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EFFECT OF METABOLIC TRANSFORMATION OF MONOTERPENES ON ANTIMUTAGENIC POTENTIAL IN BACTERIAL TESTS

BILJANA NIKOLIĆ*, DRAGANA MITIĆ-ĆULAFIĆ, OLIVERA STAJKOVIĆ-SRBINOVIĆ**, BRANKA VUKOVIĆ-GAČIĆ and JELENA KNEŽEVIĆ-VUKČEVIĆ

University of Belgrade, Faculty of Biology, Chair of Microbiology, 11000 Belgrade, Serbia
** *Permanent address: Institute of Soil Science,* 11000 Belgrade, Serbia

*Abstrac*t – The effect of metabolic transformation of the monoterpenes Linalool (Lin), Myrcene (Myr) and Eucalyptol (Euc) was evaluated on their antimutagenic potential against *t*-butyl hydroperoxide (*t*-BOOH) and 2-nitropropane (2NP) in *E. coli* WP2 and in *S. typhimurium* reversion assays, respectively. Spontaneous mutagenesis was also monitored in both assays. Mammalian metabolic transformation was provided by rat liver microsomes (S9 fraction). None of the monoterpenes was mutagenic, either with or without S9. Results obtained without S9 showed the antimutagenic potential of Lin against *t*-BOOH, of Myr against both *t*-BOOH and 2NP, and of Euc against spontaneous and mutagenesis induced with both mutagens. Mammalian enzymes significantly reduced the antimutagenic effect of Lin, completely diminished the antimutagenic effect of Myr, but did not alter the antimutagenic effect of Euc. Considering the results, metabolic transformation by host enzymes could significantly influence antimutagenic potential and should be included in antimutagenicity studies in prokaryotic assays.

Key words: Antimutagenesis, monoterpenes, *E. coli*, *S. typhimurium*, S9 fraction, *t*- butyl hydroperoxide, 2-nitropropane

INTRODUCTION

Terpenes are the largest group of natural substances biosynthetically derived from isoprene units (Wang et al., 2005). They are abundantly in fruits, vegetables and aromatic and medicinal plants, and they play an important role in protection against infections, parasites and other stress conditions (Bakkali et al., 2008). A variety of monoterpenes and their derivatives have been shown to possess cancer chemopreventive and chemotherapeutic properties (Crowell, 1999; Wang et al., 2005). Linalool (Lin), a monoterpene alcohol, myrcene (Myr), an acyclic monoterpene and eucalyptol (Euc, 1,8-cineole), a monoterpene oxide, are present in the essential oils of many medicinal and aromatic plants that are endowed with many biological activities, including antioxidant, antimicrobial, anti-inflammatory, and antitumor (Pattnaik et al., 1997; Moteki et al., 2002; Santos et al., 2004; Tepe et al., 2004). Lin and Myr are mainly used in the manufacturing of cosmetic fragranced products and detergents. Both substances have antioxidant and antibacterial properties, and Lin also has antiviral properties (Koroch et al., 2007). Euc also possesses a strong antimicrobial effect (Cermelli et al., 2008; Mitić-Ćulafić et al., 2005; Soković et al., 2010); it is traditionally used as a food-flavoring agent, in aromatherapy, as a skin stimulant, and in pharmaceutical preparations as a percutaneous penetration enhancer and as an antitussic (De Vincenzi et al., 1996; Gao and Singh, 1998; Laude et al., 1994). Recently, it has been reported that Euc systemically exerts anti-inflammatory, analgesic, gastroprotective and hepatoprotective effects (Santos et al., 2004).

In our previous studies, we demonstrated that the monoterpenes Lin, Euc and Myr displayed antimutagenic and antigenotoxic effects against the oxidative mutagen *t*-butyl hydroperoxide (*t*-BOOH) in *E. coli* strains, as well as in mammalian cells *in vitro* (Mitić-Ćulafić et al., 2009; Nikolić et al, 2011a). The observed effects were in correlation with the potential of Lin, Euc or Myr to inhibit lipid peroxidation, indicating that the mechanism of their antimutagenic activity is based on their antioxidant properties. They also suppressed UV- and 4NQO-induced mutagenesis in *E. coli* and *S. typhimurium* (Vuković-Gačić et al., 2006; Stajković et al., 2007; Stanojević et al., 2008; Nikolić et al., 2011a, b). Moreover, Euc reduced 2-nitropropane (2NP) and benzo[*a*]pyrene (B[*a*]P)-induced mutagenesis in *S. typhimurium* TA100 (Stajković et al., 2007). In addition, spontaneous mutagenesis was reduced by Lyn in the mismatch repair deficient, and by Euc in the *oxyR* deficient *E. coli* strain (Berić et al., 2008; Mitić-Ćulafić et al., 2009). Taken together, these results indicate multiple mechanisms of antimutagenicity of Lin, Euc and Myr.

