DNA-protective effects of two components of essential plant oils carvacrol and thymol on mammalian cells cultured *in vitro*

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Many components of essential volatile oils show antioxidant activity and may serve e.g. as a natural replacement of synthetic antioxidant food additives. However, it is important to evaluate such compounds also for their pro-oxidant and toxic properties as their plant origin doesn't secure their safety for living beings, including humans. The aim of this study was therefore to investigate cytotoxic, genotoxic and DNA-protective effects of the long-term (24 h) incubation of mammalian cells with two components of essential plant oils (carvacrol and thymol) in *in vitro* conditions. Cytotoxicity testing was in all cell lines (human hepatoma cells HepG2, human colonic cells Caco-2 and hamster lung cells V79) performed on the basis of trypan blue exclusion. Plating efficiency was evaluated only in V79 cells which manifest a high colony forming ability. The amount of DNA lesions induced in cells treated with hydrogen peroxide, carvacrol, thymol or combinations of carvacrol or thymol with hydrogen peroxide was measured by standard alkaline single cell gel electrophoresis in human cells HepG2 and Caco-2. Trypan blue exclusion test showed that carvacrol was mildly more cytotoxic than thymol and that Caco-2 cells were mildly more resistant to both carvacrol and thymol than HepG2 and V79 cells. At concentrations = $IC_{20–40}$, the compounds studied did not induce DNA strand breaks either in human cells HepG2 or in cells Caco-2. Incubation of HepG2 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 toward DNA strand breaks induced by a potent oxidant hydrogen peroxide.

Key words: carvacrol; thymol; HepG2, Caco-2, and V79 cells cultured in vitro; comet assay; protective effects of essential oils

The use of plants in folk medicine is as old as mankind and plant-food derived antioxidants are increasingly proposed as important dietary antioxidant factors. Volatile plant extracts and essential oils, obtained by steam or hydrodistillation of botanicals, as well as their purified or synthesized constituents, are used nowadays on a large scale in the food, cosmetic and pharmaceutical industries. Different parts of the plants can be used to obtain essential oils, including flowers, leaves, seeds, roots, stems, bark, wood, etc. Most essential oils are primarily composed of terpenes and their oxygenated derivatives. Owing to their lipophilic nature, plant volatiles appear to accumulate in the microbe cell membranes and increase their permeability, resulting in leakage of enzymes and metabolites. In addition to a characteristic flavor, many essential oils and their ingredients have been shown to exhibit a range of biological activities including anti- insecticidal, antibacterial, and antifungal activity [18, 19, 17]. Many components of essential oils show antioxidant activity [10], minimize oxidation of the lipid components in foods and thus may serve as natural replacements of synthetic antioxidant food additives. However, it is important to evaluate such compounds for their pro-oxidant properties and also to establish a complete set of toxicological data. Sometimes the chemicals in the oil, as well as the oil itself, are registered as pesticide active ingredients. It is important to remember that just because a pesticide is derived from a plant, it does not mean that it is safe for humans and other mammals or that it cannot kill a wide variety of other life.

In this study we devoted our attention to two compounds of essential plant oils, namely carvacrol (5-isopropyl-2-methyl phenol) and thymol (5-methyl-2-(1-methylethyl) phenol). Carvacrol is isomeric with thymol – both compounds interact in aqueous solution with calf thymus DNA [12]. Carvacrol and thymol are frequently found in the oil of many plants, e.g. thyme (*Thymus vulgaris L.*) or horsemint

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(*Monarda punctata L.*). The main aim of our study was to examine DNA-protective effects of carvacrol and thymol, but we thoroughly tested also cytotoxic and DNA-damaging effects of these frequently occurring components of plant oils. The DNA-protective activity of carvacrol and thymol was evaluated on the basis of their ability to reduce the genotoxic effect of hydrogen peroxide (H_2O_2).

Material and methods

Cell lines. HepG2 and Caco-2 cells were obtained from A. R. Collins (present address is: University of Oslo, Oslo, Norway). HepG2 cells were cultured in William's medium supplemented with 10% fetal calf serum and antibiotics (penicillin 200 U/ml, streptomycin 100 μg/ml, kanamycin 100 μg/ml). Caco-2 cells were cultured in Dulbecco's modified minimum essential medium (DMEM) supplemented with 10% fetal calf serum and antibiotics. V79 Chinese hamster lung fibroblasts were obtained from A. Abbondandolo (National Institute for Cancer Research, Genova, Italy). Cells were grown in DMEM supplemented with 10% fetal calf serum and antibiotics (penicillin 200 U/ml, streptomycin 100 μg/ml, and kanamycin 100 μg/ml). Cells were cultured on glass Petri dishes at 37^0C in humidified atmosphere of 5% CO_2.

