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Genotoxicity and antigenotoxicity of Origanum oil and carvacrol evaluated by Ames Salmonella/microsomal test

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Abstract

This study evaluates the genotoxic and antigenotoxic effects of the essential oil of *Origanum onites* L. and carvacrol that are used in medicine, flavouring of food and crop protection by Ames Salmonella/microsomal test. The mutagenic activity was initially screened using *Salmonella typhimurium* strains TA98 and TA100, with or without S9 metabolic activation. No mutagenicity was found in the oil to the both strains either with or without S9 mixture whereas significant mutagenic activity was induced by carvacrol generally in the absent of metabolic activity. The oil and its major constituent carvacrol were finally tested for their antimutagenic activity with 30 min standard preincubation time. It was shown that both of them strongly inhibited mutagenicity induced by 4-nitro-*o*-phenylenediamine and 2-aminofluorene in both strains with or without S9, respectively. These results indicate significant antimutagenicity of the essential oil and carvacrol in vitro, suggesting its pharmacological importance for the prevention of cancer.

Keywords: Ames test; Antigenotoxicity; Carvacrol; Genotoxicity; Origanum oil

1. Introduction

The essential oils are natural products derived from aromatic plants and have a wide range of uses in medicine, crop protection and flavouring and fragrances in the food and perfume industries (reviewed in Isman, 2000; Daferera, Ziogas, & Polissiou, 2003). Among them are *Origanum* essential oils which are known to possess a broad spectrum of in vitro and in vivo antibacterial (Lambert, Skandamis, Coote, & Nychas, 2001; Nostro et al., 2004; Sivropoulou et al., 1996), antifungal (Manohar et al., 2001), insecticidal (Isman, Wan, & Passreiter, 2001), antioxidants (Kulisic, Radonic, Katalinic, & Milos, 2004; Puertas-Mejia, Hillebrand, Stashenko, & Winterhalter, 2002; Ruberto & Baratta, 2000; Vekiari, Oreopoulou, Tzia, & Thomopoulos, 1993) and anti-carcinogenic activities (Teissedre & Waterhouse, 2000).

Chemical compositions of these oils are well documented and they include monoterpenes, biogenetically related phenolics as well as sesquiterpenes (Daferera et al., 2003; Kulisic et al., 2004; Sivropoulou et al., 1996). The major constituents are phenolic monoterpens such as thymol and carvacrol which are responsible for the main biological activities mentioned above (Aeschbach et al., 1994; Aydin, Öztürk, Beis, & Baser, 1996; Bagamboula, Uyttendaele, & Debevere, 2004; Didry, Dubreuil, & Pinkas, 1993; Nostro et al., 2004; Puertas-Mejia et al., 2002; Thompson, 1996; Ultee, Gorris, & Smid, 1998). It has been suggested that carvacrol exerts its activities by interacting with the cytoplasmic membrane via its own hydroxyl group, thus

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changing the permeability of membrane for protons and potassium ions (Ultee, Bennik, & Moezelaar, 2002).

Carvacrol inhibited DMBA-induced tumorigenesis in rats and the growth of melanomas in vitro (He, Mo, Hadisusilu, Quresni, & Elson, 1997; Zeytinoglu, Aydin, Ozturk, & Baser, 1998), on the other hand, increased the number of revertants in the Ames test by 1.5–1.7 times (Stammatia et al., 1999). In previous studies, we found that carvacrol prevented mutagenicity of known mutagens in lymphocytes and inhibited the DNA synthesis in mouse myoblasts (Ipek, Tüylü, & Zeytinoğlu, 2004; Zeytinoglu, Incesu, & Baser, 2003).

Consequently, Origanum oil and its main constituents are considered as a potential source of biologically active compounds. Therefore, this study was performed to investigate the mutagenic and antimutagenic activity of the essential oil of *Origanum onites* L. and carvacrol, using the Salmonella/mammalian microsome assay, either with or without metabolic activation. Both the oil and carvacrol exhibited a strong antimutagenic effect on TA98 and TA100 strains of *S. typhimurium* in the presence or absence of metabolic activation.

2. Materials and methods

2.1. Plant extract GC conditions

The steam distillated essential oil of *O. onites* L. obtained commercially was analysed by GC and GC/MS. GC analysis of the oil was carried out using a Shimadzu GC-9A with CR4-A integrator. Thermon 600T FSC column (50 m × 0.25 mm i.d.) was used with nitrogen as carrier gas. Oven temperature was kept at 70 °C for 10 min and programmed to 180 °C for at a rate of 2 °C/min, and then kept constant at 180 °C for 30 min. Split ratio was adjusted at 60:1. The injector and FID detector temperatures were at 250 °C.

