Antioxidant potential of aroma compounds obtained by limonene biotransformation of orange essential oil

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ABSTRACT

The antioxidant activities of a limonene biotransformation extract and of some standard monoterpenes present in the extract were assessed using four antioxidant assays: total antioxidant capacity, based on the 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay, lipid peroxidation by the thiobarbituric acid (TBA) assay, superoxide anion release by cultured leukemic cells and glutathione S-transferase (GSTs) activity. The limonene biotransformation extract had free radical-scavenging activity (EC_{50} = 2.09%, v/v) and inhibited lipid peroxidation (IC_{50} = 0.13%, v/v). The extract, perillyl alcohol and α-terpineol inhibited lipid peroxidation by $\sim$80% at a concentration of 0.02% (v/v). Perillyl alcohol and α-terpineol also reduced the release of superoxide anions by cultured leukemic cells, by 3- and 10-fold, respectively, at concentrations of <0.02% (v/v). The biotransformation extract inhibited the conversion of nitrophenyl acetate to p-nitrophenol in the glutathione assay by $\sim$50%. These results indicate that, in addition to monoterpenes, other non-volatile compounds may contribute to the antioxidant activity of the biotransformation extract.

1. Introduction

The free radical-scavenging activity of fruit (Kaur, Jabbar, Athar, & Alam, 2006), vegetable (Conforti, Marrelli, Statti, & Menichini, 2006) and medicinal plant (Gülçin, Mshvildadze, Gepdiremen, & Elias, 2006) extracts has been extensively studied. Essential oils rich in monoterpenes are recognized as food preservatives (Baratta, Dorman, Deans, Biondi, & Ruberto, 1998; Helander et al., 1998; Ruberto & Baratta, 2000), and monoterpenic essential oils are natural antioxidants (Yanishlieva, Marinova, Gordon, & Raneva, 1999) that are active against certain cancers (Kris-Etherton et al., 2002). Indeed, a number of dietary monoterpenes have antitumoral activity that can prevent the formation or progress of cancer and cause tumor regression. Limonene and perillyl alcohol have a well-established protective activity against many types of cancer (Crowell, 1999).

The biotransformation of terpenes allows the production of natural flavours and fragrances under mild reaction conditions (Adams, Demyttenaere, & De Kimpe, 2003; Carvalho & Fonseca, 2006; Maróstica Júnior & Pastore, 2007). The use of agro-industrial residues in bioprocesses provides alternative substrates while simultaneously helping to solve pollution problems (Pandey et al., 2000).

In this work, we examined the antioxidant activity of an extract from the biotransformation of limonene, as well as the activity of monoterpene standards present in this extract.

2. Materials and methods

2.1. Chemicals

Carvone (purity $\sim$98%), R-(+)-limonene ($\sim$99%), perillyl alcohol ($\sim$96%), R-(+)-α-terpineol ($\sim$99%) and the reagents for the various assays were purchased from Sigma Chemical Co. and Aldrich Fine Chemicals (St. Louis, MO, USA). All other chemicals and solvents were of the best grade available.

2.2. Extract obtained from biotransformation experiments

The biotransformation of limonene was done as described by Maróstica Júnior and Pastore (2007). Briefly, *Fusarium oxysporum*

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was grown for three days in manipueira (an effluent from commercial cassava production) and transferred to a mineral culture medium (composition, in g/l: $MgSO_4$ 0.5, $NaNO_3$ 3, K_2HPO_4 1, KCl 0.5 and Fe_2SO_4 0.01 in distilled water) (Brunerie, Benda, Bock, & Schreier, 1987). The biotransformation of limonene was started by adding orange essential oil (final concentration, 0.1% v/v) to the mineral culture medium, with two further additions after 24 and 48 h. The broths were extracted with ethyl acetate 0, 24, 48, 72 and 96 h after the first addition of orange essential oil and all of the extracts obtained were combined and concentrated.

2.3. GC and GC–MS

Gas chromatography (GC) and GC–mass spectrometric (MS) analyses were done as described elsewhere (Maróstica Júnior & Pastore, 2007). The GC analyses were done using a Chrompack CP9001 gas chromatograph equipped with a split/splitless-injector, an FID-detector and a WCOT fused silica column. The stationary phase was a CP-Sil CB column (0.25 mm i.d. × 60 m; coating thickness of 0.25 μm), and the working conditions included injector and detector temperatures of 220 °C and 250 °C, respectively, with He (1 ml/min) as the make-up gas. The oven temperature was programmed from 40 °C to 210 °C at 5 °C/min with an initial holding time of 1 min and a final holding time of 5 min. Quantification was done by comparison with the internal standard (decane).

