doi:10.4149/neo_2011_01_14

Ex vivo assessment of protective effects of carvacrol against DNA lesions induced in primary rat cells by visible light excited methylene blue (VL+MB)

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Received July 22, 2010

Carvacrol belongs to frequently occurring phenolic components of essential oils (EOs) and it is present in many kinds of plants. Biological effect of this phenol derivative on human beings is however not sufficiently known. The present study was undertaken to evaluate the level of VL+MB-induced oxidative DNA lesions in hepatocytes and testicular cells (freshly isolated from control or carvacrol-watered rats) by the modified single cell gel electrophoresis (SCGE). The results showed that carvacrol significantly reduced the level of VL+MB-induced oxidized bases (EndoIII- and Fpg-sensitive sites) only in hepatocytes but not in testicular cells. Chromosomal aberration assay of primary hepatocytes, isolated from control or carvacrol-watered rats did not testify any genotoxic activity of carvacrol. We suggest that *in vivo* applied synthetic carvacrol, whose antioxidative activity was confirmed by DPPH assay, exhibits primarily a strong hepatoprotective activity against oxidative damage to DNA.

Key words: *carvacrol, hepatocytes, testicular cells, comet assay, chromosomal aberrations, DPPH assay*

Essential oils (EOs) of plants, composed mainly of different types of terpens and phenols, represent one of the main sources for phytochemicals with chemopreventive potential. Carvacrol is the major component of the essential oil fraction of *Origanum hirtum*, oil of thyme, oil obtained from pepperwort and wild bergamot and was found also in several other EOs. This compound, isomeric with thymol (a monoterpene phenol derivative of cymene), has a long history of use for many medical applications [1]. Carvacrol exhibits a strong antibacterial activity against a wide spectrum of bacteria [2], antifungal activity [3], anticandidal activity [4] and trypanocidal activity [5]. It minimizes oxidation of the lipid components in foods and can serve as a natural replacement of synthetic antioxidant food additives [2]. EOs and aqueous tea infusions obtained from oregano, thyme and wild thyme prevent oxidation of low-density lipoproteins (LDL), that transport dangerous cholesterol and triglycerides from the liver to peripheral tissues [6]. Jukic et al. [7] found that thymol, carvacrol and their derivatives exhibited a strong inhibitory effect on acetylcholinesterase (AChE), an enzyme that degrades the neurotransmitter acetylcholine. Inhibition of AChE represents one of the therapeutic strategies developed in Alzheimer's disease treatment. Lee et al. [8] proved that carvacrol activated the human collagen type I promoter activity and the synthesis of human type I procollagen, preventing skin aging and wrinkle formation.

An increasing interest in the use of EOs and their components raises the need to evaluate such compounds for both mutagenic and antimutagenic properties. Aydin et al. [9] studied the genotoxic and antimutagenic potential of major compounds of thyme oil in human lymphocytes by SCGE technique and found a significant antimutagenicity of carvacrol *in vitro*. Possible antigenotoxic effects of EOs extracted from medicinal plants such as *Origanum compactum*, *Artemisia herba alba* and *Cinnamomum camphora*, known for their beneficial effects in humans were proved also in the yeast *Saccharomyces cerevisiae* [10]. Koparal and Zeytinoglu [11] found that higher doses of carvacrol cause in human non-small-cell lung cancer cell line A549 a decrease in cell number, apoptosis, connected with degeneration of cell morphology, and a decrease in total protein amount. This indicates that carvacrol may have a pharmacological importance for the prevention of cancer.

We demonstrated [12] that incubation of human cells in the presence of the monoterpens thymol and carvacrol led

to a significant protection of cellular DNA against a strong oxidative agent, hydrogen peroxide. On the basis of *in vitro* experiments it was however not possible to determine whether these DNA-protective effects could be operative also in the intact organism of experimental rats. This doubt was later excluded, as we found the protective activity of carvacrol added in *in vivo* conditions against DNA lesions induced by H_2O_2 in primary rat cells *ex vivo* [13]. In this report we compared primary hepatocytes and testicular cells (freshly isolated from control and carvacrol-supplemented rats) from the point of view of oxidative DNA lesions induced by visible light excited methylene blue (VL+MB). Oxidized DNA bases were detected by modified SCGE, which is able to detect EndoIII- and Fpg-sensitive sites. Genotoxic effect of carvacrol on rat hepatocytes *in vivo* was evaluated by chromosomal aberration assay. Antioxidative activity of ethanolic solutions of commercial synthetic carvacrol used in our experiments was determined by a spectrophotometric method using the stable DPPH radical.