Several recent studies have shown that certain compounds that were protective in bacterial assays were not protective in mammalian cells or were even genotoxic (Knasmüller et al., 2002). The main reason for these differences is the metabolic transformation of the compound into an inactive or genotoxic form by the enzymes involved in the activation/detoxification of xenobiotics (Knasmüller et al., 1998; Kassie et al., 2003). On the other hand, biotransformation of terpenoids by cell metabolism could also produce intermediaries with amplified antimutagenic/antigenotoxic properties. Consequently, it is important to investigate the effect of metabolic transformation on antimutagenicity/antigenotoxicity. Since it has been reported that Lin, Euc and Myr are efficiently metabolized in mammals (Chadha and Madyastha, 1984; Madyastha and Srivatsan, 1987; Miyazawa et al., 2001; Ishida, 2005; Belsito et al., 2008), in this work we investigated the effect of metabolic transformation by cytochrome P450 (CYP) enzymes on their antimutagenic potential. The effect was evaluated against mutagenesis induced by the oxidative mutagen *t*-BOOH and environmental mutagen 2NP using *E. coli* and *S. typhimurium* reversion assays, respectively. Effect was also monitored against spontaneous mutagenesis. The microsomal S9 fraction of rat liver cells was added to provide the metabolic transformation of monoterpenes in bacterial assay systems. Oxidative mutagenesis was studied because of the importance of the oxidative DNA damage and mutagenesis in the etiology of many human cancers and other degenerative disorders, including atherosclerosis, Alzheimer's disease and aging (Marnett, 2000; Olinski et al, 2002; Davydov et al., 2003; Coppede and Migliore, 2009). 2NP was chosen as an important environmental pollutant; it is an industrial chemical used as a solvent for many organic compounds including cartridges, dyes, inks, varnishes and resins, and it is also a constituent of cigarette smoke (Fiala et al., 1997; Kreis et al., 2000). The genotoxicity of 2NP in mammalian and microbial cells has been mainly attributed to DNA-reactive species arising from the CYP-independent pathway (Andrae et al., 1999).

MATERIALS AND METHODS

Media and Chemicals

Lin (Sigma-Aldrich, Cas No. 78-70-6), Euc (Fluka, Cas No. 207–431-5), Myr (Fluka, Cas No. 123-35-3) and 2NP (Sigma-Aldrich, Cas No. 79-46-9) were freshly dissolved in dimethyl sulfoxide (DMSO). *t*-BOOH (Sigma-Aldrich, Cas No. 75-91-2) was dissolved in 1 x M9 buffer immediately before use.

Bacterial strains

The *E. coli* WP2 strain IC202 *trpE65 oxyR* and *S. typhimurium* strain TA100 *hisG3052 rfa Δ(gal chl bio uvrB)*, both carrying plasmid pKM101 (Maron and Ames, 1983; Blanco et al., 1998) were used for antimutagenicity testing against *t*-BOOH and 2NP, respectively. The effect on spontaneous mutagenesis was monitored in both strains. The working cultures were prepared from frozen permanents by overnight incubation at 37°C in nutrient broth supplemented with ampicillin (20 μg/ml).

Metabolic activation

Metabolic activation was provided by an S9 mixture containing NADP (Sigma-Aldrich, Cas No. 1184-16-3), glucoso-6 phosphate (Sigma-Aldrich, Cas No. 54010-71-8), $MgCl_2$, KCl and a liver homogenate from male Wistar rats induced with Aroclor 1254 (Sigma-Aldrich, Cas No. 11097-69-1) or phenobarbital (Sigma-Aldrich, Cas No. 50-06-6) and β-naphthoflavone (Sigma-Aldrich, Cas No. 6051-87-2), for experiments with *t*-BOOH and 2NP, respectively (Maron and Ames, 1983).

