Check for updates

NPC Natural Product Communications

2012
Vol. 7
No. 12
1557 - 1560

A Comparative Study of the Antioxidant/Prooxidant Effects of Carvacrol and Thymol at Various Concentrations on Membrane and DNA of Parental and Drug Resistant H1299 Cells

Aysun Ozkan* and Ayse Erdogan

[a]*Department of Biology*, *Faculty of Science*, *Akdeniz University*, *07058*, *Antalya*, *Turkey*

aozkan@akdeniz.edu.tr

Received: March 8th, 2012; Accepted: October 16th, 2012

Carvacrol and thymol, both used as flavor agents in cosmetic and food products, have prooxidant and antioxidant activities. To clarify the mechanisms of their cytotoxicity and the factors affecting their antioxidant/prooxidant activities, we investigated cell membrane and DNA damage induced by carvacrol and thymol in parental and drug-resistant human lung cancer cell lines. After 24 and 48 hour incubation periods, the cytotoxicity of carvacrol (IC_{50} 380 and 244 μM) was found to be higher than that of thymol (IC_{50} 497 and 266 μM) in parental cells. However, thymol showed higher cytotoxic effects in drug resistant H1299 cells for three incubation periods. Also, carvacrol and thymol, at higher concentrations, increased malondealdehyde (MDA) levels causing membrane damage and 8-hydroxy deoxyguanozine (8-OHdG) levels, causing DNA damage to both parental and drug resistant cells. On the other hand, carvacrol and thymol protected the cells against H_2O_2-induced cytotoxicity, and membrane and DNA damage when the cells were preincubated with these two compounds at lower concentration ($<IC_{50}$) before H_2O_2 incubation. These findings suggest that carvacrol and thymol exhibit protective/damaging effects depending on cell resistance, concentration and time.

Keywords: Carvacrol, Thymol, DNA, Membrane, Anticancer, Antioxidant.

Monoterpenes are highly hydrophobic substances that exert a wide spectrum of biological actions of great importance in many different areas [1,2]. Thymol (5-methyl-2-(1-methylethyl) phenol) is an isomer of carvacrol (5-isopropyl-2-methyl phenol), 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 cell components [3].

Natural antioxidants are considered useful agents for the prevention of diseases [4-7]. Many studies have shown that phenolic compounds in plant essential oils display antioxidant activity as a result of their capacity to scavenge free radicals [8-10]. On the other hand, effects of antioxidant concentrations on oxidation reactions depend on many factors such as structure of the antioxidant, oxidation conditions and changing of the oxidized structure. Phenolic antioxidants lose their antioxidant effects at higher concentrations and gain a prooxidant structure. They can either protect DNA and membranes against oxidants as an antioxidant at lower concentrations or damage DNA and membranes as a prooxidant at higher concentrations. Recent studies reveal that anti-/pro-oxidant and toxic properties of these molecules change depending on concentration, the kind of cells and organism, and so are not safe for human being [11].

Tumors are heterogeneous in many respects, including chemotherapeutic susceptibility [12]. Resistance to chemo-therapeutic agents is a major problem in the treatment of patients with small cell (SCLC) and non-small cell lung cancer (NSCLC). Acquired multidrug resistance is the main obstacle for the cure of SCLC. A group of drug resistance cells can occur in tumors during chemotherapy. The development of drug resistance and detoxifying mechanisms in human and mice microsome enzymes CYP3A4 used eucalyptol as a substrate [13,14]. We tried to prove the ability of carvacrol and thymol in preventing cytotoxicity, membrane damage and DNA damage induced by the oxidative agent H_2O_2, while they also have cytotoxic and damaging effects in parental and drug resistant H1299 cells because many phenolic components have shown various protective/damaging activities in different biological systems dependant on concentration.