Components of plant volatiles. The constituents of the plant volatiles examined in this study (and their purity as given by the manufacturer, Fluka, Buchs, Switzerland) were: carvacrol (purum ≥ 97%; density = 0.974 g/ml; Mw = 150.22) and thymol (purum ≥ 99%; density = 0.965 g/ml; Mw = 149.66). Both volatiles were kept at room temperature, dissolved immediately before use in DMSO (500 mM) and diluted to the concentrations 25 – 1000 mM in culture medium. Cells were treated with different concentrations of carvacrol or thymol during 24 h.

Chemicals. Hydrogen peroxide – H_2O_2 (Sigma, USA) stock solution (10.0 M) was kept at 4°C and diluted immediately before use in phosphate-buffered saline (PBS, Ca^{2+}- and Mg^{2+}-free) at 4°C. The monolayer of control cells or cells pre-treated with volatiles during 24 h was influenced by hydrogen peroxide (250 μM) for 5 min on ice in the dark.

Cytotoxicity assays. Cytotoxic effects of carvacrol and thymol were in all cell lines evaluated by the trypan blue exclusion technique. Exponentially growing human or hamster cells (on Petri dishes in monolayer) were exposed for 24 h to different concentrations of carvacrol or thymol (100 – 1000 μM) diluted in culture medium. Control cells were kept in a fresh culture medium containing 0.1% of DMSO. After 24 h the cells were washed, trypsinized, stained by trypan blue (0.4%) and the number of viable and dead cells was scored. Hamster V79 cells treated with carvacrol or thymol were assayed also by the plating efficiency (PE) test. 500 V79 cells were plated on plastic Petri dishes (diameter = 6 cm) and incubated for 90 min. The medium was then removed and the cells were treated during further 24 h in a fresh DMEM containing thymol (100, 200, 300, 450 and 500 μM) or carvacrol (100, 150, 200, 250, 300 and 400 μM). The control cells were kept in a fresh DMEM medium with 0.1% DMSO. After the treatment, the cells were washed with DMEM and then cultivated for 7 days in a fresh DMEM to estimate both the plating efficiency and the total number of cells per 1 dish. The percentage of plating efficiency of cells was calculated after staining of colonies with methylene blue (1% solution in distilled water). The average number of cells per 1 colony was calculated by dividing the total number of cells/dish by the number of colonies grown in 1 dish.

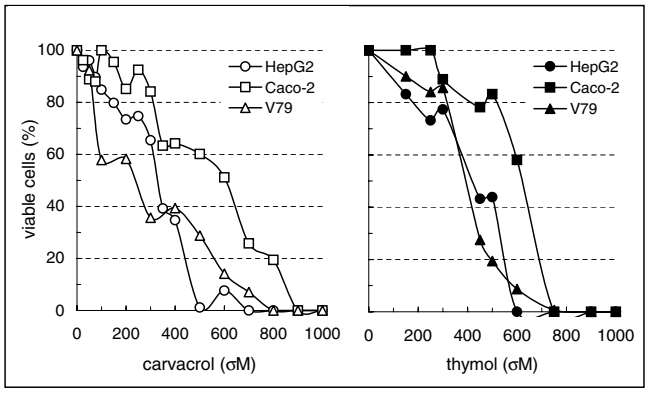

Figure 1. Effect of carvacrol (left part) and thymol (right part) on viability of HepG2 cells (circles), Caco-2 cells (squares) and V79 cells (triangles). Cells were treated with different concentrations of carvacrol or thymol for 24 h and after the treatment viability was measured by trypan blue exclusion assay. Results are mean of three independent experiments.

