Comparative study of DNA-damaging and DNA-protective effects of selected components of essential plant oils in human leukemic cells K562

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Eucalyptol, carvacrol and thymol represent components of plant essential oils characterized by a wide range of biological effects toward microorganisms, fungi, insects, etc. However, till now only a few investigations have been carried out to study the effects of essential oils and their components on human cells cultured *in vitro*. The aim of our work was therefore to compare cytotoxic and DNA-damaging effects of eucalyptol, carvacrol and thymol on human leukemic K562 cells cultured *in vitro* and to investigate their possible protective (antioxidant) effects against hydrogen peroxide-induced DNA damage. Testing of cytotoxic activity was performed by the trypan blue exclusion technique. The amount of DNA lesions in K562 cells treated with the plant volatiles studied or their combinations with hydrogen peroxide (H_2O_2) was measured by alkaline single cell gel electrophoresis (SCGE; comet assay). We found out that eucalyptol, carvacrol and thymol differed in their cytotoxic and genotoxic effects on K562 cells. As a very important we consider the finding that carvacrol and thymol significantly reduced the level of DNA damage induced in K562 cells by the strong oxidant H_2O_2. Neither DNA-damaging nor DNA-protective effect was observed using eucalyptol pre-treatment of K562 cells. We assume that DNA-protective effects of carvacrol and thymol can be accompanied by their antioxidant action.

Keywords: eucalyptol; carvacrol; thymol; antioxidant activity; comet assay; hydrogen peroxide

Herbs have been used as food and for medicinal purposes for centuries. Research interest has been focused on various herbs and herbal extracts that possess antioxidant, hypolipidemic, antiplatelet, antitumor, or immune-stimulating properties that may be useful adjuncts in helping reduce the risk of cancer and cardiovascular diseases. In different herbs, a wide variety of active phytochemicals, including terpenoids have been identified. Monoterpenes are highly hydrophobic substances present in plant essential oils. They function physiologically as chemoattractants or chemorepelents and exert a wide spectrum of biological actions of great importance in many different areas from food chemistry and chemical ecology to pharmacology and pharmaceutics [1, 2, 3, 4].

1,8-Cineole (1,3,3-trimethyl-2-oxabicyclo[2,2,2]octane), a monoterpene cyclic ether which is known as eucalyptol (Fig. 1A), is widely occuring in plants and found in essential oils like *Eucalyptus polybractea*. It is extensively used for external application in pharmaceutical preparations, e.g. in nasal spray, as disinfectant, in dental practice, in cosmetics, for cough treatment, muscular pain, neurosis, rheumatism, asthma and urinary stones [5, 6, 7]. The existing data suggest that 1,8-cineole is a substrate for CYP3A enzymes in rat and human liver microsomes [8]. Human metabolism of 1,8-cineole investigated *in vitro* and *in vivo* showed two metabolites 2α-hydroxy-1,8-cineole and 3α-hydroxy-1,8-cineole formed by human microsomes [9, 10]. Carvacrol (2-methyl-5-(1-methylethyl)phenol; Fig. 1B), a cyclic monoterpene, is a constituent of the ethereal oil of oregano or thyme, oil obtained from pepperwort and wild bergamot and reported to be found in several essential oils. The mode of action of carvacrol appears to have received considerable attention from researchers because of its use as a flavour, also as an antibacterial or antifungal agent and in food preservation methods [11, 12]. Thymol (5-methyl-2-(1-methylethyl)phenol; Fig. 1C) is an isomer of carvacrol, having the hydroxyl group at a different location on the phenolic ring. Hydrophobic nature of carvacrol and thymol enables them to react with the lipids of

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the cell membrane and mitochondria, rendering them permeable and leading to leakage of cell contents [13, 14]. Thymol is used as a food preservative, antiseptic, local anesthetic and cooling agent acting as a local irritant and anesthetic to the skin and mucous membrane. It is used in liniments, lip balms, toothpaste and mouthwash. Thymol did not increase the levels of chromosome aberrations in the system of Syrian hamster embryo cells unlike other substances used for the same purpose [15]. Both thymol and carvacrol can be bound to major and minor grooves of B-DNA. Spectroscopic evidence showed that thymol and carvacrol interaction occurred mainly through H-bonding of the thymol and carvacrol OH group to guanine N7, cytosine N3 and backbone phosphate group in DNA [16].