Thymol and carvacrol showed cytotoxic effects on parental and drug resistance H1299 cells (Figure 1 and 2). After 24 and 48 hour incubation periods, the cytotoxicity of carvacrol was found to be higher {380 and 244 μM (IC_{50}) concentrations} than that of thymol (IC_{50} 497 and 266 μM) in parental cells. However, thymol showed higher cytotoxic effects in drug resistant H1299 cells for three incubation periods. The viability of cells decreased between 25-1000 μM carvacrol and 10-1000 μM thymol concentrations. The cytotoxic effect was close to that of the control until 25 μM concentrations for carvacrol and 5 μM concentrations for thymol. So, neither compound had either an effective cytotoxic or antitumor effect on cancer cells at lower concentrations. Carvacrol and thymol exhibited dose- and incubation time-dependent cytotoxic effects on parental and drug resistant H1299 cells. Also, the effects changed depending on the drug resistance capacity of the target cells. In one study, carvacol and thymol had a dose-dependent antiproliferative effects on Hep G2 cells, which make them potentially interesting for adjuvant experimental cancer treatments.

Both compounds induced membrane damage and cytotoxicity in hepatoma G2 at relatively higher concentrations than those that mediate its anticancer activities. The induction of cytotoxic cell death can be accompanied by membrane damage [2]. Koparal and Zeytinoglu [15] 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 [16]. Also, carvacrol and thymol differed in their cytotoxic effects on K562 cells [17]. All these studies support our results.

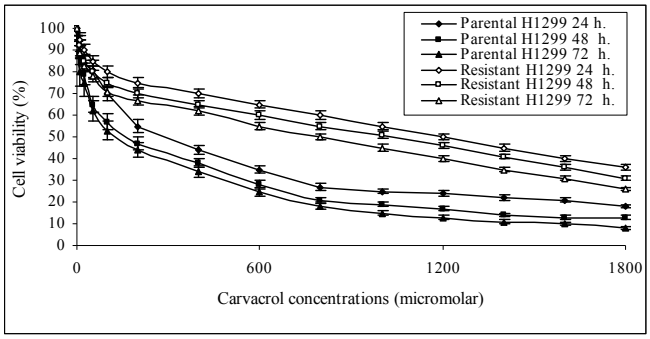


Figure 1: Cytotoxic effects of carvacrol on parental and drug-resistant H1299 cells.

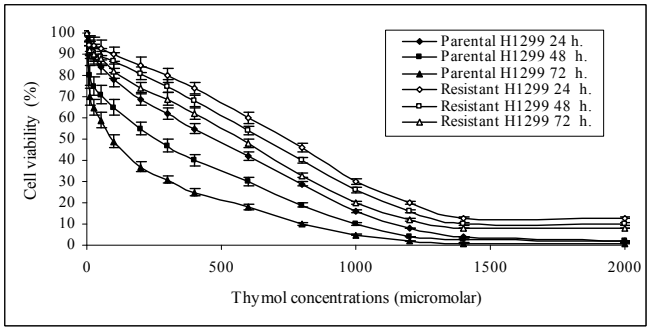


Figure 2: Cytotoxic effects of thymol on parental and drug-resistant H1299 cells.

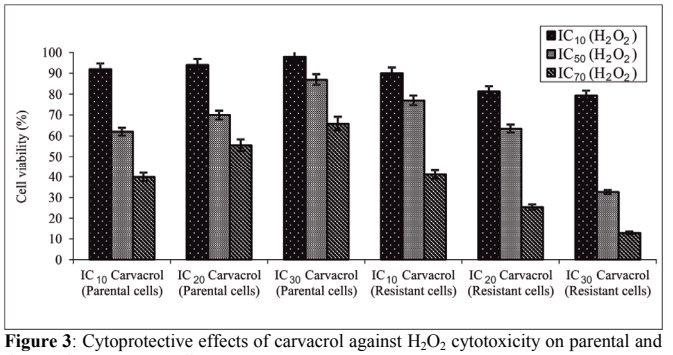


Figure 3: Cytoprotective effects of carvacrol against H_2O_2 cytotoxicity on parental and drug-resistant H1299 cells.

We measured the cytoprotective (antioxidant) effect of carvacrol and thymol against the cytotoxicity of the strong oxidant H_2O_2 in parental and drug-resistant H1299 cells. Figures 3 and 4 show the levels of H_2O_2-induced cytotoxicity in the cells pre-incubated with different concentrations of carvacrol and thymol. Carvacrol had a strong antioxidant effect at an IC_{30} concentration for parental cells and an IC_{10} concentration for resistant cells against H_2O_2 cytotoxicity (Figure 3).

