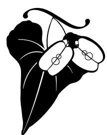
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Original Scientific Paper

Antigenotoxic potential of plant monoterpenes linalool, myrcene and eucalyptol against IQ- and PhIP- induced DNA damage

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ABSTRACT: Nutrition is recognised as one of the major factors that can influence the development of cancer. Heterocyclic amines formed during the cooking of meat are genotoxic carcinogens and consumption of meat positively correlates with certain types of human cancer. On the other hand, it has been reported that many plant components reduce the genotoxic activities of carcinogens, including HCAs. In this study we investigated the antigenotoxic potential of the monoterpenes linalool, myrcene and eucalyptol against two food-borne carcinogens: 2-amino-1-methyl-6-phenylimidazo[4-5-*b*]pyridine (PhIP) and 2-amino-3-methylimidazo[4,5-f]-quinoline (IQ). The study was performed by the comet assay method in metabolically active HepG2 human hepatoma cells. In addition, the lipid peroxidation TBA assay was used to determine the anti-oxidative potential of these monoterpenes. To evaluate the antigenotoxic potential, cells were treated simultaneously with 0.01, 0.1 or 1 μg/mL of monoterpenes and either 90 μmol/LPhIP or 0.75 mmol/L IQ. The DNA damage induced by PhIP was reduced significantly (by 40-63%) by each of the three monoterpenes, while inhibition of IQ-induced DNA damage was less efficient (12-26% reduction) and limited to myrcene and eucalyptol. Lipid peroxidation was significantly inhibited by linalool and myrcene, with IC_{50} values of approximately 30 μg/mL and 300 μg/mL, respectively. Eucalyptol showed only weak antioxidant activity (15% inhibition at 500 μg/mL). Taking into account the fact that reactive oxygen species (ROS) are formed during metabolic processing of PhIP, we assume that more efficient protection against PhIP-induced DNA damage was probably due to antioxidative activity of the monoterpenes or their metabolites, but the ability to interfere with metabolic activation of PhIP and IQ could also be involved.

Keywords: monoterpenes, heterocyclic amines, DNA damage, comet assay, HepG2 cells

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INTRODUCTION

Nutrition is recognised as a significant risk factor in certain human cancers. Common dietary mutagens include heterocyclic amines (HCAs) and polycyclic aromatic hydrocarbons (PAHs), both of which are formed during the high-temperature cooking of meat and fish (Ferguson *et al.* 2004). The most abundant HCA found in the human diet is 2-amino-1-methyl-6-phenylimidazo [4-5-*b*] pyridine (PhIP), while 2-amino-3-methylimidazo [4, 5-f]-quinoline (IQ) is usually a minor constituent (Edenharder *et al.* 1999; Nozawa *et al.* 2006; Wilson

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et al. 2007). Most HCAs are not reactive themselves and require metabolic activation, and the major pathway of the activation process is mediated by cytochrome P450 enzymes. The bioactivation of HCAs is hypothesised to occur via N-oxidation to N-hydroxy metabolites, followed by O-acetylation to form N-acetoxyarylamines. N-Acetoxyarylamines are then converted to chemically reactive nitrenium ions, ultimately leading to DNA adduct formation (Moonen *et al.* 2002; Snyderwine *et al.* 2002). However, N-OH-PhIP may be reduced by cytochrome b5 reductase, producing superoxide anions and hydroxyl radicals that cause DNA strand breaks (Wilson *et al.* 2007).

In recent years there has been growing interest in finding dietary factors with antigenotoxic effects in order to reduce the mutagenic and carcinogenic risk caused by HCAs. Furthermore, dietary factors are of interest for the prevention of lipid peroxidation, which has been implicated in several pathological disorders, such as ischaemia-reperfusion injury, coronary atherosclerosis and Alzheimer's disease, as well as in aging processes (Vijver *et al.* 1997; Smith *et al.* 2002).

In the present study, we investigated action of the plant monoterpenes linalool, myrcene and eucalyptol against PhIP- and IQ-induced DNA damage. Linalool, a monoterpene alcohol, myrcene, an acyclic monoterpene, and eucalyptol, a monoterpene oxide, are present in essential oils of many medicinal and aromatic plants and are endowed with a number of biological activities, including antimicrobial, anti-inflammatory, antitumoral and antioxidative (Pattnaik *et al.* 1997; Moteki *et al.* 2002; Santos *et al.* 2004; Tepe *et al.* 2004). As an experimental model to investigate the protective potential of monoterpenes, we used human–derived HepG2 hepatoma cells. Such cells retain many of the functions of normal liver cells and express different inducible xenobiotic metabolising enzymes (Knasmuller *et al.* 1998). To evaluate the effects of linalool, myrcene and eucalyptol on DNA damage, we performed a single-cell gel electrophoresis assay (comet assay), which is a rapid and sensitive way to analyse DNA damage at the individual cell level (Belloir *et al.* 2006).