2.2. Gas chromatography/mass spectrometry

The essential oil was analysed by gas chromatography/mass spectrometry using a Hewlett–Packard GCD system. Innowax FSC column (60 m × 0.25 mm, 0.25 μm film thickness) was used with helium as carrier gas. GC oven temperature was kept at 60 °C for 10 min and programmed to 220 °C at a rate of 4 °C/min, and then kept constant at 220 °C for 10 min and programmed to 240 °C at a rate of 1 °C min. Split flow was adjusted at 50 ml/min. The injector temperature was at 250 °C. MS were taken at 70 eV. Mass range was from *m*/*z* 35 to 425.

2.3. Identification of components

The components were identified by comparison of their mass spectra both with "Wiley GC/MS Library" and the in-house "Baser Library of Essential Oil Constituents". Relative percentage amounts of the separated compounds were calculated automatically from peak areas of the total ion chromatogrammes. *n*-Alkanes were used as reference points in the calculation of relative retention indices (RRI).

Carvacrol (2-methyl-5-(1-methyl ethyl) phenol) examined in this study was isolated from the essential oil of *O. onites* L. Carvacrol-rich fractions were bulked to obtain carvacrol with 99% purity (GC–MS). Sterilized oil and carvacrol were dissolved in dimethylsulfoxide (DMSO) and used freshly in each experiment.

2.4. Salmonella mutagenicity assay

The potential of mutagenic effects of Origanum oil and carvacrol were evaluated on two *S. typhimirium* strains TA98 and TA100 that were provided by Dr. Bruce N. Ames (University of California, Berkley, USA). The plate incorporation assay was performed according to Maron and Ames (1983), by adding 0.1 ml of the overnight bacterial culture and 0.1 ml of test compounds at different concentrations (0.01, 0.05, 0.10 and 0.50 μl/plate) to the test tubes. In the case of metabolic activation, 0.5 ml S9 mixture was supplemented. After incubation of culture for 24 h at 37 °C, 2.5 ml of top agar was added to each tube and then plated on minimal agar. His^+ revertants were counted after 72 h of incubation at 37 °C on a colony counter.

The S9 mixture was prepared from 3-methylcolanthrene (Sigma, 80 mg/kg)-induced Wistar rat microsomal fraction (Maron & Ames, 1983) and S9 mutagenicity tablets following the method of manufacturer (Boehringer–Mannheim Biochemicals, Germany). All steps were performed at 4 °C with cold and sterile solutions and glassware. S9 fraction was distributed in aliquots in small sterile tubes and stored at −80 °C. The test compounds were prepared freshly for each experiment by dissolving in DMSO. The positive controls employed were 4-nitro-*o*-phenylenediamine (4-NPD; Aldrich Chemical Co.) at 200 μg/plate and 2-aminofluorene (2-AF; Merck) at 1 μg/plate, respectively without and with 10% of S9. As a Solvent control 100 μl/plate of DMSO was run concurrently with all experiments. Three plates at two separate experiments were used for each concentration tested and for positive and negative controls.

2.5. Determination of the antimutagenic effect

The antimutagenicity assay was performed using the plate/incorporation procedure as described above, but by the preincubation of strains without or with test

substances (0.01, 0.05, 0.10 and 0.50 μl/plate) for 30 min in the presence or in the absence of S9 before the treatment with the diagnostic mutagens. Antimutagenesis was determined as the percentage of remaining mutagenesis. Each dose was assayed using triplicate plates in two independent experiments.

The mutagenicity of 4-NPD and 2-AF with DMSO as a positive control in the absence of the test compound was defined as 100% mutagenicity. The calculation of percentage remaining mutagenicity was done according to formula given by Ferrer, Sanchez-Lamar, Fuentes, Barbe, and Llagostera (2002); percentage remaining mutagenesis = $100 \times (His^+$ revertants per plate with mutagen and plant oil/His^+ revertants per plate with mutagen alone).

2.6. Statistics

Data were compared by analysis of variance followed by a Dunnett's test to compare the treated groups to the control group.

3. Results

3.1. GC analysis of origanum oil

Composition of the oil was determined by GC and GC/MS analyses using a reliable library and relative retention indices. Results of GC analysis of Origanum oil obtained by steam distillation are given in Table 1. Carvacrol was found to be a major component of the oil as 74%, and then followed by linalool, thymol and *p*-cymene 7.2%, 4.4% and 3.0%, respectively. Minor amount of β-bisabolene and caryophyllene oxide were also detected.