Also, thymol decreased H_2O_2 cytotoxicity in both cells. The maximum antioxidant effect of thymol was found at an IC_{20} concentration for parental cells and IC_{10} concentration for resistant cells (Figure 4). So, resistant cells can protect themselves with lower concentrations of thymol and carvacrol against H_2O_2 cytotoxicity than parental cells. We can assume that drug resistant cells show more resistance to cytotoxicity than parental cells.

In this study, carvacrol and thymol increased malondealdehyde (MDA) and 8-OHdG levels in both parental and drug resistant cells at different concentrations (Table 1). While increasing MDA amounts cause membrane damage, increases in 8-OHdG show the DNA damaging effect of carvacrol and thymol on the cells. The membrane and DNA damaging effect of the compounds on both

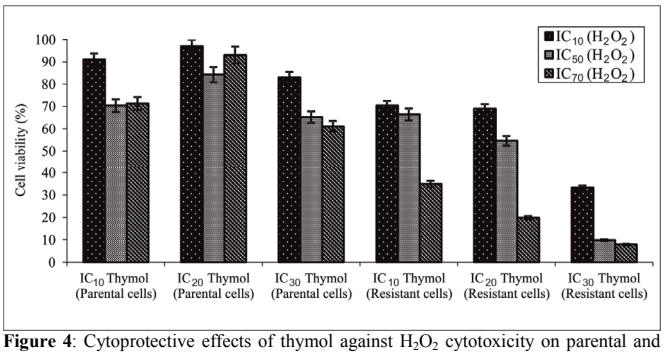


Figure 4: Cytoprotective effects of thymol against H_2O_2 cytotoxicity on parental and drug-resistant H1299 cells.

Table 1: Membrane and DNA damaging effects of carvacrol and thymol on parental and drug resistant H1299 cells

Concentrations	MDA (nmol/mg protein) X ± SE	8-OHdG (ng/mL) X ± SE
IC_{10} Carvacrol (P)	0.35 ± 0.02 a	0.09 ± 0.03 a
IC_{50} Carvacrol (P)	0.60 ± 0.03 ab	0.10 ± 0.04 a
IC_{70} Carvacrol (P)	1.67 ± 0.07 bc	0.15 ± 0.03 ab
IC_{10} Carvacrol (R)	0.31 ± 0.05 a	0.08 ± 0.03 a
IC_{50} Carvacrol (R)	0.56 ± 0.08 ab	0.09 ± 0.02 a
IC_{70} Carvacrol (R)	1.12 ± 0.12 b	0.12 ± 0.09 ab
IC_{10} Thymol (P)	0.70 ± 0.13 ab	0.08 ± 0.04 a
IC_{50} Thymol (P)	1.90 ± 0.21 bc	0.08 ± 0.03 a
IC_{70} Thymol (P)	2.71 ± 0.44 cd	0.10 ± 0.02 a
IC_{10} Thymol (R)	0.62 ± 0.45 ab	0.08 ± 0.04 a
IC_{50} Thymol (R)	1.14 ± 0.47 b	0.09 ± 0.06 a
IC_{70} Thymol (R)	2.70 ± 0.85 cd	0.15 ± 0.11 ab
Control	0.31 ± 0.02 a	0.08 ± 0.04 a
0.5% DMSO	0.33 ± 0.03 a	0.08 ± 0.03 a

Results are means of five different experiments. Values that are followed by different letters within each column are significantly different ($p \leq 0.05$). df_1=2, df_2=95, F=11.96. SE: Standard Error. P; parental, R; resistant cells

cells was found to be close to that of the control at IC_{10} concentrations. The differences between the data are given in Table 1 ($p \leq 0.05$). The higher concentration of carvacrol and thymol caused statistically important membrane and DNA damage to the cells ($p \leq 0.05$). Parental cells were found to be more sensitive than resistant cells to the membrane damaging effects of both compounds. They induced membrane and DNA damage and cytotoxicity in H1299 cells at relatively higher concentrations than those that mediate its anticancer activities.

