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Optimal antioxidant activity with moderate concentrations of Tocotrienol rich fraction (TRF) in *in vitro* assays

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Abstract

Vitamin E is known to have potent antioxidant activity and plays an important role in reducing oxidative stress, a pivotal step in atherogenesis. However, several randomised clinical trials using α-tocopherol have failed to demonstrate consistent beneficial effects of antioxidants against atherosclerosis and clinical endpoints. Tocotrienol, a vitamin E compound analogue is shown to have more potent antioxidant activity compared to tocopherol. Finding the optimal anti-oxidative dose is crucial and may effectively be applied for cardioprotection in human. The objective of this study was to determine the optimal dose of tocotrienol rich fraction (TRF) with highest antioxidant activity *in vitro* using the ferric thiocyanate (FTC), 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity and 2', 7'- dichlorofluorescein diacetate (DCFHDA) assays. It was found that TRF exhibited potent antioxidant and free radical scavenging activities with an IC_{50} of 22.10 ± 0.01 µg/ml. In all assays, TRF had optimal antioxidant activity at moderate concentrations (10-100 µg/ml). In conclusion, TRF has potent antioxidant activity, which is optimal at moderate concentrations.

Introduction

Vitamin E is an essential, fat-soluble nutrient that functions as an antioxidant in the human body. Eight compounds have been found to have vitamin E activity: α, β, γ and δ tocopherol and α, β, γ and δ tocotrienol, all of which are potent membrane soluble antioxidants (Sen *et al*., 2006). However to date, although tocotrienol has been shown to have more potent antioxidant activity compared to tocopherol, most of the vitamin E basic science and clinical studies used tocopherol with few studies using tocotrienol (Aggarwal *et al*., 2010).

High quantities of tocotrienol are derived from crude palm oil extracted from our local fruits of *Elaeis guineensis* (palm tree) (Sen *et al*., 2006). Tocotrienol is also found in wheat, rice bran, barley and annatto (Tan, 2010). Tocopherol and tocotrienol share similar structure with the exception of their side chains. Whilst tocopherol has a saturated phytyl tail, tocotrienol possesses an unsaturated isoprenoid side chain. The side chain of tocotrienol has been shown to alter its membrane distribution and metabolism considerably compared with tocopherol (Theriault *et al*., 2002). The more uniform distribution in the membrane lipid bilayer may provide a more efficient interaction of the chromanol ring of tocotrienol with lipid radicals (Serbinova *et al*., 1991).

By virtue of the antioxidant properties of vitamin E, several clinical trials have been conducted to test the effect of vitamin E supplementation on clinical end points such as cardiovascular death and all-cause mortality. When used in secondary prevention, vitamin E had shown neutral effects and lack of clinical efficacy in the improvement of cardiovascular outcomes (Tribble *et al*., 1999). There are several explanations given for the observed findings such as differences in the cohorts of subjects, timing of antioxidant treatment and the preparation type and dosage given (Vivekanathan *et al*., 2003). The appropriate optimal dose in obtaining beneficial effects in the prevention and inhibition of atherosclerosis is still unclear. Recently, high dose of vitamin E i.e. α-tocopherol (> 400 IU/day or 267mg/day) supplement has been reported to increase all-cause mortality by a group of researchers (Miller *et al*., 2005; Gee, 2011). It needs to be noted that, tocopherol but not tocotrienol was used as the vitamin E supplement for all the clinical trials included in the meta-analysis. In contrast, clinical data on tocotrienol is scarce.

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Oxidative stress and low density lipoprotein (LDL-c) oxidation are key issues in the pathogenesis of atherosclerosis. Peroxidation of LDL is known to be the first step in the development of atherosclerosis (Qureshi *et al*., 1995). It is postulated that in oxidative stress, the reactive oxygen species (ROS) is directly involved in the various mechanisms of atherogenesis such as endothelial dysfunction, monocyte migration, LDL oxidation and smooth muscle proliferation (Berliner *et al*., 1996). Although tocotrienol has been reported to have potent antioxidant activity in various *in vitro* (Kamat and Devasagayam, 1995; Das *et al*., 2005; Ahmad *et al*., 2005) and animal studies (Soelaiman *et al*., 2004) the optimal doses at which these effects are exhibited are not known.

The objectives of this study were to examine the relationship between different concentrations of tocotrienol rich fraction (TRF) with antioxidant activity, and to determine the optimal TRF concentration with maximal antioxidant activity in each assay.

Materials and Methods

Materials

TRF was supplied by Golden Hope Jomalina Sdn. Bhd, Malaysia. The composition of TRF used in this study is described in Table 1. Linoleic acid, ammonium thiocyanate, quercetin, vitamin C, butylated hydroxytoulene (BHT), α-tocopherol, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2'7'–dichlorofluorescein diacetate (DCFHDA), lipopolysaccharides (LPS) and hanks balanced salt solution (HBSS) without phenol red were purchased from Sigma Aldrich (USA). Ferrous Chloride, 3.5% hydrochloric acid and methanol was purchased from Merck (USA). Ethanol was purchased from Scharlau (Hungary). Dulbecco's Modified Eagle Media (DMEM) was purchased from Flow Lab (Australia). Fetal calf serum (FCS) and penicillin/streptomycin were purchased from PAA laboratories (Austria). Recombinant Mouse Gamma Interferon (IFN-γ) was purchased from eBioscience (San Diego, USA).