GC–MS-analyses were done using a Varian Saturn gas chromatograph equipped with an EM-IT mass selective detector, a CP-Sil 8CB Low bleed/MS capillary column (30 m × 0.25 mm × 0.25 μm) and a split/splitless CP1177 injector. The working temperatures were 280 °C for the injector and 260 °C for the transfer line to MSD. The oven temperature was programmed from 40 °C (1 min) to 160 °C (3 min) at 5 °C/min and from 160 °C to 250 °C with a final holding time of 5 min. The scan range was m/z 40–500, with a split of 1/50, an ionization of EI 70 eV, a mass range of 40–500 amu and a scan rate of 1 s^{-1}. The carrier gas was He (1 ml/min). Specific components were identified by matching their retention indices (RI) and mass spectra with those of standards analysed under identical conditions.

2.4. Total antioxidant capacity based on the DPPH radical-scavenging assay

The nitrogen radical-scavenging activity was measured by monitoring the reduction of the free nitrogen radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) in the presence of the extract, monoterpene standards and ethyl acetate (Singh, Chidambara Murthy, & Jayaprakasha, 2002). The samples (diluted in ethanol) were added to the wells of a 96-well plate and 200 μl of a DPPH solution (0.1 mM in ethanol) were added. The total antioxidant capacity was determined from the decrease in absorbance at 517 nm after 30 min incubation. The radical-scavenging activity was expressed as the % quenching of the DPPH radical, calculated as $[(Abs_{0\,min} - Abs_{30\,min})/Abs_{0\,min}] \times 100$. All determinations were done in triplicate.

2.5. Measurement of lipid peroxidation by the thiobarbituric acid (TBA) assay

The lipid peroxidation activity was determined as described by Singh et al. (2002). Aliquots (240 μl) of liver homogenate (0.5 mg of protein/ml in 50 mM Tris–HCl buffer, pH 7.4, containing 150 mM KCl) were mixed with 30 μl of extract and monoterpene standards (diluted in ethanol) and 15 μl of 4 mM ascorbic acid. Peroxidation was initiated by adding 100 μl of 0.2 mM ferric chloride and the reaction was run at 37 °C for 60 min. The reaction was stopped by adding 500 μl of an ice-cold mixture of 0.25 N HCl containing 16.8% trichloroacetic acid and 0.86% TBA, followed by heating to 80 °C for 15 min. The samples were cooled and centrifuged, and the absorbance of the supernatant was measured at 540 nm. A control experiment was done under identical conditions to determine the amount of lipid peroxidation obtained in the presence of inducing agents without extract. The percentage of antilipid peroxidative activity (% ALP) was calculated using the formula: antilipid peroxidation (%) = [1 − (sample absorbance/blank absorbance) × 100].

2.6. Synthetic monoterpene solution

The possible influence of the monoterpenes present in the extract on the DPPH and TBA assays was assessed using a solution of standard monoterpenes diluted in ethyl acetate. The concentrations of these standards were the same as those in the extract.

2.7. Superoxide release by leukemic cells

Superoxide release by leukemic cells was assayed in 96-well plates, based on the method described for monocytes by Lehrer and Cohen (1981). Myeloid leukemia K562 cells were resuspended (5×10^6 cells/ml) in Krebs–Ringer phosphate buffer with dextrose (2 mg/ml) and 80 μM cytochrome c (KRPD). Cells (10^6 cells/200 μl) were distributed in wells and 20 μl of each sample (0.2% v/v) were added immediately before the addition of phorbol myristate acetate (PMA; 2.16 μM). Cells without sample were incubated as a positive control and wells without sample and with 0.5 U of superoxide dismutase were used for the negative control. The blank consisted of KRPD with PMA, without cells. After incubation for 1 h at 37 °C in the dark, the plate was centrifuged and 150 μl of supernatant were transferred to a microwell plate and read at 550 nm. One unit of superoxide released per cell corresponded to 1 fmol of reduced cytochrome c, calculated using a molar extinction coefficient of 24 mM^{-1} cm^{-1}. The experiments were done in triplicate.