Because all three compounds have been shown to possess various protective activities in different biological systems, we tried to prove their ability to protect human cells cultured *in vitro* against DNA damage induced by oxidative agent H_2O_2. As a biological material we used rapidly proliferating human myelogenous leukemia cell line K562 which manifest a relatively high expression of xenobiotic metabolizing enzymes and is often used in molecular and genotoxicological studies.

Material and methods

Cell line. The human myelogenous leukemia cell line K562 were purchased from the American Type Culture Collection (Manassas, VA, USA). K562 cells were kept in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS), penicillin and streptomycin (100 mg/ml). Cells were incubated at 37°C in a humidified atmosphere containing 5% of CO_2. All experiments were performed during the exponential phase of cell growth.

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: eucalyptol (purum ≥ 98%; density = 0.924 g/ml; Mw = 154.25), carvacrol (purum ≥ 97%; density = 0.974 g/ml; Mw = 150.22) and thymol (purum ≥ 99%; density = 0.965 g/ml; Mw = 150.22). Volatiles were kept at room temperature and diluted to the concentrations 50 – 5000 μM either in complete RPMI 1640 medium (eucalyptol and carvacrol) or in dimethyl sulfoxide (500 mM) and then diluted in complete RPMI 1640 medium (thymol) immediately before use.

Chemicals were purchased from the following sources: RPMI 1640 medium, penicillin, streptomycin, fetal calf serum (FCS), trypan blue (0.4%), LMP (low melting point) agarose from GIBCO BRL, Paisley, UK; dimethyl sulfoxide (DMSO), NMP (normal melting point) agarose, ethidium bromide (EtBr), Triton X-100, β-NADP, glucose-6-phosphate from Sigma-Aldrich Co., Steinheim, Germany; phosphate-buffered saline (PBS, Ca^{2+}- and Mg^{2+}-free) from OXOID LIMITED, Basingstoke, UK. Other chemicals were of analytical grade from commercial suppliers.

S9 fraction. For the preparation of S9 fraction, the method described by Kuroki et al. [17] and Carver et al. [18] was employed. As co-factors for the S9 fraction 0.63 mg/ml β-NADP and 1.3 mg/ml glucose-6-phosphate were used.

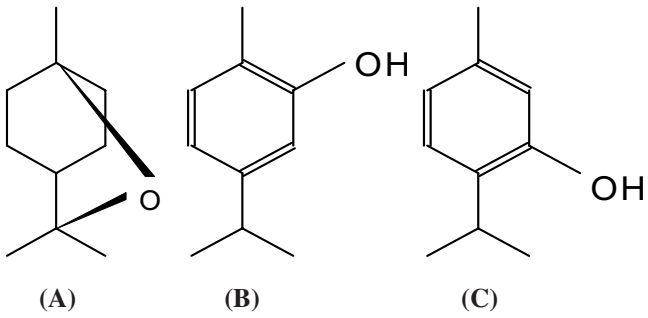

Figure 1. Structures of eucalyptol (A), carvacrol (B) and thymol (C)

Cytotoxicity assay (trypan blue exclusion). K562 cells were incubated with different concentrations of eucalyptol, carvacrol or thymol for 2 (with or without S9 fraction), 24 or 48 h at 37°C in a dark incubator together with control samples. Control cells in the sets of experiments with thymol were fed with culture medium containing 0.5% of DMSO. Samples were then centrifuged at 1000 rpm for 5 min at room temperature. After the treatment, cells were washed with PBS (Ca^{2+}- and Mg^{2+}-free) and again centrifuged at 1000 rpm for 5 min at room temperature. Cells were stained with trypan blue (0.4%) and the number of viable cells (%) was determined.

