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A comparative evaluation of antioxidant and anticancer activity of essential oil from *Origanum onites* (Lamiaceae) and its two major phenolic components

Aysun ÖZKAN, Ayşe ERDOĞAN
Department of Biology, Faculty of Science, Akdeniz University, 07058 Antalya - TURKEY

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Abstract: The objectives of the present study were to determine the effects of essential oil from Turkish oregano (*Origanum onites* L.) and its 2 major phenolic components, carvacrol and thymol, on cell viability, and to investigate their cytoprotective (antioxidant) effects against hydrogen peroxide-induced cytotoxicity and membrane damage in hepatoma G2 (Hep G2) cells. Their antioxidant properties were also evaluated with 2 in vitro complementary test systems: DPPH radical scavenging activity and linoleic acid oxidation inhibition. The aerial parts of the essential oil were isolated using a Clevenger-type apparatus (hydrodistillation) and its components were analyzed by gas chromatography/mass spectrometry (GC/MS). The essential oil of *O. onites* decreased cancer cell viability at higher concentrations. The essential oil was found to be less cytototoxic (IC50: 149.12 µg/mL) than carvacrol and thymol (IC50: 53.09 and 60.01 µg/mL, respectively) for Hep G2 cells. On the other hand, with the *O. onites* essential oil, carvacrol and thymol protected the cells against H_2O_2-induced cytotoxicity when the cells were preincubated with the oil and its components at a lower concentration (<IC50) before H_2O_2 incubation. The malondialdehyde (MDA) level increased in H_2O_2-exposed cells (IC50 and IC70), but decreased in cells preincubated with the essential oil and its components before H_2O_2 exposure. The essential oil showed a higher membrane-protective effect than thymol and carvacrol. The 3 major constituents of the essential oil were linalool (50.53%), carvacrol (24.52%), and thymol (15.66%), respectively. The DPPH radical scavenging activity (EC50: 80 µg/mL) of the essential oil was found to be higher than that of carvacrol and thymol. The linoleic acid oxidation inhibition rate of the essential oil (40%) was close to that of its 2 major components. These findings suggest that the essential oil from *O. onites* and its 2 major components exhibit antioxidant activity and carcinogenesis-reducing potential.

Key words: *O. onites*, essential oil, antioxidant, anticancer

Origanum onites (Lamiaceae)'den elde edilen uçucu yağın ve iki önemli fenolik bileşeninin antioksidan ve antikanser aktivitelerinin karşılaştırılması

Özet: Bu çalışmanın amacı, uçucu yağın ve onun iki önemli bileşeni olan karvakrol ve timol'ün hücre canlığı ve hepatoma G2 (Hep G2) hücrelerinde hidrojen peroksidin indüklediği sitotoksisite ve membran hasarına karşı koruyucu etkisinin araştırılmasıdır. Uçucu yağın ve bileşenlerinin antioksidan özelliği, birbirini tamamlayan iki in vitro test sistemi ile ortaya konmuştur, bu testler; DPPH radikalini ortadan kaldırma ve linoleik asit oksidasyonu inhibisyonu testidir. Bitknin toprak üstü organlarının uçucu yağı Clevenger (su buharı distilasyon) cihazı ile izole edilmiş, bileşenler ise gaz kromotografisi/kütle spektrometresi (GC/MS) yöntemi ile analiz edilmiştir. Çalışılan yağ, Türk kekiğinden elde edilmiştir (*Origanum onites* L.). *O. onites*'ten elde edilen uçucu yağ yüksek konsantrasyonlarda, kanser hücrelerinin