2.8. Glutathione S-transferase activity

All experiments were done in triplicate, using the assay described by Habig and Jakoby (1981), adapted for 96-well plates. Samples diluted 1:50 in buffer (0.1 M potassium phosphate, pH 7.4, containing 1 mM EDTA) (total volume of 150 μl) were transferred to wells, followed by the addition of 25 μl of 50 mM glutathione (GSH) solution in buffer and 20 μl of rat liver (2 mg of protein/ml). The mixture was incubated for 5 min at 37 °C, after which the reaction was started by adding 30 μl of 2 mM nitrophenyl acetate (NPA). The blank consisted of sample with GSH but without NPA. The occurrence of spontaneous reactions was assessed in wells containing 150 μl of buffer without sample.

2.9. Protein concentrations

All protein concentrations were determined by the Bradford dye binding method (Bradford, 1976), using bovine serum albumin as the standard.

2.10. Statistical analysis

The results were expressed as means ± S.E.M. ANOVA, followed by the Tukey test, used for statistical comparisons among groups, with a value of p<0.05 indicating significance. All calculations and comparisons were done using the software Statistica 8 (StatSoft Inc., Tulsa, OK, USA).

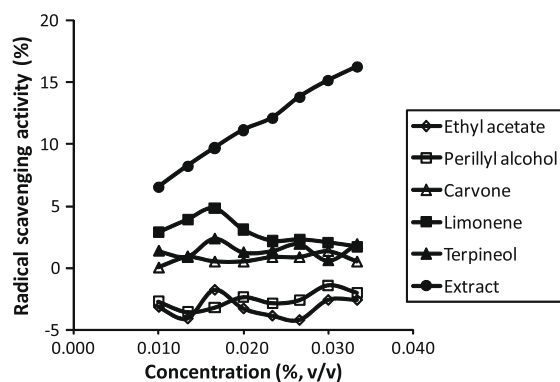**Fig. 1.** Free radical-scavenging activity of the biotransformation extract and monoterpene standards, based on the DPPH·assay. The points are the means ± SEM of three experiments. In most cases, the SEM bars are smaller than the symbols.

Fig. 2. Inhibition of lipid peroxidation by the biotransformation extract and monoterpene standards, based on the TBA assay. The points are the means ± SEM of two experiments. In some cases, the SEM bars are smaller than the symbols.

3. Results and discussion

3.1. Extract obtained from biotransformation experiments

The biotransformation extract was obtained as described in section 2.1 and analysed as described in section 2.2. The main compounds present (concentrations >0.1 mg/l) were limonene (166 mg/l), α-terpineol (6.6 mg/l), perillyl acohol (2.6 mg/l) and carvone (0.2 mg/l).

3.2. Total antioxidant capacity based on the DPPH radical-scavenging assay

(Fig. 1) shows the free radical-scavenging potential of different concentrations of the biotransformation extract and standard monoterpenes, as determined by the DPPH· assay. According to Singh et al. (2002), antioxidant compounds react with DPPH, converting it to $\alpha\alpha$diphenylβpicryl hydrazine, and the resulting degree of discoloration indicates the scavenging potential of the compounds tested. The extract showed considerable free radical-scavenging activity, whereas limonene, α-terpineol and carvone were less active. Ethyl acetate, the solvent used in the extraction (and hence the major compound present in the extract) and perillyl alcohol had slightly pro-oxidant activity. The greater free radical-scavenging activity of the extract compared to the isolated components may have resulted either from the synergistic action of a mixture of terpenes, or from the action of non-volatile compounds, other than monoterpenes, present in the extract.

3.3. Lipid peroxidation by the TBA assay

The ability of the extract and monoterpene standards to prevent lipid peroxidation was assessed by the TBA assay. Thiobarbituric acid reacts with malondialdehyde (MDA) to form a pink chromogen that can be detected spectrophotometrically. In biological systems MDA is a reactive species that participates in the cross-linking of DNA with proteins and damages liver cells (Singh et al., 2002). MDA is the major product of lipid peroxidation and is used as an indicator of this phenomenon in rat liver homogenates.

