Insecticidal and Genotoxic Activities of Mint Essential Oils

Gerasimos Franzios, Maria Mirotsou, Emmanouel Hatziapostolou, Jiri Kral, Zacharias G. Scouras, and Penelope Mavragani-Tsipidou*

Department of Genetics, Development and Molecular Biology, School of Biology, Faculty of Science, Aristotle University of Thessaloniki (AUTH), GR-54006 Thessaloniki, Greece

The essential oils (EOs) extracted from the mint species *Mentha pulegium* and *Mentha spicata*, together with their main constituents, pulegone, menthone, and carvone, were tested for insecticidal and genotoxic activities on *Drosophila melanogaster*. The EOs of both aromatic plants showed strong insecticidal activity, while only the oil of *M. spicata* exhibited a mutagenic one. Among the constituents studied, the most effective insecticide was found to be pulegone, while the most effective for genotoxic activity was menthone. Data show that both toxic and genotoxic activities of the EOs of the two studied mint plants are not in accordance with those of their main constituents, pulegone, menthone, and carvone. Pulegone is significantly more effective (9 times) as an insecticide, while menthone and carvone are less effective (6 and 2 times, respectively) insecticides when used in their authentic forms, and a mixture of authentic pulegone and menthone, in levels resembling their content in the oil of *M. pulegium*, showed that the strong toxicity of pulegone is suppressed in the presence of menthone. All the above suggest that synergistic/antagonistic phenomena may be involved that alter the toxicity of the whole EO.

Keywords: *Mint plants; pennyroyal oil; spearmint oil; essential oils; pulegone; menthone; carvone; insecticides; genotoxicity; Drosophila melanogaster*

INTRODUCTION

It is well-known that plant-derived natural products are extensively used as biologically active compounds. Among them, essential oils (EOs) were the first preservatives used by man, originally in their natural state within plant tissues and then as oils obtained by distillation. Many of these crude mixtures have been found to have antifungal, antimicrobial, cytostatic, and insecticidal activities (Kelsey et al., 1984; Janssen et al., 1987; Balandrin and Klocke, 1988; Thompson, 1989; Konstantopoulou et al., 1992; Sivropoulou et al., 1995).

Plant extracts have been used as insecticides by humans since before the time of ancient Romans. Today, there is an increasing interest in the use of "insecticidal" plants, because of the necessity of finding safer insecticides in combination with the need of preventing environmental degradation and pollution. In addition, the extensive use of several aromatic plants in the cosmetics and food industries, demands an extensive screening of EOs and their components for genotoxic activities.

The present study on the insecticidal and genotoxic activity of the essential oils of the aromatic plants *Mentha pulegium* and *Mentha spicata* as well as their main components, namely, pulegone, menthone, and carvone, was undertaken in order to confirm the toxicity of each tested compound by defining the crucial amounts of each compound that cause death to *Drosophila melanogaster*, and to determine the positive, negative, or neutral genotoxic effects that these compounds exert.

MATERIALS AND METHODS

Plant Material. Essential oils from the mint plants *M. pulegium* and *M. spicata*, species abundantly found in the Greek flora, belonging to the Lamiaceae family, were kindly provided by Dr. S. Kokkini. *M. pulegium* (designated no. 1526) contains 75.7% pulegone and 10.1% menthone; *M. spicata* (designated no. 1645) contains 32.1% carvone (S. Kokkini, unpublished results). Authentic commercially available pulegone, menthone, and carvone, used in the present study, were obtained from Aldrich Chemical Corp.

Genetic System. Two *D. melanogaster* strains (kindly provided by Dr. Marec), 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* (Marec and Gelbic, 1994), were used in the present study. Description of the genetic markers is given in Lindsley and Zimm (1992). 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 16 h light−8 h dark on a yeast−glucose medium.