MATERIAL AND METHODS

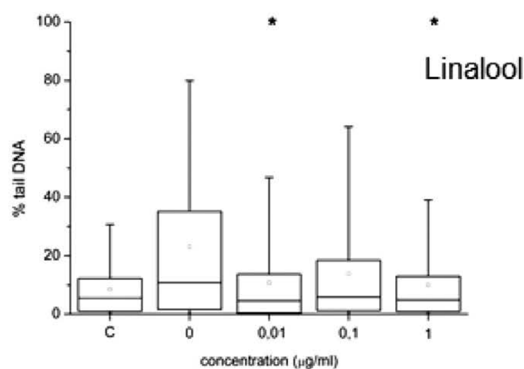
Chemicals. Williams' medium E, penicillin/streptomycin, fetal bovine serum (FBS), l-glutamine, phosphate-buffered saline (PBS), trypsin, ethidium-bromide solution (CAS 1239-45-8), dimethyl sulfoxide (DMSO, CAS 67-68-5) and EDTA (CAS 6381-92-6) were obtained from Sigma-Aldrich (St. Louis, USA). The PhIP (CAS H950765) used in the work was from Toronto Research Chemicals (Toronto, Canada), while the IQ (CAS 093-02571) was from Wako Chemical Industries (Osaka, Japan). Normal-melting-point (NMP) and low-melting-point (LMP,CAS 9012-36-6) agarose were obtained from Gibco BRL (Paisley, Scotland). IQ and PhIP were dissolved in DMSO; the final concentration of DMSO in incubation mixtures was not higher than 1%. Linalool (Aldrich, CAS L260-2), myrcene (Fluka, CAS 64643) and eucalyptol (Fluka, CAS 46090) were dissolved in 96% ethanol or in DMSO (TBA assay).

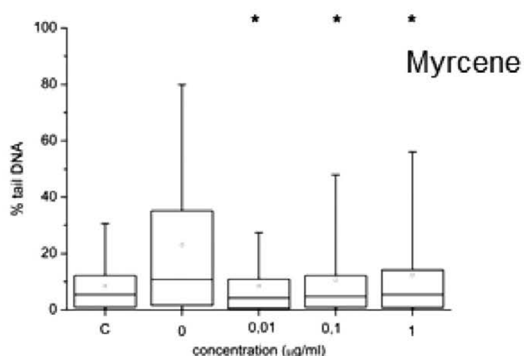
Human HepG2 cells. HepG2 cells were provided by Professor Firouz Darroudi, Department of Radiation Genetics and Chemical Mutagenesis, University of Leiden, The Netherlands. The cells were grown in Williams' medium E containing 10% fetal bovine serum, 2 mM l-glutamine and 100 U/mL penicillin/streptomycin at 37°C in 5% CO_2. Cells were used at passages between the 4th and 9th.

Antigenotoxicity testing. HepG2 cells were treated with monoterpenes and mutagens in Williams' E medium in 12-well plates (Corning Costar Corporation, New York, USA). A vehicle control and a negative control (non-treated cells) were included in each experiment. To evaluate the antigenotoxic potential of monoterpenes against PhIP andIQ, the cells were treated simultaneously with 0.01, 0.1 or 1 μg/mL of monoterpenes and either 90 μmol/LPhIP or 0.75 mmol/L IQ for 21 h at 37°C in 5% CO_2.

Comet assay. The comet assay was performed as described by Singh et al. (1988). A measured volume (30 mL) of cell suspensions (about 400,000 cells/ml) was mixed with 70 μL of 1% LMP agarose and immediately added to fully frosted microscope slides pre-coated with a layer of 1% NMP agarose. Cells were then lysed (2.5 M NaOH, 0.1 M EDTA, 0.01 M Tris and 1% Triton X-100, adjusted to pH 10) for 1 h at 4°C, rinsed with distilled water, placed in an electrophoresis buffer (1 mM EDTA, 300 mM NaOH, pH 13) for 20 min to permit DNA unwinding and electrophoresed for 20 min at 25 V and 300 mA. The slides were neutralized with 0.4 M Tris buffer (pH 7.5), stained with ethidium bromide (5 μg/mL) and analysed with the aid of a fluorescence microscope (Nikon Eclipse 800). Images of 50 randomly selected nuclei per slide were analysed with image-analysis software (VisCOMET, TillPhotonics, Germany). The percentage of DNA in the comet tail (tail intensity, TI) was used as the measure of DNA damage.