canlılığını azaltmıştır. Hep G2 hücrelerinde, uçucu yağ (IC50: 149,12 µg/mL), karvakrol ve timol'den (IC50: 53,09 ve 60.01 µg/mL) daha az toksik bulunmuştur. Diğer taraftan, Hep G2 hücrelerinin H_2O_2 uygulamasından önce, düşük konsantrasyonlarda *O. onites* uçucu yağı, karvakrol ve timol (<IC50) ile ön uygulamaya maruz bırakılması hücreleri H_2O_2 tarafından indüklenen sitotoksisiteye karşı korumuştur. Malondialdehit miktarı, H_2O_2'e (IC50 ve IC70) maruz bırakılan hücrelerde artmış, fakat H_2O_2'e maruz bırakmadan önce uçucu yağ ve bileşenleri ile ön-uygulamaya maruz bırakılan hücrelerde azalmıştır. Uçucu yağ, timol ve karvakrolden daha yüksek membrane koruyucu etki göstermiştir. Uçucu yağın en önemli üç bileşeni sırasıyla linalool (% 50,53), carvacrol (% 24,52) ve thymol (% 15,66) olarak tanımlanmıştır. Uçucu yağın DPPH radikali süpürücü aktivite (EC50: 80 µg/mL) karvakrol ve timolden daha yüksek bulunmuştur. Uçucu yağın linoleik asit oksidasyonunu inhibisyon oranı (% 40) diğer iki majör bileşene yakındır. Bu bulgular, *O. onites*'den elde edilen uçucu yağın ve onun iki önemli bileşeninin antioksidan aktiviteye ve karsinogenezi azaltıcı potansiyele sahip olduğunu göstermektedir.

Anahtar sözcükler: *O. onites*, uçucu yağ, antioxidan, antikanser

Introduction

Natural antioxidants are considered to be useful agents for the prevention of diseases (1-4). Many studies have shown that phenolic compounds in plant essential oils display antioxidant activity as a result of their capacity to scavenge free radicals (5,6). It has been shown that many species of *Origanum* have a high amount of phenolic contents in their essential oils (7-9). Herbal parts of the *Origanum* species are aromatic and are used as condiments or herbal tea. Dried *Origanum* species are also used for the production of essential oil (*Origanum* oil) and an aromatic water or hydrosol (*Origanum* water) (10). Identifying the main components of the essential oil, testing their safety, and uncovering their medicinal value in disease treatment will provide an important means for drug development and drug targeting therapy. Being lipophilic in nature, essential oils will cross the plasma membrane and may exert their effect by interacting with intracellular proteins and/or intraorganelle sites (11-13). Monoterpenes are highly hydrophobic substances present in plant essential oils. They exert a wide spectrum of biological actions of great importance in many different areas (14). The major compounds of *O. onites*' essential oil, as with many herb oils, are carvacrol (71.22%) and thymol (5.97%) (15). Carvacrol (2-methyl-5-(1-methylethyl) phenol), a cyclic monoterpene, is a constituent of the essential oil of oregano. The mode of action of carvacrol has received considerable attention from researchers because of its use in flavoring, and also as an antibacterial or antifungal agent in food preservation methods (16,17). Thymol (5-methyl-2-(1-methylethyl)phenol) is an isomer of carvacrol, having the hydroxyl group at a different location on the phenolic ring. The hydrophobic nature of carvacrol and thymol enables them to react with the lipids of the cell membrane and mitochondria, rendering them permeable and leading to leakage of the cell components (18).

We tried to prove the essential oil's ability to prevent the cytotoxicity of Hep G2 cells and membrane damage induced by the oxidative agent H_2O_2, as compared to its 2 major components, because many plant oils and their components have been shown to possess various protective activities in different biological systems.

Materials and methods

Plant material

Origanum onites samples were collected during the flowering season from Elmalı, Antalya, Turkey, in August 2006. They were identified by senior taxonomist Dr. Orhan Ünal. The voucher specimen was deposited at the Herbarium of the Department of Biology, Akdeniz University, Antalya, Turkey (Voucher No: 325-OO).

The essential oil of *O. onites* was obtained by hydrodistillation in 100 mL of H_2O for 3 h with a Clevenger apparatus (İLDAM Ltd., Ankara, Turkey) in the Molecular Biology Department of Akdeniz University. The major components of the essential oil were purchased from different manufacturers: carvacrol from Aldrich at 98% purity and thymol from Sigma at 99.5%.