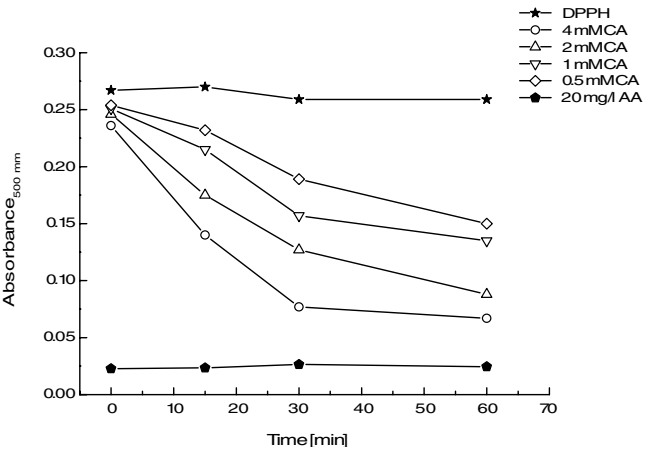


Fig. 1. Time course of DPPH radical scavenging reaction of carvacrol (0.5, 1, 2 and 4 mmol/l CA) and 20 mg/l ascorbic acid (AA).

Materials and methods

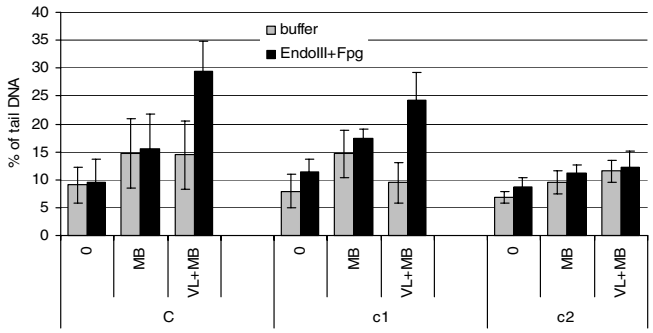
Chemicals and treatment of cells. Methylene blue (MB, Fluka, Buchs, Switzerland) was diluted shortly before use in PBS buffer to the final concentration 3.125×10^{-5} mol/l and kept at 4°C. Hepatocytes or testicular cells embedded in agarose gels were treated with visible light (60 W bulb; 180 s; 25 cm distance) + MB on ice without any other source of light. A similar light exposure regimen to induce DNA damage was used by Hartwig et al. [14]. Control cells were treated with MB for 180 s in the dark. After treatment, the cells were washed with PBS. Carvacrol – purity (as given by the manufacturer, Fluka, Buchs, Switzerland) was ≥ 97%; density = 0.974 g/ml; Mw = 150.22. This compound was kept at room temperature, diluted in drinking water (10 µl or 20 µl per 40 ml of water) and given to rats. With regard to the consumption of drinking water by rats, the amount of carvacrol equals 30 or 60 mg/day/kg b.w.

Testing DPPH radical scavenging activity of carvacrol. The antioxidative activity of carvacrol was determined with DPPH (1,1-diphenyl-2-picrylhydrazyl) radical using the spectrophotometric method by Buřičová and Réblová [15]. DPPH radical (Sigma Aldrich, St. Louis, USA) dissolved in ethanol in the concentration of 0.05 mg/ml (100 µl) was added to 50 µl of various concentrations of carvacrol. The decrease in absorbance at 500 nm was measured at 0, 15, 30, and 60 min using a spectrophotometer Multiscanreader RC, Labsystems, Finland. Ethanol of analytical grade (Microchem, Slovakia) was used to zero the spectrophotometer. Ascorbic acid (20 mg l) in H_2O was used as a positive control. All determinations were performed in triplicates at room temperature. The DPPH radical scavenging activity was calculated with the following formula: DPPH radical scavenging activity (%) = [(control absorbance – extract absorbance) / (control absorbance)] × 100.

