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ORIGINAL PAPER

Insecticidal and repellent activities of thymol from the essential oil of *Trachyspermum ammi* (Linn) Sprague seeds against *Anopheles stephensi*

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Abstract Essential oil of seeds of *Trachyspermum ammi* (Linn.) Sprague and its pure constituent thymol showed promising results when evaluated for larvicidal, oviposition-deterrent, vapor toxicity, and repellent activity against malarial vector, *Anopheles stephensi*. Thymol was 1.6-fold more toxic than the oil toward fourth-instar larvae of *A. stephensi* with LD_{50} values of 48.88 and 80.77 μg/ml, respectively. Egg laying by female adults of *A. stephensi* was much significantly reduced when exposed to vapors of thymol compared to the oil of *T. ammi* seeds, and similar effects were recorded for subsequent egg hatching and larval survival. Vapor toxicity assay showed LC_{50} value of 79.5 mg/mat for thymol against adults of *A. stephensi*, whereas the crude oil exhibited the LC_{50} value of 185.4 mg/mat. Thymol provided complete repellency toward *A. stephensi* adults at the dose of 25.0 mg/mat after 1 h duration, whereas same degree of repellency was obtained by the oil at the dose of 55.0 mg/mat, indicating its double-fold activity than the oil.

Introduction

Anopheles stephensi Liston is responsible for causing malaria in India and other western countries (Burfield and Reekie 2005). Pyrethrin-based products have been widely used to protect people from mosquito bites through their repellent and killing effects. However, these synthetic products do not provide complete protection, and their cost is also prohibitive for low socioeconomic groups. Therefore, efforts are continuing to seek natural repellents or insecticides as safer alternatives to synthetic insecticides which are nonbiodegradable, with high mammalian toxicity, and also faced vector resistance (WHO 1987).

Several phytochemicals extracted from various botanical sources have detrimental effects on mosquitoes (Syamala Devi and Vasudevan 1995). Essential oils provide a rich source of biologically active monoterpenes and are well documented for bioactivities against insect pests. Some of the essential oils with promising mosquito control potential are plant from genus *Tagetes* spp. (Vasudevan et al. 1997), *Ocimum* spp. (Bhatnagar et al. 1993), *Cymbopogon* spp. (Ansari and Razdan 1995), and *Mentha* spp. (Ansari et al. 2000) etc. Further, essential oils of cassia, camphor, wintergreen, pine, and eucalyptus are already being used in several commercial products for mosquito control (Ansari and Razdan 1994). The essential oils are generally considered nontoxic to human beings (Bagvan et al. 2008) apart from their uses in flavoring, pharmaceuticals, and confectionary industries.

Seeds of *Trachyspermum ammi* (Apiaceae) commonly known as ajowan, has widespread use as culinary spice and contains thymol as a major constituent.

Thymol has been reported as the major constituent of other medicinal plants like *Carum copticum* L. and *Semenovia tragioides* (Boiss.) Manden (Masoudi et al. 2002), *Satureja pilosa* Velen (Konakchiev and Tsankova 2002), *Nigella sativa* L. (Enomoto et al. 2001), *Oliveria decumbens* (Amin et al. 2005), *Thymus* species (Meshkatalsadat et al. 2007), *Ocimum gratissimum* (Martins et al. 1999; Koba et al. 2007), and *Aeollanthus pubescens* Benth. (Koba et al. 2007; Sonda et al. 1999) etc.

Bioactivities of thymol has been documented: acaricidal properties against mites, *Acarapis woodi*, *Tyrophagus putrescentiae* (Schrank) and *Varrora jacobsoni* (Calderone

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et al. 1997; Ellis and Baxendale 1997; Kuwahara 1982); insecticidal against beetles, *Tetranychus urticae* (El-Gengaihi et al. 1996), and *Acanthoscelides obtectus* (Say) (Regnault-Roger and Hamroui 1995); nematicidal toward, *Caenorrhabditis elegans* (Tsao and Yu 2000); molluscicidal against *Lymnacea accuminata* (Singh et al. 1999); antibacterial against bacteria, *Escherichia coli* (Calcuttawalla et al. 2002; Helander et al. 1998); toxic to slug, *Deroceras reticulatum* (Powell and Bowen 1996); fungitoxic toward *Macrophomina phaseolina* (Tassi) (Gold.) (Dwivedi and Singh 1998); and genotoxic toward *Drosophila* spp. (Karpouhtsis et al. 1998).