E. coli antimutagenicity assay

To the overnight culture of IC202 (100 μl), a monoterpene dilution (100 μl) and a solution of *t*-BOOH (100 μl) were added in 3 ml of molten top agar, mixed and poured onto ET4 plates (minimal medium containing 0.37 mM tryptophan). Metabolic activation was provided by adding 0.3 ml of S9 mixture to the molten top agar before plating. The number of revertant colonies was counted after 48 h of incubation at 37°C. To discriminate antimutagenicity from toxicity, appropriate dilutions of bacteria were treated as above and plated on LA plates. The number of surviving colonies was determined after 24 h of incubation at 37°C.

S. typhimurium antimutagenicity assay

To the overnight culture of TA100 (100 μl), a monoterpene dilution (100 μl) and a solution of 2NP (100 μl) were mixed with 3 ml of molten top agar containing 0.05 mM histidine and biotin and poured onto MG plates. Metabolic activation was provided by adding 0.3 ml of S9 mixture in the molten top agar before plating. The number of revertant colonies was counted after 48 h of incubation at 37°C. The concentrations of monoterpenes resulting in a thinner auxotrophic background lawn were considered toxic (Maron and Ames, 1983; Mortelmans and Zeiger, 2000). In addition, the concentrations that reduced the number of spontaneous revertants by more than 30% compared to the solvent controls were also considered potentially toxic and excluded from further experiments (Stajković et al., 2007).

Evaluation of antimutagenic potential

The antimutagenic potential of monoterpenes was determined by calculating the percentage of mutagenesis inhibition according to the equation: % inhibition = [1-TM/M] x100, where TM is the number of Trp^+ or His^+ revertants per plate in the presence of the mutagen and the tested monoterpene, and M is the number of Trp^+ or His^+ revertants per plate in the presence of the mutagen alone. The antimutagenic effect was considered strong when inhibition of mutagenesis was higher than 40%, moderate when it was in the range between 25% and 40%, and weak or absent when the inhibitory effect was less than 25% (Ikken et al., 1999).

Statistical analysis

Student's *t*-test was used for statistical analysis. The significance was tested at $p<0.05$ level. The results presented in the Figures are expressed as the means obtained in three independent experiments with two replicates, with the standard error of the mean.

RESULTS

The effect of mammalian P450 enzymes on the antimutagenic potential of monoterpenes Lin, Euc and Myr was evaluated in bacterial antimutagenicity tests, performed with and without a microsomal fraction of rat hepatocytes (S9 fraction). The effect was determined against mutagenesis induced by the oxidative mutagen *t*-BOOH and environmental mutagen 2NP. Spontaneous mutagenesis was also monitored.

Antimutagenicity screening against oxidative mutagenesis was performed with an *E. coli* WP2 assay using the strain IC202. The strain is deficient in the OxyR regulated response to oxidative stress and therefore more sensitive for detecting oxidative mutagens (Blanco et al., 1998). An oxidative mutagen *t*-butyl hydroperoxide (*t*-BOOH) was selected, since it induces mutations both with and without the S9

