# Antimicrobial and Cytotoxic Activities of *Origanum* Essential Oils

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Three *Origanum* essential oils, *Origanum vulgare* ssp*. hirtum, Origanum dictamnus*, and a commercially available *Origanum* oil, were analyzed by gas chromatography–mass spectrometry (GC–MS) and showed a high content of carvacrol, thymol, $\gamma$-terpinene, and *p*-cymene representing 73.7%, 92.8%, and 87.78% of the total oil, respectively. The three essential oils exhibited high levels of antimicrobial activity against eight strains of Gram-positive and Gram-negative bacteria. Among the major components of the three oils, carvacrol and thymol exhibited the highest levels of antimicrobial activity, while their biosynthetic precursors $\gamma$-terpinene and *p*-cymene were inactive. The essential oil of *O. vulgare* ssp. *hirtum* was extremely bactericidal at 1/4000 dilution and even at dilutions as high as 1/50000 caused considerable decrease in bacterial growth rates. The same essential oil also exhibited high levels of cytotoxicity against four permanent animal cell lines including two derived from human cancers.

**Keywords:** *Origanum vulgare; Origanum dictamus; essential oils; carvacrol; thymol; antimicrobial activity; cytotoxicity; terpenes; cancer*

## INTRODUCTION

Essential oils derived from many plants are known to possess antifungal (Thompson, 1989), insecticidal (Konstantopoulou et al., 1992), and antimicrobial activities (Janssen et al., 1987). Among them are the *Origanum* essential oils (Janssen et al., 1986; Valnet et al., 1978; Aifi, 1975) which are widely used in the flavoring of food products and alcoholic beverages.

Members of the genus *Origanum* (Labiatae family) are often characterized by the existence of chemical differences, with respect to both essential oil content and composition. As a result, the total (crystallizable and noncrystallizable) phenol content of their essential oils ranges from traces up to 95%, even between plants of the same species (Kokkini and Vokou, 1989; Vokou et al., 1992). The preponderance of carvacrol (noncrystallizable phenol) or thymol (crystallizable phenol) in their essential oils is responsible for their commercial classification as oregano or thyme oil, respectively (Kokkini, 1994). The variation in the chemical composition of *Origanum* essential oils is likely to have a bearing on the level of their antimicrobial properties. In this paper we report on a comparative study of the antimicrobial properties of three representative *Origanum* essential oils and their main chemical constituents. Two of them were derived from the well-known commercial herbs Greek oregano, viz. *Origanum vulgare* L. ssp. *hirtum* (Link) Ietswaart, and Dittany of Crete, viz., *Origanum dictamnus* L., whereas the third was a commercially available preparation of *Origanum* oil.

## MATERIALS AND METHODS

**Plant Material: GC and GC–MS Analyses of Essential Oils.** Aerial parts of (i) wild growing *O. vulgare* ssp. *hirtum* plants, collected from the island of Euboea, and (ii) *O. dictamnus* plants, cultivated on the island of Crete, were air-dried and grossly pulverized. Their essential oils were isolated after hydrodistillation for 2 h, using a Clevenger apparatus. A commercially available *Origanum* essential oil was also used.

The three essential oils were chromatographed using a Shimadzu GC-14A gas chromatograph equipped with a Supelcowax 10 (Supelco) capillary column (60 m $\times$ 0.25 mm i.d.). The carrier gas was helium, and the linear gas velocity was 20.4 cm/s. The injector temperature was 240 °C. The column temperature was initially 70 °C and then gradually increased at a rate of 4 °C/min up to 220 °C. For detection a flame ionization detector (FID) was used set at a temperature of 240 °C. Gas chromatography–mass spectroscopy (GC–MS) analyses were conducted using a Shimadzu GC–MS QP2000 system equipped with a Supelcowax 10 capillary column (60 m $\times$ 0.25 mm i.d.) under the same GC conditions. For GC–MS detection, an electron impact (EI) quadropolar system was used with ionization energy of 70 eV. The essential oil components were identified by comparing their relative retention times ($t_R$) and mass spectra (MS) with those of authentic samples and literature citations (Stenhagen et al., 1974; Masada, 1976; Jennings and Shibamoto, 1980) as well as a computerized MS data bank.