The induction of cytotoxic cell death can be accompanied by membrane and DNA damage. Koparal and Zeytinoglu [15] 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 [16].

The present studies show that phenolic compounds have antioxidant/prooxidant properties under different conditions. Carvacrol and thymol significantly decreased membrane and DNA damage in H_2O_2 treated H1299 cells (Table 2). The selected protective concentrations are those that showed the highest protective effect against H_2O_2 cytotoxicity. In the cytoprotective study, while carvacrol had a strong antioxidant effect at IC_{30} concentration for parental cells and IC_{10} concentration for resistant cell, thymol showed maximum antioxidant effect at IC_{20} concentration for parental cells and IC_{10} concentration for resistant cells against H_2O_2 cytotoxicity (Figures 3, 4). Also, at these cytoprotective concentrations, carvacrol and thymol showed different membrane and DNA protective effects against H_2O_2 oxidation (Table 2). The most effective membrane protective effect of three concentrations (IC_{10}, IC_{50}, and IC_{70}) was found at IC_{10} for

Table 2: Protective effects of carvacrol and thymol against H_2O_2 membrane and DNA damaging effects on parental and drug resistant H1299 cells

Concentrations	MDA (nmol/mg prot.) X ± SE	8-OHdG (ng/ml) X± SE
IC_{20} Thymol + IC_{10} H_2O_2 (P)	0.86 ± 0.03 ab	3.09 ± 0.44 d
IC_{20} Thymol + IC_{50} H_2O_2 (P)	0.90 ± 0.08 ab	8.00 ± 0.56 hi
IC_{20} Thymol + IC_{70} H_2O_2 (P)	1.10 ± 0.65 b	11.70 ± 0.98 lm
IC_{10} Thymol + IC_{10} H_2O_2 (R)	0.95 ± 0.13 ab	3.45 ± 0.22 d
IC_{10} Thymol + IC_{50} H_2O_2 (R)	1.14 ± 0.82 b	7.60 ± 0.32 hi
IC_{10} Thymol + IC_{70} H_2O_2 (R)	1.78 ± 0.96 bc	10.76 ± 0.67 kl
IC_{10} Carvacrol+ IC_{10} H_2O_2 (R)	0.56 ± 0.09 ab	2.35 ± 0.24 c
IC_{10} Carvacrol+ IC_{50} H_2O_2 (R)	0.68 ± 0.07 ab	7.10 ± 0.34 h
IC_{10} Carvacrol+ IC_{70} H_2O_2 (R)	0.96 ± 0.08 ab	10.45 ± 0.99 k
IC_{30} Carvacrol + IC_{10} H_2O_2 (P)	0.93 ± 0.08 ab	2.60 ± 0.14 cd
IC_{30} Carvacrol + IC_{50} H_2O_2 (P)	1.10 ± 0.32 b	8.00 ± 0.36 hi
IC_{30} Carvacrol + IC_{70} H_2O_2 (P)	1.70± 0.51 bc	12.00 ± 0.35 lm
IC_{10} H_2O_2 (Control P)	1.30 ± 0.03 b	2.62 ± 0.03 cd
IC_{50} H_2O_2 (Control P)	1.80 ± 0.12 bc	7.79 ± 0.50 hi
IC_{70} H_2O_2 (Control P)	2.40 ± 0.14 c	11.97 ± 0.41 lm
IC_{10} H_2O_2 (Control R)	1.20 ± 0.23 b	2.51 ± 0.14 cd
IC_{50} H_2O_2 (Control R)	1.60 ± 0.34 bc	6.65 ± 0.31 gh
IC_{70} H_2O_2 (Control R)	2.10 ± 0.98 c	10.55 ± 0.82 kl
Control	0.31 ± 0.05 a	0.10 ± 0.01 a
0.5% DMSO	0.32 ± 0.03 a	0.11 ± 0.01 a

Results are means of five different experiments. Values that are fallowed by different letters within each column are significantly different ($p \leq 0.05$). df_1=2, df_2=95, F=11.96. SE: Standard Error. P; parental, R; resistant cells.

carvacrol in resistant cells against H_2O_2 damage. Also, the IC_{10} concentration of carvacrol had the highest DNA protective effect in resistant cells against H_2O_2 damage. This means that resistant cells have more membrane protective ability than parental cells.

