Acanthoscelides obtectus* egg susceptibility to essential oils with age has been shown by Papachristos & Stamopoulos (2002). According to Thurling (1980), *T. cinnabarinus* (*T. urticae* red form now) eggs has lower metabolic rate than all active foraging life stages. On the other hand, low metabolic rate may reduce the rate of penetration (Brooks 1976). Therefore, the less effectiveness of essential oils on eggs than adults could be attributed to this phenomenon, too. However, the above mentioned reasons could not explain our finding of comparable ovicidal effect of the mixture of menthol and thymol to that of adulticidal effects of both of them when applied alone. Besides, unlike the situation mentioned for menthol and thymol, when using their mixture, the relative potency of adulticidal effect vs. ovicidal was lower (about 1.5-fold).

Comparison of efficiency of menthol and thymol revealed that menthol is much effective both on egg and adult stages than thymol. Our result is in accordance with Badawy *et al.* (2010). These authors have reported the higher contact as well as fumigant effect of menthol than thymol against *T. urticae*. However, Erler & Tunç (2005) evaluated thymol as more effective fumigant than menthol on *T. cinnabarinus*. In another work, Novelino *et al.* (2007) found thymol more potent than menthol on larvae of the tick *Boophilus microplus*.

As has been indicated by Rice & Coats (1994), different application method can lead to different bioactivities of these compounds. They found menthol more effective on the house fly, *Musca domestica* L. than thymol as fumigant. Nevertheless, thymol had more effect in contact bioassay. This difference could be related to much more volatile property of menthol than thymol due to structural difference (Novelino *et al.* 2007). Similar to our results they also reported higher ovicidal activity for menthol.

As the essential oils are composed of various constituents, it is thought that mixture of some components may enhance the overall efficiency. There are a number of studies exploring combined effects of essential oils constituents. Synergistic effect of thymol in some combinations has been shown (Hummelbrunner & Isman 2001; Passreiter *et al.* 2004; Cavalcanti *et al.* 2010; Pavela 2010; Lim *et al.* 2011). As far as we know, this is the first time to reveal the combined pesticidal effects of menthol and thymol. We found from moderate (on adults) to strong (on eggs) synergistic interaction of the two components in the mixture of 1:1 ratio (Table 8). As clearly is shown in Fig. 3, at the effect level of 0.25 (i.e. applying LC₂₅) 22.2 and 13.4-fold reductions of thymol and menthol doses is achieved, respectively when they applied in combination on the egg stage. It is evident from Fig. 3 that the DRI values depend on the lethal concentration as well as the stage tested. In addition, the combination ratio may affect the results.

Our results confirm possibility of the idea of advantageous use of mixing essential oils components. However, testing various ratios in combinations, different life stages and taking into account the effect level are suggested to better evaluation. Also, the authors suggest performing ecologically reliable methods like life table response experiments will help to get comprehensive and integrative information. Meanwhile,

testing essential oils, their individual components and especially their combinations on beneficials are needed to decide whether they are suitable for IPM programs.

Conclusion

Our results show a significant potential of synthetic menthol and thymol in management of the two-spotted spider mite damage. Results also reveal that a combination of thymol and menthol increased the lethal effect of formulated composition against *T. urticae*. In conclusion, based on the results from the current research, it can be stated that synthetic menthol and thymol are in possession of a great potential to be used for management of phytophagous mite, *T. urticae* as an integrated pest management tool.

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اثر تخم‌کشی و بالغ‌کشی متول و تیمول سنتتیک به تنهایی و مخلوط آنها علیه *Tetranychus urticae* (Acari: Tetranychidae)

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چکیده

کنه تارتن دولکه‌ای، *Tetranychus urticae* Koch، آفتی چندخوار است که به گیاهان مختلف اعم از زراعی، باغی و گلخانه‌ای خسارت می‌زند. در سال‌های اخیر بسیاری از اسانس‌های گیاهی و ترکیبات فعال آن‌ها به علت اثرات کشندگی روی بندپایان آفت مانند کنه‌های گیاهی مورد توجه قرار گرفته‌اند. در این پژوهش، اثر کشندگی تماسی سه ترکیب فرموله شده بر پایه تیمول و متول سنتتیک شامل: متول ۰.۵٪، تیمول ۰.۵٪ و متول ۰.۵٪ + تیمول ۰.۵٪ علیه مرحله تخم و ماده بالغ کنه تارتن دولکه‌ای مورد بررسی قرار گرفت. نتایج آزمایش‌های بالغ‌کشی نشان داد که متول ۰.۵٪ + تیمول ۰.۵٪ دارای کم‌ترین مقدار LC₅₀ (۶۵۶/۷۷ میکرولیتر بر لیتر)، ۲۴ ساعت پس از تیمار بود. مشابه آزمایش‌های بالغ‌کشی متول ۰.۵٪ + تیمول ۰.۵٪ بیش‌ترین کشندگی و کم‌ترین مقدار LC₅₀ (۹۶۷/۲۴) میکرولیتر بر لیتر) را علیه مرحله تخم کنه تارتن داشت. اگرچه، LC₅₀ متول ۰.۵٪ و ترکیب متول ۰.۵٪ + تیمول ۰.۵٪ اختلاف معنی‌داری با یکدیگر نداشتند اما هر دو ترکیب اختلاف معنی‌داری با تیمول ۰.۵٪ از نظر میزان کشندگی علیه مرحله تخم و بالغ کنه تارتن داشتند. هم‌چنین نتایج نشان داد که کاربرد توأم تیمول و متول باعث افزایش اثر کشندگی در مقایسه با هریک از این دو ترکیب به تنهایی شده است.

کلید واژگان: تیمول، متول، LC₅₀، زیست‌سنجی، اثر هم‌افزایی.

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