**Bacterial Strains and Media.** The following reference strains of bacteria, purchased from the National Collection of Industrial and Marine Bacteria (NCIMB, Aberdeen, Scotland), were used as test organisms in all antimicrobial assays: *Escherichia coli* (NCIMB 8879 and NCIMB 12210), *Pseudomonas aeruginosa* (NCIMB 12469), *Salmonella typhimurium* (NCIMB 10248), *Staphylococcus aureus* (NCIMB 9518 and NCIMB 8625), *Rhizobium leguminosarum* (NCIMB 11478), and *Bacillus subtilis* (NCIMB 3610). Bacteria were grown either in nutrient broth or in nutrient agar and incubated at 37 °C (*E. coli, P. aeruginosa*, and *S. typhimurium*), at 30 °C (*S. aureus* and *B. subtilis*), or at 25 °C (*R. leguminosarum*). These particular strains are standard reference strains that are routinely used for the evaluation of antimicrobial compounds.

**Antimicrobial Assay (Disk Diffusion Assay).** Filter paper disks (Whatman No. 1, 5 mm diameter) containing 5 $\mu$L of the particular essential oil or isolated compound (authentic samples, Aldrich Chemical Co., Milwaukee, WI) were

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applied on the surface of agar plates previously seeded with test organisms. The plates were incubated overnight at the appropriate temperature (see above), and the net zone of inhibition (mm) was determined. The results indicated in Table 2 and in the text represent the net zone of inhibition after substraction of the diameter (5 mm) of the paper disk.

**Determination of Bacterial Cell Growth.** Isolated bacterial colonies from overnight plates were transferred into nutrient broth and grown overnight at the appropriate temperature (see above). Tubes of nutrient broth containing varying concentrations of essential oils, initially diluted 1:10 (v/v) in ethanol, were then inoculated with appropriate aliquots of these cultures so that their optical densities ($OD_{600}$) were equal. The growth of each culture was monitored in two ways, either by measuring its optical density at 600 nm ($OD_{600}$) at 30 min intervals for a total period of 8 h (total counts) or by plating at specific time intervals suitably diluted aliquots of the culture on nutrient agar plates (viable counts).

**Cells and Cytotoxicity Assay.** Vero (African green monkey, kidney), Hep-2 (epidermoid carcinoma, larynx, human), RSC (rabbit skin), and HeLa (epitheloid carcinoma, cervix, human) cells were grown in Dulbecco's modified eagle medium (DMEM) supplemented with 5% fetal calf serum (FCS) and 5% newborn calf serum (NCS). For cytotoxicity assays, cells in DMEM were seeded into 12-well plates at a density of $2.1 \times 10^5$ cells/well and the appropriate dilution of the essential oil was then added. The essential oils were initially diluted 1:100 (v/v) in ethanol, and further dilutions were made in DMEM. After 24 h of incubation, the medium was removed; the cells were trypsinized and counted in a hemocytometer by the trypan blue-exclusion method (Hayashi et al., 1990). Growth inhibition data were plotted as a dose−effect curve (Figure 3), from which the 50% inhibitory dose ($ID_{50}$) was obtained.

## RESULTS AND DISCUSSION

Table 1 shows that the main components of the three oils analyzed, the commercial *Origanum* oil, *O. vulgare* ssp. *hirtum* oil, and *O. dictamnus* oil, were carvacrol, thymol, γ-terpinene, and *p*-cymene. Significant quantitative differences between the three oils were apparent only between the two isomeric phenols, carvacrol and thymol, and their biosynthetic precursors γ-terpinene and *p*-cymene (Poulose and Croteau, 1978). The concentration of these components varied greatly among the three oils but particularly that of carvacrol (0.43−79.58%) and thymol (0.44−31.8%). In all cases, however, the sum of the two phenols and their precursors constituted the bulk of each essntial oil: 73.7%, 92.8%, and 87.7% of the total oil, respectively. Due to its low content of carvacrol, the commercial *Origanum* oil cannot be characterized as a typical "oregano" oil (Kokkini, 1994). The high amount of carvacrol found in the *O. vulgare* ssp. *hirtum* oil has also been observed in several other Greek wild populations of this taxon. It should be noted that in some cases thymol, instead of carvacrol, is the major component of the Greek oregano essential oils (Vokou et al., 1993).

