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Modulation of genotoxicity and DNA repair by plant monoterpenes camphor, eucalyptol and thujone in *Escherichia coli* and mammalian cells

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ABSTRACT

The aim of this work was to examine the antigenotoxic potential of plant monoterpenes: camphor, eucalyptol and thujone in prokaryotic and eukaryotic cells and to elucidate their effect on DNA repair. We compared the effect of monoterpenes on spontaneous, UV- and 4NQO-induced mutagenesis in *Escherichia coli* K12 repair proficient, and MMR and NER deficient strains. Positive controls tannic acid and vanillin were included in bacterial tests. We also examined protective effect of monoterpenes against 4NQO-induced genotoxicity in Vero cell line by alkaline comet assay. The results obtained in repair proficient strain indicated antimutagenic potential of monoterpenes against UV- and 4NQO-induced mutagenesis, which was diminished with NER deficiency. Camphor and eucalyptol maintained UV-induced SOS response longer than in controls, while thujone decreased SOS response and reduced general protein synthesis and the growth rate. The three monoterpenes increased spontaneous and UV-induced recombination in *recA730* and camphor additionally in *recA*$^+$ cells. Incubation of 4NQO-pretreated Vero cells with monoterpenes resulted in significant reduction of tail moment. However, higher concentrations of monoterpenes induced DNA strand breaks. Obtained results indicate that by making a small amount of DNA lesions camphor, eucalyptol and thujone can stimulate error-free DNA repair processes and act as bioantimutagens.

1. Introduction

Terpenes $(C_5H_8)_n$ are the largest group of natural substances, biosynthetically derived from isoprene units (Wang et al., 2005). They are abundantly found in fruits, vegetables and aromatic and medicinal plants, where their main function is protection against infections, parasites and other stress conditions (Bakkali et al., 2008; Gershenzon and Dudareva, 2007). Moreover, they are endowed with many beneficial health effects and can be used to treat different health disorders (Paduch et al., 2007). It has been shown that terpenes are important cancer chemopreventive and chemotherapeutic agents (Crowell, 1999; Lampe, 2003). Numerous studies indicate protective capacity of terpenes against endogenous sources of DNA lesions, as well as against environmental genotoxic agents, indicating their possible use in primary prevention of many mutation related diseases (De Flora and Ferguson, 2005; Kaefer and Milner, 2008).

The monoterpenes investigated in this study: camphor (Cam), eucalyptol (Euc), and thujone (Thu) are widely distributed in essential oils of many medicinal and aromatic plants. They possess strong antimicrobial (Cermelli et al., 2008; Lee et al., 2011; Mahbourbi and Farzin, 2009; Mitić-Ćulafić et al., 2005; Soković et al., 2010), and Euc also cytotoxic, anti-inflamatory, analgesic, antiexudant, gastroprotective and hepatoprotective properties (Assanova et al., 2003; Moteki et al., 2002; Pattnaick et al., 1997; Santos et al., 2004). Cam is commonly applied to the skin for its antipruritic, analgesic and counterirritant properties (Burkhart and Burkhart, 2003) and used as a nasal decongestant and cough suppressant (Burrow et al., 1983). Euc is commonly used as a bronchodilator, to treat bronchitis and asthma, as well as for the treatment of sinusitis and chronic rhinitis (Juergens et al., 1998). Although the neurotoxic effect of Thu in mammals is well established (Höld et al., 2000; Rietjens et al., 2005), reported data indicate that essential oils containing Thu, i.e. *Artemisia*, *Salvia*, *Thuja* spp. can be used for medical purposes (Mayer et al., 2009; Mueller et al., 2010; Naser et al., 2005; Presti et al., 2007).

In our previous work (Vuković-Gačić et al., 2006a) we have demonstrated strong antimutagenic effect of Cam, Euc and Thu against UV-induced mutagenesis in *Escherichia coli* and *Saccharomyces cerevisiae* cells, and hypothesized that they act as bioantimutagens, the agents which modulate DNA replication and repair and

Abbreviations: Cam, camphor; Euc, eucalyptol; 4NQO, 4-nitroquinoline-1-oxide; Tan, tannic acid; Thu, thujone; Van, vannilin.