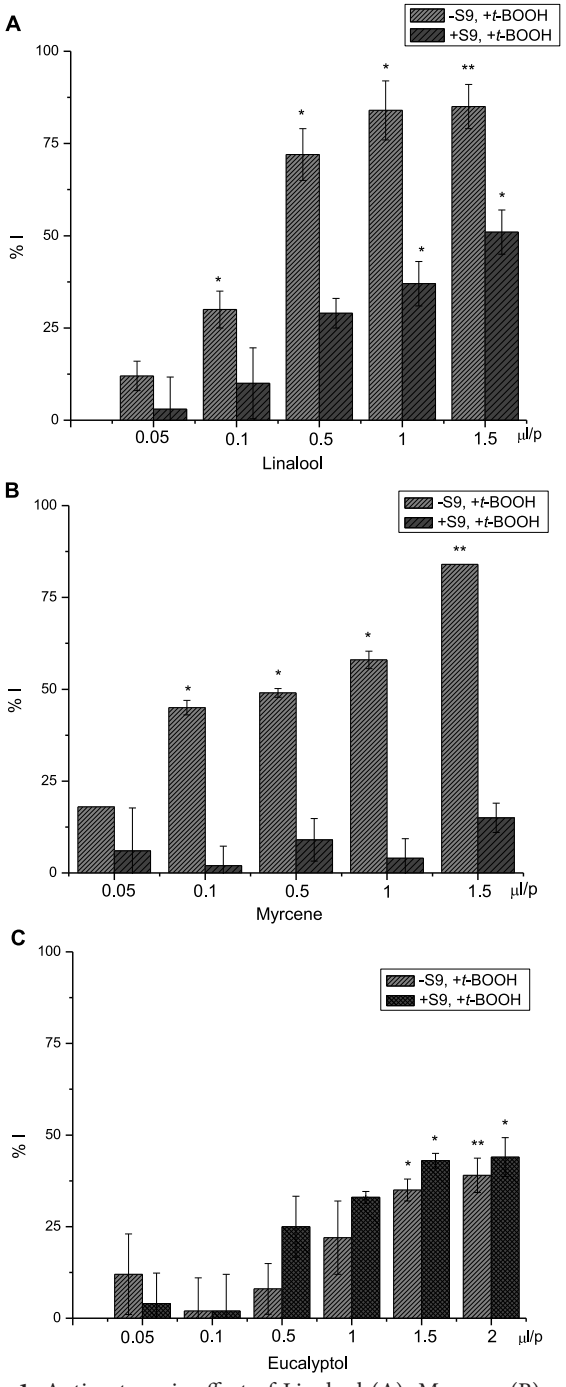

Fig. 1. Antimutagenic effect of Linalool (A), Myrcene (B) and Eucalyptol (C) against *t*-BOOH in *E. coli* IC202 strain.
%I = % of inhibition of mutagenesis
The number of *t*-BOOH-induced revertants/plate in solvent control was: 261±16 and 218±22 for Lin, 247±4 and 205±13 for Myr, 272±18 and 300±19 for Euc, without and with S9, respectively.
* $p < 0.05$ compared with corresponding solvent control
** toxic concentration

fraction. In addition, it is more stable than hydrogen peroxide, and is resistant to catalase (Urios and Blanco, 1996). The mutagen and monoterpenes were applied in the range of concentrations previously tested by Mitić-Ćulafić et al. (2009).

In experiments without the S9 mixture, all three monoterpenes inhibited *t*-BOOH-induced mutagenesis (Fig. 1), confirming our previous results (Mitić-Ćulafić et al., 2009). In the concentration range from 0.1 to 1.5 µl/plate, Myr decreased the number of *t*-BOOH-induced revertants by 45-84%. Lin was also very efficient, decreasing the number of *t*-BOOH-induced revertants by 30-85%. Euc exerted a moderate antimutagenic effect (35%) at 1.5 µl/plate. The highest tested concentration (2.0 µl/plate) was toxic. When S9 was added to provide metabolic transformation of monoterpenes, the antimutagenic effect of Lin was significantly decreased (from 85% to 51% inhibition), while the antimutagenic effect of Myr was lost (Fig. 1). On the contrary, the antimutagenic effect of Euc was slightly increased by the addition of S9.

The effect of mammalian P450 enzymes on the antimutagenic potential of Lin, Euc and Myr was also evaluated against 2NP-induced mutagenesis, using a *Salmonella*/microsome reverse mutation assay with the TA100 strain. In preliminary experiments, we confirmed that 2NP induced mutations both with and without S9. Moreover, the S9 fraction did not significantly increase the number of revertants (data not shown). For the antimutagenicity assay, we applied the mutagen and monoterpenes in the range of concentrations previously tested by Stajković et al. (2007).

In experiments without the S9 mixture, Euc and Myr inhibited 2NP-induced mutagenesis by 37 and 31%, respectively, at the highest tested concentrations, while Lin had no effect (Fig. 2). When metabolic transformation of monoterpenes was provided by S9, the antimutagenic effect of Myr was significantly decreased (down to 15% inhibition), while the antimutagenic effect of Euc was not affected.

