Insecticidal and Genotoxic Activities of Oregano Essential Oils

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The essential oils (EOs) obtained from the oregano plants *Origanum vulgare* subsp. *hirtum*, *Coridothymus capitatus*, and *Satureja thymbra* were examined by a combination of GC and GC−MS and found to be rich in carvacrol, thymol, γ-terpinene, and *p*-cymene. These EOs and their main constituents, carvacrol and thymol, were tested for insecticidal and genotoxic activities on *Drosophila*. The EO of *S. thymbra* was found to be the most effective as an insecticide, while carvacrol was found to be more toxic than thymol. The toxicities of carvacrol and thymol do not correspond to their participation in the EOs, and mixtures of these two phenols in levels resembling their content in the three oils showed that the toxicity of carvacrol was reduced in the presence of thymol, thus suggesting antagonistic phenomena. The somatic mutation and recombination test on *Drosophila* revealed that, among the five compounds studied, only thymol exhibits genotoxic activity.

Keywords: *Oregano; Origanum vulgare; Coridothymus capitatus; Satureja thymbra; essential oils; carvacrol; thymol; insecticides; genotoxicity; Drosophila melanogaster*

INTRODUCTION

In recent years, the essential oils have received much attention as resources of potentially useful bioactive compounds. Particular emphasis has been placed on their antimicrobial, antifugal, antitumor, and insecticidal action, as well as on their action on the central nervous system (Franzios et al., 1997).

The essential oils (EOs) are plant secondary metabolites mainly composed of terpenoid compounds and play an important role in the interactions between plants and insects. The effects of the EOs on insects range from an attraction or repellence to that of toxicity or even lethality. Even though the use of "insecticidal" plants is known from antiquity, only a few of these are commercially available (Balandrin and Klocke, 1988).

The aromatic plants *Origanum vulgare* subsp. *hirtum* (Link) Ietswaart, *Coridothymus capitatus* (L.) Reichenb., and *Satureja thymbra* L. are members of Labiatae and have a wide native distribution in Greece (Kokkini and Vokou, 1989). The three taxa are very important from the economical point of view, and under the commercial name "oregano", they are used as a very popular spice in the food industry (Lawrence, 1984; Kokkini, 1994; Olivier, 1997). As part of an ongoing study concerning the use of plant extracts as insecticidal factors (Konstantopoulou et al., 1992; Franzios et al., 1997), the EOs of the above aromatic plants were analyzed and together with two of their main constituents, carvacrol and thymol, were screened for insecticidal activity. The toxicity of each tested compound was found by determining the crucial amounts of each compound that cause death to 50% of *Drosophila melanogaster*. In addition, to determine if the above compounds could be safely used in cosmetics, in the food industry, and as insecticides, their genotoxic effects were tested on *D. melanogaster*.

MATERIALS AND METHODS

Plant Material. Aerial parts of fully flowered *O. vulgare* subsp. *hirtum*, *C. capitatus*, and *S. thymbra* grown wild in Mt. Taygetos (Peloponnese, Greece) were collected. Voucher specimens are kept in the Herbarium of the Laboratory of Systematic Botany and Phytogeography (University of Thessaloniki).

Essential Oil Isolation and Analysis. Air-dried, at room temperature for 10 days, plant material (25−30 plants from each species) was grossly pulverized by hand, and the essential oils were isolated after hydrodistillation for 2 h, using a Clevenger apparatus. The EO content was expressed in milliliters per 100 g of air-dried plant material. The three EOs after dilution in dichloromethane (1:4) were examined by GC and GC−MS according to the procedure previously described by Sivropoulou et al. (1996). The oil components were identified by comparing their relative retention times (RRT) and mass spectra (MS) with those of authentic samples, the literature (Cornu and Massot, 1979; Jennings and Shibamoto, 1980), and the *Wiley Registry of Mass Spectral Data* (McLafferty, 1989).