3.2. Mutagenicity and antimutagenicity of origanum oil

In order to assess the mutagenic and antimutagenic effects of Origanum oil, induction or suppression of revertant colonies was examined in *S. typhimurium* strains. The number of spontaneous revertants for both strains with or without S9 metabolism was determined in each set of experiment and indicated as untreated sample in Tables 2 and 3. The results of mutagenicity assay of Origanum oil are presented in Table 2. Different

Table 1
Results of GC analysis of the oil obtained from *O. onites* L. by steam distilation

Compound	RRI	%
Carvacrol	2239	74.0
Linalool	1553	7.2
Thymol	2198	4.4
p-Cymene	1280	3.0
β-Bisabolene	1741	1.4
Caryophyllene oxide	2008	1.3

Relative retention indices (RRI) were measured on a polar column.

Table 2
Mutagenicity: mean number of revertants induced by Origanum oil, the reference mutagen and the negative control in *Salmonella* plate incorporation test using TA98 and TA100 with or without S9

Dose level (μl/plate)	TA98	TA100
Absence of S9 metabolism		
0.01	23.6 ± 4.0	103.6 ± 11.6
0.05	19.6 ± 2.5	96.0 ± 26.2
0.10	17.6 ± 3.2	88.0 ± 16.0
0.50	15.0 ± 3.0	68.6 ± 6.8
DMSO, 100 μl	19.6 ± 5.1	105.0 ± 14.5
Untreated	25.7 ± 3.3	113.5 ± 5.2
4-NPD, 200 μg	760.0 ± 80.0	801.3 ± 143.6
Presence of S9 metabolism		
0.01	31.6 ± 3.7	64.6 ± 14.5
0.05	47.3 ± 1.5*	92.3 ± 10.5*
0.10	33.3 ± 4.1	79.6 ± 6.4
0.50	22.3 ± 2.0	40.0 ± 3.0
DMSO, 100 μl	35.0 ± 7.0	71.6 ± 10.2
Untreated	32.0 ± 8.2	97.3 ± 16.8
2-AF, 1 μg	892.3 ± 32.3	1003.0 ± 38.0

(−S9) without and (+S9) with metabolic activation. Mean and ± S.D. of three plates.
DMSO, dimethylsulfoxide, solvent control; 2-AF, 2-aminofluorene, positive control for +S9; 4-NPD, 4-nitro-*o*-phenylenediamine, positive control for −S9.
* Significantly different from the corresponding solvent control value (Dunnett's test, $P < 0.05$).

Table 3
Mutagenicity: mean number of revertants induced by carvacrol, the reference mutagen and the negative control in *Salmonella* plate incorporation test using TA98 and TA100 with or without S9

Dose level (μl/plate)	TA98	TA100
Absence of S9 metabolism		
0.01	146.3 ± 11.6**	181.3 ± 15.6**
0.05	116.8 ± 18.3**	154.3 ± 21.4*
0.10	119.3 ± 10.8**	90.5 ± 21.5
0.50	108.1 ± 7.7**	112.1 ± 15.8
1.00	24.1 ± 3.9	79.6 ± 19.4
DMSO, 100 μl	39.5 ± 6.3	95.1 ± 7.4
Untreated	48.3 ± 5.5	111.7 ± 9.7
4-NPD, 200 μg	1035.8 ± 57.6	989.3 ± 20.9
Presence of S9 metabolism		
0.01	208.0 ± 12.3**	61.3 ± 13.5
0.05	124.0 ± 13.6**	60.3 ± 10.6
0.10	54.1 ± 3.2	83.1 ± 16.6
0.50	39.0 ± 4.8	77.5 ± 13.0
1.00	28.0 ± 12.7	20.6 ± 5.6
DMSO, 100 μl	46.5 ± 12.5	105.3 ± 14.2
Untreated	39.9 ± 5.7	112.4 ± 11.1
2-AF, 1 μg	1672.0 ± 175.5	1262 ± 37.7

(−S9) without and (+S9) with metabolic activation. Mean and ±S.D. of three plates.
DMSO, dimethylsulfoxide, solvent control; 2-AF, 2-aminofluorene, positive control for +S9; 4-NPD, 4-nitro-*o*-phenylenediamine, positive control for −S9.
Significantly different from the corresponding solvent control value (Dunnett's test, $*P < 0.05$, $**P < 0.001$).

concentrations of the essential oil did not show any mutagenic effect on TA98 and TA100 strains of. In the presence of S9 microsomal fraction, a single dose (0.05 μl/plate) of the oil caused statistically significant ($p < 0.05$) mutagenic activity in the both strains. The toxic effect has been found above 0.5 μl/plate and the doses over that have been not used in the experiment. On the other hand, the lowest dose of the oil was found to be toxic in the presence of S9 mix as compared with the solvent control (DMSO).