Experimental Procedures. In order to screen the lethal effects of the tested compounds, eggs from the *Drosophila* cross mentioned above were collected during an 8 h period, and 72 ± 4 h later the larvae were removed from the food. Groups of 50 larvae, after washing with 17% NaCl solution (Graf et al., 1984), were transferred to individual Petri dishes (9 cm diameter) containing a Whatman 3 mm paper moistened with Ringer solution (Becker, 1959) and exposed to various amounts of the examined compound. Different amounts of EOs or their components were applied to a small filter paper disk (4 mm diameter) placed at the center of the petri dish (Konstantopoulou et al., 1992). Dishes were kept at 24 ± 1 °C and 60% humidity for 18 h. After the exposure period, the larvae were washed with Ringer solution and transferred to new individual vials with food until emergence of adult flies. Each experiment was repeated three times, and the number of flies 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) (see Graf et al. (1984) and Wuergler and Vogel (1986) for reviews) were applied. The experiments were carried out according to the method described in the previous paragraph. Larvae of the *flr*^{3} × *mwh* crosses were treated with concentrations slightly above the crucial dose of each individual com-

* Corresponding author (telephone 0030 31 998393; fax 0030 31 998298).

S0021-8561(96)00685-1 CCC: $14.00 © 1997 American Chemical Society

pound that causes death to 50% of the tested larvae (LD_{50}). The trans-heterozygous (mwh/flr^3) female flies that emerged from the cross mentioned above were selected and stored in 70% ethanol−glycerol (1:1). Their wings were mounted in Euparal solution and scored at 400× magnification for the presence of mosaic spots. On the basis of the size, the number, and the type of cells showing malformed wing hairs, different categories of spots were recorded by following the methods and criteria of Graf et al. (1984). Each of the five compounds tested was assayed in at least three independent experiments. For comparative analysis, a parallel experiment using only Ringer solution 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.

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.

For statistical analysis of the genotoxic effects of the tested compounds, the spots were grouped into four categories: (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 single spots. For the statistical significance of the results, the multiple-decision procedure (Selby and Olson, 1981; details are given in Frei and Wuergler (1988)) was used. The procedure is based on the conditional binomial test (Kastenbaum and Bowman, 1970; Margolin et al., 1983) and the X^2 test (K. Pearson's criterion). Each statistical test was carried out at 5% significance level.

RESULTS AND DISCUSSION

Essential oils composed by isoprenoid compounds, mainly mono- and sesquiterpenes are the carriers of the smell found in aromatic plants. These secondary metabolites are often complex biomolecules, and there are many suggestions for their biological role, as, for example, allelopathy, antiherbivore action, and interaction with microorganisms and insects (Kelsey et al., 1984; Janssen et al., 1987; Thompson, 1989; Konstantopoulou et al., 1992; Vokou, 1992; Panizzi et al., 1993; Sivropoulou et al., 1995).

The mode of action of the essential oils or their constituents, as insecticides, remains unclear. Many of them deter insects from feeding, while other have been proved to be neurotoxicant in their action or insect growth regulators, including analogs and antagonists of endogenous hormones. Moreover, constituents of EOs such as terpenoids are also present in insects, acting as pheromones, thus making the interactions between plants and insects more complex (for review see Balandrin and Klocke (1988) and Reynolds (1987) and references therein).

Mint species are very important from the economical point of view and most of them are characterized by great chemical diversity (see Kokkini (1991, 1992) for reviews). The spearmint oil (rich in carvone) extracted mainly from *M. spicata* and the pennyroyal oil (rich in pulegone) obtained exclusively from *M. pulegium* are two of the most commercially exploited EOs (see Kokkini (1992) for review).

In the present study, EOs extracted from *M. pulegium* (pennyroyal oil), rich in the monoterpenes pulegone (75.7% of the total oil) and menthone (10.1%), and *M. spicata* (spearmint oil), rich in carvone (32.1%), together with authentic commercially available pulegone, menthone, and carvone, were examined for insecticidal and genotoxic activity. The insecticidal action of all the

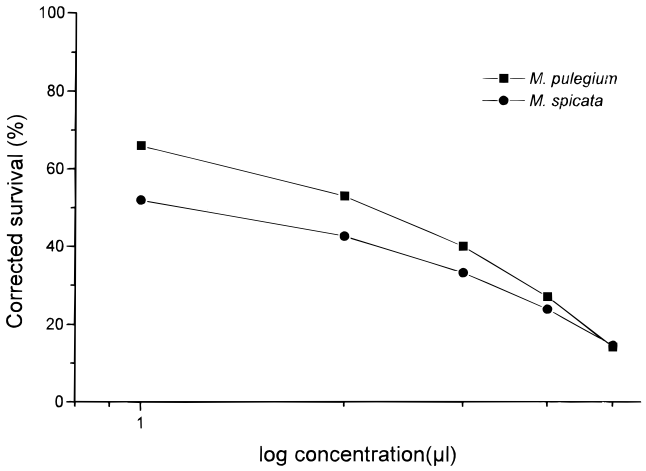

Figure 1. Percentage of larvae surviving to adulthood after exposure to different concentrations of the essential oils of *M. pulegium* and *M. spicata*.