Authentic, commercially available carvacrol and thymol, used in the present study, were obtained from Aldrich Chemical Corp. (Gillinham, U.K.).

Genetic System. Two *D. melanogaster* strains (kindly provided by Dr. F. Marec) were used: (a) the multiple wing hair strain (*mwh*), with genetic constitution *mwh e*/*mwh e*, and (b) the flare *(flr^3)* strain with genetic constitution *y w^{co}*/*y w^c; flr^3 se*/*TM2 Ubx^{130} se e* [Marec and Gelbic, 1994; see also Lindsley and Zimm (1992) for description of the genetic markers]. The stocks and the crosses were maintained at 24 ± 1 °C in 16 h light−8 h dark periods on a yeast−glucose medium.

Experimental Procedures. The experimental procedures used for screening both insecticidal and genotoxic effects of

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Table 1. Qualitative and Quantitative Analyses of the Essential Oils Obtained from *O. vulgare* subsp. *hirtum*, *C. capitatus*, and *S. thymbra*

component	*O. vulgare* subsp. *hirtum*	*C. capitatus*	*S. thymbra*
α-thujene	0.06		
α-pinene	1.22	0.65	3.80
camphene	0.10	0.16	1.21
β-pinene	0.10	0.07	1.20
sabinene	0.06	0.05	0.10
Δ−3-carene			0.02
myrcene	1.35	0.94	1.50
α-phellandrene	0.13	0.06	0.07
α-terpinene	1.04	0.67	1.55
limonene	0.17	0.13	0.70
1,8-cineole	0.19	0.14	0.29
β-phellandrene	0.03		0.05
cis-β-ocimene			0.02
γ-terpinene	5.92	2.25	13.86
trans-β-ocimene	0.17		
p-cymene	9.71	6.41	26.76
α-terpinolene	0.05	0.08	0.09
3-octanol		0.07	0.10
1-octen−3-ol			0.03
trans-sabinene hydrate	0.41	0.27	0.49
β-bourbonene			0.24
linalool	0.12	0.61	0.57
linalyl acetate		0.07	
terpinen−4-ol	0.12	0.03	0.20
cis-sabinene hydrate	0.30	0.13	0.08
β-caryophyllene	1.37	1.91	3.82
methylcarvacrol	0.09	0.03	0.15
trans-dihydrocarvone	0.04	0.06	0.04
cis-dihydrocarvone	0.06		0.10
isoborneol	0.16	0.61	1.75
α-terpineol	0.60	0.08	
γ-elemene		0.06	
β-bisabolene		0.05	0.12
γ-cadinene	0.05	0.04	
trans-carveol			0.02
calamenene	0.04		0.07
p-cymen−8-ol		0.61	1.63
carvacrol acetate	0.20		0.08
spathulenol		0.10	0.50
thymol	0.71	1.46	34.72
carvacrol	74.56	81.46	3.12
essential oil yield (mL/100 g dry weight of plant tissue)	4.5	2.3	3.2

the tested compounds were previously described in Franzios et al. (1997). Thus, to determine their toxicity, late second instar larvae (approximately 50 h old) from the cross between *flr*^{3} virgin *Drosophila* females and *mwh* males (Graf et al., 1984) were exposed to various amounts of each tested compound, and the percentage of flies that emerged (after exposure) was calculated. Each experiment was repeated three times, and the number of flies that survived per vial were counted in both control and test cultures. For screening the genotoxic activity of the tested compounds, the wing somatic mutation and recombination tests (SMART) (Graf et al., 1984; Würgler and Vogel, 1986) were applied. The experiments were carried out following the principles and the basic procedures presented by Graf et al. (1984) with some deviations (Franzios et al., 1997). Larvae of the *flr*^{3} × *mwh* crosses were treated with the crucial dose of each individual compound that causes death to 50% of the tested larvae (LD_{50}). The wings of the emerged trans-heterozygous *(flr*^{3}*/mwh)* female flies were mounted in Euparal solution and scored at 400× magnification for the presence of mosaic spots. The spots were grouped into four categories according to the methods and criteria of Graf et al. (1984): (a) small single spots (with one or two affected cells, either *mwh* or *flr*^{3}), (b) large single spots (with three or more affected cells, either *mwh* or *flr*^{3}), (c) twin spots (consisting of both *mwh* and *flr*^{3} subclones), and (d) total spots. Each of the five compounds tested was assayed in at least three independent experiments. For comparative analysis, a parallel experiment using only Ringer solution (Becker, 1959) was carried out. In all control experiments, the frequency of the spontaneous spots observed was found to be about the same, and the average frequency of the controls was used for the statistical analysis.