Gas chromatography/mass spectrometry (GC/MS) analysis

The oils, dried over anhydrous sodium sulfate, were subsequently analyzed by GC/MS and stored at –20 °C. The composition of the volatile constituents was established by GC/MS quadruple detector analyses, using the Shimadzu QP 5050 system fitted with a free fatty acid phase (FFAP) capillary column (50 m × 0.32 mm (i.d.), film thickness: 0.25 mm). The detector and injector temperatures were set at 240 °C and 250 °C. The temperature program for the FFAP column went from 60 °C (1 min) to 220 °C at a rate of 2 °C/min, and was then held at 220 °C for 20 min. Helium was used as a carrier gas at a flow of 14 psi (split 1:10) and the injection volume of each sample was 1 mL. The percentage composition was computed from the GC peak areas according to the 100% method, without using any correction factors. The identification of the components was based on the comparison of their mass spectra with those of Wiley and NIST tutorial libraries. The ionization energy was set at 70 eV.

Measurement of antioxidant capacities using the two in vitro assays

2,2'-Diphenyl-1-picrylhydrazyl (DPPH) assay: The hydrogen atoms or the electron donation abilities of the corresponding samples were measured from the bleaching of a purple-colored methanol solution of DPPH. This assay was carried out following the same method as reported elsewhere (19,20). Added to 5 mL of a 0.004% methanol solution of DPPH (2,2-diphenyl-1-picrylhydrazyl, free radicals) were 50 µL of various concentrations of the samples dissolved in methanol. After a 30-min incubation period at room temperature, the absorbance was read against a blank at 517 nm. The inhibition free radical DPPH, in percentages (I%), was calculated in the following way: $I\% = (A_{control} - A_{sample}/A_{control}) \times 100$, where A_{blank} is the absorbance of the control reaction, containing all reagents except the test compound, and A_{sample} is the absorbance of the test compound. An extract concentration providing 50% inhibition (EC_{50}) was calculated from the linear regression algorithm of the graph-plotted inhibition percentage against the extract concentration. For the calculation of these values, Microsoft Excel was used. Values are presented as means ± SD of 5 parallel measurements.

β-Carotene/linoleic acid assay: In this assay, the antioxidant capacity was determined by indirectly measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (21). A stock solution of a β-carotene/linoleic acid mixture was prepared as follows: first, 0.5 mg of β-carotene was dissolved in 1 mL of chloroform (HPLC grade), and then 25 µL of linoleic acid and 200 mg of Tween 40 were added. The chloroform was completely evaporated using a vacuum evaporator, and then 100 mL of distilled water, saturated with oxygen (30 min at a flow rate of 100 mL/min), was added with vigorous shaking. A 2.5-mL amount of this reaction mixture and a 350-µL portion of the samples (2 mg/mL) in ethanol were dispensed into each test tube and incubated for up to 48 h at room temperature. After this incubation period, the absorbance of the mixture was measured at 490 nm. Values are presented as means ± SD of 5 parallel measurements.

Cancer cell culture

The hepatoma G2 cell (Hep G2) line was purchased from the American Type Culture Collection (Rockville, MD, USA). Cells were routinely cultured in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% fetal calf serum, and 1% antibiotic-antimycotic solution in a humidified atmosphere containing 5% CO_2 at 37 °C. For subculturing, cells were harvested after a trypsin/EDTA treatment at 37 °C. Cells were used when the monolayer confluence had reached 75%.