Single cell gel electrophoresis (SCGE; comet assay). The procedure of Singh et al. [19] was used with minor modifications suggested by Slameňová et al. [20] and Gábelová et al. [21]. Briefly, a base layer of 100 μl of 1.0% NMP agarose in PBS (Ca^{2+}- and Mg^{2+}-free) was placed on microscope slides. The tested cells suspended in 0.75% LMP agarose in PBS (Ca^{2+}- and Mg^{2+}-free) were spread on the base layer on each of six slides. A cover slip was added and the agarose was allowed to solidify. The cover slips were removed, slides were treated with 50 or 250 μM H_2O_2 for 5 min on ice in the dark and they were washed twice with PBS (Ca^{2+}- and Mg^{2+}-free). After the treatment the slides were placed in lysis solution (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris, 1% Triton X-100, pH 10.0) for 1 h at 4°C to remove cellular proteins. The slides were then transferred to an electrophoresis box containing an alkaline solution (300 mM NaOH, 1 mM Na_2EDTA). They were 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, Kineting Imaging, Liverpool, UK) for determination of % DNA in the tail which is linearly related to the frequency of single strand (ss) DNA breaks.

Data analysis. For the trypan blue exclusion technique mean ± standard deviation (SD) was calculated (three deter-

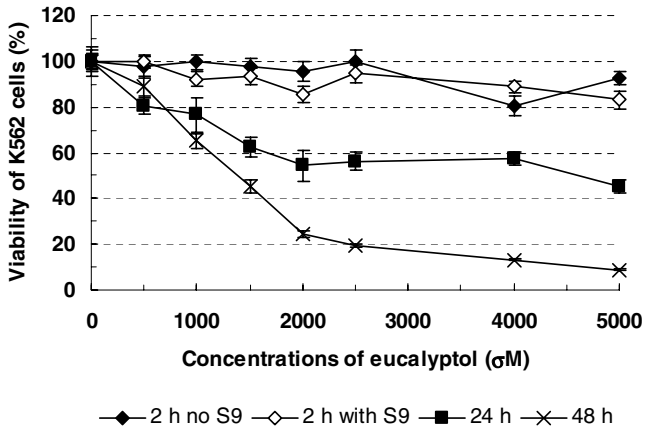

Figure 2. Percentage of viable K562 cells after 2 (with or without S9 fraction), 24 and 48 h treatment with eucalyptol evaluated by trypan blue exclusion technique. Data are means of three determinations with two replicate samples ± SD.

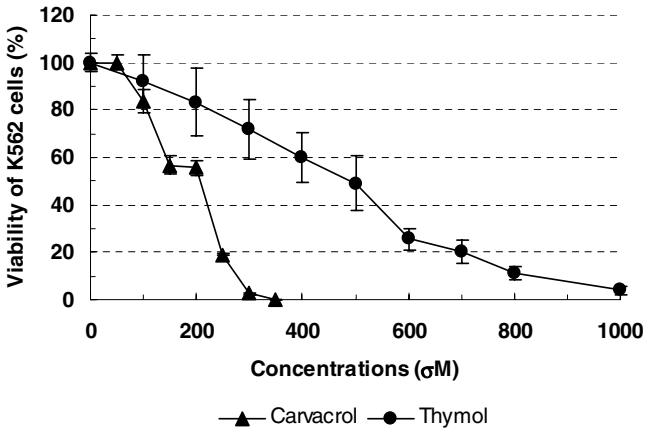

Figure 3. Percentage of viable K562 cells after 24 h treatment with carvacrol or thymol evaluated by trypan blue exclusion technique. Data are means of three determinations with two replicate samples ± SD.

minations with two replicate samples). The values of the single cell gel electrophoresis were in this study expressed as mean ± SD from three independent experiments with three replicate samples. The significance of differences between samples was evaluated by Student's *t*-test – statistically different from controls: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$ or from values for hydrogen peroxide: $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Results

Cytotoxicity of essential oil components. Cytotoxicity of the compounds studied against K562 cells was evaluated by trypan blue exclusion technique after 2 (with or without S9 fraction), 24 or 48 h treatment of cells with the given compounds. The concentrations of the compounds studied were consecutively selected for the comet assay according to the cytotoxicity of cells appointed by trypan blue exclusion technique after 24 h of treatment. The viability of K562 cells was not lower than approx. 50%. The results, depicted in Fig. 2, showed that sensitivity to toxic effects of eucalyptol was relatively low in K562 cells. 2h treatment of K562 cells with eucalyptol in the presence of S9 fraction led to no effect on their viability. The highest cytotoxic effect was observed after 48 h of treatment. K562 cells were much more sensitive to the toxic effects of carvacrol and thymol, where IC_{50} was approx. 150 – 200 μM (carvacrol) and 400 – 500 μM (thymol), respectively (Fig. 3).