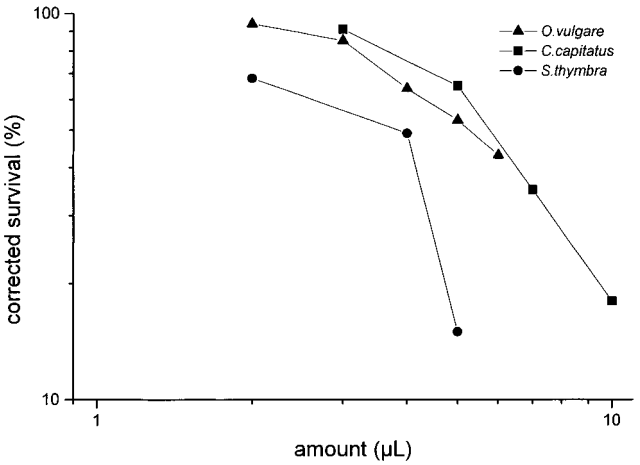

Figure 1. Percentage of larvae surviving to adulthood after exposure to different amounts of the essential oils of *O. vulgare* subsp. *hirtum*, *C. capitatus*, and *S. thymbra*.

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 surviving adults in control and test experiments, respectively.

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

RESULTS AND DISCUSSION

This study was undertaken in the frame of a more general project of screening natural products for possible biological activities. Essential oils (EOs) of the aromatic plants *O. vulgare* subsp. *hirtum*, *C. capitatus*, and *S. thymbra* were analyzed and together with their main constituents, carvacrol and thymol, were screened for toxic and genotoxic activities. In the first set of experiments, the five compounds were tested for their ability to cause death to insects. In the second set of experiments, the same compounds were screened for their ability to induce somatic mutation and recombination effects, to determine if their extensive use (as insecticides or as spices) is safe for man, animals, and generally the ecosystem. In both series of experiments, *D. melanogaster* was used as an insect species system that offers suitable genetic markers (Graf et al., 1984) and facilitates further studies on insects with great economical impact, like the pest *Bactrocera (Dacus) oleae* (P. Mavragani-Tsipidou et al., in preparation).

It is well-known that the composition of EOs may dramatically differ even within the same taxon, depending on genetic and geographical (climatic and seasonal) parameters (Kokkini and Vokou, 1989; Kokkini, 1997). Table 1 shows the results of the quantitative and qualitative analyses of the EOs isolated from the three oregano taxa studied here. *O. vulgare* subsp. *hirtum* (commercially known as Greek oregano) and *C. capitatus* (known as Spanish oregano) are characterized by the dominant occurrence of carvacrol (74.56 and 81.46% of the total oil, respectively), while *S. thymbra* is

Table 2. Crucial Amounts of the Essential Oils (EOs) (in Microliters) of *O. vulgare* subsp. *hirtum*, *C. capitatus*, and *S. thymbra*, the Compounds Carvacrol and Thymol, and 105:1, 55:1, and 1:11. Carvacrol/Thymol Mixtures, Which Cause Death to 50% of the Treated *D. melanogaster* Larvae (LD_{50})[a]

tested compound	LD_{50}
EO of *O. vulgare*	5.6
EO of *C. capitatus*	6.78
EO of *S. thymbra*	3.3
carvacrol	1.6
thymol	2.6
105:1 carvacrol:thymol	1.98
55:1 carvacrol:thymol	3.07
1:11 carvacrol:thymol	4.27

[a] LD_{50} was found after estimation of the corrected mortality (see also the text).