(Fig. 2) shows that the biotransformation extract and standard monoterpenes inhibited lipid peroxidation. The inhibition of lipid peroxidation by the monoterpene standards was concentration-dependent at the higher concentrations. Perillyl alcohol, which has antitumor and antioxidant activities (Crowell, 1999), and terpineol, were the strongest inhibitors of lipid peroxidation. The extract showed strong inhibition at low concentrations, with little
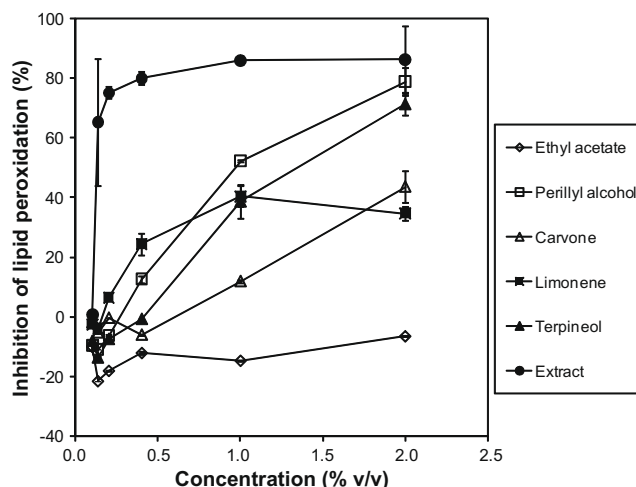
concentration-dependence at dilutions between 0.13% and 2.0%. The possible causes of this greater inhibitory activity are probably the same as those indicated in section 3.2 above.

3.4. Comparison of the antioxidant activity of the extract and synthetic monoterpenes in the DPPH· and TBA assays

To examine whether synergism among the monoterpenes present in the extract could account for the antioxidant activity of the extract, we prepared a mixture of monoterpenes consisting of limonene, perillyl alcohol, carvone and α-terpineol in the same concentrations as found in the extract. Ethyl acetate was used as the solvent. (Fig. 3) shows that, in the DPPH· and TBA assays, the free radical-scavenging activity of the mixture of synthetic monoterpenes was less potent than that of the extract. This finding suggested that other unidentified compounds present in the extract also contributed to the antioxidant activity.

3.5. Superoxide released by leukemic cells

Phorbol myristate acetate (PMA) is a potent tumor promoter with diverse effects on cellular growth, differentiation and metabolism. PMA also stimulates superoxide anion ($O_2^{-\bullet}$) production in neutrophils by activating NADPH oxidase (Lehrer & Cohen, 1981). This assay was initially designed to detect $O_2^{-\bullet}$ released by leucocytes, but leukemic K562 cells are also sensitive to PMA. (Fig. 4) shows that the extract had little effect on $O_2^{-\bullet}$ release by K562 cells when compared to the controls, whereas carvone, perillyl alcohol and α-terpineol markedly inhibited $O_2^{-\bullet}$ production; in contrast, limonene stimulated this formation. Since the extract contains a large amount of limonene and smaller amounts of carvone, perillyl alcohol and α-terpineol, the mild effect of the extract on $O_2^{-\bullet}$ production probably reflects a balance between the opposing actions of these monoterpenes.

3.6. Glutathione S-transferase activity

Glutathione S-transferases (GSTs) are the most important enzymes in phase II reactions of drug biotransformation and are involved in the end-stage metabolism of alkylating agents, xenobiotics and products of lipid peroxidation. Reduced glutathione (GSH) is a cofactor for GST. The transferases also catalyse the glutathione-dependent release of p-nitrophenol from p-nitrophenyl
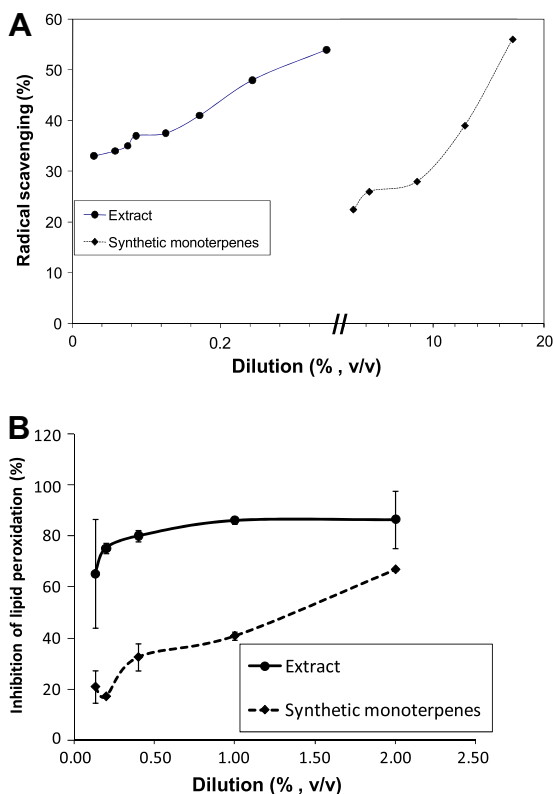
Fig. 5. Inhibition of *p*-nitrophenol formation by the biotransformation extract and monoterpenes. The points are the means ± SEM of three experiments.