However, the essential oil of *T. ammi* seed and its major constituent, thymol has not been evaluated against insect-pests of public health importance. For the first time, the present investigation is aimed to study the efficacy of *T. ammi* oil and thymol against malarial vector, *Anopheles stephensi* as larvicidal, oviposition-deterrent, vapor toxicant, and repellent.

Materials and methods

Essential oil extraction

Seeds (500 g) of *T. ammi* were steam distilled for 4–5 h in a clevenger type apparatus to extract the oil (Senthilkumar et al. 2008). The gas chromatography of the essential oil was done on a Varian Gas Chromatogram, model CX-3400, under the conditions: carrier gas hydrogen, injector (detector FID) temperatures, 220°C and 225°C, respectively, capillary column (Supelcowax –10, 30 m×0.32 mm, film thickness 0.25 μm); and temperature programmed from 2 min at 40°C to 270°C at 5°C/min. The area percentage was obtained on Varian 4400 integrator. The identity of the component was assigned by comparing their retention time with those of authentic samples. Thymol (99.5 % purity) was purchased from M/s SIGMA Co., USA.

Test organism

Malarial vector mosquito, *A. stephensi* was reared in the laboratory. The larvae were fed on 5% yeast suspension. Adults were provided with 10% sucrose solution and rabbit for blood meal. Gravid females were used to obtain egg, larva, and adult. All the bioassays were conducted at 28±2°C, 70–88% relative humidity, with a photoperiod of 12:12 (L/D).

Bioassay

Larvicidal test

Essential oil of *T. ammi* and thymol were evaluated at the level of 0.0, 25.0, 50.0, 75.0, 100.0, 125.0, and 150.0 μg/ml in tap water. Tween-80 was used as emulsifier at a concentration of 0.001%. Tap water mixed with Tween-80 was used as control. Standard WHO test (WHO 1981) was employed with slight modification in the test procedure. A single fourth-instar larva of *A. stephensi* was put into each of 20 vials containing 5.0 ml of the test solution of each concentration. Observation on larval mortality was recorded after 24 h. Larvae were considered dead, when they did not react to touching with a needle. Data recorded on larval mortality were analyzed statistically for LC_{50} and LC_{95} values.

Oviposition-deterrence

The effect of *T. ammi* seed oil and thymol on oviposition and subsequent egg hatching by *A. stephensi* were studied by introducing 20 gravid females (fed on rabbit blood) and unlimited number of males from a laboratory colony in a 25 × 25 × 25 cm oviposition cages under choice conditions. The cages contained seven 100-ml glass dishes with 0.0, 10.0, 25.0, 50.0, 75.0, 100.0, and 125.0 μg/ml concentrations of the oil and 0.0, 5.0, 10.0, 20.0, 40.0, 80.0, and 100.0 μg/ml of the compound. Each cage had a control glass dish having only tap water with 0.001% of Tween-80. The test was replicated four times. The number of eggs was counted for 7 days. The laid eggs were observed for hatching and subsequent survival up to second larval instar only. Oviposition inhibition percentage was calculated according to Mulla et al. (1974).

Vapor toxicity

The essential oil of *T. ammi* and thymol were dissolved in acetone to make desired concentrations. Aliquot (0.50 ml) of the test solution was dispensed over a cardboard sheet (mat) of size (22×35 mm) and thickness (2.5 mm) equal to commercially available mosquito mats, so that amount of the oil and thymol received per mat were 0, 50, 100, 200 300, 400, and 500 mg and 0, 25, 50, 100, 150, 200, and 250 mg, respectively. The solvent was allowed to evaporate at room temperature. The treated cardboard sheet was placed on a mosquito mat machine, and machine was kept on for 15 min. The cardboard used in the control was dispensed with acetone only. Vapor toxicity was evaluated in a specially designed apparatus (Tripathi et al. 2004).

Fifty 6- to 8-day-old females of *A. stephensi* were used. Cardboard mat treated with essential oil of *T. ammi* along with mat machine was kept at the corner in the cage. Observations on adult mortality at varying dosages were recorded at 1 h after the treatment. The experiment was repeated three times. Before the start of every experiment, all the chambers were thoroughly washed with soap water and

Table 1 GC analysis of the essential oil of *T. ammi* used for bioassay studies

Major constituents	Percentage
β-Pinene	2.26
p-Cymene	17.39
γ-Terpenene	10.12
Linalool	1.03
Thymol	66.96
Others	2.24

detergent, and dried properly. Data recorded on adult mortality were analyzed statistically for LC_{50} and LC_{95} values.