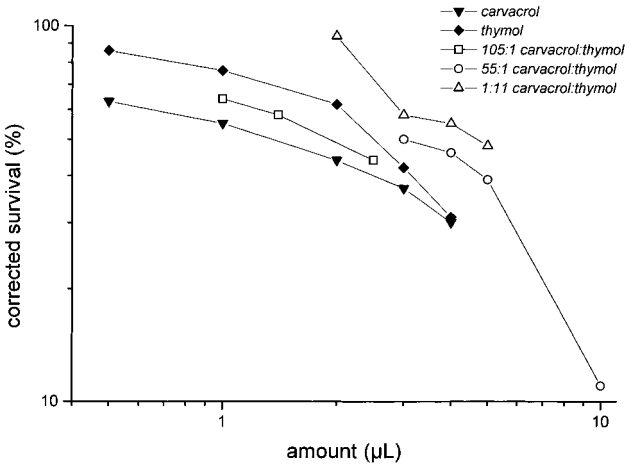

Figure 2. Percentage of larvae surviving to adulthood after exposure to different amounts of the authentic compounds carvacrol and thymol and 105:1, 55:1, and 1:11 carvacrol/thymol mixtures.

characterized by the dominant occurrence of thymol (34.72% of the total oil). Among the other EO constituents, the two monoterpene hydrocarbons γ-terpinene and *p*-cymene, the biosynthetic precursors of thymol and carvacrol (Poulose and Croteau, 1978), were found in considerable amounts in the three oils. However, in *S. thymbra*, the sum of these precursors is higher (40.62% of the total oil) than that of the phenolic compounds (37.84% of the oil), while in *O. vulgare* subsp. *hirtum* and *C. capitatus*, the sum is lower (Table 1).

After preliminary experiments, five different amounts of each compound that cause death to insects at levels lower than 100% were used in our experiments. After the toxicity was determined as the fraction of the adult flies that did not emerge from the treated larvae, the number of larvae surviving to adulthood was recorded in both control and tested cultures. Taking into account the fact that the average survival in the controls was 88%, the results, expressed as the percentage of surviving larvae, after correction (see also statistical analysis), are given in Figures 1 and 2. From these curves, the amount of each individual compound that allowed 50% of the larvae to develop to the adult stage (LD_{50}) was estimated (Table 2). As is shown in Table 2, among the oregano plants studied, the EO of *S. thymbra* was found to be the most effective as an insecticide (LD_{50} = 3.3), followed by those of *O. vulgare* (LD_{50} = 5.6) and *C. capitatus* (LD_{50} = 6.78). Both authentic compounds tested, carvacrol and thymol, showed strong toxicity, with the former being the stronger one. Taking into account the insecticidal activity of the two phenolic compounds (Table 2 and Figure 2), as well as their content in each oil (Table 1), it is obvious that the insecticidal activity of the whole EOs of the three aromatic plants is not linearly dependent on these two constituents. Data show that EOs of *O. vulgare* and *C. capitatus* (Table 1) with an extremely high content of carvacrol (74.56 and 81.46%, respectively) exhibit less insecticidal activity than that of *S. thymbra* that contains a very small amount of this phenol (3.12%). The same is true if we take into account the total content of the two phenolic compounds, carvacrol and thymol, which in the EO of *S. thymbra* was found to be much lower (37.84%) than in the other two EOs (*O. vulgare*, 75.27%; and *C. capitatus*, 82.92%). All the above could indicate either that other constituents of the EOs of the aromatic plants are responsible for their toxicity or that synergistic and/or antagonistic phenomena exist that alter the toxicity of the whole EO. To directly demonstrate the interactions between the two pure compounds, we calculated the LD_{50} of 105:1, 55:1, and 1:11 mixtures of authentic carvacrol/thymol, which represent the relative levels of these compounds as found in the EOs of *O. vulgare*, *C. capitatus*, and *S. thymbra*, respectively. Data show (Table 2) that the toxicity of the 105:1 mixture (which corresponds to the relative content of these compounds in the EO of *O. vulgare*) is slightly lower than that of the pure carvacrol, but quite different from that of *O. vulgare*. The LD_{50} of the 55:1 mixture (corresponding to the content of carvacrol/thymol in the EO of *C. capitatus*) is 3.07 μL, meaning that 3 μL of carvacrol of the mixture is able to cause death to 50% of the treated insects while half of this amount (1.6 μL) is needed when pure carvacrol is used. The above data suggest that antagonistic phenomena exist that alter the toxicity of carvacrol in the presence of thymol. The 1:11 mixture, which corresponds to the content of carvacrol/thymol in the EO of