Single cell gel electrophoresis (SCGE; comet assay). The procedure of Singh et al. [15] was followed with minor modifications made by Slameňová et al. [16] and Gábelová et al. [6]. Briefly, the assayed cells were suspended in 0.75% LMP agarose and spread on a base layer (100 μl of 1% NMP agarose in Ca^{2+}- and Mg^{2+}- free PBS buffer) on a microscope slide. When the agarose was solidified, the slides were placed in lysis mixture for 1 h at 4°C to remove cellular proteins. The slides were then transferred to an electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH 13.0) and kept in this solution for 40 min unwinding time at 4°C. A current of 25 V (300 mA) was then applied for 30 min. The slides were removed, neutralized with Tris-HCl (0.4 M, pH 7.5) and stained with 20 μl ethidium bromide (EtBr, 5 μg/ml). EtBr stained nucleoids were evaluated with an Olympus fluorescence microscope. For each sample, 100 comets were scored by computerized image analysis (Komet 5.5, Kinetic Imaging, Liverpool, UK) for determination of DNA in the tail, which is linearly related to the frequency of single strand DNA breaks (ss DNA breaks).

Statistics. The results represent a mean of 3–5 experiments ± standard deviation (SD). The significance of differences between samples was evaluated by Student's *t*-test: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

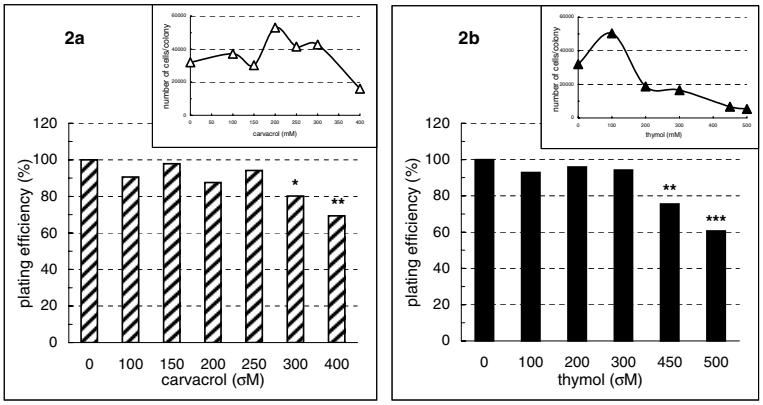

Figure 2a and 2b Effect of 24 h treatment with carvacrol or thymol on plating efficiency of V79 cells. Fig.2a and 2b inset – the influence of carvacrol or thymol on the number of cells per colony (size of colonies). Results are mean of two independent experiments.

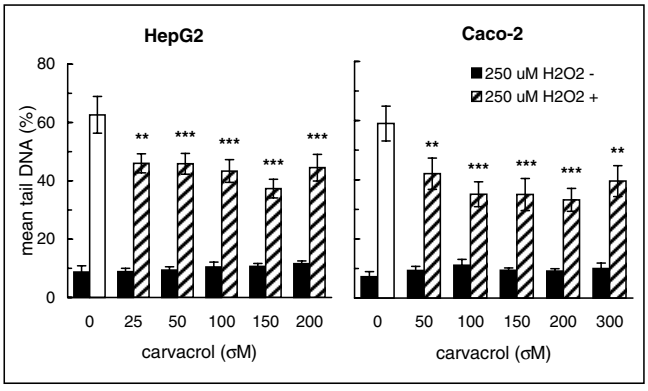

Figure 3. Level of DNA lesions induced in HepG2 or Caco-2 cells either by 250 μM H_2O_2 (open bars) or different concentrations of carvacrol (black bars). Stripped bars represent the level of DNA lesions in cells that were treated with H_2O_2 after 24 h pre-treatment with different concentration of carvacrol. Level of DNA lesions (DNA strand breaks) was measured by the single cell gel electrophoresis. Results are mean of three independent experiments ± SD. ** $p < 0.01$; *** $p < 0.001$ refers to differences between cells treated with H_2O_2 and cells pretreated with carvacrol and then exposed to H_2O_2.