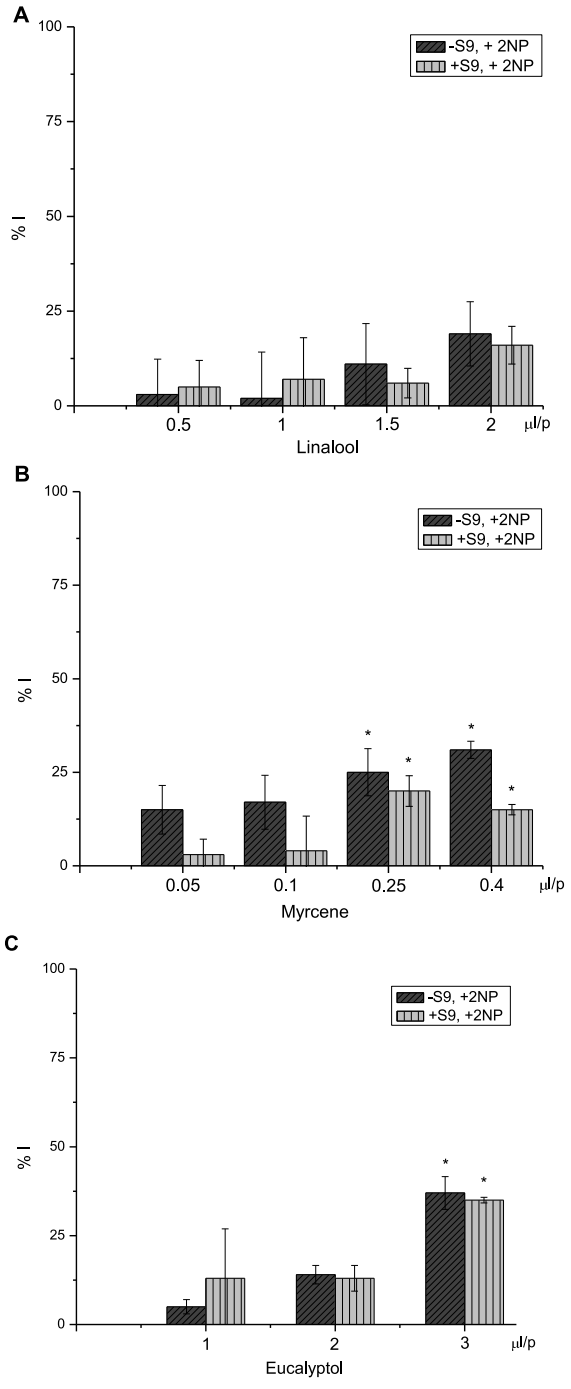

Fig. 2. Antimutagenic effect of Linalool (A), Myrcene (B) and Eucalyptol (C) against 2NP in *S. typhimurium* TA100 strain.
%I = % of inhibition of mutagenesis
The number of 2NP-induced revertants/plate in solvent control was: 548±42 and 634±80 for Lin, 549±17 and 479±54 for Myr, 576±83 and 603±16 for Euc, without and with S9, respectively.
* $p < 0.05$ compared with corresponding solvent control