Table 3. Summary of Results in the Wing Somatic Mutation and Recombination Test (SMART) on *D. melanogaster* after Treatment with the Essential Oils (EOs) of *O. vulgare* subsp. *hirtum*, (O.v.) *C. capitatus* (C.c.), and *S. thymbra* (S.th.) and the Authentic Compounds Carvacrol and Thymol (See Also the Text)

		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
control (Ringer)	74	0.74 (55)	0.06 (5)	0.08 (6)	0.89 (66)
EO of O.v. (5.6 μL)	60	0.73 (44) −	0.06 (4) −	0.06 (4) −	0.86 (52) −
EO of C.c. (6.78 μL)	60	0.82 (49) −	0.08 (5) −	0.06 (4) −	0.95 (58) −
EO of S.th. (3.3 μL)	60	0.76 (46) −	0.06 (4) −	0.08 (5) −	0.91 (55) −
carvacrol (1.6 μL)	62	0.9 (56) −	0.05 (3) −	0.06 (4) −	1.03 (63) −
thymol (2.6 μL)	63	1.22 (77) +	0.06 (4) −	0.09 (6) −	1.42 (87) +

[a] Statistical diagnosis according to Frei and Wuergel (1988): +, positive; −, negative; and m, multiplication factor. Probability levels: $\alpha = \beta = 0.05$.

S. thymbra, exhibits insecticidal activity lower than those of the two other mixtures (LD_{50} = 4.27). This result is not in accordance with the toxicity caused either by this phenol or by the whole EO. In addition, the toxicity of thymol is also altered in the presence of carvacrol, a result that may suggest that antagonistic phenomena do exist. However, these antagonistic phenomena could not be exclusively responsible for the differences observed in the toxicity of the EOs of the tested aromatic plants. The high insecticidal activity of *S. thymbra* may be attributed to other components of the remaining 62.16% of the whole oil, or even to synergistic phenomena that may exist among some constituents. Recently, Lee et al. (1997) reported that the larvicidal activity of carvacrol was increased when it was used as a mixture with phosphomidon, suggesting a possible synergistic effect. We note that the quantities of the constituents of the studied oils do not significantly differ in *O. vulgare* and *C. capitatus*, but they do differ in *S. thymbra* (e.g. in the carvacrol and thymol content, in their biosynthetic precursors γ-terpinene and *p*-cemene, and in other compounds such as α-pinene, camphene, β-pinene, and β-caryophylene) (Table 1).