The great variability in the concentration of the main compounds present in the three essential oils led us to evaluate the antimicrobial activities of the isolated authentic compounds and the three essential oils. Antimicrobial activity was evaluated by the disk diffusion assay against a panel of eight bacterial strains. The results presented in Table 2 show that the the oils exhibited a variable degree of antimicrobial activity against all the bacteria tested with the exception of *P. aeruginosa* which was resistant to the three oils and all compounds tested. Similarly, essential oils from other *Origanum* species have also been shown to possess high levels of antimicrobial activity (Janssen et al., 1986; Deans and Ritchie, 1987).

**Table 1. Quantitative and Qualitative Composition of the Three Essential Oils: *Origanum* Commmercial Oil, *O. vulgare* ssp. *hirtum*, and *O. dictamnus***

| component | composition (%) | | |
|---|---|---|---|
| | commercial *Origanum* oil | *O. vulgare* ssp. *hirtum* | *O. dictamnus* |
| 1, α-thujene | 0.13 | –[b] | – |
| 2, α-pinene | 2.40 | 0.88 | 1.56 |
| 3, camphene | 5.36 | 0.15 | 0.13 |
| 4, β-pinene | 0.34 | 0.08 | 0.14 |
| 5, sabinene | 1.00 | 0.04 | 0.06 |
| 6, myrcene | 0.75 | 0.61 | 1.21 |
| 7, α-phellandrene | 0.12 | 0.07 | 0.15 |
| 8, α-terpinene | 1.53 | 0.62 | 1.88 |
| 9, limonene | 4.12 | 0.14 | 0.23 |
| 10, 1,8-cineole | 0.68 | 0.18 | 0.19 |
| 11, β-phellandrene | tr[a] | 0.08 | 0.06 |
| 12, γ-terpinene | 1.32 | 2.07 | 11.41 |
| 13, β-ocimene | tr | 0.09 | 0.03 |
| 14, *p*-cymene | 40.15 | 8.76 | 13.49 |
| 15, α-terpinolene | 0.40 | 0.05 | 0.08 |
| 16, 6-methyl-3-heptanol | 0.11 | – | – |
| 17, nonanal | 0.09 | – | – |
| 18, 3-octanol | – | – | 0.08 |
| 19, 1-octen-3-ol | 0.04 | 0.37 | 0.38 |
| 20, *trans*-sabinene hydrate | tr | 0.15 | 0.63 |
| 21, β-bourbonene | 0.05 | – | – |
| 22, *cis*-sabinene hydrate | 4.32 | 0.22 | 1.01 |
| 23, linalool | | 0.12 | 0.23 |
| 24, linalyl acetate | 0.06 | – | 0.02 |
| 25, terpinen-4-ol | tr | – | – |
| 26, β-caryophyllene | 0.62 | 1.50 | 1.63 |
| 27, methylcarvacrol | 0.14 | 0.05 | 0.05 |
| 28, *trans*-dihydrocarvone | 0.13 | 0.03 | 0.03 |
| 29, *cis*-dihydrocarvone | 0.02 | tr | tr |
| 30, isoborneol | 0.09 | 0.10 | 0.17 |
| 31, α-terpineol | 2.31 | 0.42 | 0.44 |
| 32, γ-elemene | 0.07 | 0.20 | 0.02 |
| 33, β-bisabolene | 0.09 | 0.15 | 0.17 |
| 34, γ-cadinene | – | 0.02 | 0.06 |
| 35, *trans*-carveol | 0.06 | 0.10 | 0.18 |
| 36, calemene | – | 0.03 | 0.08 |
| 37, *p*-cymen-8-ol | – | 0.08 | 0.09 |
| 38, carvacrol acetate | – | 0.36 | 0.52 |
| 39, spathulenol | 0.05 | 0.05 | 0.22 |
| 40, thymol | 31.80 | 2.45 | 0.44 |
| 41, carvacrol | 0.43 | 79.58 | 62.44 |

[a] tr, $<0.01\%$. [b] Not determined.