Cell viability assay: The cancer cells (500 cells/well, monolayer) were plated in a 96-well plate. The next day, the cells were treated with different concentrations of essential oil, carvacrol, and thymol (10-3000 µg/mL) in the medium for 24 h. At the end of the incubation period (24 h), the cytotoxicity of the essential oil and its 2 major components on cancer cells was determined with the CellTiter-Blue® Cell Viability Assay (Promega, Madison, WI, USA). The assay is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent

end product (resorufin). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal (22). Following cellular reduction, fluorescence was recorded at 560 nm (excitation) and 590 nm (emission). The data were expressed as average values obtained from 8 wells for each concentration. The IC_{50} value was calculated from the graphical equation. H_2O_2 cytotoxicity on the cancer cells was measured the same way. For measuring the antioxidant effect of the essential oil and its 2 major components against H_2O_2 cytotoxicity, the cells were preincubated at different concentrations (<IC_{50}) for 1 h before hydrogen peroxide treatment (IC_{50} and IC_{70}) for 24 h. The essential oil and its 2 main components were dissolved in 0.1% DMSO.

Determination of malondialdehyde level: Hep G2 cells were plated at a density of (5-10) × 10^5 cells/dish in 100-mm dishes. The cells were preincubated at maximum cytoprotective concentrations of the essential oil and its 2 major components for 1 h before hydrogen peroxide treatment (IC_{50} and IC_{70}) for 24 h. Cells were scraped off culture plates with culture medium and were centrifuged at 400 × *g* for 10 min. The cell pellets were washed with PBS and then sonicated (3 × 15 s) in 50 mM potassium phosphate (pH 7.2; containing 1 mM PMSF (Sigma) and 1 µg/mL of leupeptin (Sigma)) and centrifuged at 150,000 × *g* for 1 h. The supernatant was used for the determination of malondialdehyde levels.

Malondialdehyde levels in the Hep G2 cells were assayed as described by Wasowicz et al. (23). This fluorometric method for measuring thiobarbituric acid-reactive substances (TBARS) in supernatant is based on the reaction between malondialdehyde and thiobarbituric acid. The product of this reaction was extracted into butanol and measured at 525 nm (excitation) and 547 nm (emission) spectrofluorometrically.

Protein was determined by the Bradford method (24) with bovine serum albumin as a standard.

Data analysis

The results of the replicates were pooled and expressed as mean ± SD. Analysis of variance and Student's t-test were carried out. Significance was accepted at $P \leq 0.05$ (25).

Results and discussion

Antioxidant activity

The components included in the essential oil investigation were classified into 3 groups depending on their chemical nature. Group 1 includes alcohols, group 2 includes hydrocarbons, and group 3 includes phenols such as carvacrol and thymol. GS/MS analysis of the crude oil isolated from dried aerial parts of *O. onites* resulted in the identification of some compounds, as shown in Table 1. According to the GS/MS analysis results, 12 (99.44%) compounds were identified. The 6 major components of the essential oil were linalool (50.53%), carvacrol (24.52%), thymol (15.66%), cymene (2.68%), terpinen-4-ol (2.06%), and gamma-terpinene (1.41%), respectively. Thymol and carvacrol were found to be the 2 major phenolic components in the essential oil of *O. onites*. Phenolic components in the essential oil were the main source of antioxidant activity. These results are supported by many studies (26-29). In the present study, it can be concluded that the antioxidant activity of the essential oil can come from its phenolic components and/or from their interaction with other components in the essential oil. The essential oil and its 2 main components were individually assessed for their possible antioxidant activities

Table 1. Chemical composition of *O. onites* essential oil determined by GC/MS.

Components	Composition (%)
alpha-Pinene	0.15
Myrcene	0.63
alpha-Terpinene	0.54
gamma-Terpinene	1.41
Cymene	2.68
1-Octen-3-ol	0.29
Linalool	50.53
Terpinen-4-ol	2.06
Linalyl propionate	0.38
Borneol	0.59
Thymol	15.66
Carvacrol	24.52

by employing 2 complementary tests: free radical-scavenging activity (DPPH) and β-carotene/linoleic acid antioxidant assays. Their antioxidant capacities were determined by comparison with the activities of known antioxidants, such as BHT, ascorbic acid, and α-tocopherol.