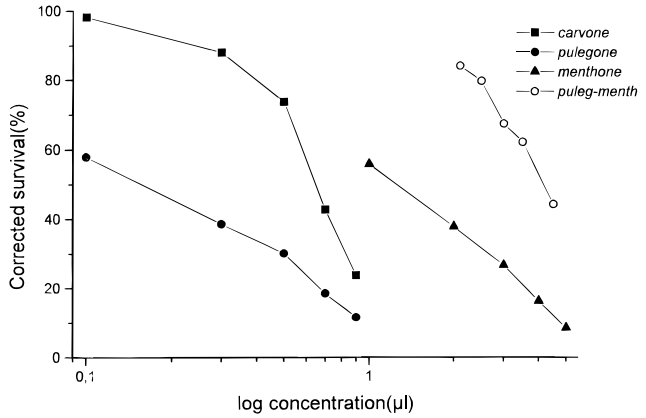

Figure 2. Percentage of larvae surviving to adulthood after exposure to different concentrations (in microliters) of the authentic compounds, pulegone, menthone, carvone and 7.5:1 pulegone−menthone mixture.

compounds tested was confirmed after a series of preliminary experiments. Five different amounts of each compound were determined, which caused death to *Drosophila* lower than 100%. Determining the toxicity as the fraction of the adult flies that emerged from the treated larvae, the number of larvae surviving to adulthood was recorded, in control and tested cultures. The average survival in the controls was 86%. The results, expressed as the percentage of survived larvae, after correction (see also the statistical analysis), are shown in Figures 1 and 2. From these curves, the LD_{50} of each compound was estimated (Table 1).

According to the present results, the EOs derived from the mint plants *M. pulegium* and *M. spicata* are very effective as insecticides since small amounts of both EOs cause death to a great number of insects (Figure 1; Table 1). The observed strong toxicity of the above oils on *D. melanogaster* is in accordance with that previously reported on *Drosophila auraria* (Konstantopoulou et al., 1992). However, contrary to the previous report, the EO of *M. spicata* proved to be the most effective one (Figure 1; Table 1). Such differences are rather expected, since both the EOs and the insects used in the above studies are different. It is well-known that the chemical composition of the EOs of mint species may differ significantly within the same taxon, depending on genetic and geographical parameters (e.g., climatic, seasonal) (see Kokkini (1992) for a review), and their

Table 1. Crucial Concentration of the Essential Oils (EOs) (μL) of *M. pulegium* and *M. spicata*, the Authentic Compounds, Pulegone, Menthone, and Carvone, and a 7.5:1 Pulegone−Menthone Mixture, Which Causes Death to 50% of the Treated *D. melanogaster* Larvae (LD_{50})[a]

tested compound	LD_{50}
EO of *M. pulegium* (pennyroyal oil) (containing 75.7% pulegone and 10.1% menthone)	2.09
EO of *M. spicata* (spearmint oil) (containing 32.1% carvone)	1.12
pulegone	0.17
menthone	1.29
carvone	0.67
mixture pulegone−menthone (7.5:1)	4.06

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

toxic effects may be species-specific (see Kelsey et al. (1984) as a review). In addition to their insecticidal activity, it was found that the EOs of *M. pulegium* and *M. spicata* present also strong antimicrobial activity (Sivropoulou et al., 1995).