The possible antimutagenic potential of Origanum oil was examined against 4-NPD and 2-AF in the same tester strains using plate incorporation assay. Prior to the experiment, pre-incubation time for antimutagenicity assay at 0.5 μl/plate of test oil was determined in both strains (data are not shown). In the assay, pre-incubation time for Origanum oil was chosen as 30 min, which was found to be the most effective on the inhibition of mutagenic activity induced by 4-NDP. The results of antimutagenic effects of the essential oil are presented in Fig. 1(A) as plots of the percentage of the remaining mutagenicity. Origanum oil strongly reduced the mutagenicity of both 4-NPD and 2-AF in the absence or presence of metabolic activation, respectively. Remaining mutagenicity was around 40% and 50% for TA98 and 60% for TA100 with or without S9, respectively. The remaining mutagenicity was observed slightly less in TA98 without metabolic activation than the others.

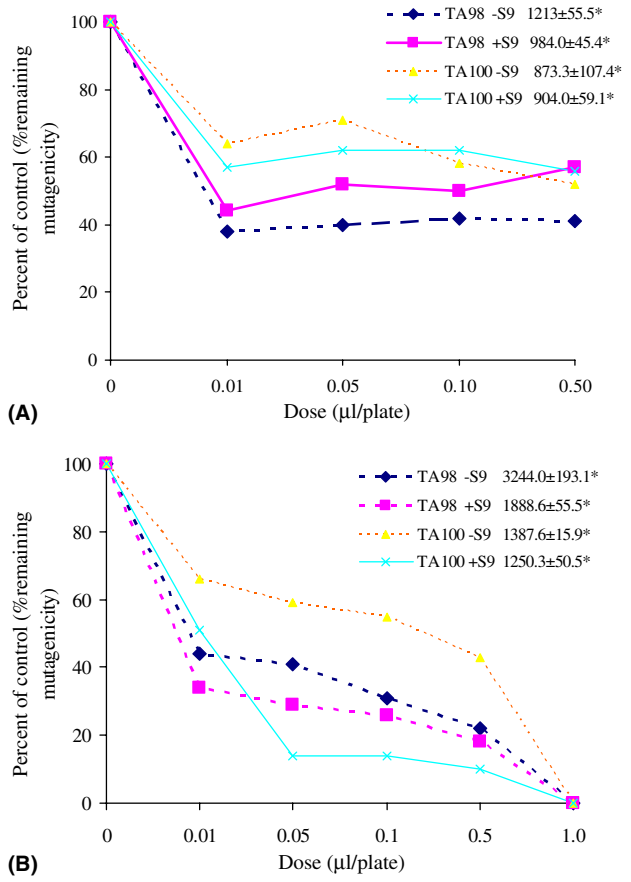

Fig. 1. Inhibitory effect of Origanum oil (A) and carvacrol (B) against the mutagenicity of 4-NPD (200 μg/plate) and 2-AF (1 μg/plate) to *S. typhimurium* TA98 and TA100 with or without S9 metabolic activity, respectively.

3.3. Mutagenicity and antimutagenicity of carvacrol

Further carvacrol as a major constituent of Origanum oil was investigated for its involvement in the antimutagenic activity and also its possible mutagenicity in the same assay. The results of antimutagenicity testing of carvacrol are given in Fig. 1(B). Carvacrol also strongly reduced the mutagenicity of 4-NDP and 2-AF in the absence or presence of metabolic activation, respectively. Remaining mutagenicity was from 60% to 80% and 65% to 80% for TA98 and from 55% to 60% and 50% to 90% for TA100 with or without S9, respectively. The effects of carvacrol were in a dose-dependent manner. At the highest dose (1 μl/plate), not any colony was observed at all, indicating its toxicity as the similar effects observed from the oil (data not shown).

On the other hand, in the absence of metabolic activation (S9 mix), all doses of carvacrol significantly ($P < 0.001$) induced the number of revertants colonies of TA98 and only 0.01 and 0.05 μl/plate doses caused significant induction of TA100 revertants as shown in Table 3. However, in the presence of S9 metabolism, only 0.01 and 0.05 μl/plate carvacrol exhibited mutagenicity for TA98 when compared to the solvent (DMSO) control. It was not mutagenic for TA100 at all. One μl/plate of carvacrol was found slightly toxic for the both strains.

