Food Chemistry 114 (2009) 1386–1390

Contents lists available at ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

Antioxidant and antiproliferative properties of a tocotrienol-rich fraction from grape seeds

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ARTICLE INFO

Article history:
Received 8 July 2008
Received in revised form 2 November 2008
Accepted 6 November 2008

Keywords:
Tocotrienol-rich fraction
Grape seeds
Antioxidant activity
Antiproliferative activity

ABSTRACT

The antioxidant and antiproliferative activities of a tocotrienol-rich fraction (TRF) obtained from grape seeds were evaluated. TRF, a mixture of γ-tocopherol and α- and γ-tocotrienol, was prepared from a methanol-soluble fraction of grape seed oils by eluting with 10% ether (v/v) using silica gel chromatography. TRF had significantly higher antioxidant and antiproliferative activities compared to other fractions. TRF showed 3.5-, 40.0-, and 39.0-fold higher ABTS radical scavenging activity, inhibition of lipid peroxidation, and reducing power, respectively, compared to α-tocopherol fraction (5% diethyl ether fraction). TRF had higher antiproliferative activity against MCF7 (81%) and NCI-H460 (76%) cells at a concentration of 1.0 mg/ml. The results suggest that TRF from grape seeds has significant health-promoting effects, having excellent antioxidant and anticancer activities.

1. Introduction

In recent years, there has been much interest and research into the influence of diet on chronic diseases including cancer, coronary heart disease, atherosclerosis, and diabetes (Halliwell & Gutteridge, 1984; Steinberg, Parthasarathy, Carew, Khoo, & Witztum, 1989). Recent epidemiological studies have suggested that increased consumption of whole grains, legumes, fruits, and vegetables is inversely associated with the risk of chronic diseases (Hu, 2002). This association may be attributed to natural antioxidants such as vitamin C, vitamin E, polyphenol, and flavonoids, which prevent free radical damage (Diplock et al., 1998; Shahidi, 2004).

Agricultural and industrial residues are attractive sources of potential natural antioxidants. Grape seeds, a byproduct of the wine-making or juice-processing industry, constitute about 5% by weight of the grape and contain 10–20% oil with a high vitamin E content, which is important for human health. Commercial grape seed oil contains 399–785 mg/kg vitamin E, depending on the variety and environmental growing conditions (Crews et al., 2006).

Vitamin E is a generic term for tocopherols and tocotrienols, which possess a saturated phytyl tail and an unsaturated isoprenoid side chain, respectively. Tocopherols and tocotrienols are closely related chemically; however, they have widely varying degrees of biological activities (Theriault, Chao, Wang, Gapor, & Adeli, 1999). α-Tocopherol is regarded as intracellular antioxidants due to their activity in inhibiting the peroxidation of polyunsaturated fatty acids in biological membranes. Although α-tocopherol is the most active form in the vitamin E group *in vivo*, hypocholesterolemic, antitumor, neuroprotective, and antioxidant activities of tocotrienols or a tocotrienol-rich fraction (TRF) have recently received much attention (Khanna et al., 2003; Nesaretnam, Yew, & Wahid, 2007; Qureshi, Mo, Packer, & Peterson, 2000). Recent papers have only demonstrated the biological effects of TRF from barley, palm, and rice bran oils. Qureshi, Burger, Peterson, and Elson (1986) reported that α-tocotrienol from barley was an inhibitor of HMG-CoA reductase, which is the rate-limiting enzyme of the cholesterol biosynthetic pathway. In several recent studies, γ- and δ-tocotrienols from palm oil were shown to inhibit the growth of human breast cancer cells in culture (Nesaretnam et al., 2004). Furthermore, Serbinova, Tsuchiya, Goth, Packer, and Kagan (1993) reported higher antioxidant activity with tocotrienol than with α-tocopherol against lipid peroxidation in rat liver microsomes. However, no study has examined the biological effects of grape seed-derived tocotrienol-rich fraction (TRF) compared to those of tocopherols. Here we aimed to evaluate the antioxidant and antiproliferative activities of TRF obtained from grape seeds in relation to those of α-tocopherol.