Among the monoterpenes studied, pulegone was found to be the most toxic, causing death to *Drosophila* even in extremely small amounts, followed by carvone and menthone (Table 1; Figure 2). The strong toxicity of pulegone was also proved for *Spodoptera eridania* larvae (Gunderson et al., 1985). According to our data presented in Table 1, the insecticidal activity of the studied EOs is not linearly dependent upon the content of their main constituents. Taking into account the toxicity of the authentic component pulegone (LD_{50} = 0.17) as well as its content (75.7% of the oil) in the whole EO of *M. pulegium*, the oil would be expected to be 9 times more effective than it is. On the contrary, the same oil was expected to be about 6 times less effective, if its lethal effects were linearly dependent on its constituent menthone (LD_{50} = 1.29, content 10.1%). This is also the case of the EO of *M. spicata* (LD_{50} = 1.12) and its main constituent carvone (LD_{50} = 0.67, content 32.1%). Carvone is 2 times less effective when used in its pure form. Given the above results are accurate, synergistic/antagonistic phenomena may exist that alter the toxicity of the EOs. In an effort to check this possibility, we tried to have direct demonstration of the interactions between the two pure compounds of *M. pulegium*. So, we performed the LD_{50} of a mixture 7.5:1 of authentic pulegone:menthone (relative levels of these compounds as found in the extract of *M. pulegium* oil). Data show (Figure 2; Table 1) that the toxicity of this mixture is not in accordance either with the toxicity of the whole essential oil or with that of its constituents. Indeed, the mixture is found to be about 2 times less effective than the oil of *M. pulegium* and 17 times less so than the authentic pulegone. Taking into account that 4.06 μL of the above mixture (Table 1) that contains about 3.75 μL of pulegone causes death to 50% of the tested *Drosophila* while 0.2 μL of this constituent presents the same insecticidal activity, when it is exclusively used, it is obvious that antagonistic phenomena do exist that alter the toxicity of pulegone in the presence of menthone. Moreover, the compounds that constitute the remaining 14.2% of *M. pulegium* seem to play an important role in the final toxicity of the oil, increasing its insecticidal activity. This is probably due to synergistic phenomena. Similar phenomena have also been reported for many constituents of natural products (Harborne, 1982). In the case of carvone, it was proved that its presence alters the insecticidal activity of pyrethrins (Bestmann et al., 1988).

Aiming to find out if the crucial amounts (LD_{50}) of each tested compound that causes death to 50% of the tested *D. melanogaster* larvae exert mutations and/or mitotic recombination effects, the somatic mutation and recombination tests (SMART) were used. These tests proved to be very sensitive assays for screening chemical compounds for genotoxic activity. When *Drosophila* embryos or larvae are exposed to different doses of chemical compounds, various types of somatic mutation or even mitotic recombination effects may be induced in a number of imaginal disk cells (see Wuergler and Vogel (1986) for a review). A good genetic system for detecting wing somatic mutations and recombination effects is the one that uses larvae trans-heterozygous for the third chromosome markers *mwh* (Lindsley and Grell, 1968) and *flr*^{3} (Garcia-Bellido and Dapena, 1974). In the case of mutagenesis, recessive phenotypes are re-expressed as mosaic spots on the wings of the F1 progeny flies. Any increase in the clone frequency as compared to control reflects mutagenic activity, the extent of which is characterized by the frequency of the clone induction (Szabad et al., 1983; Graf et al., 1984).

Table 2 shows the ability of the EOs of *M. pulegium* and *M. spicata* as well as their main constituents, pulegone, menthone, and carvone, to induce somatic mutation or recombination effects. Each of the five compounds was tested with concentrations slightly above LD_{50}. As is shown from the statistical analysis,

Table 2. Summary of Results Obtained in the Wing Somatic Mutation and Recombination Test (SMART) on *D. melanogaster* after Treatment with the Essential Oils (EOs) of *M. pulegium (M.pul)* and *M. spicata (M.spi)*, and the Authentic Compounds, Pulegone, Menthone, and Carvone

		spots per wing (no. of spots) diagnosis[a]			
treatment	wings analyzed	small single spots (1−2 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 *M.pul* 2.1 μL	60	0.81 (49) −	0.13 (8) i	0.08 (5) −	1.03 (62) −
EO of *M.spi* 1.2 μL	60	1.56 (94) +	0.11 (7) i	0.15 (9) −	1.83 (110) +
pulegone 0.2 μL	60	1.08 (65) +	0.04 (3) −	0.05 (4) −	1.2 (72) w
menthone 1.3 μL	62	1.48 (92) +	0.13 (8) i	0.09 (6) −	1.71 (106) +
carvone 0.7 μL	61	0.93 (56) −	0.08 (5) −	0.08 (5) −	1.08(66) −