Induction of DNA strand breaks in K562 cells treated with eucalyptol, carvacrol or thymol and effects of these compounds on the level of DNA damage induced by H_2O_2. We measured both the level of single strand (ss) DNA breaks in K562 cells treated for 24 h with the volatiles selected as well as the level of DNA damage in cells pre-incubated with these compounds and then treated with H_2O_2. The concentrations of H_2O_2 in our experiments were 50 or 250 μM and the damage induced by H_2O_2 was regarded as positive controls.

Fig. 4 represents the level of ss DNA breaks in cells incubated for 24 h with eucalyptol (black bars) and the combination of 24 h pre-treatment of cells with eucalyptol with a short-term (5 min) treatment with 250 μM H_2O_2 (white bars). The concentration range of eucalyptol was 2000 – 5000 μM. Eucalyptol did not induce any DNA strand breaks and had no effect on the level of DNA damage induced by H_2O_2.

Fig. 5 shows the levels of carvacrol-induced ss DNA breaks (black bars) and H_2O_2-induced DNA strand breaks in K562 cells pre-incubated with different concentrations of carvacrol (stripped and white bars). 24 h pre-treatment with carvacrol slightly but significantly increased the level of DNA damage compared to negative control. In comparison with positive control a significant decrease of DNA lesion levels induced with 50 or 250 μM H_2O_2 by 10.50 – 16.47% ($*p < 0.05$ – $***p < 0.001$) can be observed in samples pre-treated with 150 and 200 μM of carvacrol.

Fig. 6 presents both the level of strand breaks induced by thymol (black bars) and the protective activity of 24 h pre-treatment of K562 cells with thymol in combination with a short-term treatment of cells with 50 or 250 μM H_2O_2 (stripped and white bars). 24 h pre-treatment with thymol did not induced any ss DNA breaks but significantly decreased the level of H_2O_2-induced DNA lesions starting from the lowest concentration of thymol applied (100 μM). The percentage of thymol protective effect was 5.12 – 19.22% ($*p < 0.05$ – $***p < 0.001$) at the concentration range of 100 – 400 μM.

Discussion

Many components of essential volatile oils serve e.g. as a flavouring, aroma, natural replacement of synthetic food ad-

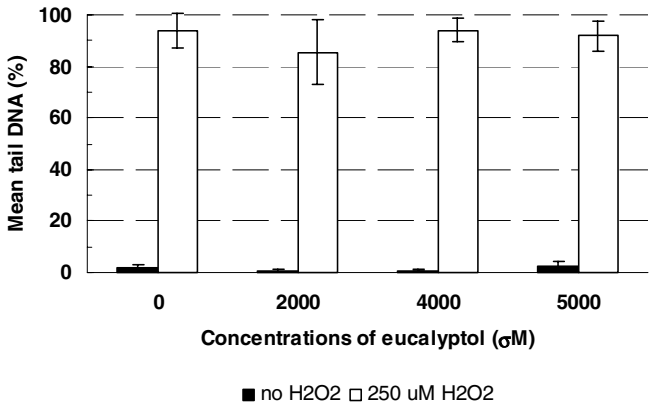

Figure 4. Incidence of DNA damage in K562 cells treated with eucalyptol for 24 h, with 250 μM H_2O_2 for 5 min or pre-treated with eucalyptol and then treated with H_2O_2. Data represents three independent experiments with three replicate samples ± SD. Statistically different from untreated control: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$ or from value for hydrogen peroxide: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

