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The antioxidant and antigenotoxic properties of citrus phenolics limonene and naringin

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ABSTRACT

Phenolic compounds not only contribute to the sensory qualities of fruits and vegetables but also exhibit several health protective properties. Limonene and naringin are the most popular phenolics found in Citrus plants. In this study, we investigated the antioxidant capacities of limonene and naringin by the trolox equivalent antioxidant capacity (TEAC) assay and the cytotoxic effects by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay in Chinese hamster fibroblast (V79) cells. The genotoxic potentials of limonene and naringin were evaluated by micronucleus (MN) and alkaline COMET assays in human lymphocytes and V79 cells. Limonene and naringin, were found to have antioxidant activities at concentrations of 2–2000 μM and 5–2000 μM respectively. IC_{50} values of limonene and naringin were found to be 1265 μM and 9026 μM, respectively. Limonene at the concentrations below 10,000 μM and naringin at the all concentrations studied, have not exerted genotoxic effects in lymphocytes and in V79 cells. Limonene and naringin at all concentrations revealed a reduction in the frequency of MN and DNA damage induced by H_2O_2.

1. Introduction

Phenolic compounds are secondary metabolites which are the derivatives of the pentose phosphate, shikimate, and phenylpropanoid pathways in plants (Balasundram et al., 2006; Harborne and Williams, 2000). They contribute to the sensory qualities of fruits and vegetables: color, astringency, bitterness, and aroma (Alasalvar et al., 2001). Consumption of great amounts of fruits and vegetables rich in phenolic compounds has been associated with health benefits such as anti-allergenic, anti-artherogenic, anti-inflammatory, anti-microbial, antioxidant, anti-thrombotic, cardioprotective and vasodilatory effects (Balasundram et al., 2006; Moure et al., 2001).

Reactive oxygen species (ROS), produced in the course of several biochemical reactions, are extremely reactive intermediates and can cause damage to various biologic targets, such as proteins, DNA, and lipids. Phenolic compounds have been regarded as possible antioxidants, so they have been used in the food industry and in the prevention of diseases resulting from oxidative stress (Block et al., 1992; Nakatani, 2000). But on the other hand it is suggested that various phenolic antioxidants can display pro-oxidant properties at high doses (Wong and McLean, 1999).

Limonene (p-Mentha-1,8-diene) is a major component of oils obtained from *Citrus* plants, orange, lemon and grape fruit (Arruda et al., 2009; Del Toro-Arreola et al., 2005). Limonene is listed in the Code of Federal Regulation as generally recognized as safe (GRAS) for a flavoring agent (Sun, 2007). It is commonly used as an additive in foods, soaps and perfumes (Whysner and Williams, 1996). Limonene, which has been used to prevent gastric diseases, such as to dissolve gallstones (Sun, 2007), is also suggested to exert antiproliferative effects in various cancer cell types (Crowell et al., 1992; Swenberg, 1991).

Naringin (4′,5,7-trihydroxyflavanone 7-rhamnoglucoside), the predominant flavanone found in grapefruit and related *Citrus* species, is the main cause of bitterness in some *Citrus* fruits (Cavia-Saiz et al., 2010; Jagetia and Reddy, 2002). When naringin is administrated orally, it is hydrolyzed to naringenin which is the major absorbable metabolite (Kim et al., 1998). Naringin and naringenin have been suggested as the potential inhibitors of CYP enzymes (Ho et al., 2000). Naringin has showed several health promoting effects such as antioxidant, lipid-lowering, antimicrobial, anti-inflammatory and anticancer and the protective role of naringin against many pathological disorders depends on its antioxidant properties (Jeon et al., 2004).

The aim of this study is to evaluate the antioxidant activities of two phenolic compounds, limonene and naringin, commonly found in *Citrus* plants, as well as their cytotoxicity, genotoxic effects and antigenotoxic effects against hydrogen peroxide (H_2O_2).

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2. Materials and methods

The study was approved by Hacettepe University Local Ethical Committee (GO 13/578-16).