2. Materials and methods

2.1. Chemicals

1,1-Diphenyl-2-picrylhydrazyl (DPPH), diammonium salt of 2,2-azino-bis-(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), potassium ferricyanide, ferric chloride, ferrous chloride, ferozine [3-(2-pyridyl)-5,6-bis-(4-phenylsulphonic acid)-1,2,4-triazine], linoleic

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doi:10.1016/j.foodchem.2008.11.018

acid, and thiazolyl blue terazolium bromide (MTT) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Tocopherol and tocotrienol standards were obtained from Merck (Darmstadt, Germany). All other reagents and solvents used were of analytical and HPLC grade. The human tumor cell lines MCF 7 (breast), NCI-H460 (lung), HCT116 (colon), and MKN45 (gastric) were obtained from the Korean Cell Line Bank (KCLB).

2.2. Purification and analysis of tocopherol and tocotrienols

Purification of TRF from grape seeds was carried out using silica gel chromatography as described by Qureshi et al. (2000) with some modifications. Briefly, Two kilograms of ground grape seeds (variety: *Campbell early*) were extracted with 5 l of hexane by shaking for 24 h at room temperature and filtered through Toyo No. 2 filter paper, and the combined extracts were evaporated under vacuum (hexane-soluble fraction). The oily residue (100 g) was extracted with 2 l of methanol by stirring for 24 h, and the methanol layer containing tocopherols and tocotrienols was separated and evaporated under vacuum (methanol-soluble fraction). The residue was redissolved in 50 ml of hexane for tocol analysis and silica gel (Merck, 230–400 mesh, 60 Å) chromatography. Silica gel activated with 500 ml of hexane was poured into a glass funnel and washed with 1 l of hexane prior to being loaded with methanol-soluble fraction. The tocopherols and tocotrienols were eluted with 5%, 10%, 15%, and 20% diethyl ether in hexane. The eluates were evaporated under vacuum, the residues were redissolved in 50 ml of hexane, and tocols were analysed using HPLC.

Analysis of tocopherols and tocotrienols was performed on a LiChrosphere® Diol 100 column (250 × 4 mm, i.d., 5 μm) using a mobile phase of hexane/isopropanol (98.7:1.3, v/v) at a flow rate of 1.0 ml/min. Peaks were detected by fluorescence using an excitation wavelength of 290 nm and an emission wavelength of 330 nm (Choi, Jeong, & Lee, 2007).

2.3. Determination of antioxidant activities of TRF

The scavenging activity of TRF on the ABTS radical cation was estimated according to the method of Re et al. (1999) with some modifications. The ABTS radical cation was generated by adding 7 mM ABTS to 2.45 mM potassium persulphate solution, and the mixture was left to stand overnight in the dark at room temperature. The ABTS radical cation solution was diluted with ethanol to obtain an absorbance of 1.0 at 734 nm. Diluted ABTS radical cation solution (1 ml) was added to 20 μl of sample fractions or Trolox® standard solution. The absorbance was measured at 734 nm after 30 min. The ABTS radical cation scavenging activity was expressed as Trolox® equivalent antioxidant capacity (TEAC) and defined as mg Trolox® equivalents per 1 g residue. α-Tocopherol (95% purity) was also assayed as a positive control.

The scavenging activity of TRF on the DPPH radical was measured according to the method of Kim, Lee, Lee, and Lee (2002) with some modifications. The 0.2 mM DPPH radical solution (1 ml) was added to 20 μl sample fractions or Trolox® standard solution. After 30 min, the absorbance was measured at 520 nm using a spectrophotometer (Beckman Instruments Inc., Fullerton, CA, USA). The DPPH radical scavenging activity was expressed as TEAC and defined as mg Trolox® equivalents per 1 g residue. α-Tocopherol was also assayed as a positive control.