Chromosomal aberrations. Slides were prepared using the standard air-drying method and stained with 10% aqueous Giemsa solution. The classification of aberration was carried out as described by Venitt and Parry [16]. The metaphases were analyzed for the following structural aberrations: chromatid gaps and breaks and exchanges (dicentrics, quadriradials, triradials, and double minutes). Since the genetic significance of gaps is not clearly understood, they were not included in the assessment of the chromosomal damage and thus were not evaluated statistically.

Enzymes. Endonuclease III (EndoIII) and formamidopyrimidine-DNA-glycosylase (Fpg) were a generous gift of Prof. A. R. Collins, University of Oslo, Norway. The crude extracts of EndoIII and Fpg were diluted in a buffer containing 40 mmol/l HEPES; 0.1 mol/l KCl; 0.5 mmol/l EDTA, 0.2 mg/ml BSA, pH 8.0 prior to use. The dilutions used were 1:1000 for EndoIII and 1:3000 for Fpg.

Animals. Nine sexually mature male Sprague-Dawley (SD) rats, obtained from ANLAB, Czech Republic, with a mean body weight (b.w.) of 300 g, were used (by one) in nine individual experiments. The animals were obtained from an in-house strain and were maintained in a room with 12 h light/dark cycle, room temperature 22±2°C, relative humidity 55±5%. They were housed in plastic cages on hardwood bedding (1 rat per cage). A standard diet (containing nutriment, amino acids, biofactors, and minerals) was given. Food consumption for each cage was recorded daily and individual body weights were recorded at the beginning and at the end of experiment. The compound tested, carvacrol, was added to drinking water in two different concentrations (30 or 60 mg/day/kg b.w.) during 7 days. The basis for the choice of these carvacrol concentrations were results published by Azirak and Rencuzogullari [17]. Water (+/- carvacrol) was provided *ad libitum*. After 7 days the total b.w. gain was ~ 40 g in both control and carvacrol drinking rats. No degeneration of inner organs or worsening of the overall health condition was observed in carvacrol sup-



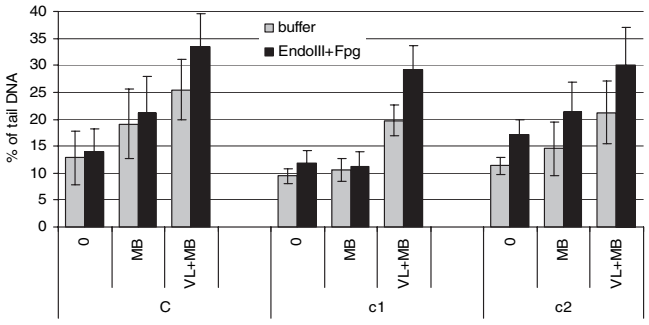


Figure 2 and 3. Percentage of tail DNA in hepatocytes (Fig. 2) and testicular cells (Fig. 3) isolated from control rats (C) and rats supplemented with 30mg/kg/day (c1) or 60mg/kg/day (c2) carvacrol and treated with methylene blue (MB) and visible light+methylene blue (VL+MB). Grey bars represent samples in the absence and black bars in the presence of repair enzymes EndoIII and Fpg.
Results of statistical analysis:
Figure 2. C/VL+MB<C/VL+MB+e (p<0.001); c2/VL+MB+e< C/VL+MB+e (p<0.001); c2/VL+MB+e< c1/VL+MB+e (p=0.001).
Figure 3. C<C/MB (p=0.029); C<C/VL+MB (p<0.001); C/MB<C/VL+MB (p=0.015); C/VL+MB<C/VL+MB+e (p<0.001); c1/MB<C/MB (p<0.001); c1/VL+MB<C/VL+MB (p=0.024);

plemented rats. The rats were ethically sacrificed by i.p. dose of thiopental and from each animal hepatocytes and testicular cells determined for further processing were isolated. The experiments were conducted under the guidelines of the Animal Ethics Committee and were approved by the State Veterinary and Food Administration of the Slovak Republic.

Isolation of cells. Hepatocytes were isolated by the *in situ* two-step collagenase perfusion technique as described by Michalopoulos et al.[18]. Testicular cells were isolated from testes of sexually mature male SD rats by enzymatic digestion as described by Bradley and Dysart [19]. Flow cytometry of testicular cells isolated by this method showed that the total testicular pool contains approximately 59.42% of haploid round spermatids, 16.8% of elongating spermatids, 14.0% of diploid (both somatic and germ) cells, 2.6% of S-phase cells and 7.2% of tetraploid cells [20].