Repellency

The essential oil of *T. ammi* and thymol were dissolved in acetone to make desired concentrations. Repellency was also evaluated in a specially designed apparatus (Tripathi et al. 2004) as mentioned in vapor toxicity assay, but with further attachments. For repellency studies, the criteria was migration of mosquitoes from one chamber to another connected by tunnel after 1 h for assessment of true repellency. A rabbit (anaesthetized) was placed on the copper wire mesh surface of chamber. Thirty adult female mosquitoes were used for the test.

Aliquot (0.50 ml) of the test solution of the oil and compound were dispensed separately over a cardboard sheet (mat) of size (22×35 mm) and thickness (2.5 mm) equal to commercially available mosquito mats so that each mat received 0, 5, 15, 25, 35, 45, and 55 mg and 0, 2, 5, 10, 15, 20, and 25 mg of the oil and thymol, respectively. The solvent was allowed to evaporate at room temperature. The treated cardboard sheet was placed on a mosquito mat machine, and the machine was kept on for 15 min. The cardboard used in control was dispensed with acetone only. After 1 h experimental duration, the number of mosquitoes present in both two chambers was counted, and percent repellency was calculated as:

$$\%\ \text{repellency} = \frac{\%\ \text{mosquitoes observed in chamber 'C'} - \%\ \text{mosquitoes observed in chamber 'B'}}{100 - \%\ \text{mosquitoes observed in chamber 'B'}} \times 100$$

Statistical analysis

Probit analysis (Finney 1971) was used to analyze lethal doses (LC_{50}/LD_{50} and LC_{99}/LD_{99}) of the oil and thymol. Linear regression was used to describe the relationship between dosage–mortality (SPSS 1999).

Results

Oil extraction

GC analysis of the oil revealed thymol (66.96%) as major constituent (Table 1).

Larvicidal

T. ammi seed oil and thymol showed LD_{50} values of 80.77 and 48.88 µg/ml, respectively, whereas LD_{99} values were observed as 172.12 and 105.49 µg/ml, respectively (Fig. 1). Thus, thymol was 1.65-fold more toxic than the oil itself toward the fourth-instar larvae of *A. stephensi*. At the dose of 100.0 µg/ml, the thymol gave 100.0% mortality, whereas *T. ammi* seed oil resulted into 63.0% larval mortality only at the same dose. Further, regression analysis of the data also showed significant (F=38.90, df=6, P<0.01; F=281.24, df=6, P<0.01) dose-dependent toxicity toward the fourth-instar larvae exposed to thymol and *T. ammi* seed oil, respectively (Fig. 1).

Oviposition-deterrence

Thymol and *T. ammi* seed oil both significantly (F=29.49, df=6, P<0.01; F=341.56, df=6, P<0.01, respectively)

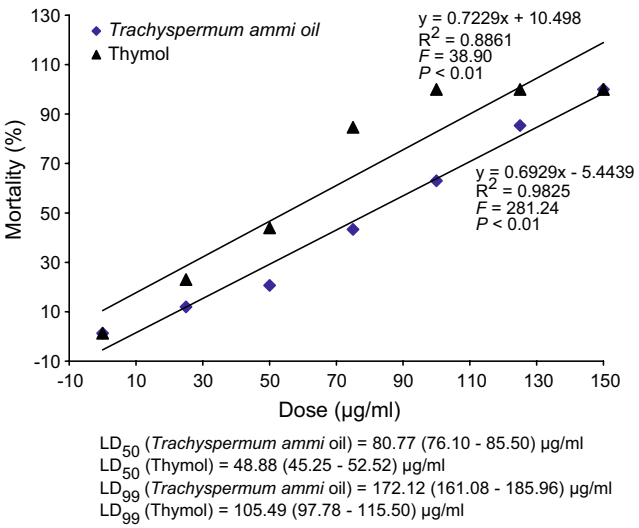

Fig. 1 Dose–response relationships of toxicity of *T. ammi* seed oil and thymol toward mortality of the fourth-instar larvae of *A. stephensi*

Table 2 Effect of *T. ammi* seed oil and thymol on fecundity and fertility of *Anopheles stephensi*