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

EO of *M. pulegium* did not show any mutagenic or recombinogenic effects, since no significant differences were found at the frequency of the total single or twin spots, between the experimental and control series. On the contrary, *M. spicata* shows strong mutagenic activity. The statistically significant increase in the frequency of single spots could mean that *M. spicata* is able to induce point mutations, deletions, and somatic recombination effects. On the other hand, the negative result obtained from the statistical analysis of the twin spots indicates that the compound does not exhibit recombinogenic activity (Graf et al., 1984). Taking into account the concentrations of the EOs used (Tables 1 and 2), it is obvious that the EO of *M. spicata* is a very strong toxic and genotoxic (mutagenic but not recombinogenic) oil while that of *M. pulegium* exhibits strong toxic but not genotoxic activities. The observed negative mutagenic and recombinogenic effects of *M. pulegium* essential oil in correlation with its strong insecticidal activity should mean that this compound can be used as a potent substance for insect pest control. These results give also signs that this mint oil could possibly be used in food and cosmetics, although additional studies, using different bioassays, are needed.

Among the constituents studied (Table 2), carvone shows negative genotoxic activity, pulegone shows a very weak positive one, and menthone is found to be a potent mutagenetic but not recombinogenic inducer. However, since the concentrations of the above substances used are different, depending on their insecticidal doses (Table 1; Figure 2), only comparative conclusions between their toxic and genotoxic activities can be emerged. Nevertheless, data presented here show that both toxic and genotoxic activities of the essential oils of the studied aromatic plants, *M. pulegium* and *M. spicata*, are not in accordance with those of their major constituents, pulegone, menthone, and carvone, due to synergistic/antagonistic phenomena. The high insecticidal activity of the studied pennyroyal and spearmint oil in cooperation with the increasing interest in the use of "insecticidal" natural products calls for the extension of these studies to insects with great economical impact like *Bactrocera (Dacus) oleae* (Mavragani-Tsipidou et al., in preparation). Moreover, the extensive use of the aromatic plants in the cosmetics and food industries calls for further screening of all the used aromatic plants for mutagenic and recombinogenic activities.

ABBREVIATIONS USED

EO, essential oil; *flr*, flare; LD_{50}, lethal dose 50%; μL, microliter; *mwh*, multiple wing hair; SMART, somatic mutation and recombination test.

ACKNOWLEDGMENT

We thank very much Dr. S. Kokkini for providing the essential oils and for sharing her results before publication. We also thank Dr. Marec for providing us the *D. melanogaster* strains.

LITERATURE CITED

Balandrin, M. F.; Klocke, J. A. Medicinal, Aromatic, and Industrial Materials from Plants. In *Biotechnology in Agriculture and Forestry, Medicinal and Aromatic Plants I;* Bajaj, Y. P. S., Ed.; Springer-Verlag: Berlin, 1988.

Becker, H. J. Die Puffs der Speicheldrusen-chromosomen von *Drosophila melanogaster* I. Beobachtungen zum Verhalten des Puffmusters in Normalstamm und bei zwei Mutanten, *giant* und *lethal-giant*-larvae. *Chromosoma* **1959**, *10*, 654−678 (in German).

Bestmann, H. J.; Classen, B.; Vostrowsky, O.; Klingauf, F.; Stein, U. Botanical insecticides, VIII: the synergistic effect of carvone and pyrethrin I in the essential oil of *Chrysanthemum balsamita* L. *J. Appl. Entomol.* **1988**, *106*, 144−149.

Frei, H.; Wuergler, F. E. Statistical methods to decide whether mutagenicity test data from *Drosophila* assays indicate a positive, negative or inconclusive result. *Mutat. Res.* **1988**, *203*, 297−308.

Garcia-Bellido, A.; Dapena, J. Induction, detection and characterization of cell differentiation mutants in *Drosophila*. *Mol. Gen. Genet.* **1974**, *128*, 117−130.

Graf, U.; Wuergler, F. E.; Katz, A. J.; Frei, H.; Juon, H.; Hall, C. B.; Kale, P. G. Somatic mutation and recombination test in *Drossophila melanogaster*. *Environ. Mutagen.* **1984**, *6*, 153−188.

Gunderson, C. A.; Samuelian, J. H.; Evans, C. K.; Brattsten, L. B. Effects of the mint monoterpene pulegone on *Sporoptera eridania* (Lepidoptera: Noctuidae). *Environ. Entomol.* **1985**, *14*, 859−863.