Modified single cell gel electrophoresis. The basis of this test is the conventional procedure suggested by Singh et al. [21]. The modified comet assay version enables detection of oxidative DNA damage using specific enzymes [22]. The gels containing control or VL+MB-treated cells were placed in lysis solution for 1 h at 4°C to remove cellular proteins. After lysis the cells were washed twice for 10 min with endonuclease buffer (40 mmol/l HEPES; 0.1 mol/l KCl; 0.5 mmol/l EDTA, pH 8.0) and incubated with a cocktail of repair enzymes EndoIII and Fpg for 30 min at 37°C. The control slides were incubated with endonuclease buffer containing BSA. The subsequent steps of unwinding and electrophoresis are identical in both the conventional and modified procedures. EtBr stained nucleoids were evaluated with an Olympus BX-51 fluorescence microscope. For each sample, 100 comets were scored using computerized image analysis (Komet 5.5, Kinetic Imaging, Liverpool, UK) for determination of DNA in the tail. The percentage of DNA in the tail is directly proportional to the frequency of DNA strand breaks [23].

Statistics. The results represent a mean from 3 sets of experiments. The significance of differences between individual groups and individual samples presented in Figs. 2 and 3 was evaluated by a one-way analysis of variance (ANOVA) followed by Bonferroni or Tamhan test for multiple comparisons in dependence on the absence or presence of a statistically significant difference in variances among individual groups. We calculated at the significance level α=0.05 and used a statistical software SPSS 16.0. Chromosomal aberrations were statistically evaluated by testing the difference of two relative values.

Results

Antioxidative activity of ethanolic solutions of carvacrol was investigated using DPPH assay. When DPPH, which is a stable free radical with dark violet color (absorbed at 517 nm) reacts with an antioxidant compound which can donate hydrogen, it is reduced and changes its color to yellow. The results showed (Fig. 1) that all concentrations of carvacrol tested (0.5, 1, 2 and 4 mmol/l) exhibited a notable antioxidative potential.

Genotoxic effects of carvacrol were analysed on the basis of the level of chromosomal aberrations in hepatocytes isolated from control and carvacrol-treated rats by the chromosomal aberration assay (Table 1). We did not observe any cytogenetic effects of this compound.

Protective effects of carvacrol against DNA damage induced by VL+MB. Figs. 2 and 3 show % of DNA lesions induced by methylene blue (MB) and visible light+methylene blue (VL+MB) in the presence or absence of repair enzymes in hepatocytes (Fig. 2) and testicular cells (Fig. 3). Both kinds of primary cells were isolated from control rats (C) and carvacrol-supplemented rats (c1 = 30 mg/kg/day and c2 = 60 mg/kg/day). Statistical evaluation of the results showed that in both kinds of cells isolated from control rats there was the percentage of tail DNA significantly increased after VL+MB+e treatment (induc-

Table 1. Chromosomal aberrations induced in primary rat hepatocytes by 7 day carvacrol supplementation

Sample	Number of scored metaphases	Number of aberrant metaphases	Number of chromosomal aberrations								Total number of CA
			Chromatid		Isochromatid		Exchange				
			g	b/f	g	b/f	dic	qr	tr	dmin	
SC	140	8	6	8	2	-	-	-	-	-	8
30 mg/kg	154	12	3	7	1	5	-	-	-	-	12
60 mg/kg	183	12	1	10	1	2	-	-	1	-	13

SC, solvent control (PBS); CA, chromosomal aberrations; g, gap; b/f, break and/or fragment; dic, dicentric; qr, quadriradial; tr, triradial; dmin, double minute not significantly different from the SC ($p>0.05$)

The numbers of chromatid and isochromatid gaps were recorded for each treatment group; however, since their genetic significance is not clearly understood, they are not included in our assessment of chromosomal damage.

tion of oxidative DNA lesions), in comparison with samples treated by VL+MB. In hepatocytes isolated from control rats there was found no significant differences in the percentage of tail DNA in control (C) and samples exposed to methylene blue (MB) or visible light+methylene blue (VL+MB). On the other side, in testicular cells isolated from control rats there were statistically significant differences ($p<0.001$) in the percentage of tail DNA between control (0) and samples exposed to MB or VL+MB. This fact suggests a genotoxic effect of MB (0.01%) and VL+MB on testicular cells. Genotoxic effects of MB on breast cells were reported by Masannat et al. [24].

