Insecticidal and Genotoxic Effects of Essential Oils of Greek sage, *Salvia fruticosa*, and Mint, *Mentha pulegium*, on *Drosophila melanogaster* and *Bactrocera oleae* (Diptera: Tephritidae)[1]

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ABSTRACT The essential oils (EOs) extracted from Greek sage, *Salvia fruticosa,* and mint, *Mentha pulegium*, together with their main constituents, 1,8-cineole, thujone, and camphor and pulegone and menthone, respectively, were tested for insecticidal effects on the olive fruit fly, *Bactrocera oleae* (Diptera: Tephritidae). The EO of Greek sage plant and its main constituents also were screened for toxic and genotoxic activities in *Drosophila melanogaster* (Diptera: Tephritidae). Genotoxic activity was determined using the somatic mutation and recombination (SMART) test. Results showed that mint EO and its main constituents are the most effective insecticides against *B. oleae*. Among the tested constituents of Greek sage oil, 1,8-cineole was found to be the most toxic for *B. oleae*, whereas thujone and 1,8-cineole did not significantly differ in their toxicity against *D. melanogaster.* For both insects, camphor was found to be the weakest insecticide. Greek sage EO, 1,8-cineole and thujone showed negative genotoxic activity, whereas camphor exhibited mutagenic activity. Mixtures of authentic 1,8-cineole–thujone, 1,8-cineole–camphor, and pulegone–menthone, in proportions resembling those in the corresponding oils, showed no synergistic or antagonistic interactions among the main constituents of Greek sage oil, whereas the toxicity of pulegone was suppressed in the presence of menthone, indicating antagonistic interactions. Pennyroyal oil and the compounds pulegone, menthone, 1,8-cineole, and camphor were significantly more effective as insecticides against the pest *B. oleae* than *D. melanogaster*.

KEY WORDS *Bactrocera oleae*, *Drosophila* wing spot test, genotoxicity, *Salvia fruticosa*, *Mentha pulegium*, 1,8-cineole, thujone, camphor, pulegone, menthone

Bactrocera (*Dacus*) *oleae* (Gmelin) (Diptera: Tephritidae) is one of the major agricultural pests of olive-producing countries and causes extremely high annual losses in olive crops (Drew 1989, White & Wang 1992). Although studies of its

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biology, ecology, and genetics (see Mavragani-Tsipidou 2002 and refs. therein) are focused on developing procedures and methods for the management of this pest, to date control has been based mainly on synthetic chemical insecticides. The heavy reliance on these chemicals, however, with the widespread environmental pollution and their possible implications for human welfare and health (Denholm & Rowland 1992) emphasizes the need of more environment-friendly approaches.

In recent years, essential oils (EOs) of aromatic plants have received much attention as potentially useful bioactive compounds with particular emphasis on their antimicrobial, antifungal, cytostatic, and insecticidal activities. Although their insecticidal properties have been known since antiquity, only recently have these plant products been applied as alternative botanical pesticides (Kelsey et al. 1984, Mansour et al. 1986, Balandrin & Klocke 1988, Regnault-Roger et al. 1993, Rice & Coats 1994, Lee et al. 1997, Isman 2000).

In the present study, the insecticidal activity of two of the most commercially exploited oils, from Greek sage *Salvia fruticosa* Mill, and pennyroyal oil extracted exclusively from mint, *Mentha pulegium,* together with their main constituents 1,8-cineole (known as eucalyptol), thujone, camphor, pulegone, and menthone were tested on *B. oleae*. In a second set of experiments, the compounds were screened for their genotoxic effects using the somatic mutation and recombination test (SMART) in *Drosophila melanogaster* (Graf et al. 1984).

Materials and Methods

Plant material. Essential oils from Greek sage containing 51.5% 1,8-cineol, 11.4% thujone (α-thujone 8.3%, β-thujone 3.1%), and 0.9% camphor (Karousou et al. 1998) and mint containing 75.7% pulegone and 10.1% menthone (Franzios et al. 1997) were provided by Dr. Kokkini (University of Thessaloniki, Greece). Authentic commercially available constituents 1,8-cineole, thujone, camphor, pulegone, and menthone, used in the present study, were supplied by Aldrich Chemical Corp. (Gillinham, United Kingdom).