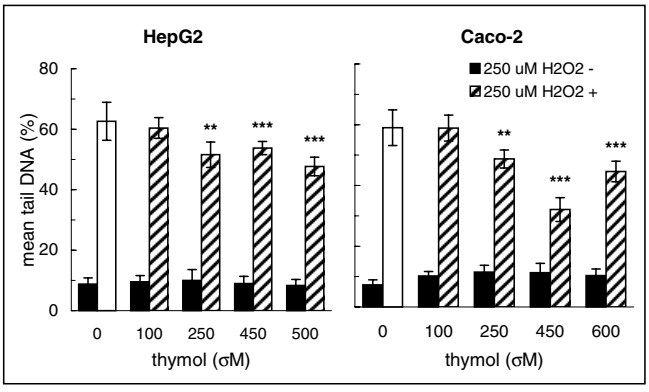

Figure 4. Level of DNA lesions induced in HepG2 or Caco-2 cells either by 250 μM H_2O_2 (open bars) or different concentrations of thymol (black bars). Stripped bars represent in both figures the level of DNA lesions in cells that were treated with H_2O_2 after 24 h pre-treatment with different concentration of thymol. Level of DNA lesions (DNA strand breaks) was measured by the single cell gel electrophoresis. Results are mean of three independent experiments ± SD. ** $p < 0.01$; *** $p < 0.001$ refers to differences between cells treated with H_2O_2 and cells pretreated with thymol and then exposed to H_2O_2.

Results

Cytotoxicity testing of carvacrol and thymol. Trypan blue exclusion assay showed that HepG2 and V79 cells reacted on 24 h treatment with carvacrol (25 – 1000 μM) more sensitively than did Caco-2 cells (Fig. 1 – left side). IC_{50} of carvacrol represented in V79 cells 250 μM, in HepG2 cells 350 μM and in Caco-2 cells 600 μM. A total loss of viability (IC_{100}) was observed in HepG2 cells at 500 μM, in V79 cells at 700 μM and in Caco-2 cells at 900 μM. Thymol (Fig. 1 – right side) was tested in the scale of concentrations 150 – 1000 μM. IC_{50} of thymol represents in both V79 and HepG2 cells approximately 400 μM, and in Caco-2 cells 700 μM. IC_{100} was in HepG2 cells observed at 600 μM, in V79 cells at 700 μM and in Caco-2 cells at 800 μM. The cytotoxic effects of both carvacrol and thymol increased gradually in parallel with increasing concentrations. Fig. 2a and 2b represent plating efficiency of V79 cells treated during 24 h with 100 – 500 μM carvacrol or thymol. The reduction in plating efficiency should represent reduction in proliferation capacity of treated cells. Though the decrease of PE was evident in V79 cells influenced with higher concentrations of both carvacrol and thymol, the excise IC_{50} values cannot be determined as in parallel with the decrease of the number of colonies decreased also the size of colonies, i.e. the number of cells/colony (Fig. 2a and 2b, inset).

Induction of ss DNA breaks and DNA–protective effects of carvacrol and thymol. Measuring of single strand DNA breaks

induction in human cells incubated for 24 h with different concentrations of carvacrol or thymol (Fig. 3 and Fig. 4 – black bars) was secured by single cell gel electrophoresis, which represents a sensitive method for measuring DNA damage at the level of single cells. At concentrations = $IC_{20–40}$, the studied compounds did not induce any DNA strand breaks. Selected oxidative agent hydrogen peroxide (H_2O_2; 250 μM), which belongs to the group of strong oxidants, induced in both cell types a significant increase of DNA strand breaks (Fig. 3 and Fig. 4 – open bars). The comet assay was used also for evaluation of protective effects of carvacrol and thymol against DNA-damaging effects of H_2O_2 (Fig. 3 and Fig. 4 – striped bars). Obtained results showed that the level of H_2O_2-induced DNA strand breaks was in human cells pre-treated with carvacrol or thymol significantly reduced.

Discussion

Carvacrol and thymol belong to very frequently occurring phenolic components of essential plant oils often used e.g. in clinical dentistry as sedatives for the dental pulp, as disinfectants for caries, and as root canal medications. Chang et al. [9] investigated the pathobiological effects of various phenolic compounds (guaiacol, phenol, eugenol, and thymol) on human dental pulp fibroblasts with Hoechst 33258 fluorescence assay and DNA precipitation assay. They found that phenolic compounds-treated human dental pulp fibroblasts expressed cytotoxic changes and inhibited cellular DNA values in a concentration-dependent manner, but they did not cause DNA single-strand breaks.

