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

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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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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

Table 1
Concentration (mg/g) of vitamin E in grape seeds and their hexane-soluble fraction, methanol-soluble faction, and TRF.

Samples[a]	Tocols (mg/g of sample)					Purity of tocols (%)
	αT[b]	αT3	γT	γT3	Total	
Grape seeds	0.00	0.04	0.00	0.07	0.12	0.01
Hexane-soluble fraction	0.07	0.20	0.00	0.46	0.75	0.07
Methanol-soluble fraction	0.86	2.87	0.16	8.61	12.50	1.24
5% Fraction	1.26	–[c]	–	–	1.26	0.13
10% Fraction	–	5.06	0.27	16.20	21.53	5.64
15% Fraction	–	–	–	–	–	–
20% Fraction	–	–	–	–	–	–

[a] Mean of duplicate determinations expressed as mg/g of sample.
[b] Corresponding tocopherols and tocotrienols.
[c] Not detected.

total lipid material and is more effective in extracting tocotrienols than other non-polar solvents (Budin, Breene, & Putnam, 1995).

The 5% diethyl ether (v/v) elution only contained α-tocopherol. The TRF, a mixture of γ-tocopherol and α- and γ-tocotrienol, was prepared from the methanol-soluble fraction by eluting with 10% diethyl ether (v/v). The purity of TRF determined by HPLC was 5.64%. No tocopherols or tocotrienols were detected in the 15% and 20% diethyl ether fractions.

3.2. Antioxidant activity of TRF

Whereas numerous studies have investigated the antioxidant properties of tocopherols, much less is known about tocotrienols, particularly TRF, from grape seeds. In this study, we investigated the antioxidant activities of TRF, a mixture of γ-tocopherol and α- and γ-tocotrienol purified from grape seeds. Our results clearly demonstrate that TRF is more effective than other fractions in neutralising free and lipid peroxy radicals and in chelating prooxidant metals.

The antioxidant activities of the TRF compared to αT, as determined by the scavenging of ABTS and DPPH radicals, are presented in Fig. 1. The ABTS and DPPH radical scavenging activities of α-tocopherol were 29 and 19 TEAC (mg/g residue), respectively. The 10% fraction (TRF) had significantly higher ABTS (14 TEAC) and DPPH (13 TEAC) radical scavenging activity than other fractions (0.2–0.8 and 0.3–0.8 TEAC, respectively). Several *in vitro* and *in vivo* studies have shown that TRF is a more potent antioxidant than α-tocopherol (Nesaretnam, Devasagayam, Singh, & Basiron, 1993; Serbinova et al., 1993). This property of tocotrienols is believed to be related to the presence of an unsaturated side chain, which can more easily be incorporated into cells, and higher recycling efficiency from chromoxyl radicals (Serbinova, Kagan, Han, & Parker, 1991). We found that α-tocopherol possessed higher free radical scavenging activity than TRF. This might be related to the purity of TRF, which contains approximately 6% tocols.

The reducing power of α-tocopherol and TRF is presented in Fig. 2. In the method used, the ferric–ferricyanide complex is reduced to the ferrous form with dependence on the presence of antioxidants (Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004). α-Tocopherol (A_{700} = 1.7) and TRF (A_{700} = 1.2) also had significantly higher reducing power than other samples (A_{700} = 0.02–0.4), showing a significant correlation with ABTS (R^2 = 9229, $p < 0.05$) and DPPH (R^2 = 9847, $p < 0.05$) radical scavenging activities.