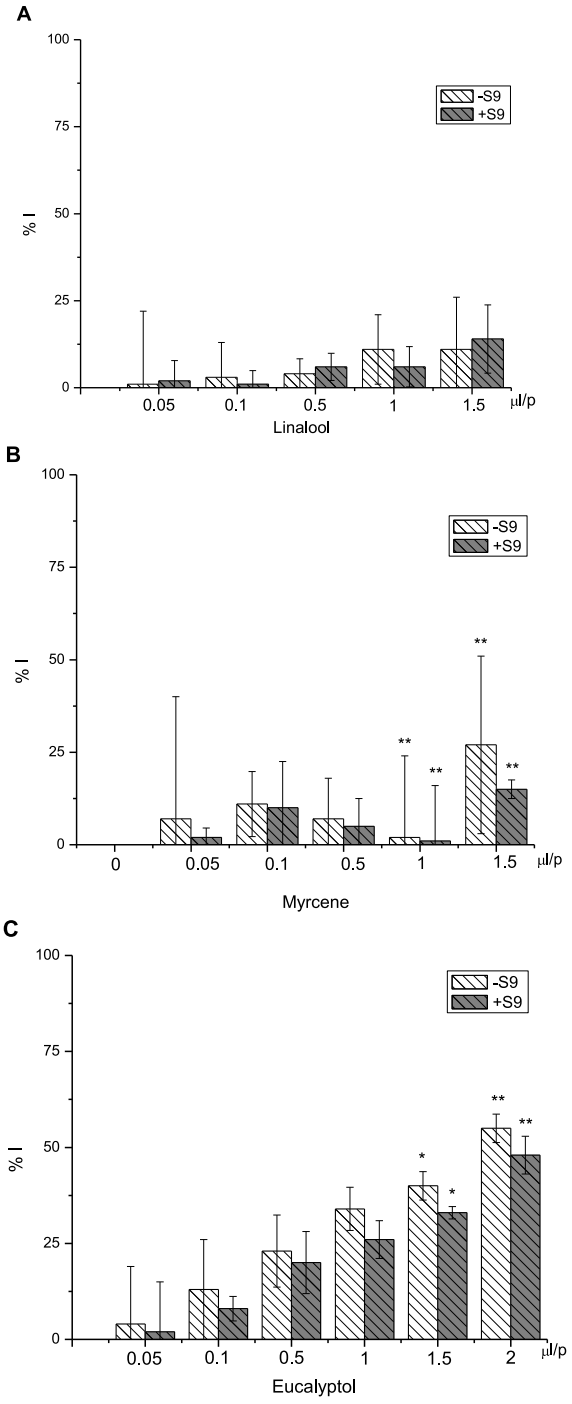

Fig. 3. Antimutagenic effect of Linalool (A), Myrcene (B) and Eucalyptol (C) against spontaneous mutagenesis in *E. coli* IC202 strain. %I = % of inhibition of mutagenesis
The number of spontaneous revertants/plate in solvent control was: 46±12 and 51±4 for Lin, 45±3 and 40±4 for Myr, 53±2 and 61±2 for Euc, without and with S9, respectively.
* $p < 0.05$ compared with corresponding solvent control
** toxic concentration

The effect of monoterpenes on spontaneous mutagenesis was also monitored in *E. coli* WP2 and *Salmonella*/microsome reverse mutation assays. In both assays, none of the monoterpenes was mutagenic, either with or without S9. Moreover, Euc decreased the number of spontaneous revertants in *E. coli* IC202, by 33% and 40% with and without S9, respectively (Fig. 3). Interestingly, the addition of S9 reduced the toxicity of Euc in IC202 (data not shown).

DISCUSSION

To study the effect of metabolic transformation in the bacterial assay, a mammalian microsomal preparation with cofactors (S9 mixture) is widely used. The metabolizing capacity of S9 is significantly affected by the differences in enzyme inducers, species or even organs used (Shahin et al., 1983). A mixture of polychlorinated biphenyls, designated as Arochlor 1254 (ARO), was initially used to increase the level of total CYP enzymes in hepatic microsomes (Clare, 1989). However, due to the detrimental environmental impact of ARO and the high risk of manipulating it, phenobarbital/β-naphthoflavone (PB/NF) was proposed as an alternative inducer (Matsushima et al., 1976). Although there are differences in the CYP subfamilies induced by ARO and PB/NF, they are more quantitative than qualitative (Escobar-Garcia et al., 2001): CYP1A1/2, CYP2B1/2, CYP2E1 and CYP3A enzyme subfamilies are all induced both by ARO and PB/NF (Clare, 1989; Okey, 1990; Miyazawa et al., 2001; Meredith et al., 2003).

Monoterpenes are efficiently metabolized in mammals. Their oxidation products have the capacity to participate in a range of nucleophilic and electrophilic addition reactions with biological material, including DNA. Oxidation is mediated by CYP-dependent monooxigenases, mainly in the liver (Belsito et al., 2008).

Study of the CYP-mediated oxidative metabolism of monoterpene alcohol Lin, using mammalian CYP enzymes, identified the 8-hydroxy-Lin, 8-carboxy-Lin, as well as the cyclic ethers pyranoid-Lin oxide and furanoid-Lin oxide as the enzymatic products (Chadha and Madyastha, 1984; Meesters et al., 2007). After treatment of rats with Lin, the CYP activity in the liver microsomes was increased in a typically PB-inducible manner (Boutin et al., 1985). Since the S9 fraction used in our experiments was prepared with PB/NF or ARO, the enzymes for the metabolic transformation of Lin were provided.

The obtained results reveal that Lin strongly reduced *t*-BOOH-induced mutagenesis, and this is in accordance with previously reported data (Mitić-Ćulafić et al., 2009; Nikolić et al, 2011a). However, its antimutagenic potential was significantly diminished by S9, indicating that the antioxidant capacity of the obtained metabolic products had probably been decreased.