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prevent processing of premutagenic lesions into mutations (Kada et al., 1986). The aim of this work was to further evaluate antimutagenic potential of Cam, Euc and Thu and to elucidate their effect on DNA repair processes. We first determined and compared the effect of monoterpenes on spontaneous, UV- and 4-nitroquinoline-1-oxide (4NQO)-induced mutagenesis in *E. coli* K12 repair proficient strain and mutants deficient in mismatch (MMR) or nucleotide excision (NER) repair. We then examined the capacity of monoterpenes to induce and modulate SOS response and recombination. The effect on SOS induction was monitored in repair proficient and NER deficient strains, both with *lacZ* gene put under control of SOS gene *sfiA* (Vuković-Gačić et al., 2006b). The effect on recombination was elucidated in *recA*$^+$ strain and *recA730* mutant, constitutive for SOS response (Simić et al., 1998). Tannic acid (Tan) and vanillin (Van) were used as positive controls, since their protective effect against UV- and 4NQO-induced mutagenesis has already been established, and the molecular mechanisms of their bioantimutagenic action have been proposed (Shimoi et al., 1985; Ohta et al., 1986, 1988; Shaughnessy et al., 2006). We also examined protective effect of monoterpenes in mammalian cells *in vitro*. We used the alkaline comet assay in Vero cell line to monitor the effect of Cam, Euc and Thu on the repair of DNA damage induced by 4NQO.

2. Materials and methods

2.1. Chemicals

D,L-Camphor (Cas No. 76-22-2, Alfa Aesar), eucalyptol (Cas No. 207–431-5, Fluka), α,β-thujone (Cas No. 76231-76-0, Sigma–Aldrich), tannic acid (Cas No. 1401-55-4, Sigma–Aldrich) and vanillin (Cas No. 121-33-5, Fluka) were freshly dissolved in DMSO for bacterial assay, or in ethanol for comet assay. For antimutagenicity assay, 4NQO (Cas No. N-8141, Sigma–Aldrich) was dissolved first in DMSO and then tenfold diluted in distilled water immediately before use. For comet assay, 4NQO was dissolved in DMSO and then diluted in PBS buffer (PAA Laboratories GmbH, Austria) immediately before use.

2.2. Bacterial and eukaryotic cell cultures

Bacterial strains used in this study are listed in Table 1. The *uvrA*::Tn*10* mutation was introduced into strain IB111 by P1 transduction to give IB127 strain. The Vero cell line obtained from the kidney of a normal adult African green monkey (ECACC No: 88020401) was a gift from the Institute of Virology, Vaccines and Sera – Torlak, Belgrade, Serbia.

2.3. Media and growth conditions

Bacteria were grown in LB medium (5 g yeast extract, 10 g Bacto tryptone, 5 g NaCl, 3 ml 1 M NaOH, 1000 ml distilled water) at 37 °C. Semi-enriched minimal medium (minimal medium supplemented with 3% (v/v) nutrient broth) – SEM (Witkin, 1976) and top agar (5 g NaCl, 6 g agar, 1000 ml distilled water) were used for *E. coli* K12 reversion assay. The formation of Lac$^+$ papillae in recombination assay was monitored on MacConkey lactose plates (Konrad, 1977). The Vero cells were grown in MEM medium (PAA Laboratories GmbH, Austria) supplemented with 10% fetal bovine serum (PAA Laboratories GmbH, Austria), 2 mM L-glutamine, penicillin G (100 U/ml), streptomycin (100 μg/ml) and amphotericin B (25 μg/ml) (Sigma–Aldrich), at 37 °C, 5% CO_2 and 100% humidity.

2.4. UV-irradiation conditions

UV-irradiation was carried out with a germicidal lamp (Benda, NU-8 KL) having maximum output at 254 nm. Dose rates were measured with the Latarjet dosimeter. Cell suspensions in 0.01 M $MgSO_4$ were irradiated in glass Petri dishes at a thickness of less than 1 mm (Simić et al., 1985). UV-treated cell suspensions were kept in dark to prevent photoreactivation.

2.5. E. coli K12 reversion assay

For detection of spontaneous mutagenesis, the overnight cultures of SY252, IB103 and IB105 strains were washed by centrifugation at 1700g and resuspended in the same volume of 0.01 M $MgSO_4$. Samples (0.1 ml) of cell suspension, appropriately diluted for determination of cell survival and Arg$^+$ revertants, were added to 3 ml of molten top agar, mixed and poured in triplicates onto 3% SEM plates containing different concentrations of test substance and incubated at 37 °C for 48 h.

Table 1
E. coli K12 strains.