The inhibitory effect of TRF on lipid peroxidation (Fig. 3) was evaluated using a β-carotene and linoleic acid model system. Inhibitory effects of TRF and the methanol-soluble fraction on lipid peroxidation were higher than in other fractions. The order of activity was TRF > αT > methanol-soluble fraction > 15% fraction > 20% fraction > 5% fraction. In a recent study (Serbinova et al., 1993), tocotrienols showed remarkably higher antioxidant activity than αT against lipid peroxidation in rat liver microsomes. Moreover, Pearce, Parker, Deason, Qureshi, and Wright (1992) reported the

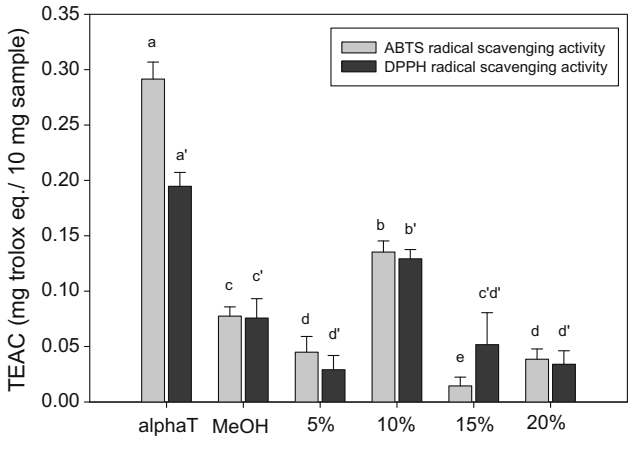

Fig. 1. Scavenging effect of tocotrienol-rich fractions (10 mg/ml) from grape seeds on ABTS and DPPH radical. Alpha T = α-tocopherol; MeOH = methanol-soluble faction; 5% = fraction eluted with 5% diethyl ether; 10% = fraction eluted with 10% diethyl ether (TRF); 15% = fraction eluted with 15% diethyl ether; 20% = fraction eluted with 20% diethyl ether. Values with different letters above bar graphs are significantly different at the 5% level by one-way ANOVA and Duncan's test.

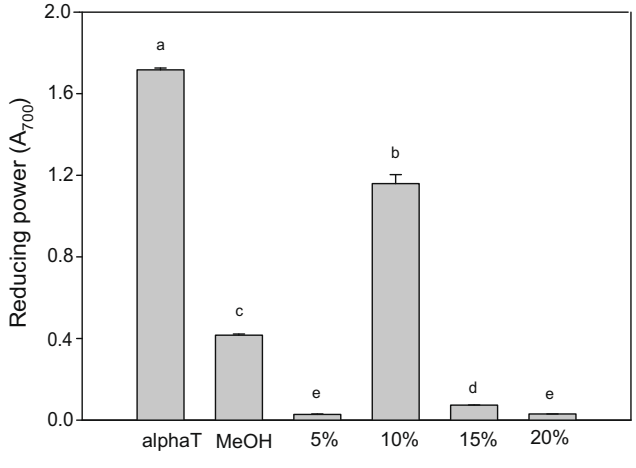

Fig. 2. Reducing power of tocotrienol-rich fractions (10 mg/ml) from grape seeds. Alpha T = α-tocopherol; MeOH = methanol-soluble faction; 5% = fraction eluted with 5% diethyl ether; 10% = fraction eluted with 10% diethyl ether (TRF); 15% = fraction eluted with 15% diethyl ether; 20% = fraction eluted with 20% diethyl ether. Values with different letters above bar graphs are significantly different at the 5% level by one-way ANOVA and Duncan's test.