Free radical scavenging capacities of the corresponding samples were measured by DPPH assay and the results are shown in Table 2. According to the results, essential oil (EC_{50}: 80.00 μg/mL) was less active than the positive controls of BHT (EC_{50}: 18.45 μg/mL), ascorbic acid (EC_{50}: 3.21 μg/mL), and α-tocopherol (EC_{50}: 7.25 μg/mL), but more active than carvacrol (EC_{50}: 248 μg/mL) and thymol (EC_{50}: 163 μg/mL). The essential oil of *O. onites* had higher antiradical activity than some other plant essential oil (30). As shown in Table 2, oxidation of the linoleic acid was effectively inhibited by the essential oil of *O. onites* (40.00%). On the other hand, the oxidation capacity of the essential oil was not superior to positive controls BHT (95.02%), ascorbic acid (92.92%), or α-tocopherol (96.20%), but was close to carvacrol (52.02%) and thymol (57.11%). Moreover, it was found to be close to the essential oils of *Salvia limbata* C.A.Mey. and *Salvia sclarea* L. (31).

Table 2. Antioxidant capacities of the essential oil of *O. onites*, its 2 major components, and positive controls.

Samples	DPPH[1]	β-Carotene/ linoleic acid[2]
Essential oil	80.00 ± 2.11^{d}	40.00 ± 1.07^{x}
Carvacrol	248 ± 31.11^{f}	52.02 ± 1.30^{b}
Thymol	163 ± 25.11^{e}	57.11 ± 1.27^{bc}
BHT	18.45 ± 1.07^{c}	95.02 ± 1.61 ^{d}
Ascorbic acid	3.21 ± 0.19^{a}	92.92 ± 1.72^{d}
α-Tocopherol	7.25 ± 1.130^{ab}	96.20 ± 1.81^{d}

Results are the means of 5 different experiments. Values that are followed by different letters within each column are significantly different ($P \leq 0.05$).

[1]EC_{50} value of DPPH assay (as μg/mL).

[2]Given as percentage of % inhibition of the linoleic acid.

Antioxidant effect of the essential oils on Hep G2 cells

We measured the protective (antioxidant) effect of the essential oil and its 2 main phenolic components against strong oxidant H_2O_2 in Hep G2 cells. Figure 1 shows the levels of H_2O_2-induced cytotoxicity in Hep G2 cells, preincubated with different concentrations of essential oil. The essential oil has a strong antioxidant effect (<90 μg/mL) against H_2O_2 cytotoxicity. The maximum protective concentration of essential oil was found to be 20 μg/mL. Under the same conditions, carvacrol and thymol reduced the cytotoxic effects and induced the strong oxidant H_2O_2 in Hep G2 with 10.62 μg/mL (IC_{10}) and 24μg/mL (IC_{20}), respectively, for maximum levels (Figures 2 and 3).

The essential oil and its 2 major components significantly decreased membrane damage in H_2O_2-treated Hep G2 cells (Table 3). The Hep G2 cells, preincubated with 20 μg/mL of essential oil, 24 μg/mL of carvacrol, and 10.62 μg/mL of thymol for 1 h before hydrogen peroxide treatment (IC_{10}, IC_{20},

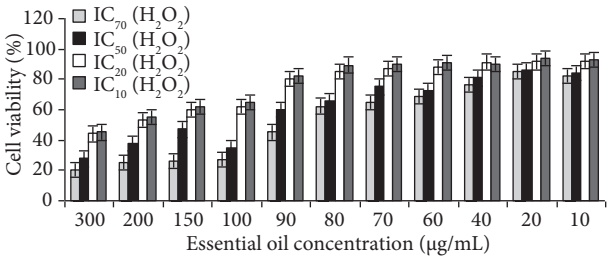

Figure 1. Antioxidant effect of essential oil of *O. onites* on Hep G2 cancer cells against H_2O_2 cytotoxicity.