4. Discussion

Plant essential oils including Origanum oil which are widely used as food additive and crop protective agents are known to possess strong antimicrobial and antioxidants activities (Isman, 2000; Kulisic et al., 2004; Ruberto & Baratta, 2000). Therefore, the mutagenic or antimutagenic properties of essential oils are important. To the best of our knowledge, no previous genotoxicity study has been performed with Origanum oil whereas a few papers have been published on its constituents (Azizan & Blevins, 1995; Stammatia et al., 1999).

We have shown that Origanum oil has strong antimutagenic activity in TA98 and TA100 strains of *S. typhimurium*. Inhibition of mutagenicity was around 60% and 50% for TA98 and 40% for TA100 in the presence or absence of the metabolic activity. The inhibition was found to be slightly more in TA98 with frame-shift mutation than in TA100 with base-substituted. This effect was slightly significant in the absence of metabolic activation,

indicating antimutagenicity of the oil against the direct-acting mutagen 4-NDP. This is the first data about the genotoxicity and more importantly antimutagenicity of Origanum oil with one exception of a previous work on its anticarcinogenic activity (Teissedre & Waterhouse, 2000). Mechanism of this antimutagenic activity may be due to its antioxidant activity suggested by several other works (Kulisic et al., 2004; Ruberto & Baratta, 2000; Teissedre & Waterhouse, 2000).

In the essential oil, the antimutagenic effect might be related to the presence of some major components such as carvacrol, thymol and thymoquinone (Başer, Özek, Tümen, & Sezik, 1993; Daferera et al., 2003; Kulisic et al., 2004). We have recently showed that carvacrol inhibits DNA synthesis in myoblast cells (Zeytinoglu et al., 2003). Furthermore, our group has also shown that carvacrol possessed a strong antimutagenic activity in human lymphocytes by inhibiting the induction of sister chromatid exchange (SCE) formation. On the other hand, it has antitumorigenic activity in in vitro and in vivo systems (He et al., 1997; Zeytinoglu et al., 1998), supporting our findings. Antitumor and hepatoprotective activities of thymoquinone as an glycosidically bound volatile have been also reported (Badary, 1999; Daba & Abdelrahman, 1998; Worthen, Ghosheh, & Crooks, 1998). Antimutagenic activity of carvacrol had not yet been well studied.

Little is known about the genotoxicity of its components. Our finding indicates that carvacrol is a strong direct acting mutagen in the bacterial system and its metabolites show weaker mutagenicity at lower concentrations at least for TA98 strains of *S. typhimurium*. The genotoxic potential of carvacrol and thymol was reported to be very weak in the Ames assay but negative in SOS chromo test by Stammatia et al. (1999). This weak mutagenic activity was found only in TA100 strains independently from the metabolic activation on the contrary to our findings. However, in other cell system, similar doses of carvacrol did not cause the formation of SCE in human lymphocytes while it inhibited the mitomycin C-induced formation of SCE (Ipek et al., 2004). All these data on the genotoxicity of carvacrol suggest that it may act by a base substitution mutagen and not cause strand breaks or any other damage lead the formation of SCE or induction of SOS response. Further studies for the genotoxic potential of carvacrol must be performed using different test system.

Present results show that carvacrol has also a strong antimutagenic effect similar to that of the oil, supporting its antimutagenic and antitumorigenic activities observed in different assays (He et al., 1997; Ipek et al., 2004; Zeytinoglu et al., 1998). Mechanism of antimutagenic activity appears to be due to its mutagenic potential at least in Ames test system, since many anticancer agents are known to be also mutagenic (reviewed in Bertram, 2001). The antimutagenic activity also might be related to on its ability to change membrane lipids and permeability of ion channels or to the antioxidant nature as suggested by others (Aeschbach et al., 1994; Ultee et al., 2002). Therefore, these may be further substantiated by the in vitro antioxidant studies and other relevant assays. The antimutagenic activity of the essential oil may be attributed mainly to its other compounds such as linalool, thymol and *p*-cymene, therefore each of them or in combinations must be further tested.

The results have shown that it is safe to use Origanum oil and may be carvacrol at lower doses, both found in nature and used as food additives. Their ability to protect human health against genotoxic agents is an added advantage of these materials. The data presented here are supportive of the fact that they are considered as leads for new anticancer agents.

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