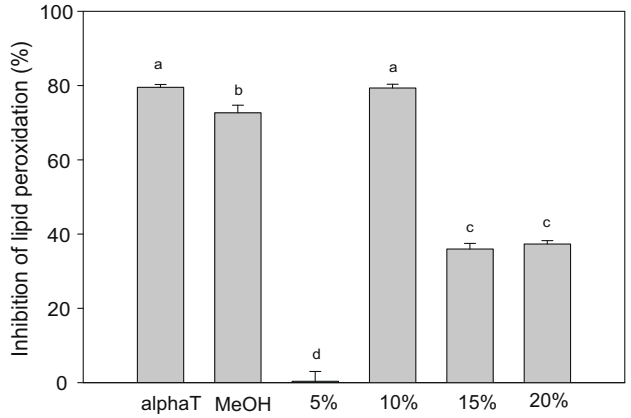

Fig. 3. Inhibition of lipid peroxidation of tocotrienol-rich fractions (10 mg/ml) from grape seeds. Alpha T = α-tocopherol; MeOH = methanol-soluble faction; 5% = fraction eluted with 5% diethyl ether; 10% = fraction eluted with 10% diethyl ether (TRF); 15% = fraction eluted with 15% diethyl ether; 20% = fraction eluted with 20% diethyl ether. Values with different letters above bar graphs are significantly different at the 5% level by one-way ANOVA and Duncan's test.

Table 2
Antiproliferative activity of tocotrienol-rich fractions from grape seeds against human tumor cell lines.

Sample (mg/ml)	Human tumor cell lines (% of cytotoxicity)			
	MCF7 (Breast)	NCI-H460 (Lung)	HCT116 (Colon)	MKN45 (Gastric)
Alpha T				
0.5	33 ± 3.9	19 ± 1.1	15 ± 7.3	16 ± 8.1
1.0	23 ± 1.9	32 ± 5.9	4 ± 3.2	4±3.8
MeOH Fraction				
0.5	28 ± 4.2	20 ± 4.8	26 ± 5.9	14 ± 1.7
1.0	20 ± 6.0	21 ± 6.3	27 ± 7.8	17 ± 8.3
5% Fraction				
0.5	31 ± 3.2	21 ± 11.2	17 ± 2.2	9 ± 8.0
1.0	32 ± 4.1	28 ± 3.5	10 ± 7.2	32 ± 8.2
10% Fraction				
0.5	30 ± 14.2	25 ± 13.8	16 ± 3.3	32 ± 4.8
1.0	81 ± 3.3	76 ± 0.8	17 ± 3.3	47 ± 11.0

Alpha T = α-tocopherol; MeOH = methanol-soluble faction; 5% = fraction eluted with 5% diethyl ether; 10% = fraction eluted with 10% diethyl ether (TRF).

relative antioxidant capacity of different vitamin E isomers *in vitro*, by determining their ability to protect against Cu-induced LDL lipoprotein oxidation. They found that the order of the inhibitory effect of LDL oxidation was γT3 > γT > αT3 > αT. In our results, the inhibitory effect of the methanol-soluble fraction on lipid peroxidation was similar to that of αT and TRF. This suggests that there might be synergistic effects and/or additive interactions between the various antioxidants including tocols, polyphenols and phytosterols that give rise to the net antioxidant capacity in the methanol-soluble fraction (Qureshi et al., 2000).

Although iron is essential for oxygen transport, respiration, and activity of enzymes, it is a reactive metal that catalyzes oxidative damage in living tissues and cells (Miller, 1996). The chelating effects (%) of α-tocopherol and TRF (10 mg/ml) on ferrous ion are presented in Fig. 4. TRF showed the highest chelating effect (58%) compared to the other samples (13–51%). However, no correlation was observed with four different methods. This suggests that the relative antioxidant activities depend on the experimental conditions and may vary significantly according to the conditions and evaluation methods used (Yoshida, Niki, & Noguchi, 2003).