maximum activity occurring at a concentration of 2.5%. This finding suggests that these compounds may be metabolized by phase II reactions *in vivo*.

4. Conclusions

The results of this study show that the limonene biotransformation extract used here had important antioxidant activity that was apparently mediated by monoterpenes and other components of the extract. The possibility of using a crude extract as an antioxidant would greatly reduce the need to obtain pure compounds via expensive industrial purification techniques. Our findings also suggest that some monoterpenes may be useful as 'functional aroma compounds' in health supplements and nutraceuticals. However, studies *in vivo* are needed to assess the true protective capacity of these compounds and to determine the metabolic pathways involved in their degradation.

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Fig. 3. Antioxidant activity of the biotransformation extract and synthetic monoterpenes. (A) DPPH·assay and (B) TBA assay. EC_{50} values for the DPPH·assay: Extract = 2.10%, v/v, synthetic monoterpenes = 16.0%, v/v. EC_{50} values for the TBA assay: Extract = 0.13%, v/v, synthetic monoterpenes = 1.4%, v/v. The points are the means ± SEM of three experiments. In some cases, the SEM bars are smaller than the symbols.
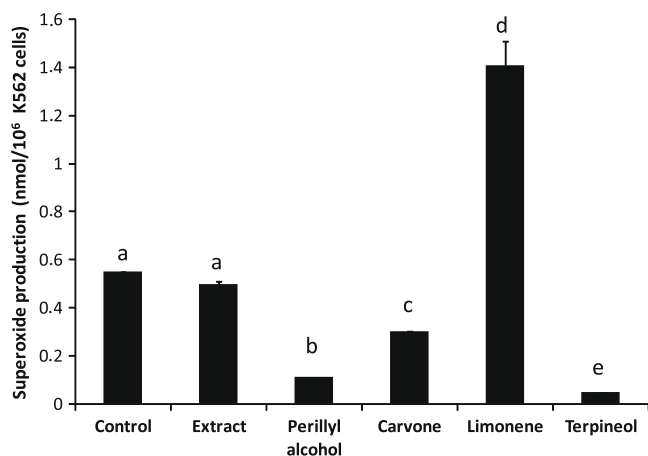

Fig. 4. Effect of the biotransformation extract and synthetic monoterpenes on superoxide (O_2^-) production by leukemic cells stimulated with phorbol myristate acetate. The final concentration of extract and standard monoterpenes was 1.33% (v/v) per well. The columns are the means ± SEM of three experiments. Columns with different letters are significantly different from each other ($p < 0.05$).

acetate and *p*-nitrophenyl trimethylacetate, and this principle is used in the colorimetric assay for GST activity. (Fig. 5) shows that the biotransformation extract and monoterpenes inhibited the formation of *p*-nitrophenol from *p*-nitrophenyl acetate. The extract inhibited the formation of *p*-nitrophenol to a greater extent than did the monoterpene standards, perillyl alcohol and limonene, with
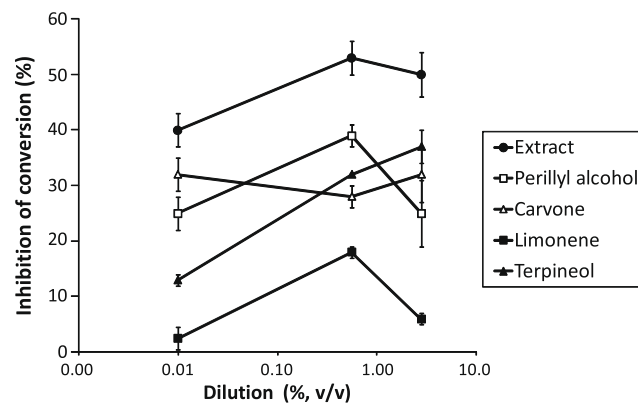
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