2.1. Chemicals

The chemicals used in the experiments were purchased from the following suppliers: giemsa, hydrogen peroxide (35%) (H_2O_2), from Merck Chemicals (Darmstadt, Germany), Dulbecco's Phosphate Buffered Saline, Fetal Calf Serum (FCS), Fetal Bovine Serum (FBS) and phytohaemagglutinin-M (PHA-M) from Biological Industries (Kibbutz Beit-Haemek, Israel), D-limonene from Fluka (St. Gallen, Switzerland), penicillin-streptomycin from PAA The CellCulture Company (Cansera, Canada), 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), potassium peroxodisulfat, (±)6-hydoxy-2,5,7,8-tetramethylchromon-2-carboxylic acid (trolox) (purity >97%), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), acridine orange, low melting point agarose (LMA), normal melting point agarose (NMA), ethidium bromide (EtBr), formamidopyrimidine DNA glycosylase (Fpg), L-glutamine, phosphate buffered saline (PBS) tablets, minimum essential medium (MEM), *N,N*-dimethylformamide, RPMI-1640, bovine serum albumin, cytochalasin-B (Cyt-B), sodium dodecyl sulfate (SDS), trypsin-EDTA and naringin from Sigma-Aldrich Chemicals (St. Louis, MO, USA).

2.2. Trolox equivalent antioxidant capacity (TEAC) assay

The antioxidant activities of limonene and naringin were determined in a cell-free system by TEAC assay as previously described by Miller et al. (1993). TEAC was measured spectrophotometrically at 734 nm by analyzing the decolorization of stable radical cation, ABTS, in the presence of different concentrations of limonene and naringin solutions or the same concentrations of the synthetic antioxidant trolox solutions in ethanol.

2.3. Cell culture

V79 cells were seeded in 75 cm^2 flasks in 20 ml MEM supplemented with 10% FCS and 1% penicillin-streptomycin and then grown for 24 h in an incubator at 37 °C in an atmosphere supplemented with 5% CO_2.

2.4. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay

MTT assay was performed by the method of Mosmann (1983) with the modifications of Hansen et al. (1989) and Kuźma et al. (2012). Following disaggregation of cells with trypsin/EDTA and resuspension of cells in medium, a total of 10^5 cells/well were plated in 96 well tissue-culture plates. After 24 h incubation, cells were exposed to the different concentrations of limonene and naringin (10, 100, 500, 1000, 2000, 5000, 10,000 and 20,000 μM) in medium for 24 h at 37 °C in 5% CO_2 in air. After exposure, the medium was aspirated and MTT (5 mg/ml of stock in PBS) was added (10 μl/well in 100 μl of cell suspension), and cells were incubated for an additional 4 h with MTT dye. At the end of incubation period, the dye was carefully taken out and 100 μl of SDS and *N,N*-dimethylformamide solution (pH 4.7) was added to each well. The absorbance of the solution in each well was measured in a microplate reader at 570 nm. Results were expressed as the mean percentage of cell growth from three independent experiments.

2.5. Single cell gel electrophoresis (COMET) genotoxicity assay in lymphocytes

Lymphocytes from whole heparinized blood were separated by Ficoll-Hypaque density gradient and centrifugation (Bøyum, 1976) then the cells were washed with PBS buffer. The concentration of the lymphocytes was adjusted to approximately 2×10^5 cells/ml in PBS buffer. The cells were treated with the increasing concentrations of limonene (500, 1000, 2000, 5000 and 10,000 μM) and naringin (50, 100, 500, 1000 and 2000 μM) for 30 min at 37 °C for the assessment of genotoxic effects of limonene and naringin. After the pretreatment with limonene and naringin, oxidative DNA damage was induced by replacing the medium with PBS containing 50 μM H_2O_2 and then incubating for 5 min on ice to assess the antigenotoxic effects of limonene and naringin. Then the lymphocytes were centrifugated and washed with PBS for removing the H_2O_2 solution. A negative control (1% DMSO) and a positive control (50 μM H_2O_2) were also included in the experiments.

Alkaline Comet assay technique of Singh et al. (1988), as further described by Collins (2009) and Aydın et al. (2013) was followed. The alkaline comet assay using Fpg, a lesion-specific enzyme, was used to detect oxidized purines as a result of oxidative stress-induced DNA damage as described with some modifications (Collins et al., 1993). The details of the alkaline comet assay with and without formamidopyrimidine-DNA glycosylase (Fpg) protocol was given previously (Taner et al., 2014). The cells were embedded on agarose gel, lysed, and fragmented DNA strands were drawn out by electrophoresis to form a comet. After electrophoresis, the slides were neutralized and then incubated in 50%, 75% and 98% of alcohol for 5 min each. The dried microscopic slides were stained with EtBr (20 μg/ml in distilled water, 60 μl/slide) with a Leica® fluorescence microscope under green light. The microscope was connected to a charge-coupled device camera and a personal computer-based analysis system (Comet Analysis Software, version 3.0, Kinetic Imaging Ltd, Liverpool, UK) to determine the extent of DNA damage after electrophoretic migration of the DNA fragments in the agarose gel. In order to visualize DNA damage, 100 nuclei per slide were examined at 40× magnification. Results were expressed as the length of the comet ("tail length"), the product of the tail length and the fraction of total DNA in the tail ("tail moment") and percent of DNA in tail ("tail intensity").