Determination of lipid peroxidation. The extent of lipid peroxidation (LP) was determined by the TBA assay (Mimica-Dukić *et al.* 2004), This was done by measuring colour of the adduct produced in the reaction between thiobarbituric acid (TBA) and malondialdehyde (MDA), a product of lipid peroxidation. Commercially prepared "PRO-LIPO S" liposomes (Lucas-Meyer, Hamburg, Germany, pH 5-7) were used as a model system of biological membranes. Liposomes 225-250 nm in diameter were obtained by dissolving the commercial preparation in demineralised water (1:10) in an ultrasonic bath. In Fe^{2+}/ascorbate-induced LP, 60 mL of a suspension of liposomes





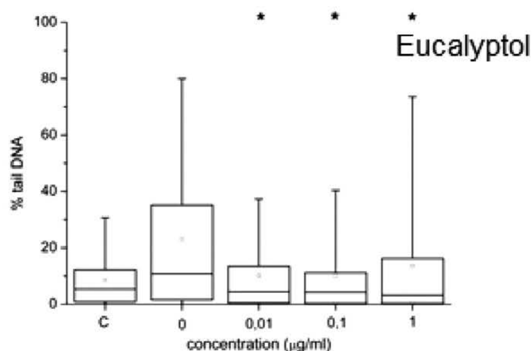
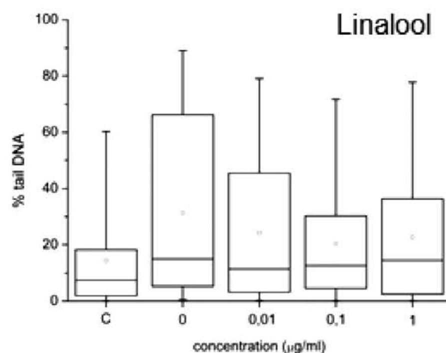
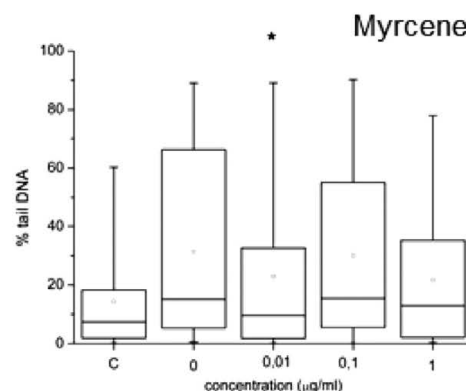


Figure 1. Antigenotoxic effect of monoterpenes against PhIP-induced DNA damage in HepG2 cells.
The level of DNA damage is expressed as the percentage of DNA in the comet tail. * Significantly different from the group treated with PhIP (Kruskal-Wallis test, Dunn's post test, p<0.05), c-control.





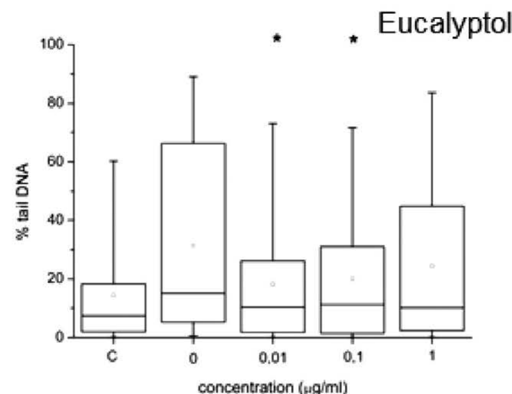


Figure 2. Antigenotoxic effect of monoterpenes against IQ-induced DNAdamage in HepG2 cells.
The level of DNA damage is expressed as the percentage of DNA in the comet tails.* Significantly different from the group treated with IQ (Kruskal-Wallis test, Dunn's post test, p<0.05), c-control.

was incubated with 20 mL of 0.01 M $FeSO_4$, 20 mL of 0.01 M ascorbic acid and 10 mL samples in 2.89 mL of 0.05 M KH_2PO_4-K_2HPO_4 buffer, pH 7.4, for 1 h at 37°C. Lipid peroxidation was terminated by adding 2 mL of TBA and 0.2 mL of 0.1 M EDTA and incubating at 100°C for 20 min. After the solution was cooled and precipitated, proteins were removed by centrifugation for 10 min at 4000 rpm and the content of the MDA was determined by measuring absorbance of the adduct at 532 nm(A_{532}). All reactions were carried out in triplicate with inclusion of positive and negative controls.