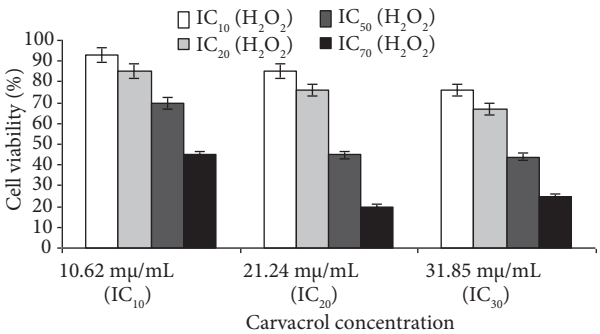

Figure 2. Antioxidant effect of carvacrol on Hep G2 cancer cells against H_2O_2 cytotoxicity.

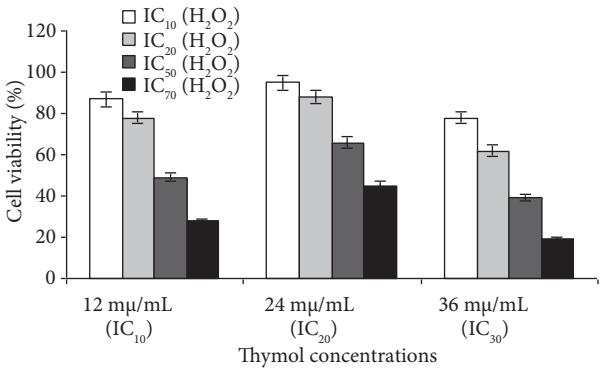

Figure 3. Antioxidant effect of thymol on Hep G2 cancer cells against H_2O_2 cytotoxicity.

IC_{50}, and IC_{70}) for 24 h had lower MDA levels than unpreincubated cells. The amount of MDA increased almost 2.8-fold in the IC_{50} H_2O_2-treated Hep G2 cells and 4.7-fold in the IC_{70} H_2O_2-treated Hep G2 cells compared to the control cells. The preincubation of cells with the essential oil and its 2 major components decreased the MDA amount compared to the Hep G2 cells treated with H_2O_2 alone. The membrane-protective effect of the essential oil was found to be higher than that of carvacrol and thymol at the highest cytoprotective concentration. The data in Table 3 reveal a membrane-protective effect with the essential oil decreasing the MDA amount by 54%, carvacrol decreasing it by 38%, and thymol by 29% in the IC_{50} H_2O_2-treated cells, and by 48%, 41%, and 34%, respectively, in the IC_{70} H_2O_2-treated Hep G2 cells.

We assume that decreasing H_2O_2 levels induced a cytotoxic effect; membrane damage can be accompanied by antioxidant action of the essential oil and its 2 major components with concentrations lower than IC_{50}. The essential oil from *O. onites*, carvacrol, and thymol showed a protective (antioxidant) effect on Hep G2 cells, depending on the concentrations. Aydin et al. (32) also observed that the phenolic compounds thymol and carvacrol, at concentrations below 0.2 and 0.1 mM, respectively, significantly reduced the oxidative damage in human lymphocytes. In another study, the incubation of Hep G2 and Caco-2 cells in the presence of the whole scale of concentrations of carvacrol or thymol led, in both cases, to a significant protection of the cells studied from DNA strand breaks induced by the potent oxidant hydrogen peroxide (33). Further understanding of the underlying mechanism of their protective effects in reducing intracellular oxygen radicals in Hep G2 cell death may lead to the development of new therapeutic treatments for cancer, since the essential oil and its main components have protection against H_2O_2 insult. Their hepatoprotective effects against H_2O_2 toxicity might be of importance and may contribute in part to their clinical efficacy for the treatment of hepatocellular carcinoma. These

Table 3. Amount of malondialdehyde in Hep G2 cells preincubated with *O. onites* essential oil and its 2 major components before hydrogen peroxide treatment.

