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Comparative study of genotoxic, antigenotoxic and cytotoxic activities of monoterpenes camphor, eucalyptol and thujone in bacteria and mammalian cells

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ABSTRACT

Genotoxic/antigenotoxic, mutagenic/antimutagenic and cytotoxic effects of monoterpenes camphor, eucalyptol and thujone were determined in bacteria and mammalian cells using alkaline comet assay, *Escherichia coli* K12 reversion test and MTT assay, respectively. When applied in low doses (up to 200 μM in bacterial assay and 50 μM in comet assay) monoterpenes protected repair proficient *E. coli* and Vero cells against UV-induced mutagenesis and 4NQO-induced DNA strand breaks, respectively. Antimutagenic response was not detected in nucleotide excision repair (NER) deficient bacteria. When monoterpenes were applied in higher doses, a weak mutagenic effect was found in mismatch repair (MMR) and NER deficient *E. coli* strains, while induction of DNA strand breaks was evident in human fetal lung fibroblasts MRC-5, colorectal carcinoma HT-29 and HCT 116 cells, as well as in Vero cells. Moreover, the involvement of NER, MMR and RecBCD pathways in repair of DNA lesions induced by monoterpenes was demonstrated in *E. coli*. Camphor, eucalyptol and thujone were cytotoxic to MRC-5, HT-29 and HCT 116 cells. The most susceptible cell line was HCT 116, with IC_{50} values of 4.5 mM for camphor, 4 mM for eucalyptol and 1 mM for thujone. Observed effects of monoterpenes are consistent with hormesis response, characterized by a low dose beneficial effect and a high dose adverse effect of a stressor agent, and provide a basis for further study of both chemopreventive and chemotherapeutic potential of camphor, eucalyptol and thujone.

1. Introduction

Terpenes are plant secondary metabolites formed from five-carbon isoprene units (C_5H_8). They are endowed with many beneficial health effects including antimicrobial, antiparasitic, spasmolytic, hypoglycemic, anti-inflammatory, anti-allergenic and immune-modulatory, and consequently they are constituents of many botanical dietary supplements, formulated to treat different health disorders [1,2]. Numerous studies indicated their possible use in primary prevention and therapy of cancer [3–5]. Although some literature data show the antigenotoxic effect of terpenes [6], there are also reports showing their genotoxicity [7,8].

Monoterpenes (C_{10} terpenes) are the major components of plant essential oils. The monoterpenes investigated in this study: camphor (Cam), eucalyptol (Euc), and thujone (Thu) are constituents of essential oils of many aromatic and medicinal plants, including *Cinnamomum*, *Eucalyptus*, *Artemisia*, *Salvia* and *Thuja* species [9]. Although the neurotoxic effect of Thu in mammals is well established [10], plant derivatives containing Thu are constituents of many dietary supplements and herbal medicinal products [11,12]. Recently, the neurotoxicity of α-Thu in experimental animals has been more accurately evaluated and the current view rather downgrades its risk to humans [12].

Available data regarding genotoxic/antigenotoxic and mutagenic/antimutagenic features of Cam, Euc and Thu and their derivatives are controversial, depending on the cell type, genetic background, experimental setup and concentrations applied.

Abbrevations: Cam, Camphor; Euc, Eucalyptol; MMR, mismatch repair; NER, nucleotide excision repair; 4NQO, 4-Nitroquinoline-1-oxide; Thu, Thujone.

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Numerous literature data showed no mutagenicity of Cam and Euc in the *Salmonella*/microsome assay and in bone marrow cells of pregnant rats [13–15]; however Cam and Euc were genotoxic in Somatic Mutation and Recombination Test (SMART) on *Drosophila melanogaster* [16] and Euc also in comet test on human and hamster cells [17]. Literature data about mutagenicity/genotoxicity of Thu are also controversial: Pelkonen et al. [12] reported no genotoxicity in the micronucleus test on peripheral blood erythrocytes in male mice; however they found small, but significant increase of micronucleated erythrocytes in female animals. In addition, Thu was not mutagenic in Ames test and in SMART test on *D. melanogaster* [12,16], but was co-mutagenic with aflatoxin B_1 in Ames test, i.e. enhanced its mutagenic potential [18]. On the other hand, several reports indicate significant antimutagenicity and antigenotoxicity of Cam, Euc and Thu [14,18–20]. In addition, recent literature data indicate the cytotoxic potential of monoterpenes against some human cancer cell lines. Cytotoxicity of Cam against human lung carcinoma cells was reported by Őzkan and Erdoğan [21], while Rodenak Kladniew et al. [22] found cytotoxic potential of Euc against human hepatoma and lung carcinoma cells. As far as we know, there are no available data concerning cytotoxicity of pure Thu, but cell viability of malignant melanoma cell line was significantly decreased by Thu-rich fractions of *Thuja occidentalis* [23].