Genetic system. The standard *Bactrocera oleae* laboratory strain, obtained from the olive fruit fly colony of "Democritos" Nuclear Research Center, Athens Greece, was used. This strain has been maintained in our laboratory for more than 10 years and reared on an artificial medium based on yeast hydrolysate, sucrose, and egg yolk in water (Tzanakakis & Economopoulos 1967, Tsitsipis 1977) at 24 ± 1°C with a photoperiod of 12:12 (L:D) h and 60% RH.

Two *Drosophila melanogaster* strains (provided by Dr. F. Marec, Institute of Entomology ASCR, Ceske Budejovice, Czech Republic), the multiple wing hair strain (*mwh*), with genetic constitution *mwh e*/*mwh e* and the flare (*flr*^{3}) strain with genetic constitution *y w*^{co}/*y w*^{co}*; flr*^{3} *se*/*TM2 Ubx*^{130} *se e*, were used in this study (Marec & Gelbic 1994, see also Lindsley & Zimm 1992 for description of the genetic markers). Larvae from the cross between *flr*^{3} virgin females with *mwh* males were used for testing (Graf et al. 1984). The stocks and the crosses were maintained at 24 ± 1°C in photoperiod of 16:8 (L:D) h on a yeast-glucose medium.

Toxicity test on *D.* melanogaster and B. *oleae* larvae. The experimental procedures used for screening the tested compounds for insecticidal effects against *D. melanogaster* were used as described previously (Franzios et al. 1997, Karpouhtsis et al. 1998). Eggs from the *Drosophila* (*flr*^{3} X *mwh*) cross were collected during a 4-h period, and 72 h later (Graf et al. 1984), groups of 30 larvae

were transferred to individual Petri dishes (9-cm diameter) containing a Whatman 3-mm paper, moistened with 1 mL of the desired concentration of the tested compound (dissolved in acetone and diluted with Ringer solution (Becker 1959) to the desired concentration, with the final acetone concentration 2%, v/v). Dishes were kept at 24 ± 1°C for 18 h. After the exposure period, the larvae were transferred to new individual vials with food until emergence of the adult flies. The control consisted of 2% acetone in Ringer solution. All treatments were replicated at least five times. The fractions of the adult flies that emerged in both control and test cultures were counted, and the LD_{50} of each individual compound was calculated.

For *B. oleae*, we followed the same experimental procedures described for *Drosophila*, modifying only the developmental stage of larvae exposed to the tested compounds. Eggs were collected for a 4-h period, and 192 h later, groups of 30 larvae were exposed to the tested compounds for 18 h. Taking into account the life cycle of the two insects used in the present study, we consider that *D. melanogaster* larvae 72 ± 4 h after oviposition (late second instar larvae) correspond to *B. oleae* larvae 192 ± 4h (Mavragani-Tsipidou, unpublished data). After the exposure, the larvae were transferred to fresh food until emergence of the adult flies. The adult flies that emerged in both control and test cultures were counted. Each experiment was repeated at least five times and 30 larvae were screened each time. The control consisted of 2% acetone in Ringer solution.

Genotoxicity test on *Drosophila*. The wing SMART was used to screen the genotoxic activity of the tested compounds, (Graf et al. 1984, Würgler & Vogel 1986). The experiments were modeled after the procedures of Graf et al. (1984) with modifications suggested by Franzios et al. (1997). Larvae of the flr^3 x *mwh* crosses were treated with the concentration of each compound represented by LD_{50} values. The trans heterozygous (mwh/flr^3) female flies that emerged from the cross were stored in 70% ethanol-glycerol (1:1) solution and their wings were mounted in Euparal solution. They were scored at 400x magnification for the presence of mosaic spots. Following the procedure of Graf et al. (1984), the spots were grouped into four categories based on the size, the number, and the type of cells showing malformed wing hairs: 1) small single spots (with one or two affected cells, either *mwh* or flr^3), 2) large single spots (with three or more affected cells, either *mwh* or flr^3), 3) twin spots (consisting of both *mwh* and flr^3 subclones), and 4) total spots. The control consisted of Ringer solution. The frequency of the observed spontaneous spots was approximately the same in all control experiments, and for statistical analysis, the average frequency of spots of the controls was used.

