Protective effects of d-limonene and linalool on female rats intoxicated with 7,12 dimethylbenz(a)anthracene (DMBA)

S. TANYILDIZI1, K. SERVI1, O. CIFTCI1,2*

1Department of Pharmacology and Toxicology, Faculty of Veterinary Medicine, University of Firat, 23 119 Elazig, TURKEY.
2Department of Pharmaceutical Toxicology, Faculty of Pharmacy, University of Inonu, 44 280 Malatya, TURKEY.

*Corresponding author: osmciftci@gmail.com or ociftci@inonu.edu.tr

SUMMARY

This study was carried out to investigate the potential antioxidant effects of 2 monoterpenes, linalool and d-limonene, in female rats orally treated or not with dimethylbenz(a)anthracene (DMBA). For this purpose, 70 female Wistar Albino rats (7-8 weeks old) were randomly divided into 10 equal groups according to the modalities of monoterpene dietary supplementation and/or oral DMBA (65 mg/kg) treatment. Negative controls were not supplemented and untreated, positive controls received only DMBA, whereas all the other groups were fed with diets enriched by 5% limonene or linalool for 150 days and received no DMBA (groups 2 and 3) or DMBA administration 15 days after (groups 5 and 6), in the same time (groups 7 and 8) or 6 days before (groups 9 and 10) the beginning of terpene supplementation. Plasma concentrations of MDA (considered as a marker of oxidative stress) were measured in all animals on days 15, 30, 45, 60, 75 and 150. This marker was dramatically increased by DMBA treatment alone then gradually declined. By contrast, the dietary monoterpene supplementation has induced significant decreases of spontaneous MDA formation and also has markedly inhibited DMBA-induced lipid peroxidation, the best protocol being initiation of supplementation before DMBA administration. Furthermore, linalool has appeared as a more potent antioxidant than limonene. These results evidence the protective in vivo effects of monoterpenes on an experimentally induced oxidative stress and suggest that dietary supplementation would be beneficial for the prevention of free radical damage.

Keywords: Dimethylbenz(a)anthracene, malondialdehyde, rat, d-limonene, linalool, dietary supplementation, anti-oxidant.

Introduction

Monoterpenes are dietary components found in the oils of citrus oil and some edible plants. These compounds are derived from the mevalonate pathway in plants but are not produced by mammals [3]. D-limonene is found in orange and other citrus peel oil, caraway and dill. In addition, it is a prevalent flavouring agent for fruit juices and soft drinks [5]. Linalool is a component of a number of essential oils including lavender, coriander [34], and sweet basil [23]. It was reported that it has antinociceptive, gastro protective [2] and exhibited immunostimulatory effects [4]. Both compounds have chemopreventive activity against mammary, lung and fore stomach cancers when rats were dietary supplemented with the terpene compounds during the initiation phase [7, 9, 11, 27]. Furthermore, some previous studies have reported the antioxidant properties of D-limonene and linalool [12, 25].

Lipid peroxidation, widely recognized as a primary toxicological event, is caused by the generation of free radicals from a variety of sources including organic hydroperoxides and redox processes [10, 13]. The secondary events include changes in membrane structure, permeability and fluidity, lysosomal destabilization, and stimulation of apoptosis [6]. One of the effects of many antioxidants is to scavenge radicals, and consequently to inhibit lipid peroxidation. The 7,12 dimethylbenz(a)anthracene (DMBA) is a chemical carcino-
genic compound strongly involved in the generation of reactive oxygen species, such as peroxides, hydroxyl and superoxide anion radicals, leading to cellular oxidative damage through DNA strand breaks and lipid peroxidation [29]. When various primary and secondary products of lipid peroxidation in biological systems are decomposed, aldehydes are formed. One intensely studied aldehyde is malondialdehyde (MDA), and it is commonly used as a marker for the lipid peroxidation process [19]. However, no information is available about the potential protective effects of d-limonene and linalool on plasma MDA concentrations to our knowledge. As a consequence, the aims of the present study were to investigate and to compare the eventual beneficial effects of these 2 terpene compounds on lipid peroxidation induced by DMBA in female rats.

Materials and Methods

CHEMICALS

D-Limonene (CAS no: 138-86-3, > 97%) and linalool (CAS no: 78-70-6, >95%), were purchased from Merck Co (Germany), the 7,12-dimethylbenz[a]anthracene and the other chemicals from Sigma Chemicals Co (St Louis, USA).