Groups	**MDA levels (nmol/mg protein ± SD)**
Control	0.25 ± 0.11a
0.5% DMSO	0.22 ± 0.20a
H_2O_2 (IC_{50})	0.69 ± 0.55ef
EsO pretreated (20 μg/mL) + H_2O_2 (IC_{50})	0.32 ± 0.10b
Carvacrol pretreated (10.62 μg/mL) + H_2O_2 (IC_{50})	0.43 ± 0.13c
Thymol pretreated (24 μg/mL) + H_2O_2 (IC_{50})	0.49 ± 0.15cd
H_2O_2 (IC_{70})	1.18 ± 1.42jk
EsO pretreated (20 μg/mL) + H_2O_2 (IC_{70})	0.61 ± 0.44e
Carvacrol pretreated (10.62 μg/mL) + H_2O_2 (IC_{70})	0.70 ± 0.56ef
Thymol pretreated (24 μg/mL) + H_2O_2 (IC_{70})	0.78 ± 0.86fg

Values that are followed by different letters within column are significantly different ($P \leq 0.05$). Each datum in the table is an average of 5 repetitions of MDA amount. MDA: malondialdehyde, EsO: essential oil, SD: standard deviation.

results suggest that *O. onites* may be a potentially valuable source of natural therapeutic agents. Thus, it is becoming increasingly evident that certain phytochemicals, particularly those included in our daily diet, have important cancer chemopreventive properties (34).

Cytotoxic effect of essential oils on Hep G2 cells

The cytotoxicity of the essential oil and its 2 major components, thymol and carvacrol, studied in Hep G2 cells, was evaluated with the CellTiter-Blue® Cell Viability Assay after 24-h treatment of the cells with the compounds given in Figure 4. Hep G2 cells were much more sensitive to the toxic effect of carvacrol and thymol, with IC_{50} values of 53.09 and 60.01 µg/mL, respectively, than the essential oil (IC_{50}: 149.12 µg/mL). The essential oil was found to be less toxic than carvacrol and thymol for Hep G2 cells. The viability of the Hep G2 cells decreased when the cells were exposed to the essential oil, carvacrol, and thymol at increasing concentrations between 20-170 µg/mL, and did not change at concentrations of 170 µg/mL or higher (Figure 1). The essential oil from *O. onites* and its 2 components had dose-dependent antiproliferative effects on Hep G2 cells, which makes them potentially interesting for adjuvant experimental cancer treatments. They induced membrane damage and cytotoxicity in Hep G2 cells at relatively higher concentrations than those that mediate its anticancer activities. The induction of cytotoxic cell death can be accompanied by membrane damage. Koparal and Zeytinoglu (35) also observed that carvacrol was a very potent inhibitor of cell growth in the A549 cancer cell line. In another study, carvacrol and thymol had dose-dependent antiproliferative effects on human uterine carcinoma cells (36).

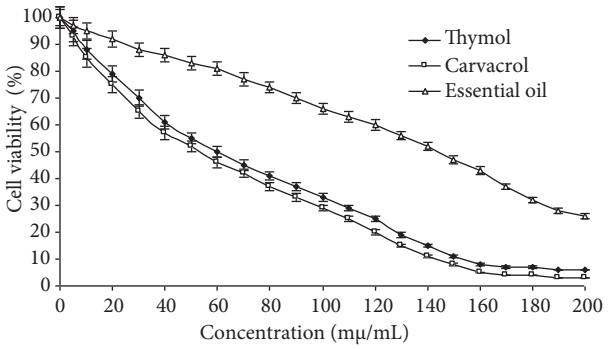

Figure 4. Dose-dependent cytotoxicity of essential oil from *O. onites*, carvacrol, and thymol on Hep G2 cells.

Taken together, these findings suggest that the essential oil and its 2 main components exhibit antioxidant activity at lower concentrations and anticancer effects at higher concentrations in cells. Further studies will be needed to identify the active compounds in plant extracts that have antioxidant and/or carcinogenesis-reducing potential.

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Corresponding author:

Aysun ÖZKAN

Department of Biology,

Faculty of Science,

Akdeniz University,

07058 Antalya - TURKEY

E-mail: aozkan@akdeniz.edu.tr

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