Statistical analysis. The mortality caused by the tested compounds was corrected according to the following equation: $(a - b)\ 100/a$, where a and b correspond to the number of the surviving adults in the control and test experiments, respectively. Data were subjected to ANOVA analysis (LSD-test) using Statistica and Excel packages. The significance level was 5%.

For statistical analysis of the genotoxic activities of the tested compounds, the multiple-decision procedure (Selby & Olson 1981, Frei & Würgler 1988) was used. The procedure is based on the conditional binomial test (Kastenbaum & Bowman 1970, Margolin et al. 1983) and the x^2 test (K. Pearson's criterion). Each statistical test was carried out at the 5% significance level.

Results

Toxicity of Greek sage essential oils. The insecticidal action of the Greek sage EO and its main constituents 1,8-cineole, thujone, and camphor was screened on *B. oleae* and *D. melanogaster* late second instar larvae. After preliminary experiments, at least four different amounts of each compound that cause death to insects at levels lower than 100% were used (Table 1). Taking into account the average survival in the controls (80% for *B. oleae* and 86% for *D. melanogaster*) the LD_{50} values of each individual compound was estimated (Table 2).

Analysis of variance of the LD_{50} values (Table 2) of the tested compounds showed that the insecticidal activity of Greek sage EO and authentic thujone did not significantly differ in the two insects. In contrast, 1,8-cineole and camphor exhibited a significantly higher toxicity for the olive fruit fly. Among the tested constituents of sage oil, 1,8-cineole was found to be the most toxic to *B. oleae* and thujone to *D. melanogaster.* However, in the latter species the toxicity of thujone did not significantly differ from that of 1,8-cineole. For both insects, camphor was found to be the weakest insecticide.

LD_{50} values of the mixtures 4:1 1,8-cineole:thujone and 50:1 1,8-cineole:camphor (Table 2), which represent the relative levels of these compounds in the sage oil, showed no significant differences in the toxicity of these mixtures and the authentic constituents 1,8-cineole and thujone for both insects.

Toxicity of mint essential oils. Pennyroyal oil and its main constituents, pulegone and menthone (75.7% and 10.1% of the total oil, respectively) were tested for their toxic effects against *B. oleae* larvae. In a recent study, the same compounds have been screened for both insecticidal and genotoxic activities on *D. melanogaster* (Franzios et al. 1997). All experiments in this study, and in Franzios et al. (1997) were carried out with the same "batch" of essential oil, for comparative analysis.

Given that the average survival in controls was 80%, the LD_{50} values (Table 2) showed that pennyroyal oil and its main constituents, pulegone and menthone, were very effective insecticides against *B. oleae* causing death even in extremely small amounts. Pulegone was found to be the most effective compound. The LD_{50} values of the mixture 7.5:1 pulegone:menthone, which represents the relative levels of these compounds in the pennyroyal oil, show that the toxicity of the mixture is not in accordance either with the toxicity of whole essential oil or with that of pulegone (Table 2), since the mixture was found about two times less effective than pure pulegone ($P < 0.05$).

Genotoxic activity of Greek sage essential oils. The results of the SMART for Greek sage EO and constituents 1,8-cineole, thujone and camphor are given in Table 3. Each of the four compounds was tested with concentrations slightly above their *D. melanogaster* LD_{50} values (Table 2). Comparative screening for spontaneous and induced mutagenesis showed no significant differences at the frequency of the total single spots between the experimental and the control series, in the case of *S. fruticosa,* 1,8-cineole and thujone. On the contrary, exposure of larvae to camphor caused an increase of single spots. Statistical analysis showed that among the tested compounds only camphor exhibited mutagenic activity (Table 3). The higher frequency of twin spots recorded after treatment of larvae with all the tested compounds give inconclusive results about their recombinogenic activity.