The reducing power of TRF was determined according to the method of Oyaizu (1986) with some modifications. Sample fractions (100 μl), 200 mM sodium phosphate buffer (250 μl, pH 6.6), and 1% potassium ferricyanide (250 μl) were mixed and incubated in a water bath at 50 °C. After 20 min, 250 μl 10% trichloroacetic acid (w/v) were added to the mixture and centrifuged at 10,000 rpm (9800*g*) for 3 min. The supernatant (500 μl) was then mixed with an equal volume of distilled water and ferric chloride solution (0.1%, w/v). The intensity of blue–green colour was measured at 700 nm using a spectrophotometer. α-Tocopherol was also assayed as a positive control.

The inhibition of lipid peroxidation of the TRF, based on coupled oxidation of linoleic acid and β-carotene, was evaluated following the method of Taga, Miller, and Pratt (1984) with some modifications. β-Carotene (25 mg) was dissolved in 50 ml chloroform. A 3 ml aliquot of the β-carotene solution was mixed with 40 mg linoleic acid and 400 mg Tween 20. The chloroform was evaporated under vacuum at 30 °C, and distilled water (100 ml) was added to the dried mixture. Sample fractions (200 μl, 10 mg/ml) were added to 2 ml β-carotene emulsion, and the mixtures were incubated in a water bath at 50 °C. After 20 min, the absorbance of the mixtures was measured at 470 nm using a spectrophotometer. Inhibition of lipid peroxidation was expressed as the percentage activity relative to the control. α-Tocopherol was also assayed as a positive control.

The chelating activity of TRF was determined according to the method of Dinis, Madeira, and Almeida (1994). Sample fractions (100 μl) were reacted with 100 μl ferrous chloride (1 mM) and ferrozine (5 mM) for 10 min, and the absorbance of the mixture was measured at 562 nm. α-Tocopherol was also assayed as a positive control.

2.4. Determination of antiproliferative activities of TRF

Breast (MCF7), colon (HCT 116), lung (NCI-H460), and gastric (MKN 45) tumor cells were grown in RPMI containing 10% fetal bovine serum (FBS), 2 mM glutamine, 100 unit/ml penicillin, and 50 μg/ml streptomycin. The cultures were maintained in a humidified incubator with 5% CO_2 and seeded onto 75-cm^2 culture flask. Antiproliferative activities of grape seed TRF on tumor cells were measured by evaluating cell viability using the MTT assay (Mosmann, 1983). The cells were seeded at a density of 5×10^3 cells/well for MKN 45 and 2×10^3 cells/well for the other cell lines using a brief trypsinization, and then the α-tocopherol and sample fractions (1.0 and 0.5 mg/ml) were added into a 96-well plate. After 48 h of incubation, 20 μl of MTT reagent (5 mg/ml) were added and incubated for a further 4 h, and the absorbance of formazan was determined at 550 nm. The cell viability (%) was obtained by comparing the absorbance between the samples and a negative control. α-Tocopherol was also assayed as a positive control at the concentration of 1.0 and 0.5 mg/ml for antiproliferative activity.

2.5. Statistical analysis

The results were reported as means ± standard deviation (SD). The significance of differences among treatment means was determined by one-way analysis of variance (ANOVA) using SAS version 8.1 (SAS Institute, Cary, NC, USA) with a significance level of 0.05.

3. Results and discussion

3.1. Yields of methanolic extracts

The vitamin E isomers present in grape seeds (var. *Campbell early*) include α-tocopherol (αT), α-tocotrienol (αT3), γ-tocopherol (γT), and γ-tocotrienol (γT3). The vitamin E profiles and purities of hexane- and methanol-soluble fractions and TRF are presented in Table 1. Hexane was chosen to extract lipid-soluble substances from grape seeds such as tocopherols, tocotrienols, sterols, fatty acid esters, and triglycerides. Methanol was chosen to extract vitamin E from the hexane-soluble fraction because it extracts less