The statistical analysis showed (1) that in hepatocytes isolated from rats supplemented by carvacrol (c1 or c2) and exposed to VL+MB+e there was found a statistically significant decrease in the percentage of tail DNA (compared to control rats). This suggests a reduction of oxidative DNA lesions in hepatocytes isolated from carvacrol-supplemented rats; (2) that in testicular cells isolated from control or carvacrol (c1 or c2)-supplemented rats no significant differences were found between samples VL+MB and VL+MB+e, what suggests that supplementation of rats by carvacrol does not reduce the level of oxidative DNA lesions induced by VL+MB.

Discussion

Humans and animals are constantly confronted with genotoxic or potentially genotoxic compounds occurring in the environment. In somatic cells DNA damage is considered to be the most significant stimulus of initiation of the multistep process of carcinogenesis. In germinal cells can DNA lesions besides have a destructive effect on the next and successive generations. Freshly isolated rat hepatocytes (somatic cells) and testicular cells (mostly germinal cells) are provided with a wide spectrum of protective mechanisms that shield important cellular biomacromolecules. Their use in *ex vivo* conditions seems to be very useful in investigating the ability of natural compounds to reduce or eliminate genotoxic effects of oxidative carcinogens. Liver is the main organ for the metabolism of foreign compounds, therefore liver cells represent a suitable system for the evaluation of different effects which could raise or reduce the response to mutagens. The examination of sperm or testicular cells seems to be an easier and more logical choice for genotoxicity studies on germinal cells than female germ cells, as these are more complicated to obtain. We investigated the effect of dietary carvacrol supplementation from the point of view of resistance of freshly isolated rat somatic and germinal cells towards DNA-damaging oxidative agents. Our assumption that carvacrol given to rats in drinking water reaches different rat organs including testes is supported by several findings, e.g. that *Origanum majorana* L. oil containing carvacrol minimizes hazard effects of ethanol toxicity on male fertility as well as toxic effects of ethanol on liver and brain tissues [25] or that huge molecules of lignin biopolymer could be found by FTIR (fourier-transformed infrared) spectroscopy in lyophylized blood lymphocytes and testicular cells isolated from rats fed lignin enriched food [26].

The oxidative agent used in our study was visible light excited methylene blue. It is known [27] that excited molecules of photosensitizer (methylene blue) react with DNA mainly via singlet oxygen ($^1O_2$), the lowest energy-excited state of molecular oxygen, more reactive than the triplet ground-state molecule. The generating of $^1O_2$ in the cytoplasm might be able to react with DNA in the nucleus, provided it is not intercepted by one of its biological scavengers [28]. DNA damage induced by $^1O_2$ is dominated by base modifications sensitive to repair enzymes [29]. A statistically significant increase of the percentage of tail DNA (ss DNA breaks) in both hepatocytes and testicular cells (isolated from control animals) by VL+MB in the presence of repair enzymes EndoIII+Fpg manifests the induction of oxidative DNA base modifications recognized by repair enzymes. The level of these DNA lesions was significantly lowered in hepatocytes isolated from carvacrol-supplemented animals, however it was not changed in testicular cells. It seems that supplementation of rats by carvacrol did not increase the resistance of testicular cells towards DNA lesions induced by singlet oxygen. There were found also further differences between hepatocytes and testicular cells. While MB or VL+MB did not induce any DNA lesions in hepatocytes, they induced statistically significant increase of DNA lesions in testicular cells. This category of

DNA lesions was in carvacrol-supplemented animals (mainly in group c1) reduced, similarly as were DNA lesions induced by H_2O_2 [13].

To date we do not know the exact mechanism in which carvacrol reduces genotoxic effects of H_2O_2 in both kinds of cells and genotoxic effects of singlet oxygen ($^1O_2$) only in liver cells. We consider several possibilities: that carvacrol will not necessarily be distributed in the same quantities and/or ratios to all organs throughout the body; that the antioxidative status of cytoplasm in hepatocytes and testicular cells of carvacrol-supplemented rats can be different in comparison with liver cells; that differential patterns of the antioxidant defense systems are required for reduction of oxidative damage induced in cells by H_2O_2 and $^1O_2$; or that the different reaction of hepatocytes and testicular cells (isolated from carvacrol-supplemented rats) are associated with differences in cellular DNA repair. The antioxidant status of cytoplasm of hepatocytes and testicular cells as well as activity of base excision repair in both kinds of cells isolated from control and carvacrol-supplemented rats will be investigated in our further studies.