ANIMALS AND PROTOCOL DESIGN

Seventy seven healthy young adult female Wistar Albino rats (7–8 weeks old), weighing between 150-180g at the start of the study (Virology Institute, Elazığ-Turkey) were used for this experiment. Animals were housed in sterilized polypropylene rat cages, in 12-h light-dark cycle, at an ambient temperature of 21°C. Animal diet and drinking water were given ad libitum. Experiments on animals were performed based on animal ethics guidelines of Institutional animals Ethics Committee.

Rats were randomly divided into ten equal groups (n = 7 in each group). A first group served as negative control and only powdered rodent diet was given to these rats. Rats of 2 other groups (limonene and linalool groups, respectively) received a powdered rodent diet enriched with 5% limonene and 5% linalool respectively during the whole experimental period (150 days). Based on the assumption that a 200 g female rat consumes ~ 8 g of food per day, the daily intake for both of limonene and linalool would be 2 g/kg. In the fourth group (DMBA group or positive control), DMBA suspended in olive oil was orally administered at the dose of 65 mg/kg [5, 28, 31] using an 8 French feeding tube to rats fed with the not supplemented rodent diet. Animals of the 5th and 6th groups respectively for 150 days and received the DMBA administration 15 days after the beginning of the limonene or linalool supplementation, whereas rats from the groups 7 and 8 were submitted to the DMBA treatment as soon as dietary limonene or linalool supplementations were started. Finally, in the 2 last groups (groups 9 and 10), oral DMBA administration was committed 6 days before the onset of terpene supplementation. Blood samples were collected from puncture of the tail vein with a lancet on days 15, 30, 45, 60, 75 and 150 into sterile tubes containing EDTA. Plasmas were obtained after whole blood centrifugation (1 000 g, 10 minutes, at 4°C) and were stored at -20°C until analysis.

BIOCHEMICAL ANALYSIS

Plasma protein-bound malondialdehyde concentrations were measured with thiobarbituric acid reaction in rat blood using the method described by LEFEVRE et al. [18]. Plasma proteins were precipitated by adding 2.5 mL of 200 g/L trichloroacetic acid to 0.5 mL of plasma and centrifugation (1 000g for 10 min, 20°C). Then 3 mL of thiobarbituric acid (2 g/L in 2 M Na2SO4) was added to the pellet resuspended in 2.5 mL of 10% acetic acid solution. This mixture was heated in a boiling water bath for 30 min, and then quickly cooled in an ice bath to stop the reaction. The chromophore was extracted in 4 mL of n-butanol and the supernatant isolated by centrifugation at 3 000g for 10 min, 20°C. The absorbance of supernatant was measured at 532 nm, a solution of 1.1% (w/v) 1,1,3,3- tetraethoxypropane was used for calibration. For that, appropriate dilutions of 1,1,3,3- tetraethoxypropane given concentrations ranged between 0.05 to 15 μM were prepared and heated in a boiling water bath for 30 min. After the extraction with n-butanol, absorbances were measured as in plasma samples.

STATISTICAL ANALYSIS

Results are expressed as mean ± S.E.M. The Student t test was applied to determine statistically significant differences between the malondialdehyde concentrations of control and treatment groups. The malondialdehyde concentrations were compared between treatments and within time points using one-way analysis of variance (ANOVA) and post hoc Duncan’s test. All analyses were carried out by SPSS statistical program (Win 10.0). Differences were considered as significant when p values were less than 0.05.