Our previous study on *Escherichia coli* and Vero cells demonstrated that Cam, Euc and Thu induced opposite effects on DNA repair and mutagenesis depending on the concentrations applied: although considerably genotoxic at high concentrations, in small amounts they reduced UV- and 4-nitroquinoline-1-oxide (4NQO)-induced mutagenesis [24]. The results were consistent with the hormesis response. Hormesis is a term used by toxicologists to refer to a biphasic dose response to an environmental agent, characterized by low dose stimulation or beneficial effect and a high dose inhibitory or toxic effect [25,26]. In order to test the hormesis hypothesis and to investigate the underlying mechanism, in this work we further examined genotoxic/antigenotoxic effect of monoterpenes applied in broad range of concentrations and in different genetic backgrounds. Effect was monitored in *E. coli* K12 strains differing in DNA repair capacity and in mammalian cell lines: Vero, MRC-5, HT-29 and HCT 116. Bearing in mind the genotoxicity of high doses of Cam, Euc and Thu, we additionally estimated their cytotoxicity against colorectal carcinoma HT-29 and HCT 116 cells, in order to examine their potential use in chemotherapy.

2. Material and methods

2.1. Chemicals

D, L-camphor (Cas No. 76-22-2, Alfa Aesar), eucalyptol (1,8-cineole, Cas No. 207-431-5, Fluka) and α,β-thujone (Cas No. 76231-76-0, Sigma Aldrich), were freshly dissolved in dimethyl sulphoxide (DMSO) for bacterial tests, or in ethanol for MTT and comet assays. 4NQO (Cas No. N-8141, Sigma Aldrich) was dissolved first in DMSO and then, immediately before use, ten-fold diluted in distilled water for antimutagenicity assay or in Dulbecco's Phosphate Buffered Saline (PBS buffer without Ca and Mg, PAA Laboratories GmbH, Austria) for comet assay.

2.2. Bacterial and eukaryotic cell cultures

Bacterial strains used in this study are listed in Table 1. The Vero cell line, obtained from the kidney of a normal adult African green monkey (ECACC No: 88020401), was used in antigenotoxicity assay. The human cell lines used in cytotoxicity and genotoxicity assays were fetal lung fibroblasts MRC-5 (ECACC No. 84101801), and colorectal carcinoma HT-29 (ATCC HTB-38) and HCT116 (ATCC CCL-247) cells. Both bacteria and mammalian cells were grown as previously reported [24].

Table 1
E. coli K12 strains.

Strain	Relevant genotype	Reference
AB1157	*rec*$^+$	[27]
AB2470	AB1157 *recB21*	[27]
DL131	AB1157 *recF143*	[28]
SY252	*argE3 mutS*$^+$ *uvrA*$^+$	[29]
IB103	SY252 *mutS*::Tn*10*	[30]
IB105	SY252 *uvrA*::Tn*10*	[31]

2.3. UV-irradiation conditions

UV-irradiation was carried out as previously described by Nikolić et al. [24]. Briefly, cell suspensions in 0.01 M $MgSO_4$ were irradiated in glass Petri dishes at a thickness of less than 1 mm, and kept in dark to prevent photoreactivation. Maximum output of the used UV-lamp (Benda, NU-8 KL) was at 254 nm.

2.4. Mutagenicity and antimutagenicity assays

To monitor the mutagenic effect, overnight cultures of SY252, IB103 andIB105 strains were diluted 15 × in LB medium and incubated for 120 min with or without monoterpenes at 37 °C, with aeration (150 rpm). Afterwards, the cells were washed by centrifugation at 1700 *g* and re-suspended 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 and incubated at 37 °C for 48 h.

To monitor the antimutagenic effect, overnight cultures of SY252 and IB105 strains were diluted 15x in LB medium and incubated for 120 min with aeration (150 rpm) at 37 °C, with or without monoterpenes. Afterwards, the cells were washed by centrifugation at 1700 *g*, re-suspended in the same volume of 0.01 M $MgSO_4$ and irradiated with appropriate UV dose (28 J/m^2 for SY252 and 3 J/m^2 for IB105). Samples (0.1 ml) of control and 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 and incubated at 37 °C for 48 h.

2.5. Sensitivity of E. coli repair deficient mutants to Cam, Euc and Thu

The overnight cultures of AB1157, AB2470, DL131, SY252, IB103 and IB105 were diluted 50x and incubated at 37 °C with aeration (150 rpm) until early exponential phase (OD_{600} ~ 0.4). Cultures were divided into portions and treated with monoterpenes for 120 min at 37 °C with aeration (150 rpm). Samples (0.1 ml) of cell suspension, appropriately diluted for determination of viable cells, were plated in triplicate on LA plates. Fraction of cell survival was determined after incubation at 37 °C for 24 h.

2.6. Genotoxicity and antigenotoxicity assays

Genotoxic effect of monoterpenes was monitored in Vero, MRC-5, HT-29 and HCT116 cells, whilst antigenotoxic effect against 4NQO was monitored in Vero cells. Each cell line was inoculated into 12-well plates at a density 4 × 10^5 cells/well and incubated 4 h to attach. For the genotoxicity testing, medium was replaced with the