Table 1. Concentrations (μL/mL) of the essential oils (EO) of Greek sage and mint; the authentic compounds, 1,8-cineole, thujone, camphor, pulegone, and menthone; and the mixtures 4:1 1,8-cineole–thujone, 50:1 1,8-cineole–camphor and 7.5:1 pulegone–menthone used for tests.

Tested compound	*B. oleae* Concentration, μL/mL	*D. melanogaster* Concentration, μL/mL
Greek sage EO	0.5, 1.0, 1.5, 2.0	0.5, 1.0, 1.5, 2.0
1,8-cineole	0.1, 0.3, 0.5, 1.0	0.5, 1.0, 1.5, 2.0
Thujone	0.1, 0.3, 0.5, 1.0	0.5, 1.0, 1.5, 2.0
Camphor	0.5, 1.0, 1.5, 2.0	1.0, 2.0, 4.0, 5.0
Mixture 1,8-cineole–thujone 4:1	0.1, 0.3, 0.5, 1.0	0.5, 1.0, 1.5, 2.0
Mixture 1,8-cineole–camphor 50:1	0.1, 0.3, 0.5, 1.0	0.5, 1.0, 1.5, 2.0
Mint EO	0.1, 0.2, 0.3, 0.4	1.0, 2.0, 3.0, 4.0[a]
Pulegone	0.05, 0.07, 0.1, 0.2	0.1, 0.2, 0.4, 0.5[a]
Menthone	0.05, 0.1, 0.2, 0.5	1.0, 1.5, 2.0, 3.0[a]
Mixture pulegone–menthone 7.5:1	0.05, 0.1, 0.2, 0.5	1.0, 2.0, 3.0, 4.0[a]

[a]Data from Franzois et al. 1997.

Discussion

Plant-derived insecticides may represent alternative pest control and management tactics (see Isman 2000). They may degrade more rapidly than the synthetic insecticides, may be more specific in their action and have no genotoxicity. As part of a more general project of our laboratory in screening natural products for possible insecticidal activity (Konstantopoulou et al. 1992, Franzios et al. 1997, Karpouhtsis et al. 1998), Greek sage EO and its main constituents 1,8-cineole, thujone, and camphor (Karousou et al. 1998) were screened for their toxic effects against *D. melanogaster* and *B. oleae.* In addition, one of the most popular essential oils, the pennyroyal oil (Kokkini 1991, 1992, Franzios et al. 1997),and its main constituents, pulegone and menthone (75.7% and 10.1% of the total oil, respectively), which have recently been screened for insecticidal activity against *D. melanogaster* (Franzios et al. 1997), were tested for their toxic effects against *B. oleae*.

Results showed that pennyroyal oil and its principal constituents, pulegone and menthone, were the most effective insecticides against *B. oleae* (Table 2). Pennyroyal oil was eight times more toxic than sage oil, whereas pulegone and menthone were at least four times more toxic than those of the sage oil. Pulegone, the most toxic monoterpenoid against *B. oleae,* also was the most effective insecticide among the main constituents of sage, mint and oregano oils, tested by the same bioassay technique, against *D. melanogaster* larvae (Franzios et al. 1997, Karpouhtsis et al. 1998, present data). These results agree with previous reports that referred to the strong insecticidal toxicity of the pennyroyal oil and pulegone (Gunderson et al. 1985, Grundy & Still 1985, Lee et al. 1997, Lamiri et al. 2001). On the other hand, the monoterpenoid with the weakest insecticidal activity for

Table 2. Concentrations (μL/mL; mean ± SD) of the essential oil (EO) of Greek sage and mint; the authentic compounds 1,8-cineole, thujone, camphor, pulegone, and menthone; and the mixtures 4:1 1,8-cineole–thujone, 50:1 1,8-cineole–camphor, and 7.5:1 pulegone–menthone, which cause death to 50% of the treated *B. oleae* and *D. melanogaster* larvae.