Previous studies have shown that carvacrol and thymol, as well as EOs of aromatic plants rich in the two phenolic compounds, exhibit strong insecticidal (Konstantopoulou et al., 1992; Lee et al., 1997), antimicrobial (Janssen et al., 1987; Didry et al., 1993; Panizzi et al., 1993; Sivropoulou et al., 1996), and antifugal activity (Thompson, 1989; Shimoni et al., 1993; Daouk et al., 1995; Müller-Riebau et al., 1995). In agreement with our data, among the three oregano species, the EO of *S. thymbra* was found to be the most effective insecticide against another *Drosophila* species, *Drosophila auraria* (Konstantopoulou et al., 1992). Similarly, carvacrol has been reported to be a very potent larvicidal compound, more toxic than thymol, when tested against *Thecodiplosis japonensis* (Diptera, Cecidomyiidae) (Lee et al., 1997). On the other hand, thymol was found to be more toxic than carvacrol at least against some bacteria (Didry et al., 1993; Sivropoulou et al., 1996), and carvacrol possesses higher antimicrobial activity in the presence of thymol (synergistic phenomena) (Didry et al., 1993). Both carvacrol and thymol are also characterized by high antifungal activity (Müller-Riebau et al., 1995).

Besides the above, differences do also exist in the activity of the EOs and their main constituents in oregano and mint plants. Using the same experimental procedure and organism, the EOs of mint plants (Franzios et al., 1997) were found to be more toxic as insecticides than those of the oregano plants (this study). On the other hand, Müller-Riebau et al. (1995) in fungi and Sivropoulou et al. (1995, 1996) in bacteria reported that EOs rich in carvacrol and thymol were shown to have higher fungicidal and bactericidal activities than those rich in pulegone (mint EOs).

The differences observed in the toxicity of the EOs and the mixtures of compounds may be due at least to four parameters: the essay technique, the growth medium, the composition of the EO, and the organism used in each experiment (Janssen et al., 1987). It is well-known that the EOs are often species-specific in their activities [see Kelsey et al. (1984) as a review and references therein] and that synergistic and/or antagonistic phenomena do exist among the constituents of the oil (Harborne, 1982; Bestmann et al., 1988; Didry et al., 1993; Franzios et al., 1997). Concerning the insecticidal activity, the developmental stage of the tested organism must be of great importance since many of the constituents of EOs are found in the secretions of insects and proved to be analogues or antagonists of endogenous hormones (Balandrin and Klock, 1988). Mortality of larvae exposed to "insecticidal" compounds may be a result of many parameters such as the structure, function, and biochemistry of the insect cuticle in relation to the moulting cycle (Reynolds, 1987). Many substances could also be neurotoxic or act as insect growth regulators, thus disrupting the normal process of morphogenesis (Reynolds, 1987; Balandrin and Klock, 1988).

The genotoxic activity of the tested compounds was performed using the wing somatic and recombination tests (SMART) in *D. melanogaster* (Graf et al., 1984). These tests proved to be very sensitive assays for screening compounds for their ability to induce mutations or mitotic recombination effects, in a number of imaginal disk cells (Würgler and Vogel, 1986) of *D. melanogaster*. These effects are expressed as mosaic spots on the wings of trans-heterozygous female flies (see also Materials and Methods). A compound may reduce or increase the spontaneous mutation rate or may even be ineffective depending on its genotoxic activity. In these experiments, the concentration of each compound that allowed 50% of the *Drosophila* larvae to develop to the adult stage (LD_{50}) was used (Table 2). As is shown in Table 3, the oils of the three oregano plants did not show any mutagenic or recombinagenic activity, while among the two phenols, carvacrol showed negative genotoxic activity and thymol was found to be a potent mutagenic but not recombinagenic inducer. Comparative screening for spontaneous or induced mutagenesis showed that the EO of *O. vulgare* subsp. *hirtum* is the only oil that shows reduced mutagenicity. The putative anticancer potential of the *Origanum* oil proposed by Lam and Zheng (1991) should be investigated further. Taking into account the fact that the consumption of oregano plants and their EOs in food (pizzas, sauces, salads, etc.) is continuously increasing (Olivier, 1997), the results of this study suggest that their use in food and cosmetics is safe. Moreover, present data suggest that the oregano EOs are safer than those of the mint plants (Franzios et al., 1997).

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