Results

As shown in Table I, plasma MDA concentrations remained low and stable according to time in untreated and not supplemented rats (negative controls). By contrast, DMBA oral treatment has dramatically induced strong increases of plasma MDA concentrations compared to negative controls for all time points (p < 0.001). Nevertheless, the lipid peroxidation intensity has significantly decreased according to time (p < 0.05). On the other hand, the dietary supplementation with 5% terpene (limonene or linalool) markedly reduced plasma MDA concentrations of untreated rats with DMBA compared to negative controls for all time points (p < 0.001). Moreover, linalool supplementation appeared significantly more potent than limonene supplementation (p < 0.001). However, the antioxidant effects of these terpene compounds significantly varied during the whole experimental period and were maximal on days 60 and 150 for limonene and between the 45th and the 75th days for linalool (p < 0.05). Furthermore, plasma MDA concentrations of DMBA treated rats were dramatically reduced when the animals were supplemented with limone-
ne or linalool compared to positive controls (DMBA treated rats) for all time points (Table I) (p < 0.001) whatever the delay between DMBA administration and the onset of dietary terpene supplementation. Nevertheless, the lipid peroxidation intensity remained very low throughout the whole experimental period compared to positive and negative controls (p < 0.001) when terpene supplementation was started 15 days before the oral DMBA treatment, even if some significant fluctuations (p < 0.05) were observed according to time: plasma MDA concentrations were minimal on days 15 and 30 when limonene was used and on days 30, 45 and 75 when linalool was used. When the dietary supplementation was started on the same day that DMBA treatment (groups 7 and 8) or 6 days after (groups 9 and 10), plasma MDA concentrations appeared significantly more elevated than those measured in negative controls and in supplemented untreated rats (groups 2 and 3 for limonene and linalool respectively) (p < 0.001) and progressively declined according to time (p < 0.05) (Table I). But, this marker of oxidative stress was significantly less diminished (p < 0.001) when DMBA treatment was performed after the beginning of the terpene supplementation. The comparative efficiency of the different supplementation protocols was summarized in the Table II: whereas limonene or linalool supplementation for 150 days and eventually orally treated by DMBA (65 mg/kg). Results are expressed as means ± SEM.

### Table I: Plasma MDA concentrations (µmol/l) in female adult rats dietary supplemented or not with terpene compounds (5% limonene or 5% linalool) for 150 days and eventually orally treated by DMBA (65 mg/kg). Results are expressed as means ± SEM.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Plasma MDA concentrations (µmol/l)</th>
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<tr>
<td></td>
<td>Day 15</td>
</tr>
<tr>
<td>No terpene supplementation</td>
<td></td>
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<tr>
<td>Negative control</td>
<td>2.48 ± 0.14&lt;sup&gt;C&lt;/sup&gt;</td>
</tr>
<tr>
<td>Positive control (DMBA group)</td>
<td>218.71 ± 5.71&lt;sup&gt;AH&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% limonene supplementation</td>
<td></td>
</tr>
<tr>
<td>No DMBA (group 2)</td>
<td>1.01 ± 0.03&lt;sup&gt;B&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ DMBA</td>
<td>Group 5&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Group 7&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>Group 9&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>5% linalool supplementation</td>
<td></td>
</tr>
<tr>
<td>No DMBA (group 2)</td>
<td>0.33 ± 0.41&lt;sup&gt;AB&lt;/sup&gt;</td>
</tr>
<tr>
<td>+ DMBA</td>
<td>Group 61</td>
</tr>
<tr>
<td></td>
<td>Group 82</td>
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<td></td>
<td>Group 103</td>
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</tbody>
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Different superscripts a,b,c,d,e,f in a same row indicate significant differences (p < 0.05) of plasma MDA concentrations according to time for the same group.
Different superscripts A-J in a same column indicate significant differences (p < 0.001) between experimental groups.

### Table II: Comparative efficiency (%) of the different protocols of dietary terpene (limonene or linalool) supplementation for reducing plasma MDA concentration increases induced with oral DMBA treatment (65 mg/kg) of rats. Results are expressed as mean SEM.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Efficiency (%)</th>
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<tbody>
<tr>
<td></td>
<td>Day 15</td>
</tr>
<tr>
<td>Protocol 1</td>
<td></td>
</tr>
<tr>
<td>Limonene</td>
<td>99.8 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Linalool</td>
<td>99.6 ± 0.3&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protocol 2</td>
<td></td>
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<tr>
<td>Limonene</td>
<td>96.5 ± 0.4&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Linalool</td>
<td>98.0 ± 0.3&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Protocol 3</td>
<td></td>
</tr>
<tr>
<td>Limonene</td>
<td>41.9 ± 0.7&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Linalool</td>
<td>73.5 ± 0.6&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Different superscripts a,b,c,d,e,f in a same row indicate significant differences (p < 0.001) between experimental groups.

Protocol 1: the dietary terpene supplementation was started 15 days before the DMBA treatment; Protocol 2: the dietary terpene supplementation was started the same day that DMBA treatment; Protocol 3: the dietary terpene supplementation was started 6 days after the DMBA treatment.
99%, the observed reduction factors were mainly dramatically depressed (p < 0.001) when DMBA administration has been performed before the initiation of supplementation, reaching 73.5% (at day 15) and 95.0% (at day 150) with linalool and 41.9% (at day 15) – 83.3% (at day 150) with limonene. When DMBA treatment and terpene supplementation were simultaneously performed, the reduction factor remained above 90%. Moreover, except for the first protocol (terpene supplementation was initiated before DMBA treatment) for which a comparable efficiency was obtained between the 2 terpene compounds, the linalool supplementation appeared more efficient in reducing the DMBA induced lipid peroxidation.