Strain	Relevant marker	Reference
SY252	*argE3*	Berić et al. (2008)
IB103	as SY252 *mutS*::Tn*10*	Berić et al. (2008)
IB105	as SY252 *uvrA*::Tn*10*	Stanojević et al. (2008)
IB111	as SY252 [λp(*sfiA*::*lacZ*)c*Iind1*] PHO^c	Stanojević et al. (2008)
IB127	as IB111 *uvrA*::Tn*10*	This work
GY7066	*lacMS286* Φ80dII*lacBK1*Δ*recA306* *srl*::Tn*10*	Dutreix et al. (1989)
GY8281	as GY7066/mini*FrecA*$^+$	Dutreix et al. (1989)
GY8252	as GY7066/mini*FrecA730*	Dutreix et al. (1989)

For detection of UV-induced mutagenesis, the overnight cultures of SY252 and IB105 strains were washed by centrifugation at 1700g, resuspended in the same volume of 0.01 M $MgSO_4$ and UV-irradiated. The UV doses were 28 J/m^2 for SY252 and 3 J/m^2 for IB105. Samples (0.1 ml) of UV-irradiated cells, appropriately diluted for determination of cell survival and Arg$^+$ revertants, were added to 3 ml of molten top agar, mixed and poured in triplicates onto 3% SEM plates containing different concentrations of test substance and incubated at 37 °C for 48 h.

For detection of 4NQO-induced mutagenesis, the overnight cultures of *SY252* and IB105 strains were pre-treated with 4NQO for 40 min at 37 °C, with aeration at 150 rpm. The final concentration of 4NQO was 50 μg/ml for SY252 and 5 μg/ml for IB105. After 4NQO-treatment, the cells were washed by centrifugation at 1700g and resuspended in the same volume of 0.01 M MgSO4. 4NQO-treated cell suspensions, appropriately diluted for determination of cell survival and Arg$^+$ revertants, were added to 3 ml of molten top agar, mixed and poured in triplicates onto 3% SEM plates containing different concentrations of test substance and incubated at 37 °C for 48 h.

2.6. Evaluation of SOS induction and protein synthesis

The effect of monoterpenes on SOS induction and general protein synthesis was determined as described by Quillardet and Hofnung (1993). The exponential cultures of *E. coli* strains IB111 and IB127 were washed by centrifugation, resuspended in 0.01 M $MgSO_4$ and irradiated with 28 J/m^2 and 3 J/m^2, respectively. The cell suspension was added in equal volume of 2×LB medium, with or without test substance, and incubated for 200 min at 37 °C with aeration at 150 rpm. At regular time intervals, samples were washed by centrifugation at 1700g, resuspended in minimal medium supplemented with 10% casamino acids and incubated 20 min on ice. After incubation, optical density at 600 nm (OD_{600}) was measured, the samples were diluted in appropriate buffer and cells were lysed with chloroform and 0.1% SDS, for β-galactosidase assay, or with 0.1% SDS, for alkaline phosphatase assay. The buffer used for determination of β-galactosidase contained $Na_2HPO_4 \cdot 12H_2O$ (21.2 g), $NaH_2PO_4 \cdot H_2O$ (5.5 g), KCl (0.75 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), SDS (1 g), β-mercaptoethanol (2.7 ml), per 1000 ml of distilled water (pH 7). The buffer used for determination of alkaline phosphatase contained tris(hydroxymethyl)aminomethane 121 g and SDS 1 g, per 1000 ml of distilled water (pH 8.8). The mixtures were incubated at 28 °C for 5 min and enzymatic reactions were started by adding appropriate substrates: 2-nitophenyl-β-D-galctopyranoside (CAS No. 73660 Sigma–Aldrich) for β-galactosidase, or p-nitrophenyl phosphate (CAS No. 104-0, Sigma–Aldrich) for alkaline phosphatase. The enzymatic reactions were stopped by adding 1 M NA_2CO_3 for β-galactosidase, or 2.5 M HCl and 2 M Tris for alkaline phosphatase. Optical densities were determined at 420 nm (OD_{420}) and 550 nm (OD_{550}). The enzymes activity was calculated by the equation:

$$EU = \frac{1000 \times (OD_{420} - 1.75 \times OD_{550})}{t \times V \times OD_{600}}$$

where t was reaction time until the color has developed, and V was the volume of the culture (Miller, 1972).

2.7. Evaluation of recombination

Intrachromosomal recombination was measured in *E. coli* strains GY8281 (*recA*$^+$) and GY8252 (*recA730*) by monitoring Lac$^+$ recombinants on MacConkey-lactose plates containing different concentrations of test substance. Samples (0.01 ml) of bacterial exponential cultures (3×10^8 cells/ml) were spread in the form of patches (2 × 2 cm) in triplicate on the same plate and irradiated with split UV-doses (5 + 5 J/m^2 for *recA*$^+$; 1 + 1 J/m^2 for *recA730*). The first UV exposure was immediately after plating, and the second after 3 h of incubation at 37 °C. The number of Lac$^+$ papillae was determined after incubation at 37 °C for 48 h. The strain GY7066 (Δ*recA*), which formed no papillae, was used as a negative control in all experiments.