Statistical evaluation. The Student *t*-test was used to compare mean values of absorbance in the LP assay; $P < 0.05$ was considered to be statistically significant. For results of the comet assay, one-way analysis of variance

(non-parametric ANOVA, Kruskal–Wallis test) was used to analyse differences between treatments within each experiment. Dunn's post test was used to compare median values of TI for all treatments; $P < 0.05$ was considered to be statistically significant.

RESULTS

Protective action of linalool, myrcene and eucalyptol against PhIP- and IQ-induced DNA damage. In a previous study, we determined that exposure of HepG2 cells to myrcene, linalool or eucalyptol (concentrations of 0.01, 0.1 and 1 μg/mL) for 20 h did not affect their viability. Moreover, the highest non-cytotoxic concentration of monoterpenes (1 μg/mL) did not induce DNA strand breaks in comparison with untreated cells (Mitić-Ćulafić *et al.* 2009). Therefore, in the present study the non-cytotoxic and non-genotoxic concentration range of up to 1 μg/mL was employed to test the protective action of linalool, myrcene and eucalyptol against PhIP- and IQ-induced DNA damage. Cells were exposed to three different concentrations of myrcene, linalool or eucalyptol (0.01, 0.1 and 1 μg/mL) and either PhIP (90 mmol/L) or IQ (0.75 mmol/L) for 21 h. With the exception of linalool at 0.1 μg/mL, monoterpenes at all of the tested concentrations significantly prevented PhIP-induced DNA damage (Fig. 1). The extent of inhibition was similar (40-63%) for all three substances. The DNA damage induced by IQ was moderately reduced by eucalyptol (0.01 and 0.1 μg/mL) and myrcene (0.01 μg/mL), while linalool was ineffective (Fig. 2). The highest inhibition of IQ-induced genotoxicity was obtained with eucalyptol and amounted to 26%.

Antioxidant activity of linalool, myrcene and eucalyptol. The antioxidant activity of monoterpenes was compared by measuring inhibition of Fe^{2+}/ascorbate-induced LP in the TBA assay. In the range of tested concentrations, linalool and myrcene significantly inhibited LP, with IC_{50} values of approximately 30 μg/mL and 300 μg/mL, respectively. On the other hand, eucalyptol showed only weak antioxidant activity (15% inhibition at 500 μg/mL). The obtained result is in accordance with previously published data (Mitić-Ćulafić *et al.* 2009).

DISCUSSION

There is now much evidence from epidemiological studies linking high consumption of fruits, vegetables and spices with reduced risk of cancer (Edenharder *et al.* 1999; Wu *et al.* 2004; Arora *et al.* 2005). Studying and understanding the mechanisms of action of plant substances is important in order to establish their role in protection of human health. In the present work, we examined the protective capacity of monoterpenes present in many medicinal and aromatic plants, viz., linalool, myrcene and eucalyptol, against two food-borne mutagens and carcinogens (IQ and PhIP). Protective capacity was monitored in HepG2 cells using the comet assay.

In a previous study of ours, we demonstrated that linalool, myrcene and eucalyptol showed no toxic or genotoxic effect in HepG2 cells at concentrations up to 1 mg/mL (Mitić-Ćulafić *et al.* 2009), a finding that was used to limit the concentration range used in the present work. This was necessary because the existence of a protective effect should be tested in a non-cytotoxic concentration range and also because many substances can act as mutagens or antimutagens depending on concentration, cell type, experimental conditions, etc. (Borstel & Higgins 1998). In the present work, we demonstrated a strong protective capacity of myrcene, linalool and eucalyptol against PhIP-induced genotoxicity and moderate protective action against IQ-induced genotoxicity in HepG2 cells. The HepG2 cell line was found to retain many specialised functions, such as the capacity for synthesis of several plasma proteins normally lost by primary hepatocytes in culture (Lin & Yang 2007). In addition, HepG2 cells possess a variety of xenobiotic metabolising phase 1 and 2 enzymes (Kassie *et al.* 2003) and have been extensively used for genotoxicity studies.

The activation of dietary HCAs can proceed via different metabolic pathways, and the hepatic pathway has been studied extensively (Moonen *et al.* 2002). N-Hydroxy-PhIP/IQ is the first product in the activation of PhIP and IQ. After further esterification, N-hydroxy-PhIP may be converted to nitrenium ions (Durling & Abramsson-Zetteberg 2005) or be reduced by cytochrome b5 reductase, producing superoxide anions and hydroxyl radicals, which could cause DNA strand breaks or bulky DNA adducts (Wilson *et al.* 2007). In contrast, *O*-acetylation by NAT2 is a major phase 2 metabolic pathway involved in the activation N-hydoxy-IQ (Muckel *et al.* 2002).