2.6. Single cell gel electrophoresis (COMET) genotoxicity assay in V79 cells

V79 cells were cultured as described in section 2.3. Following disaggregation of cells with trypsin/EDTA and resuspension of cells in medium, a total of 2×10^5 cells/well were plated in 6 well tissue-culture plates. After 24 h incubation, different concentrations of limonene (500, 1000, 2000, 5000 and 10,000 μM) and naringin (50, 100, 500, 1000 and 2000 μM) solutions were added to each plate and cells were incubated for an additional 1 h at 37 °C. After the pretreatment with limonene and naringin, oxidative DNA damage was induced by replacing the medium with PBS containing 50 μM H_2O_2 and then incubating for 5 min on ice to assess the antigenotoxic effects of limonene and naringin. Then the cells were centrifugated and washed with PBS for removing the H_2O_2 solution. A negative control (1% DMSO) and a positive control (50 μM H_2O_2) were also included in the experiments.

Cell suspension with LMA, lysis, electrophoresis, washing, staining and evaluation of DNA damage were performed as described in section 2.6. The experiments were repeated for three times.

2.7. Micronucleus (MN) genotoxicity assay in lymphocytes

The presence of MN in a binucleated cell using the protocol of Fenech (2000) was detected with minor modifications. 0.5 ml of heparinized blood samples were placed in sterile culture tubes containing 5 ml of RPMI 1640 medium supplemented with 10% FCS, 2% L-glutamine, 2% penicillin-streptomycin and 2.5% PHA-M. The samples were incubated at 37°C in a 5% CO_2 in air. After 24 h, different concentrations of limonene (500, 1000, 2000, 5000 and 10,000 μM) and naringin (50, 100, 500, 1000 and 2000 μM) solutions were added to the samples alone and also in combination with 50 μM H_2O_2 and they were incubated for another 48 h at 37°C in a 5% CO_2 in air. In all sets of experiments, an untreated negative control (1% DMSO) and a positive control (50 μM H_2O_2) were also used. Cytochalasin-B (Cyt-B) was added to the samples at a final concentration of 6 μg/ml at 44 h of the incubation. After the incubation and preparation of slides, 1000 binucleated cells were scored for the presence of MN. 500 lymphocyte cells from each slide were scored to evaluate the percentages of the cells with 1–4 nuclei. The nuclear division index (NDI) was calculated as follows: $NDI = [(1 \times N1) + (2 \times N2) + (3 \times N3) + (4 \times N4)]/N$, where N1–N4 represent the number of cells with 1–4 nuclei, respectively, and N is the total number of cells scored. The experiments were repeated for three times.

2.8. Micronucleus (MN) genotoxicity assay in V79 cells

V79 cells were plated in 6 well tissue-culture plates. After 24 h incubation, different concentrations of limonene (500, 1000, 2000, 5000 and 10,000 μM) and naringin (50, 100, 500, 1000 and 2000 μM) solutions were added to the samples alone and also in combination with 50 μM H_2O_2 and they were incubated for another 18 h at 37 °C in a 5% CO_2 in air. In all sets of experiments, an untreated negative control (1% DMSO) and a positive control (50 μM H_2O_2) were also used. The cells were fixed with cold ice-cold methanol:glacial acetic acid solution (3:1, v/v) for 15 min and fixation procedure was repeated for five times. The fixed cells were dropped onto slides previously cleaned with nitric acid and stayed in 70% ethanol. The dried microscopic slides were stained with acridine orange, covered with a cover-glass prior to analysis with a Leica® fluorescence microscope under green light. 1000 cells were scored for the presence of MN. The experiments were repeated for three times.

2.9. Statistical analysis

For statistical analysis of the MN assay results, the z-test was applied for the percentage of MN and NDI. The results were given as the mean ± standard error. For alkaline COMET and TEAC assays statistical analysis was performed by SPSS for Windows 20.0 computer program. The results were expressed as the mean ± standard deviation. Differences between the means of data were compared by the one way variance analysis (ANOVA) test and post hoc analysis of group differences by least significant difference (LSD) test. p value of less than 0.05 was considered as statistically significant.

3. Results

3.1. Antioxidant capacities of limonene and naringin

The antioxidant capacity of the different concentrations of limonene and naringin as measured by the TEAC assay is shown in Fig. 1. Limonene and naringin were found to have antioxidant activities at concentrations of 2–2000 μM and 5–2000 μM respectively.