Of the main compounds tested, γ-terpinene and *p*-cymene did not show any activity against the bacterial strains tested, whereas their biosynthetic products carvacrol and thymol exhibited high levels of antimicrobial activity against all bacteria with the exception of *P. aeruginosa*. In agreement, Didry et al. (1993) also observed high levels of antimicrobial activity for thymol and carvacrol against several bacterial strains but not against *P. aeruginosa*. This bacterium exhibits resistance to many antimicrobial agents but was found to be sensitive to pulegone, isopulegol, and piperitone as well as to oils rich in these compounds (Sivropoulou et al., 1995; Panizzi et al., 1993). Essential oils rich in phenolic compounds (carvacrol, thymol) are reported to posses high levels of antimicrobial activity (Panizzi et al., 1993; Pellecuer et al., 1980; Gergis et al., 1990). Carvacrol has also been reported to posses antifungal (Thompson, 1989) and antioxidant activity (Lagouri et al., 1993). The results presented in Table 2 indicate that thymol was more active than carvacrol against Gram-negative bacteria, in agreement with the data presented by Didry et al. (1993). The antibacterial activity of both thymol and carvacrol was variable even between different strains of the same bacterial species, e.g., *S. aureus* strain NCIMB 8625 was more susceptible to both compounds than *S. aureus* strain NCIMB 9518.

**Table 2. Antimicrobial Activity of Three *Origanum* Essential Oils (Commercial *Origanum* Oil, *O. vulgare* ssp. *hirtum*, and *O. dictamnus*) and Their Main Components[a]**

| | bacterial strains according to NCIMB[c] number | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| oil/compound | 8879 | 12210 | 10248 | 12469 | 11478 | 8625 | 9518 | 3610 |
| commercial *Origanum* | 12 | 15 | 11 | 2 | 33 | 17 | 13 | 29 |
| *O. dictamnus* | 19 | 17 | 13 | 2 | 24 | 9 | 13 | 30 |
| *O. vulgare* | 15 | 19 | 16 | 3 | 24 | 15 | 12 | 27 |
| *p*-cymene | –[b] | – | – | – | – | – | – | – |
| γ-terpinene | – | – | – | 2 | – | – | – | – |
| carvacrol | 16 | 18 | 18 | 3 | 25 | 23 | 13 | 26 |
| thymol | 26 | 25 | 23 | 3 | 34 | 16 | 12 | 24 |

[a] The diameter (mm) of the inhibition zone is the mean of the three independent experiments (the diameter of the paper disk, 5 mm, is not included). [b] no activity. [c] NCIMB, National Collection of Industrial and Marine Bacteria (see Materials and Methods for details).

*R. leguminosarum* was the most sensitive among the bacteria tested. *R. leguminosarum* was more susceptible to thymol than to carvacrol and also to the *Origanum* commercial oil among the three essential oils tested. The higher suseptibility of this bacterium to *Origanum* commercial oil must be attributed to its higher thymol content: 37.9% versus 2.45% for *O. vulgare* ssp. *hirtum* and 0.44% for *O. dictamnus.*

Generally the three oils showed comparable levels of antimicrobial activity, even though the total content of phenolic compounds was lower in *Origanum* commercial oil (32.23%) than in the other two oils (*O. vulgare*, 82.03%, and *O. dictamnus*, 62.88%). This may be attributed at least in part to the fact that the oils contained other components with antimicrobial activity such as 1,8-cineole, linalool, linalyl acetate, α-terpineol, isoborneol (unpublished data), *trans*-dihydrocarvone, *cis*-dihydrocarvone, and *trans*-carveol (Sivropoulou et al., 1995). Some of these compounds, such as 1,8-cineole, *trans*-dihydrocarvone, *cis*-dihydrocarvone, and α-terpineol, were present in higher concentrations in *Origanum* commercial oil than in the other two oils, while the rest of the compounds were present at very low concentrations in all three oils. In addition, synergistic or antagonistic effects between some components may also affect the observed antimicrobial activity of the oil. Synergistic (partial or total) antimicrobial activity of carvacrol and thymol against some bacteria has been recently reported (Didry et al., 1993).