Acknowledgement. This work was supported by the VEGA grant 2/0072/09. The authors wish to thank Mrs. Alžbeta Vokáliková, Lívia Šebová, and Viera Dytrichová for their excellent technical assistance.

References

[1] ROMMELT H, ZUBER A, DIRNAGL K, DREXLER H. The absorption of terpenes from bath additives [author's transl]. Munch Med Wochenschr 1974; 116: 537–540.

[2] BURT S. Essential oils: their antibacterial properties and potential applications in foods. A review. Int J Food Microbiol 2004; 94: 223–253. doi:10.1016/j.ijfoodmicro.2004.03.022

[3] PINTO E, PINA-VAZ C, SALGUEIRO L, GONÇALVES MJ, COSTA-DE-OLIVEIRA S et al. Antifungal activity of the essential oil of Thymus pulegioides on Candida, Aspergillus and dermatophyte species. Journal of Medical Microbiology 2006; 55: 367–1373. doi:10.1099/jmm.0.46443-0

[4] CHAMI N, CHAMI F, BENNIS S, TROUILLAS J, REMMAL A. Antifungal treatment with carvacrol and eugenol of oral candidiasis in immunosuppressed rats. Braz J Infect Dis 2004; 8: 217–226. doi:10.1590/S1413-86702004000300005

[5] SANTORO GF, DAS GRACAS CARDOSO M., GUIMARAES LG SALGADO AP, MENNA-BARRETO R et al. Effect of oregano (Origanum vulgare L.) and thyme (Thymus vulgaris L.) essential oils on Trypanosoma crusi (Protozoa: Kinetoplastida) growth and ultrastructure. Parasitol Res 2007; 100: 783–790. doi:10.1007/s00436-006-0326-5

[6] KULISIC T, KRISKO A, DRADOVIĆ-UZELAC V, MILOS M, PIFAT G. The effects of essential oils and aqueous tea infusions of oregano (Origanum vulgare L. spp. hirtum), thyme (Thymus vulgaris L.) and wild thyme (Thymus serpyllum L.) on the copper-induced oxidation of human low-density lipoproteins. Int J Food Sci Nutr 2007; 58: 87–93. doi:10.1080/09637480601108307

[7] JUKIC M., POLITEO O, MAKSIMOVIC M, MILOS M, MILOS M. In vitro acetylcholinesterase inhibitory properties of thymol, carvacrol and their derivatives thymoquinone and thymohydroquinone. Phytother Res 2007; 21: 259–261. doi:10.1002/ptr.2063

[8] LEE J, JUNG E, YU H, KIM Y, HA J et al. Mechanisms of carvacrol-induced expression of type I collagen gene. J Dermatol Sci 2008; 52: 160–169. doi:10.1016/j.jdermsci.2008.06.007

[9] AYDIN S, BASARAN AA, BASARAN N. The effects of thyme volatiles on the induction of DNA damage by the heterocyclic amine IQ and mitomycin C. Mutat Res 2005; 581: 43–53.

[10] BAKKALI F, AVERBECK S, AVERBECK D, IDAOMAR M. Biological effects of essential oils. A review. Food Chem Toxicol 2008; 46: 446–475. doi:10.1016/j.fct.2007.09.106

[11] KOPARAL AT, ZEYTINOGLU M. Effects of carvacrol on a human non-small cell lung cancer (NSCLC) cell line A549. Biomed Subject Life Sci 2003; 43: 149–154.

[12] SLAMENOVA D, HORVATHOVA E, SRAMKOVA M, MARSALKOVA L. DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured in vitro. Neoplasma 2007; 54: 108–112.

[13] SLAMENOVA D, HORVATHOVA E, MARSALKOVA L, WSOLOVA L. Carvacrol given to rats in drinking water reduces the level of DNA lesions induced in freshly isolated hepatocytes and testicular cells by H2O2. Neoplasma 2008; 55: 394–399.