Carvacrol and thymol have effective DNA protective effects in both cells at different concentrations. Aydin *et al*. [18] also observed that these compounds, 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 a range of concentrations of either 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 [19]. Epigallocatechin-3-gallate (1 μM), a polyphenol abundant in tea, was shown to significantly reduce MDA production due to H_2O_2/Fe^{2+} exposure, indicating a protection of cells from oxidative stress [20]. The malondialdehyde level increased in H_2O_2 exposed (IC_{50} and IC_{70}) hepatoma G2 cells, but decreased in these cells preincubated with carvacrol and thymol before H_2O_2 exposure [2]. Carvacrol and thymol differed in their cytotoxic and genotoxic effects on K562 cells, and reduced the level of DNA damage induced by the strong oxidation of H_2O_2 [17]. Further understanding of the underlying mechanism of their protective effects in reducing intracellular oxygen radicals in H1299 cell death may lead to the development of new therapeutic treatments for cancer, since carvacrol and thymol provide protection against H_2O_2 insult. Their lung protective effects against H_2O_2 toxicity might be of importance and may contribute in part to their clinical efficacy for the treatment of lung carcinoma. These results suggest that carvacrol and thymol may be potentially valuable sources 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. In the present study, carvacrol and thymol induced DNA and membrane damage, and cytotoxicity in H1299 cells at relatively higher concentrations than those that mediate its anticancer activities. These findings suggest that carvacrol and thymol exhibit anticancer/antioxidant effects dependant on cell resistance, concentration and time.

Experimental

Cancer cell culture: The H1299 cell line was purchased from the American Type Culture Collection (Rockville, MD). Cells were routinely cultured in RPMI 1640 medium supplemented with10% fetal calf serum, 1% antibiotic-antimycotic solution in a humidified atmosphere containing 5% CO_2 at 37°C. For subculturing, cells were harvested after trypsin/EDTA treatment at 37°C. Cells were used when monolayer confluence had reached 75%. The drug resistant (Epirubicin-resistant) H1299 tumor cells were derived from the parental line by stepwise selection in increasing concentrations of Epirubicin until the cells were capable of propagating in 220 ng/mL drug, as described previously [21,22].

Cell viability assay: The cancer cells (10,000 cells/well, monolayer) were plated in a 96-well plate. The next day, the cells were treated with different concentrations of carvacrol and thymol in the medium for 24, 48 and 72 h. At the end of the incubation periods, the cytotoxicity of thymol and carvacrol on cancer cells was determined by the the cell titer-blue-cell viability assay. 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 [23]. Following cellular reduction, fluorescence is recorded at 560 nm excitation / 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 equation of the graph. H_2O_2 cytotoxicity on cancer cells was measured in the same way. For measuring the antioxidant effect of carvacrol and thymol against H_2O_2 cytotoxicity, the cells were preincubated with them at different concentrations (10–150 μg/mL) for 1 h, before hydrogen peroxide treatment for 24 h.

Determination of malondealdehyde level: The cells were plated at a density 5–10 x 10^5 cells/100 mm dish. The cells were preincubated with maximum cytoprotective concentrations of carvacrol and thymol for 1 h, before hydrogen peroxide treatment (IC_{10}, IC_{50} and IC_{70}) for 24 h. Cells were scraped off the culture plates with culture medium and centrifuged at 400 × *g* for 10 min. The cell pellets were washed with PBS and then sonicated (3 × 15 sec) 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 malondealdehyde level, as described by Wasowicz *et al*. [24]. 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 *n*-butanol and measured spectrofluorometrically at 525 nm (excitation) and 547 nm (emission). Protein was determined by the Bradford method [25] with bovine serum albumin as a standard.