We studied the effects of carvacrol and thymol on three histopathologically different types of mammalian cells, namely human hepatoma cells HepG2, human colonic cells Caco-2 and hamster lung cells V79. HepG2 cells are considered to posses a great spectrum of metabolizing enzymes involved in activation/detoxification process [20] and to reflect the *in vivo* metabolism more than incompetent cells, e.g. hamster lung cells V79, which do not contain any monooxygenase activity [21] and require the exogenous addition of metabolizing enzymes. Caco-2 cell line, isolated from a human colon carcinoma retained in *in vitro* conditions the phase I enzyme CYP1A1 and of the phase II enzymes glutathione-S-transferases, glucuronidase, and sulfotransferases [3, 7]. Metabolic activity of mammalian cells could be increased during incubation with carvacrol and thymol, as these phenolic compounds belong to bifunctional inducers, i.e. substances capable of inducing phase I and phase II enzymes [13]. Our results presented in Fig. 1 and Fig 2 showed that the sensitivity to carvacrol and thymol was approximately equal in human hepatoma cells HepG2 and hamster lung cells V79 while human colonic cells Caco-2 were more resistant to cytotoxic effects of these compounds. Similar cytotoxicity of carvacrol and thymol against cells which are not able to synthesize metabolizing enzymes at all (V79) and cells which synthesize a high level of metabolizing enzymes (HepG2) suggests that cytotoxic effects of these compounds are not connected with their biotransformation. To the best of our knowledge, metabolic activation of carvacrol and thymol *in vitro* has been described till now only in cultured plant cells of *Eucalyptus perriniana* [14].

In hamster V79 cells, characterized by a high colony-forming ability (PE), we measured also a loss of proliferation after carvacrol or thymol treatment (Fig. 2). As it is evident from this figure, loss of proliferation corresponds only roughly with results of the trypan blue exclusion technique (Fig. 1) and suggests that the significant uptake of the vital dye do not need to be an unequivocal indicator of membrane lysis and of irrevocable cell death. It seems that some cells that took up the vital dye did not loss completely their proliferative activity. On the other side, the decrease in the number of colonies (Fig. 2) was accompanied by the decrease of the cell number/colony, (Fig. 2 inset), so it is not possible to evaluate the IC values so exactly as in the trypan blue exclusion assay.

To evaluate DNA-damaging effects of carvacrol and thymol, as well as hydrogen peroxide (H_2O_2), which was in our experiments applied as a positive control, we used the comet assay. Fig. 3 and Fig 4 show that at concentrations = $IC_{20–40}$ neither carvacrol nor thymol induced DNA strand breaks in human hepatoma or colonic cells. These data indicate that the phenolic compounds studied represent cytotoxic agents that do not have any genotoxic effects on the human cells studied. On the other hand, Aydin et al. [1] found by the same technique a mild increase of DNA lesion in human lymphocytes treated with carvacrol or thymol (100 – 200 μM). These discrepancies could be explained by finding of Moteki et al. [11] who described a specific induction of apoptosis by the phenolic compound 1,8-cineole in human leukemia Molt 4B and HL-60 cells but not in human stomach cancer KATO III cells.

Collins and Horváthová [4] stated that oxidation of mammalian DNA is a useful marker of oxidative stress, and this can be decreased by supplementation with pure antioxidants or with foods rich in antioxidants. As both components of essential oils studied are considered to be antioxidants [10], they can be expected to exert DNA-protective antimutagenic effects. Recently some investigations were carried out to study antimutagenic effects of thyme and its major ingredients in human lymphocytes [1, 2]. The authors showed that a short-term treatment of human lymphocytes with low concentrations of phenolic compounds protect DNA against some genotoxins [(2-amino-3-methylimidazo[4,5-f]-quinoline (IQ), mitomycin C (MMC), and hydrogen peroxide (H_2O_2)]. We evaluated the potential preventive effect of a long-term (24 h) pre-incubation of human cells with carvacrol and thymol against DNA strand breaks induced by H_2O_2. Instead of human lymphocytes we used two different types of human cells exhibiting different levels of enzyme activities, namely human hepatoma cells HepG2 and human colonic cells Caco-2. Our results presented in Fig. 3 and Fig. 4 showed that after 24 h pre-incubation of HepG2 or Caco-2 cells with non-toxic concentrations of carvacrol or thymol, the cells expressed a significantly higher resis-

tance to the DNA-damaging effect of hydrogen peroxide than did the cells which were not pre-incubated. We conclude that the protective efficiency of carvacrol and thymol against DNA lesions induced by a strong oxidant (hydrogen peroxide) confirm their antioxidant properties.

To sum up, the mild cytotoxic effect of carvacrol and thymol ($IC_{20–40}$) on human hepatoma or colonic cells had no DNA-damaging effect but reduced the level of DNA lesions induced in these cells by an oxidative agent and thus acted as antioxidants. Our data as well as findings published earlier [8, 5] indicate that components of some essential oils can be considered as potentially active antimutagenic drugs.

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