Tested compound	*B. oleae* LD_{50}	*D. melanogaster* LD_{50}
Greek sage EO (51.5% 1,8-cineole, 11.4% thujone, and 0.9% camphor)	1.79 ± 0.10	1.93 ± 0.13
1,8-cineole	0.50 ± 0.06	1.19 ± 0.14
Thujone	0.82 ± 0.05	0.95 ± 0.12
Camphor	1.45 ± 0.09	4.82 ± 0.58
Mixture 1,8-cineole–thujone 4:1	0.59 ± 0.05	1.22 ± 0.03
Mixture 1,8-cineole–camphor 50:1	0.69 ± 0.04	1.14 ± 0.10
Mint EO (75.7% pulegone and 10.1% menthone)	0.22 ± 0.050	2.09 ± 0.025[a]
Pulegone	0.09 ± 0.026	0.17 ± 0.050[a]
Menthone	0.13 ± 0.023	1.29 ± 0.045[a]
Mixture pulegone-menthone 7.5:1	0.16 ± 0.035	4.06 ± 0.090[a]

[a]Data from Franzois et al. 1997.

both insects was camphor (Table 2). Camphor was also found to be almost inactive as an antimicrobial, cytotoxic and virucidal agent, while pulegone presented strong antimicrobial activity (Sivropoulou et al. 1995, 1997).

Most of the tested compounds were significantly more effective as insecticides against *B. oleae* than *D. melanogaster* (Table 2). With the exception of the whole Greek sage EO and thujone, the toxicity of which was not significantly different among the two insects, the rest of the tested compounds were 2 to 10 times (1,8-cineole and pulegone were about two times, camphor three times and pennyroyal oil and menthone about 10 times) more toxic to *B. oleae* than to *D. melanogaster* larvae (present study, Franzios et al. 1997). The LD_{50} values showed that mortality caused by the latter compounds was significantly different in the two species. Taking into account that the assay technique (present study, Franzios et al. 1997) and the developmental stage of *B. oleae* and *Drosophila* larvae are the same, the observed differential insecticidal activity seems to be the result of inherent interspecific differences, suggesting a species specific mode of action. This unexpected result (because *B. oleae* is about three times heavier than *D. melanogaster)* may be beneficial for the management of the olive fruit pest.

The toxicity of pennyroyal oil and sage oil significantly differ from that of their main constituents (Table 2). For direct demonstration of the interactions of these monoterpenoids, we assessed the LD_{50} values of mixtures of the authentic constituents in levels representing their relative content in the two oils. Data show that the toxicity of the mixtures (4:1 1,8-cineole:thujone and 50:1 1,8-

Table 3. Summary of results in the wing somatic mutation and recombination test (SMART) on *D. melanogaster* after treatment with the essential oil (EO) of Greek sage and the authentic compounds, 1,8-cineole, thujone, and camphor.

		Spots per wing (no. of spots) diagnosis[a]			
Treatment	Wings analyzed	Small single spots (1 or 2 cells), m = 2.0	Large single spots (>2 cells), m = 5.0	Twin spots, m = 5.0	Total spots, m = 2.0
Control	80	0.45 (36)	0.08 (6)	0.03 (1)	0.56 (45)
Greek sage EO 2.0, μL/mL	78	0.50 (39)–	0.01 (1)–	0.043 (3)i	0.55 (43)–
1,8-cineole 1.2 μL/mL	61	0.50 (30)–	0.05 (3)–	0.03 (2)i	0.59 (35)–
Thujone 1.0 μL/mL	56	0.62 (35)i	0.05 (3)–	0.04 (2)i	0.71 (40)–
Camphor 4.8 μL/mL	52	0.98 (51)+	0.04 (2)–	0.11 (6)i	1.15 (59)+

[a]Statistical diagnosis according to Frei & Würgler (1988).
+, positive; –, negative; i, inconclusive; m, multiplication factor.
Probability levels: $\alpha = \beta = 0.05$.

cineole:camphor) of the main constituents of the sage oil did not significantly differ from that of the authentic 1,8-cineole and thujone. However, the LD_{50} values of the mixture 7.5:1 pulegone:menthone showed that the toxicity of authentic pulegone was suppressed in the presence of menthone (Table 2), suggesting antagonistic phenomena. This antagonism between the two monoterpenes also was found in previous experiments with *Drosophila* (Table 2, Franzios et al. 1997). However, this phenomenon does not explain the differences in the toxicity of the mixture and the whole essential oil of *M. pulegium* (Table 2). Indeed, the mortality caused by the mixtures differ significantly from the whole oils (pennyroyal and sage oil), suggesting that compounds in the remaining 14.2% and 36.2% of *M. pulegium* and *S. fruticosa*, respectively, play an important role in the determination of the final toxicity of the oils.