Determination of 8-OHdG level: After DNA purification [26] from the cultured cells (Genomic DNA Mini Kit, Invitrogen), the genomic DNA samples were used to determine 8-OHdG with a competitive ELISA kit (Highly sensitive 8-OHdG Check New, Japan Institute for Control of Aging, Fukuroi, Shizuoka, Japan). Micro titer ELISA plates were precoated with 8-OHdG. Fifty μL of the sample and primary antibody were added to each well and incubated at 4°C overnight. The wells were washed 3 times. Then 100 μL secondary antibody was added to each well and incubated for 1 h at room temperature. The wells were again washed 3 times. After that, enzyme substrate solution was added and the wells incubated at room temperature for 15 min. Terminating solution stopped the reaction. The absorbance was read at a wavelength of 450 nm [27,28].

Data analysis: The results of the replicates were pooled and expressed as mean ± standard error. Analysis of variance and Student's t-test were carried out. Significance was accepted at $p \leq 0.05$ [29].

Acknowledgements - This work was supported by the Scientific Research Projects of the Administration Unit of Akdeniz University (2008.01.0105.011). The authors wish to thank Akdeniz University Scientific Research Projects Unit for financial support of this work.

References

[1] Ozbek T, Gulluce M, Sahin F, Ozkan H, Sevsay S, Baris O. (**2008**) Investigation of the antimutagenic potentials of the methanol extract of *Origanum vulgare* L. subsp *vulgare* in the Eastern Anatolia region of Turkey. *Turkish Journal of Biology*, ***32***, 271–276.

[2] Ozkan A, Erdogan A. (**2011**) A comparative evaluation of antioxidant and anticancer activity of essential oil from *Origanum onites* (Lamiaceae) and its two major phenolic components. *Turkish Journal of Biology*, ***35***, 735-742.

[3] Lambert RJV, Skandamis PN, Coote PJ, Nychas GJE. **(2001)** A study of the minimum inhibitory concentration and mode of action of oregano essential oil, thymol and carvacrol. *Journal of Applied Microbiology*, ***91***, 453-462.

[4] Duthie GG, Brown KM. (**1998**) Reducing the risk of cardiovascular disease. In *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals*. Goldberg I. (Ed.), Chapman & Hall, New York, NY, USA, 19-38.

[5] Milner JA. (**1998**) Reducing the risk of cancer. In *Functional Foods: Designer Foods, Pharmafoods, Nutraceuticals.* Goldberg I. (Ed.). Chapman & Hall, New York, NY, USA, 39-70 pp.

[6] Gulçin I, Kireçci E, Akkemik E, Topal F, Hisar O. (**2010**) Antioxidant, antibacterial, and anticandidal activities of an aquatic plant: duckweed *(Lemna minor* L., Lemnaceae). *Turkish Journal of Biology*, ***34***, 175-188.

[7] Kutlu T, Durmaz G, Ateş B, Yılmaz I, Cetin MS. (**2011**) Antioxidant properties of different extracts of black mulberry (*Morus nigra* L.). *Turkish Journal of Biology*, ***35***, 103-110.

[8] Seyoum A, Asres K, El-Fiky FK. (**2006**) Structure-radical scavenging activity relationship of flavonoids. *Phytochemistry*, ***67***, 2058-2070.

[9] Ozkan A, Erdogan A, Sokmen M, Tugrulay S, Unal O. (**2010**) Antitumoral and antioxidant effect of essential oils and *in vitro* antioxidant properties of essential oils and aqueous extracts from *Salvia pisidica. Biologia*, ***65***, 990-996.

[10] Ozkan A, Gubbuk H, Gunes E, Erdogan A. (**2011**) Antioxidant capacity of juice from different papaya (*Carica papaya* L.) cultivars grown under greenhouse conditions in Turkey. *Turkish Journal of Biology*, ***35***, 619-625.

[11] Moteki H, Hibasami H, Yamada Y, Katsuzaki H, Imai K, Komiya T. (**2002**) Specific induction of apoptosis by 1,8-cineole in two human leukemia cell lines, but not in human stomach cancer cell line. *Oncology Reports*, ***9***, 757-760.

[12] Rihova B, Strohalm J, Kobackova K. (**2002**) Acquired and specific immunological mechanisms co-responsible for efficacy of polymer-bound drugs. *Journal of Controlled Release*, ***78***, 97-114.