Harborne, J. B. *Introduction to Ecological Biochemistry*; Academic press: London, 1982.

Janssen, A. M.; Scheffer, J. J.; Baerheim Svendsen, A. Antimicrobial activity of essential oils: a 1976−1986 literature review. Aspects of the test methods. *Planta Med.* **1987**, *53*, 395−398.

Kastenbaum, M. A.; Bowman, K. O. Tables for determining the statistical significance of mutation frequencies. *Mutat. Res.* **1970**, *9*, 527−549.

Kelsey, R G.; Reynolds, G. W.; Rodriguez, E. The chemistry of biologically active constituents secreted and stored in plant glandular trichomes. In *Biology and Chemistry of Plant trichomes*; Rodriguez, E., Healey, P. L., Mehta, I., Eds.; Plenum Press: New York, 1984.

Kokkini, S. Chemical races within the genus *Mentha* L. In *Modern Methods of Plant Analysis, New Series 12;* Linskens, H. F., Jackson, J. F., Eds.; Spinger: Berlin, 1991.

Kokkini, S. Essential oils as taxonomic markers in *Mentha*. In *Advances in Labiatae Science*; Harley, R. M., Reynolds, T., Eds.; Royal Botanic Gardens: Kew, U.K., 1992.

Konstantopoulou, I.; Vassilopoulou, L.; Mavragani-Tsipidou, P.; Scouras, Z. G. Insecticidal effects of essential oils. A study of the effects of essential oils extracted from eleven Greek aromatic plants on *Drosophila auraria*. *Experientia* **1992**, *48*, 616−619.

Lindsley, D. L.; Grell, E. H. Genetic variations of *Drosophila melanogaster*. Garnegie Institute Washington Publication No 627; 1968.

Lindsley, D. L.; Zimm, G. G. *The genome of Drosophila melanogaster*; Academic Press: San Diego, 1992.

Marec, F.; Gelbic, I. High recombinagenic activities of three antiviral agents adenine derivatives, in the *Drosophila* wing spot test. *Mutat. Res.* **1994**, *311*, 305−317.

Margolin, B. H.; Collings, B. J.; Mason, J. M. Statistical analysis and sample-size determinations for mutagenicity experiments with binomial responses. *Environ. Mutagen.* **1983**, *5*, 705−716.

Panizzi, L.; Flamini, G.; Cioni, P. L.; Morelli, I. Composition and antimicrobial properties of essential oils of four Mediterranean Lamiaceae. *J. Ethnopharmacol.* **1993**, *39*, 167−190.

Reynolds, S. E. The cuticle, growth and moulting in insects: the essential background to the action of acylurea insecticides. *Pestic. Sci.* **1987**, *20*, 131−146.

Selby, P. B.; Olson, W. H. Methods and criteria for deciding whether specific-locus mutation-rate data in mice indicate a positive, negative, or inconclusive result. *Mutat. Res.* **1981**, *83*, 403−418.

Sivropoulou, A.; Kokkiki, S.; Lanaras, T.; Arsenakis, M. Antimicrobial activity of mint essential oils. *J. Agric. Food Chem.* **1995**, *43*, 2384−2388.

Szabad, J.; Soos, I.; Polgar, G.; Hejja, G. Testing the mutagenicity of malondialdehyde by the *Drosophila* mosaic and the

sex-linked recessive lethal tests. *Mutat. Res.* **1983**, *113*, 117−133.

Thompson, D. P. Fungitoxic activity of essential oils components on food storage fungi. *Mycologia* **1989**, *81*, 151−153.

Vokou, D. The allelopathic potential of aromatic shrubs in phryganic (east Mediterranean) ecosystems. In *Allelopathy: Basic and Applied aspects*; Rizvi, S. J. H., Rizvi, V., Eds.; Chapman and Hall: London, 1992.

Wuergler, F. E.; Vogel, E. W. In *vivo* mutagenicity testing using somatic cell of *Drossophila melanogaster*. In *Chemical, Principles and Methods for Their Detection*; de Serres, F. J., Ed.; Plenum: New York, 1986.

Received for review September 9, 1996. Accepted April 15, 1997.[⊗] The present work was supported by an ECLAIR grant from EC (AGRE CT91-0060, Project 0019).

JF960685F

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1997.