It has been shown that PhIP possesses higher genotoxicity in mammalian cells than IQ (Pfau *et al.* 1999), possibly because PhIP can induce reactive oxygen species (ROS), causing DNA strand breaks (Wilson *et al.* 2007). Taking into account our results together with data on genotoxicity of PhIP, we speculate that the tested monoterpenes act in an antioxidant manner by scavenging ROS formed during metabolic processing of PhIP. The results obtained with myrcene and linalool in the LP test support this hypothesis. On the other hand, eucalyptol exerted strong protective action against PhIP-induced genotoxicity, although its anti-oxidative potential in the LP test was poor. However, published data show that eucalyptol is metabolised to 2-exo-hydroxy-1,8-cineole by microsomes from human and rat liver (Myazawa *et al.* 2001; Soares *et al.* 2005) and judging from its chemical structure it seems likely that this product may act as an antioxidant. In addition, protective action of monoterpenes may be based on their ability to

interfere with the metabolic activation of mutagens, since monoterpenes are efficiently metabolised in mammalian cells (Nikolić *et al*. 2012). Further studies are required to elucidate mechanisms of the observed protective action of linalool, myrcene and eucalyptol.

Understanding the risk of human exposure to HCAs is very important because the compounds vary widely in carcinogenic potency and quantities present in food. In this connection, it is significant that linalool, myrcene and eucalyptol were more effective against PhIP, the most abundant and potent food-borne mutagen, than against IQ.

CONCLUSION

The plant monoterpenes linalool, myrcene and eucalyptol possess an antigenotoxic potential against the food-borne mutagens/carcinogens PhIP and IQ. The monoterpenes were more effective against PhIP than against IQ. Further studies are required to elucidate mechanisms governing the observed protection provided by linalool, myrcene and eucalyptol.

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REZIME

Antigenotoksični potencijal biljnih monoterpena Linaloola, Mircena i Eukaliptola na IQ- i PhIP-indukovana DNK oštećenja

Dragana Mitić-Ćulafić, Bojana Žegura, Metka Filipič, Biljana Nikolić, Marina Jovanović i Jelena Knežević-Vukčević

Hrana može biti jedan od važnih faktora koji dovode do nastanka kancera. Prilikom termičke obrade mesa mogu nastati aromatični heterociklični amini (HCAs) koji spadaju u grupu genotoksičnih/karcinogenih agenasa, i potrošnja mesa je u pozitivnoj korelaciji sa pojavom određenih tipova kancera kod ljudi. Sa druge strane brojni biljni konstituenti mogu redukovati genotoksičnu aktivnost karcinogena uključujući i HCAs. U ovom radu ispitivan je antigenotoksični potencijal monoterpena Linaloola, Mircena i Eukaliptola prema dva karcinogena koji mogu nastati za vreme termičke obrade hrane: 2-amino-1-metil-6-fenilimidazo[4-5-*b*]piridin (PhIP) i 2-amino-3-metilimidazo[4,5-f]-kinolin (IQ). Studija je urađena primenom Komet testa u metabolički aktivnim humanim ćelijama jetre (HepG2). Pored toga, primenom TBA testa određen je i antioksidativni potencijal monoterpena. Da bi se utvrdio antigenotoksični potencijal, ćelije su tretirane simultano sa 0.01, 0.1 i 1 μg/mL monoterpena i sa 90 μmol/LPhIP ili 0.75 mmol/L IQ. Oštećenja molekula DNK indukovana PhIP-om su značajno bila redukovana (40-63%) sa sva tri monoterpena, dok je inhibicija IQ-indukovanih DNK oštećenja bila manje efikasna (12-26% redukcije) i bila je ograničena na Mircen i Eukaliptol. Lipidna peroksidacija je značajno bila inhibirana Linaloolom i Mircenom, i IC_{50} vrednosti su aproksimativno bile dostignute na koncentracijama 30 μg/mL i 300 μg/mL, respektivno. Eukaliptol je pokazao umerenu antioksidativnu aktivnost (15% inhibicije na 500 μg/mL). Pošto se za vreme metaboličke obrade PhIP-a formiraju reaktivne kiseonične vrste (ROS) smatramo da je efikasnija zaštita prema PhIP-indukovanim DNK oštećenjima verovatno nastaje zbog antioksidativne aktivnosti monoterpena i njihovih metabolita, ali i inhibicija metaboličke aktivacije PhIP i IQ, takođe, može biti uključena.

Ključne reči: monoterpeni, heterociklični amini, DNK oštećenja, Komet test, HepG2 ćelije