Oil/compound (μg/ml)	Total no. of eggs laid	Mean percent egg hatching	Mean percent survival (2nd instar)	Percent oviposition-deterrence
Trachyspermum ammi				
0	352.0±12.70	98.04±1.89	90.27±1.22	–
10	295.6±5.64	83.50±1.58	54.31±2.80	15.79±1.59
25	244.4±6.01	66.63±3.06	17.50±1.45	30.40±1.19
50	190.8±5.89	38.14±3.08	4.64±1.52	45.72±0.81
75	148.0±10.70	24.00±2.30	0.0±0.00	58.12±1.77
100	73.8±6.86	17.45±2.21	0.0±0.00	79.12±1.51
125	13.6±3.24	0.0±0.00	0.0±0.00	96.20±0.82
Thymol				
0	357.2±12.46	91.90±1.69	90.89±3.23	–
5	275.8±9.18	82.90±2.19	48.29±2.67	22.48±1.02
10	220.8±13.16	55.98±2.04	15.64±2.89	38.13±3.27
20	163.0±17.35	37.20±3.46	0.0±0.00	54.60±4.23
40	102.4±10.42	11.75±1.84	0.0±0.00	71.30±2.78
80	42.6±9.34	0.0±0.00	0.0±0.00	88.11±2.51
100	5.2±2.82	0.0±0.00	0.0±0.00	98.57±0.76

Regression analysis of data *Trachyspermum ammi*: no. of eggs laid: Y=327.8–2.54X, df=6, R^2 =0.99, F=341.6, P<0.01; percent egg hatch: Y=88.5−0.76X, df=6, R^2 =0.95, F=99.9, P<0.01; percent larval survival: Y=56.7−0.59X, df=6, R^2 =0.64, F=8.83, P<0.01; percent oviposition-deterrent: Y=6.75−0.72X, df=6, R^2 =0.99, F=358.1, P<0.01; thymol: no. of eggs laid: Y=275.7−2.99X, df=6, R^2 =0.86, F=29.5, P<0.01; percent egg hatch: Y=71.5−0.87X, df=6, R^2 =0.79, F=18.6, P<0.01; percent larval survival: Y=42.8−0.57X, df=6, R^2 =0.41, F=3.35, P<0.01; percent oviposition deterrent: Y=22.7−0.84X, df=6, R^2 =0.85, F=29.4, P<0.01.

reduced egg laying (oviposition) by *A. stephensi* as concentration increased (Table 2). At the dose of 100 μg/ml, thymol-exposed *A. stephensi* female adults laid only <5.2 eggs, whereas *T. ammi* seed oil exposed adults laid 25.8 times less number of eggs compared to control. Thus, both the compound and oil reduced oviposition by *A. stephensi* adults significantly (F=29.37, df=6, P<0.01; F=358.0, df=6, P<0.01, respectively) as evidenced from percent oviposition-deterrence values (Table 2). Similarly, both the compound and oil significantly (F=18.61, df=6, P<0.01; F=99.80, df=6, P<0.01, respectively) reduced the viability of eggs laid. Further, both the compound and oil significantly (F=3.35, df=6, P<0.01; F=8.83, df=6, P<0.01, respectively) suppressed survival of larvae emerged from laid eggs (Table 2).

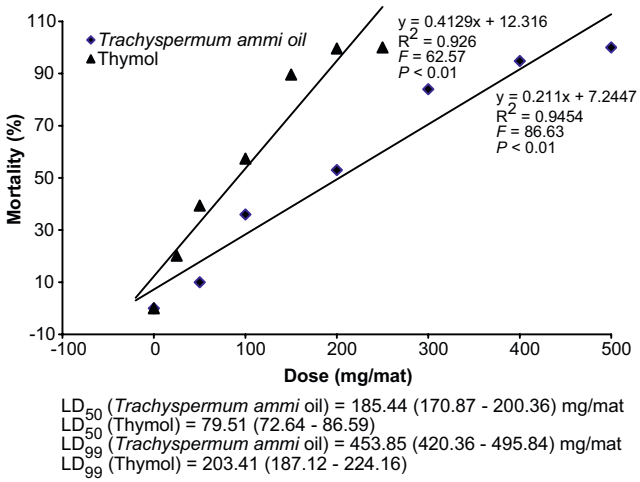

Fig. 2 Dose–response relationships of vapor toxicity of *T. ammi* seed oil and thymol toward adults mortality of *A. stephensi*