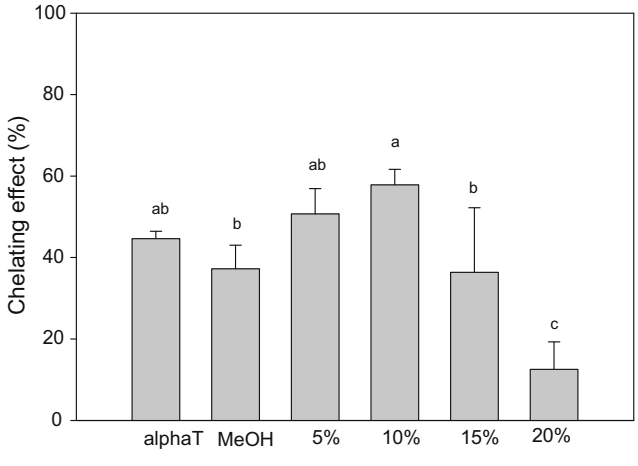

Fig. 4. Chelating effect of tocotrienol-rich fractions (10 mg/ml) from grape seeds. Alpha T = α-tocopherol; MeOH = methanol-soluble faction; 5% = fraction eluted with 5% diethyl ether; 10% = fraction eluted with 10% diethyl ether (TRF); 15% = fraction eluted with 15% diethyl ether; 20% = fraction eluted with 20% diethyl ether. Values with different letters above bar graphs are significantly different at the 5% level by one-way ANOVA and Duncan's test.

In this study, the 15% and 20% fractions, which contain no tocols, showed similar ABTS and DPPH radical scavenging activities and reducing power and a much better inhibitory effect on lipid peroxidation compare to the 5% fraction (αT fraction). Although αT is the most active form in the vitamin E group, the 5% contains only 100 ng/ml of αT in the assay mixture due to the low purity of tocols (0.13% purity), which is insufficient to provide significant antioxidative activities compared to αT positive control (95% purity). Moreover, the 15% and 20% fraction might contain unidentified vitamin E analogues and unsaponifiable matters such as low molecular weight polyphenols and phytosterols, which had been shown antioxidant activity (Minhajuddin, Beg, & Iqbal, 2005). Further research on isolation, identification and characterisation of unidentified tocols is needed to understand this phenomenon.

3.3. Antiproliferative activity of TRF

The antiproliferative effects of TRF on breast (MCF7), lung (NCI-H460), colon (HCT116), and gastric (MKN45) cancer cells were quantified in terms of cytotoxicity (%). The 15% and 20% diethyl ether fractions were not evaluated for antiproliferative activity because these two fractions did not contain any tocols and had lower antioxidant activities as evaluated by five different methods. TRF had the highest cytotoxicity (Table 2) at a concentration of 1 mg/ml against MCF7 (81%) and NCI-H460 (76%) cells, whereas it had lower antiproliferative activity against HCT116 (17%) and MKN45 (47%) cells. αT had relatively lower antiproliferative activities than TRF against all tumor cells. Nesaretnam, Stephen, Dils, and Darbre (1998) reported that TRF of palm oil inhibits growth of MCF7 cells completely at 8 μg/ml with no inhibitory effect of αT. In another study, Campbell et al. (2006) were shown that γ-tocopherol results in significant cell death (approximately 15%) for HCT116 cell lines at a 200 μM.

During the past decade, experimental studies, both *in vivo* and *in vitro*, have suggested that tocotrienols exhibit antitumor activities by inhibiting the growth and proliferation of many cancer cells, such as breast, lung, and liver cancer cells (Nesaretnam et al., 2007; Wada et al., 2005). In particular, TRF from palm oil was shown to inhibit the growth of two human breast cancer cell lines (MDA-MB-231 and MCF7) by modulating the c-Myc-binding protein MM-1 and the interferon-inducible protein 9-27 (IFITM-1) gene (Nesaretnam et al., 2004). Moreover, tocotrienols prevent the

formation of new blood vessels, thus preventing the growth and proliferation of cancer, and they induce apoptosis of cancer cells (Miyazawa, Inokuchi, Tsuzuki, Nakagawa, & Igarashi, 2003).

In conclusion, TRF, a mixture of γ-tocopherol and α- and γ-tocotrienol, purified from grape seeds showed significantly higher antioxidant activity and antiproliferative activity against breast and colon cancer cells than other samples. Our results suggest that TRF from grape seeds has significant health-promoting effects through its excellent antioxidant and anticancer activities and provides a source of antioxidants for the development of functional foods.

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