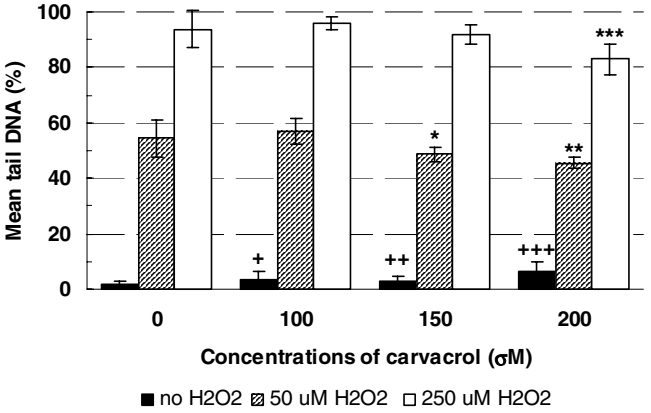

Figure 5. Incidence of DNA damage in K562 cells treated with carvacrol for 24 h, with 50 or 250 μM H_2O_2 for 5 min or pre-treated with carvacrol and then treated with H_2O_2. Data represents three independent experiments with three replicate samples ± SD. Statistically different from untreated control: $^{+}p < 0.05$; $^{++}p < 0.01$; $^{+++}p < 0.001$ or from values for hydrogen peroxide: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

ditives, or may have a potential in clinical medicine. In the last couple of decades there is an increasing emphasis for reliable evaluation of such compounds for their anti-/pro-oxidant and toxic properties as their plant origin does not secure the safety for living beings, including humans. Many investigators have been working on the explanation of mechanisms of terpenoid actions. It has been recognized that many components of essential oils have antioxidant properties [22, 23, 24] and reduce the uncontrolled production of ROS which contributes to the pathogenesis of cancer and cardiovascular diseases [25]. H_2O_2, a well-defined and characterized oxidative agent, does not react directly with DNA. Biological membrane-crossing H_2O_2 is thought to penetrates into the cell nucleus and react with ions of iron or copper to form $^{\bullet}OH$ radicals. Attack of $^{\bullet}OH$ radicals on DNA results in breaks of nucleotide sugar moieties and a terminal sugar residue fragment [26, 27]. Direct rejoining repairs these single strand breaks of DNA very quickly in cultured cells [28]. In addition, $^{\bullet}OH$ radicals attack DNA bases and produce thymine glycol, 8-hydroxyguanine or 2,6-diamino-4-hydroxy-5-formamidopyrimidine. Most of these oxidative DNA lesions are repaired by base excision repair. However, some forms of oxidative damage of bases are repaired by systems that apparently recognize elements of DNA helix distortion rather than specific base damage [29].

The aim of our work was to compare cytotoxic, DNA-damaging, and mainly DNA-protective (antioxidant) effects of three components of essential oils: eucalyptol, carvacrol and thymol towards DNA damage induced by H_2O_2 in human leukemic K562 cells. K562 cell line was chosen for its extensive application in the fields of cancer and genotoxicology research and for its relatively high expression of cytochrome P450 enzymes, namely CYP1A1, CYP1B1, CYP2A6, CYP2A7, CYP2D6, CYP2E1, CYP3A4 and CYP3A5 [30] and detoxification enzymes, e.g. glutathione S-transferases [31]. Expressions of aryl hydrocarbon (Ah) receptor and Ah receptor nuclear translocator, which mediate induction of the CYP1 family, were also detected in these cells [30]. The results presented in Fig. 2 showed that sensitivity of K562 cells toward the toxic effects of eucalyptol was relatively low despite of CYP3A4 and CYP3A5 enzymes content needed for metabolism of eucalyptol [8]. Even addition of external S9 activation fraction from rat livers during 2 h treatment of cells with eucalyptol does not affect the viability of K562 cells. Long-term, 24 and 48 h, incubations of K562 cells with eucalyptol led to an increased cytotoxicity. IC_{50} concentration after 24 h treatment of K562 cells with eucalyptol was approx. 5000 μM. We suggest that this relatively low cytotoxicity of eucalyptol on the cells studied could be connected with its lower penetration into cells. K562 cell line was much more sensitive to the toxic effects of 24 h treatment with carvacrol or thymol (Fig. 3).