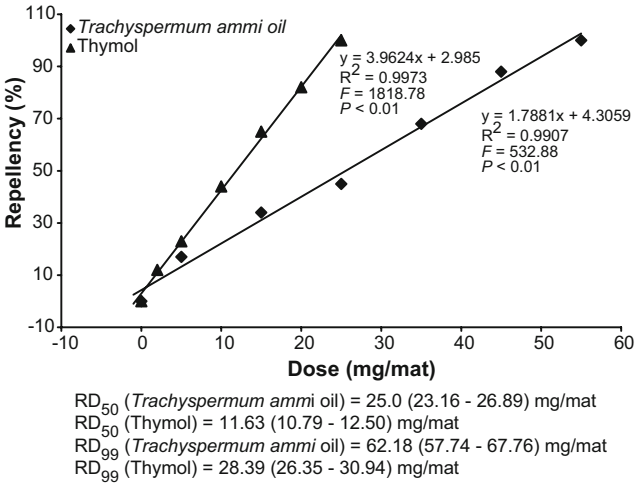

Fig. 3 Repellent responses of adults of *A. stephensi* to the doses of *T. ammi* seed oil and thymol

Thymol caused 37.20% egg hatching at a dose of 20.0 µg/ml and *T. ammi* seed oil caused 24.0% egg hatching at a dose of 75.0 µg/ml, but the emerged larvae could not survive up to the second instar at these doses.

Vapor toxicity

Thymol was highly toxic toward the adults of *A. stephensi* in vapor toxicity assay, as it provided complete adult mortality at two times less dose rate compared to *T. ammi* seed oil (Fig. 2). LC_{50} values of thymol and *T. ammi* seed oil were found to be 79.5 and 185.4 mg/mat, respectively, whereas LD_{99} values observed were 203.41 and 453.85 mg/mat, respectively. Thus, the lethal toxicity of thymol was 2.3 times more to that of *T. ammi* seed oil (Fig. 2). Further, regression analysis of the data also showed significant (F=62.57, df=6, P<0.01; F=86.63, df=6, P<0.01) dose-dependent mortality toward adults exposed to thymol and *T. ammi* seed oil, respectively (Fig. 3).

Repellency

Exposure of thymol to *A. stephensi* adults showed complete repellency at 2.2 times less dose to that of *T. ammi* seed oil after 1 h duration (Fig. 3). At the dose of 25.0 mg/mat, thymol provided complete repellency, whereas *T. ammi* seed oil could achieve a repellency of 45.0% only (Fig. 3). The repellent doses (RD_{50}) observed were 25.02 and 11.63 mg/mat for *T. ammi* seed oil and thymol, respectively.

Discussion

The results of the present investigation showed that pure constituent thymol was twofold more active than essential oil of *T. ammi* seed in vapor toxicity and repellent assays against adults of *A. stephensi*. However, as a larvicidal, thymol was only 1.65-fold more active than the *T. ammi* oil. The importance of toxic and growth-retarding influence of the thymol may have better practical significance, if such effects can also be observed when applied to natural habitat like larval breeding sites. Present investigation revealed the promising potential of thymol as larvicidal, oviposition-deterrent, adulticidal, and repellent against *A. stephensi*. Toxic and growth-retarding activities of thymol makes its wide applications both in larval breeding nitches and household conditions. Thymol has also been reported to be highly toxic (LD_{50}= 25.4 µg/larva) toward the larva of *Spodoptera litura* (Hummelbrunner and Isman 2001) and tracheal mites, *Acarapis woodi* with a LC_{50} value of 0.90 µg/ml (Ellis and Baxendale 1997), whereas it gave LD_{50} value of 48.8 µg/ml toward fourth-instar larvae of *A. stephensi* in our studies. The difference in LD_{50} values may be attributed to the mode of application of the compound. In the case of *A. stephensi* larvae, thymol was mixed in water, whereas in the case of *S. litura* larvae, it was applied topically.

The dose–response relationships reported in this study provide a foundation for future investigations of thymol and *T. ammi* seed oil as vapor toxicant and repellent against adults of *A. stephensi*. Length of exposure, temperature, and humidity are factors that can influence the activity of the test chemicals as vapor toxicants under household conditions and merit further investigations. Likewise, mode of action studies can provide insight on to how best to use *T. ammi* seed oil and thymol. The potential use of these selective and fully biodegradable materials in management of malarial vector is encouraging.

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