[13] Miyazawa M, Shindo M. (**2001**) Biotransformation of 1,8-cineole by human liver microsomes. *Natural Product Letters*, ***15***, 49-53.

[14] Duisken M, Sandner F, Blomeke B, Hollender J. (**2005**) Metabolism of 1,8-cineole by human cytochrome P450 enzymes: identification of a new hydroxylated metabolite. *Biochimica et Biophysica Acta*, ***1722***, 304–311.

[15] Koparal AT, Zeytinoglu M. (**2003**) Effects of carvacrol on a human nonsmall cell lung cancer (NSCLC) cell line, A549. *Cytotechnology*, ***43***, 149-154.

[16] Mastelic J, Jerkovic I, Blazevic I, Poljak-Blazi M, Borovic S, Ivancic-Bace I, Smrecki V, Zarkovic N, Brcic-Kostic K, Vikic-Topic D, Muller N. (**2008**) Comparative study on the antioxidant and biological activities of carvacrol, thymol, and eugenol derivatives. *Journal of Agricultural and Food Chemistry*, ***56***, 3989-3996.

[17] Horvathova E, Turcaniova V, Slamenova D. **(2007**) Comparative study of DNA-damaging and DNA-protective effects of selected components of essential plant oils in human leukemic cells K562. *Neoplasma*, ***54***, 478-483.

[18] Aydin S, Basaran AA, Basaran N. (**2005**) Modulating effects of thyme and its major ingredients on oxidative DNA damage in human lymphocytes. *Journal of Agricultural and Food Chemistry*, ***53***, 1299-1305.

[19] Slamenova D, Horvathova E, Sramkova M, Marsalkova L. (**2007**) DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured *in vitro*. *Neoplasma*, ***54***, 108-112.

[20] Peng IW, Kuo SM. (**2003**) Flavonoid structure affects the inhibition of lipid peroxidation in Caco-2 intestinal cells at physiological concentrations. *Journal of Nutrition*, ***133***, 2184-2187.

[21] Jonsson O, Motlagh PB, Persson M, Henriksson R, Grankvist K. (**1999**) Increase in doxorubicin cytotoxicity by carvedilol inhibition of P-glycoprotein activity. *Biochemical Pharmacology*, ***58***, 1801-1806.

[22] Ozkan A. (**2007**) Lymphokine-activated killer cell susceptibility in epirubicin resistant and parental human non-small cell lung cancer (NSCLC). *Biologia*, ***62***, 232-237.

[23] Gloeckner H, Jonuleit T, Lemke HD. (**2001**) Monitoring of cell viability and cell growth in a hollow-fiber bioreactor by use of the dye Alamar Blue (TM). *Journal of Immunological Methods*, ***252***, 131-138.

[24] Wasowicz W, Neve J, Peretz A. (**1993**) Optimized steps in fluorometric determination of thiobarbituric acid-reactive substances in serum; importance of extraction pH and influence of sample preservation and storage. *Clinical Chemistry*, ***39***, 2522-2526.

[25] Bradford MM. (**1976**) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein dye binding. *Analytical Biochemistry*, ***72***, 248-254.

[26] www.invitrogen.com

[27] Toyokuni S, Tanaka T, Hattori Y, Nishiyama Y, Yoshida A, Uchida K, Hiai H, Oci H, Osawa T. (**1997**) Quantitative immunohistochemical determination of 8-hydroxy-2'-deoxyguanosine by a monoclonal antibody N45. 1: its application to ferric nitrilotriacetate-induced renal carcinogenesis model. *Laboratory Investigation*, ***76***, 365-374.

[28] Garçon G, Dagher Z, Zerimech F, Ledoux F, Courcot D, Aboukais A, Puskarıc E, Shirali P. **(2006)** Dunkerque city air pollution particulate matter-induced cytotoxicity, oxidative stress and inflammation in human epithelial lung cells (L132) in culture. *Toxicology in Vitro*, ***20***, 519–528.

[29] Kirkman TW. (**1996**) Statistics to use [Online]. Available:http://www.physics.csbsju.edu/stats/1996 [17 August 2008].