At concentrations $\leq IC_{50}$, only carvacrol slightly but statistically significantly increased the level of DNA damage in human leukemic K562 cells in comparison to negative control samples (Fig. 5). No DNA damage formation was observed in sets of experiments with different concentrations of eucalyptol or thymol $\leq IC_{50}$ (Figs. 4 and 6). Similar results obtained Ribeiro and co-workers who reported no increase in the level of DNA damage induced by 3 h eucalyptol-treatment neither in mouse lymphoma cells [32] nor in Chinese hamster ovary cells [33]. Our observations are also in good agreement with the results of Fabian et al. [34] who described a slight carvacrol-induced increase in incidence of apoptotic cell death connected with DNA breaks in the system of human colon carcinoma Caco-2 cells. Similar results on flavonoids com-

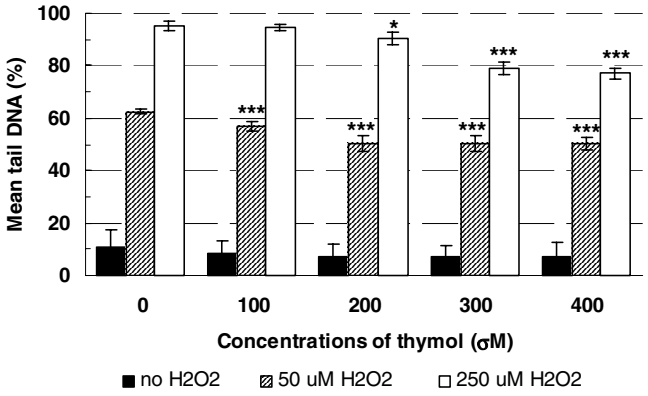

Figure 6. Incidence of DNA damage in K562 cells treated with thymol for 24 h, with 50 or 250 μM H_2O_2 for 5 min or pre-treated with thymol and then treated with H_2O_2. Data represents three independent experiments with three replicate samples ± SD. Statistically different from control treated with 0.5% DMSO: $^{+}$ $p < 0.05$; $^{++}$ $p < 0.01$; $^{+++}$ $p < 0.001$ or from values for hydrogen peroxide: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

monly present in plant foods were observed by Collins [35] who reported that quercetin induced DNA damage in human peripheral blood lymphocytes when present alone, though the effect was significant only at concentrations above those that would be present in the body. But when administered in combination with H_2O_2, quercetin significantly decreased the amount of damage done. Specific induction of apoptosis by 1,8-cineole was revealed in human leukemic Molt 4B and HL-60 cell lines but not in human stomach cancer KATO III cells [36]. In our previous studies we reported no increase in the level of DNA damage either in human hepatoma cell line HepG2 or in colon carcinoma Caco-2 cells treated with carvacrol or thymol at concentrations ≤ IC_{50} [37, 38]. Grouping together these results suggest that the induction of DNA lesions and apoptosis by selected plant volatiles is cell line specific.

DNA protective effects of carvacrol and thymol after a short-term pre-treatment of human lymphocytes were recently described by Aydin et al. [23, 39] and after a long-term pre-treatment of human hepatoma HepG2 cells and human colonic Caco-2 cells by Horváthová et al. [37] and Slameňová et al. [38]. The results presented in Figs. 5 and 6 showed that after 24 h pre-treatment of human leukemic K562 cells with concentrations ≤ IC_{50} of carvacrol or thymol, K562 cells manifested a significantly higher resistance to the DNA-damaging effect of H_2O_2 than did the cells which were not pre-treated. Comparison of the percentage extent of DNA-protective activity of carvacrol and thymol determined by the SCGE was similar. We conclude that the protective efficiency of carvacrol and thymol against DNA lesions, induced by the strong oxidant hydrogen peroxide, testify to their antioxidant properties.

In conclusion, eucalyptol had no effect on the level of DNA damage induced by H_2O_2-treatment, whereas carvacrol and thymol were characterized by similar degree of DNA-protective activities against H_2O_2-induced DNA damage in K562 cells. Carvacrol and thymol in concentrations ≤ IC_{50} could contribute to the protection of organisms against oxidative agents in the environment, but further investigations to explore even the mechanisms of action of these components of essential plant oils may prove to be worthwhile.

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