Discussion

DMBA is a synthetic polycyclic aromatic hydrocarbon (PAH), which has been used extensively as a prototype carcinogen and causes the production of malondialdehyde (MDA) in organism [33]. It was reported that the occurrence of MDA-DNA adducts in patients with breast and larynx cancer was higher than in controls [9, 32]. Whatever the administration route, DMBA is oxidized to 3,4-epoxide by the phase I enzyme cytochrome P450 [16, 24]. The epoxide hydratase is another phase I enzyme which converts the epoxide form to 3,4-diol, the proximate carcinogen. Then, DMBA-3,4-diol-1,2-epoxide is produced depending on the further oxidation by cytochrome P450 enzyme [16, 24]. Conjugation of these oxidized DMBA metabolites to hydro-soluble glucuronate or glutathione by the phase II enzymes UDP glucuronol transferase and glutathione-S-transferase respectively can then occur [5]. Consequently, DMBA can be considered as carcinogenic via the MDA production and lipid peroxidation induction and via formation of highly genotoxic metabolites. In agreement with that, strong increases of plasma MDA concentrations were observed in female rats receiving an unique oral dose (65 mg/kg) of DMBA compared to untreated controls in the present study. In the same way, numerous studies have demonstrated that 7,12-dimethylbenz[a]anthracene (DMBA) efficiently induced breast and mammary cancers and lipid peroxidative changes in female rats [15, 22, 26]. Dissolved in corn oil, DMBA at the dose of 50 mg/kg body weight was able to induce lung and kidney tumours in rats [30]. Additionally, it was suggested that a single intragastric dose (80 mg/kg body weight) of DMBA could cause breast cancer in female rats [1]. Therefore, the oral DMBA delivery to rats would be considered as a model mimicking the dietary intake of carcinogens in humans, and has been intensively used for carcinogenic studies since the 1960s [14]. The doses of DMBA vary according to investigations depending on the aim of studies. In this study, the dose of 65 mg/kg of DMBA was chosen because it was known to induce lipid peroxidation [29].

By contrast, the dietary terpene (limonene and linalool) supplementation negatively controlled spontaneous MDA formation since plasma MDA concentrations in supplemented animals were significantly lower than in not supplemented rats and also greatly inhibited the oxidative stress induced by DMBA intoxication because the concentrations of the bio-chemical marker were dramatically depressed in DMBA treated and terpene supplemented rats compared to those observed in DMBA treated rats (positive controls). It has been reported that antioxidant properties of limonene would be related to induction of total cytochrome P450 [20] and of phase II enzymes glutathione-S-transferase and UDP glucuronol transferase [7]. In the current study, limonene may inhibit MDA accumulation by promoting the elimination of DMBA and oxidized compounds through amplification of conjugation steps. As far as linalool is concerned, it has been suggested that its anti-inflammatory and immunomodulatory activities [4, 8] would be linked to the reduction of MDA formation throughout the arachidonate oxidation pathway [17, 19]. Indeed, PEANA et al. [21] observed a significant reduction of PGE2 formation and COX2 expression in macrophages exposed to linalool (10-3M). It would be possible that in the present study the linalool antioxidant efficiency would be coupled to the COX2 repression and to the consequent prevention of the arachidonate oxidation. However, whatever the considered terpene compounds, the most potent protocol for limiting oxidative stress occurrence was to initiate the antioxidant dietary supplementation before the DMBA administration rather than to start it in the same time that DMBA treatment or after. As terpenes acts to a long term by regulating enzyme expression, their efficiency on counteract DMBA genotoxicity would be exacerbated if enzymes involved in phase II reactions would be already over-expressed (limonene) or if enzymes indirectly involved in MDA formation would be already repressed (linalool). Furthermore, the factors of reduction of the MDA formation compared to positive controls (rats only treated to DMBA) were significantly higher when rats were supplemented with linalool rather than with limonene for the 3 supplementation protocols tested in the present study, suggesting that linalool exhibits a more potent MDA scavenging effect than d-limonene.

As a conclusion, this in vivo study demonstrate the antioxidant effects of terpene (limonene and linalool) compounds incorporated to diets as well as their preventive effects against DMBA toxicity. These results suggest that monoterpenes in daily diet would be relevant for offering some protection against lipid peroxidation and inherent mutagenic risks.

References