Myr has been shown to be metabolized by β-glucuronidase/arylsulfatase to several conjugates of dioles and hydroxyl acids, formed via epoxide intermediates (Ishida, 2005). 10-hydroxyLin was detected as a major metabolite excreted in the urine of Myr-treated rats (Madyastha and Srivatsan, 1987; Miyazawa and Murata, 2000). Data provided by De-Oliviera et al. (1997a, b) indicated that Myr is both inducer and substrate for the CYP2B1 subfamily in rats. In light of these data, we assume that the S9 used in our experiments, which contained the CYP2B1 enzyme subfamily, could metabolically process Myr, leading to intermediates with significantly decreased antimutagenic potential against *t*-BOOH, and without effect against 2NP.

Our previous research, reported by Mitić-Ćulafić et al. (2009), confirmed that the metabolic transformation of both Lin and Myr, catalyzed with mammalian liver enzymes, decreased their antigenotoxic potential against *t*-BOOH-induced DNA damage. They noticed that the antigenotoxic potential of Lin was stronger in metabolically less active NC-NC cells than in hepatic HepG2 cells, which retained the activity of many enzymes involved in the activation/detoxification of xenobiotics. Moreover, the antigenotoxic potential of Myr was obtained only in NC-NC cells, but not in hepatic HepG2 cells.

Although the antimutagenic potential of Lin and Myr was decreased with metabolic transformation, it is important that neither monoterpenes nor their metabolic products were mutagenic. This is in line with our previous study where even higher doses of Lin (up to 5 μl/plate) and Myr (up to 10 μl/plate) were not mutagenic (Nikolić et al., 2011a). Numerous reported data also indicate no mutagenicity/genotoxicity of Lin and Myr (Ishidate et al. 1984; Yoo, 1986; Heck et al., 1989; Kauderer et al., 1991; Letizia et al., 2003; Bickers et al., 2003; Di Sotto et al., 2010). However, Stajković et al. (2007) showed that Lin was co-mutagenic with B[*a*]P.

Euc has been found to be oxidized by rat and human liver microsomal CYP2B1 and CYP3A enzymes, mainly to 2-*exo*-hydroxy-1,8 cineole, but also to (±) 3-*endo*-hydroxy-1,8 cineole, (±) 3-*exo*-hydroxy-1,8 cineole, 2-oxo-1,8 cineole and 3-oxo-1,8 cineole (Miyazava et al., 1989; 2001; De-Oliviera et al., 1999). According to these reports, Euc was probably metabolized by the S9 mixture used in our experiments, but its antimutagenic potential against both mutagens was not significantly affected. Similar result was obtained by Mitić-Ćulafić et al. (2009) who reported a comparable antigenotoxic potential of Euc against *t*-BOOH-induced genotoxicity in both human cell lines: metabolically active HepG2, and metabolically less active NC-NC cells.

Our results show that Euc also equally reduces the rate of spontaneous mutagenesis in *E. coli* IC202, both with and without S9. However, this result could not be confirmed in *S. typhimurium* TA100 because viability was not quantitatively measured, but was only estimated through the monitoring of the auxotrophic background lawn. This means that reduction of the number of revertants could result not only from the inhibition of spontaneous mutagenesis, but also from the toxicity of the tested concentration. Furthermore, as we previously mentioned, the concentrations that reduced the number of spontaneous revertants by more than 30% compared to the solvent controls were considered potentially toxic and excluded from further experiments, as recommended by Stajković et al. (2007). It is important, however, that no evidence of Euc mutagenicity/genotoxicity was obtained in either assay, as well as in available literature data (Gomes-Carneiro et al., 1998; Horvathova et al., 2007; Stajković et al., 2007; Nikolić et al., 2011a).

In conclusion, this study confirms that Lin, Myr and Euc possess a significant antimutagenic potential in bacteria. Lin and Myr strongly reduced *t*-BOOH-induced mutagenesis, while Myr additionally induced a moderate reduction of 2NP-induced mutagenesis. Euc was moderately effective against both mutagens, as well as against spontaneous mutagenesis. We showed that metabolic transformation significantly decreased the antimutagenicity of Lin and completely diminished the antimutagenicity of Myr. In contrast, the antimutagenic potential of Euc was not significantly affected by metabolic transformation. Considering our results, metabolic transformation by host enzymes could significantly influence antimutagenic potential and should be included in antimutagenicity studies in prokaryotic assays.

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