Mortality of larvae exposed to "insecticidal" compounds may be the result of many parameters, including the structure, function, and biochemistry of the insect cuticle in relation to the molting cycle (Reynolds 1987). It is well known that many EOs and their constituents affect biochemical processes, which specifically disrupt the endocrinologic balance of insects. They may be neurotoxic or may act as insect growth regulators, disrupting the normal process of morphogenesis (Reynolds 1987, Balandrin & Klocke 1988). Observations on the mortality caused by the compounds used in the present study showed that most of the larvae of both insects were acutely poisoned (death occurred during the exposure period), whereas a small proportion died at a later larval stage. No lethal effects were observed during the pupal stage. Among the dead larvae, many kinds of malfor-

mations were observed, including blackened long larvae, black spots in the larval body and puparium, and blackened salivary glands and gut. However, the acute insecticidal effects of the tested compounds suggest a neurotoxic mode of action.

Because a "good" pesticide must be safe for humans, animals, and generally the ecosystem, the genotoxic activity of the sage oil and its main monoterpenoids was determined by their ability to induce somatic mutation and recombination effects in the wing spot essay in *Drosophila* (Graf et al. 1984, 1998). The results of the SMART are given in Table 3. Each of the four compounds was assayed with concentrations represented by the *D. melanogaster* LD_{50} values (Table 2). Comparative screening for spontaneous (negative control, water) and induced mutagenesis showed that no significant differences were found at the frequency of the total single spots between the experimental and the control series, in the case of Greek sage, 1,8-cineole, and thujone. On the contrary, exposure of larvae to camphor caused an increase of single spots. Statistical analysis showed that among the tested compounds only camphor exhibited mutagenic activity (Table 3). However, because the quantities of the above substances used are different, depending on their LD_{50} values, only comparative conclusions can be drawn. In accordance with our results, sage oil (Simic 1995) and 1,8-cineole were reported as nonmutagenic agents (Gomes-Carneiro et al. 1998), whereas there is no information about the mutagenic activity of thujone. Camphor has been found to be nonmutagenic in the Ames test (Gomes-Carneiro et al. 1998), although it has been reported that this monoterpene had a radiomodifying ability (Goals et al. 1989) and that entodontic products containing camphor presented a positive result in the 'rec-assay' (Kanematsu & Shibata 1990). Even though camphor has a very low insecticidal activity and, thus, it is unlikely to be used as an insecticide, much attention could be given to the use of this compound.

Previous studies on the genotoxic activities of pennyroyal oil and its main constituents (Franzios et al. 1997) showed that the pennyroyal oil was a negative mutagenic and recombinogenic agent, pulegone a very weak negative one, whereas menthone was found to be a potent mutagenic but not recombinogenic inducer. The observed negative mutagenic and recombinogenic effects of pennyroyal oil, sage oil, 1,8-cineole, and thujone in correlation with their strong insecticidal activity should mean that they are potent compounds for pest control. However, the extensive exposure of humans to higher amounts of these compounds is a matter of concern. Contrary to the alleged safety of plant derived substances, many EOs and constituents are reported to produce serious effects on the nervous central system (Burkhard et al. 1999, Bishop & Sanders 2000, Hold et al. 2000). It is apparent that the safety of a compound can be only reliably determined by carrying out many studies using different bioassays (Lazutka et al., 2001).

The results of this study demonstrate significantly higher insecticidal properties of most of the tested compounds against *B. oleae* than *D. melanogaster*, suggesting species specificity mode of their action and suggesting benefits for the use of these plant extracts as insecticides against the olive fruit fly. Our study also showed the suppression of the toxicity of pulegone in the presence of menthone, indicating antagonistic interactions, and revealed the safety of the sage oil, which is extensively used as culinary herbs in folk remedies and in the food, drug and fragrance industries (Rivera et al. 1994, Boelens & Boelens 1997).

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