The disk diffusion assay can not differentiate between the bactericidal and bacteriostatic effects exerted by the oils. Consequently we examined the effect of one representative oil, *O. vulgare,* containing the highest amounts of carvacrol and thymol (total 82.03%) on *S. aureus* (NCIMB 8625) against cummulative time. The oil at 1/4000 dilution killed all the bacteria within 60 min after exposure (Figure 1). The high bactericidal action of the oil was even more obvious when the bacteria were in the stationary phase of growth (data not shown). Similar results were obtained for *O. dictamnus* (data not shown).

The high bactericidal action of the *O. vulgare* oil prompted us to examine its effect on bacterial growth rates at high dilutions (1/5000, 1/10000, and 1/50000) and over longer periods of exposure. The results (Figure 2) showed that the oil abrogates bacterial replication at dilutions of up to 1/10000, while 1/50000 dilution caused a considerable decrease in the growth rate of *S. aureus* over the 8-h incubation period. Similar results were obtained for *O. dictamnus* oil.

Lam and Zheng (1991) reported that *Origanum* oil fed to mice induces the activity of glutathione *S*-transferase (GST) in various tissues. GST is thought to play an important role in detoxifying chemical carcinogens. The observed high biological activity of *Origanum* oil against several bacteria, coupled with the putative anticancer potential of the oil suggested by Lam and Zheng (1991), leads us to examine the effect of *O. vulgare* oil on the replication of eukaryotic cells. For that purpose four continuous cell lines, Vero (African green monkey, kidney), Hep-2 (epidermoid carcinoma, larynx, human), RSC (rabbit skin), and HeLa (epitheloid carcinoma, cervix, human), were exposed to varying concentrations of the oil, and the cell viability was evaluated after 24 h of exposure. The oil caused complete cell death in all cell lines tested at dilutions of up to 1/10000 (Table 3) and continued to cause a small decrease in cell viability even at concentrations as high as 1/50000. The oil did not seem to have any specific action on Hep-2 and HeLa cells. The $ID_{50}$ doses of *O. vulgare* oil on Vero cells was 1/36000, as calculated from the curve presented in Figure 3.

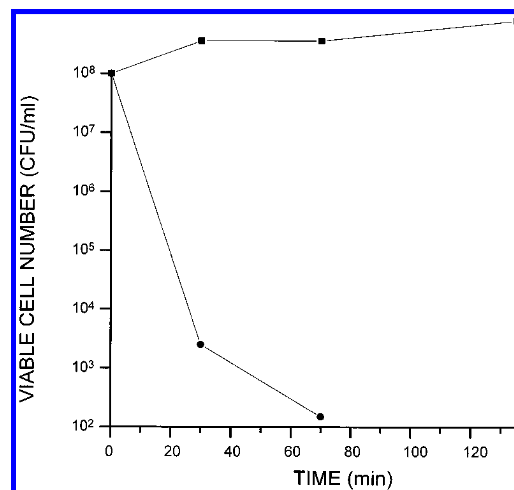


**Figure 1.** Time dependent effect of *O. vulgare* ssp. *hirtum* essential oil on the viability of *S. aureus* (NCIMB 8625). Equal aliquots of overnight bacterial cultures were inoculated in equal amounts of nutrient broth supplemented with *O. vulgare* ssp. *hirtum* essential oil at 1/4000 dilution or with diluent alone. At specific time intervals, suitably diluted aliquots of the two cultures were plated on nutrient agar and the viable cell number was counted. Each point represents the average of three values. These experiments were repeated independently three times and yielded essentially the same results: control (■) and 1/4000 (●).