[14] HARTWIG A, DALLY H, SCHLEPEGRELL R. Sensitive analysis of oxidative DNA damage in mammalian cells: Use of the bacterial Fpg protein in combination with alkaline unwinding. Toxicol Lett 1990; 88: 85–90. doi:10.1016/0378-4274(96)03722-8

[15] BURICOVA L, REBLOVA Z. Czech Medicinal Plants as Possible Sources of antioxidants. Czech J Food Sci 2008; 26: 132–138.

[16] VENITT S, PARRY JM. Mutagenicity testing "A Practical Approach". 1984; pp. 353. In Oxford, Washington DC, IRL Press.

[17] AZIRAK S, RENCUZOGULLARI E. The in vivo genotoxic effects of carvacrol and thymol in rat bone marrow cells. Environ Toxicol 2008; 23: 728–735. doi:10.1002/tox.20380

[18] MICHALOPOULOS G, CIANCILLI HD, NOVOTNY A, KLINGERMAN AD, STROM SC et al. Liver regeneration studies with rat hepatocytes in primary culture. Cancer Res 1982; 42: 4673–4682.

[19] BRADLEY MO, DYSART G. DNA single-strand breaks, double-strand breaks, and crosslinks in rat testicular germ cells: measurements of their formation and repair by alkaline and neutral filter elution. Cell Biol Toxicol 1985; 1: 181–195. doi:10.1007/BF00120163

[20] WELLEJUS A, BORNHOLDT J, VOGEL UB, RISOM L, WIGER R et al. Cell-specific oxidative DNA damage induced by estrogen in rat testicular cells in vitro. Toxicol Lett 2004; 150: 317–323. doi:10.1016/j.toxlet.2004.02.010

[21] SINGH NP, McCOY MT, TICE RR, SCHNEIDER EL. A simple technique for quantitation of low levels of DNA damage in individual cells. Exp Cell Res 1988; 175: 184–191. doi:10.1016/0014-4827(88)90265-0

[22] COLLINS AR, DUTHIE SJ, DOBSON VL. Direct enzymatic detection of endogenous oxidative base damage in human

lymphocyte DNA. Carcinogenesis 1993; 14: 1733–1735. doi:10.1093/carcin/14.9.1733

[23] McKELVEY-MARTIN VJ, GREEN MH, SCHMEZER P, POOL-ZOBEL BL, DE MEO M P et al. The single cell gel electrophoresis assay (comet assay): a European review. Mutat Res 1993; 288: 47–63. doi:10.1016/0027-5107(93)90207-V

[24] MASANNAT YA, HANBY A, HORGAN K, HARDIE LJ. DNA damaging effects of the dyes used in sentinel node biopsy: possible implications for clinical practice. J Surg Res 2009; 154: 234–238. doi:10.1016/j.jss.2008.07.039

[25] El-ASHMAWY IM, SALEH A, SALAMA OM. Effects of marjoram volatile oil and grape seed extracty on ethanol toxicity in male rats. Basic Clin Pharmacol 2007; 101: 320–327. doi:10.1111/j.1742-7835.2007.00125.x

[26] LABAJ J, SLAMENOVA D, LAZAROVA M, KOSIKOVA B. Lignin-stimulated reduction of oxidative DNA lesions in testicular cells and lymphocytes of sprague-dawley rats in vitro and ex vivo. Nutrition and Cancer 2004; 50: 198–205. doi:10.1207/s15327914nc5002_10

[27] PFLAUM M, KIELBASSA C, GARMYN M, EPE B. Oxidative DNA damage induced by visible light in mammalian cells: extent inhibition by antioxidants and genotoxic effects. Mutat Res 1998; 408: 137–146.

[28] DAHL TA, MIDDEN WR, HARTMAN PE. Some prevalent biomolecules as defenses against singlet oxygen damage. Photochem Photobiol 1988; 47: 357–362. doi:10.1111/j.1751-1097.1988.tb02737.x

[29] BOITEUX S, GAJEWSKI E, LAVAL J, DIZDAROGLU M. Substrate specificity of the Escherichia coli Fpg protein (formamidopyrimidine-DNA glycosylase): excision of purine lesions in DNA produced by ionising radiation or photosensitization. Biochemistry 1992; 31: 106–110. doi:10.1021/bi00116a016