**Table 3. Cytotoxicity of *O. vulgare* ssp. *hirtum* Essential Oil on Cell Lines (RSC, Hep-2, HeLa, Vero)[a]**

| | oil dilution | |
|---|---|---|
| cell line | 1/10000 | 1/50000 |
| RSC | ++++ | – |
| Hep-2 | ++++ | – |
| HeLa | ++++ | – |
| Vero | ++++ | + |

[a] ++++, all cells were dead after 24 h; –, no effect; +, small decrease in the number of cells.

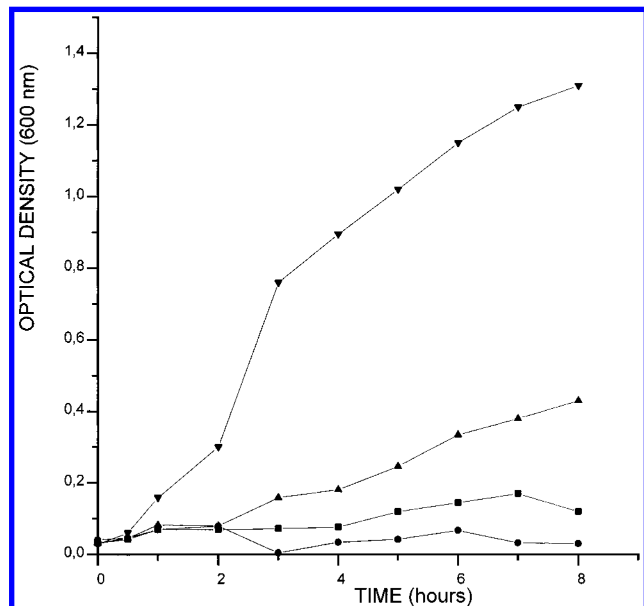


**Figure 2.** Time and concentration dependent effect of *O. vulgare* ssp. *hirtum* essential oil on the rate of growth of *S. aureus* (NCIMB 8625). Equal aliquots of overnight bacterial cultures were inoculated in equal amounts of nutrient broth supplemented with or without varying concentrations of essential oil (1/5000, 1/10000, and 1/50000), and cell growth was monitored spectrophotometrically at 600 nm at specific time intervals. Each point represents the average of three values. These experiments were repeated independently three times and yielded essentially the same results: control (▼), 1/5000 (●), 1/10000 (■), and 1/50000 (▲).

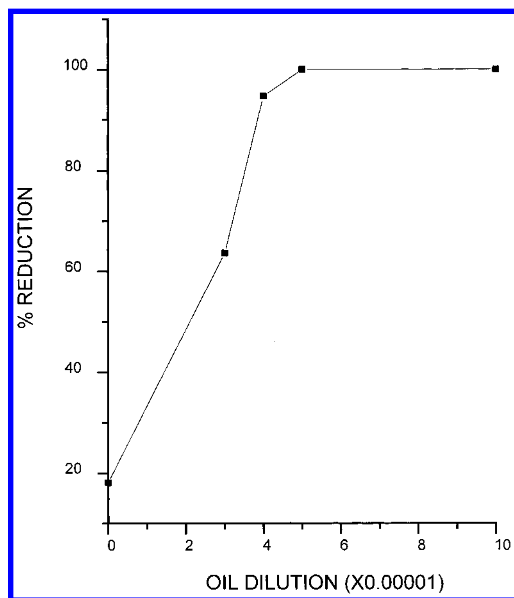


**Figure 3.** Effect of *O. vulgare* ssp. *hirtum* essential oil on the growth of Vero cells. Percent reduction in cell numbers relative to the untreated control is plotted as a function of oil dilution. Each point represents the average of three values. These experiments were repeated independently three times and yielded essentially the same results.

Consequently *Origanum* oil is an interesting prospect as a potential source of biologically active compounds.

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Received for review August 10, 1995. Revised manuscript received December 27, 1995. Accepted February 16, 1996.[⊗] This work was financially supported, in part, by EEC (AGRE CT91-0060, Project 0019).

JF950540T

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1996.