Ferric thiocyanate (FTC) assay

The peroxide level at the primary stage of linoleic acid oxidation was measured by FTC method which was adapted from Osawa and Namiki (1981). A mixture of 4 ml of different concentrations of TRF (0.1–20,000 µg/ml), 4.1 ml of 2.5% linoleic acid in absolute ethanol, 8 ml of 0.05 M phosphate buffer (pH 7.0) and 3.9 ml of water was placed in a vial with a screw cap and then placed in an oven at 40°C in the dark. Then, 0.1 ml of this solution was added to a reaction mixture containing 9.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate and 0.02 M ferrous chloride in 3.5% hydrochloric acid. The peroxide level was then determined by reading the absorbance at 500 nm using a spectrophotometer (Cary UV 50) for every 24 hours until the absorbance of the control reached maximum value. During linoleic acid oxidation, peroxides are formed which oxidise Fe^{2+} to Fe^{3+}. The latter ions form a red colour complex with thiocyanate and this complex has a maximum absorbance at 500 nm. Quercetin, BHT and tocopherol were used as positive controls. The controls were subjected to the same procedures as the samples except that solvent or control compounds were added in the negative and positive controls respectively. All samples were prepared in triplicates. The percentage inhibition of lipid peroxidation in linoleic acid emulsion was calculated by the following equation:

Table 1. The composition of tocotrienol rich fraction (TRF) used in *in vitro* assays

Vitamin E components	Percentage (%)	Weight (mg/g)
α- Tocopherol	34.5	233.4
α - Tocotrienol	26.7	180.7
β - Tocotrienol	2.2	14.6
γ - Tocotrienol	22.8	154.5
δ - Tocotrienol	13.8	93.3
Total	100	676.5

$$\text{Inhibition of lipid peroxidation (\%)} = 100 - [(A_1/A_0) \times 100],$$

Where A_0 = Absorbance of the control reaction
A_1 = Absorbance in the presence of the TRF or standards.

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity

The DPPH assay measures the ability of different concentrations of TRF to donate hydrogen, thus indicates its free radical scavenging activity. DPPH is maximally absorbed at 517 nm, and decreases upon reduction by antioxidants. A solution of DPPH was prepared by dissolving 5 mg of DPPH in 2 ml of methanol (MeOH) and the solution was kept in the dark at 4 °C. A stock solution of TRF at a concentration of 1000 µg/ml was prepared by diluting the crude TRF extract in absolute methanol. An amount of 100 µl of TRF stock solution (1000 µg/ml) was added into the well and it was further down diluted to the lowest concentration (7.8 µg/ml) by performing a serial two fold dilution in a 96-well microtiter plate. A row of negative DPPH control was developed in the same 96-well microtiter plates by adding 100 µl of MeOH into each well. Quercetin, vitamin C and tocopherol were used as positive controls. Then, 5 µl

of methanolic DPPH was added into each well and reaction was allowed to proceed for 30 minutes in dark. The absorbance was measured at 517 nm by a microplate reader (Micro Quant, Biotek Instruments). The lower the absorbance reading of the reaction mixture, the higher the free radical scavenging activity. The DPPH scavenging effect (%) was calculated using the following formula:

$$\text{DPPH scavenging effect (\%)} = \frac{\text{OD (DPPH)} - \text{OD (DPPH + samples)}}{\text{OD (DPPH)}} \times 100\ \%$$

The IC_{50} value was defined as the concentration of TRF which reduced the initial DPPH concentration by 50% as compared to the negative control. All samples were performed in triplicates.

2', 7'- dichlorofluorescin diacetete (DCFHDA)

Preparation of different concentrations of TRF

A stock solution of TRF at 50 g/l was prepared in absolute ethanol and stored at -80°C. The TRF stock was then mixed with FCS at a ratio of 1:20 and incubated at 37°C for 15 minutes during which time a brief vortex was conducted every 5 minutes. By using this method, it has been shown that there was an increased of cellular vitamin E uptake in a dose-dependent manner (Martin *et al*., 1997). The FCS-TRF was then diluted into various concentrations of TRF with DMEM by performing a serial 2 fold dilution in 96-well microtiter plates, with 'U' bottom (BD Falcon, UK). The concentration of TRF was started from 290 µg/ml, which was added into the first well. Each TRF concentration was prepared in triplicates.

Cell culture

Murine monocytic cell lines (RAW 264.7) was obtained from American Type Culture Collection (ATCC), USA. Cells were cultured in DMEM supplemented with 10% FCS and antibiotics (50 U/ml penicillin and 50 µg/ml streptomycin). Cells were grown in 75 cm2 flasks (Corning, USA) at 37°C in a humidified CO_2 incubator. Cells were subcultured by gentle scrapping and sub cultivation ratio 1:3 to 1:6 (culture:medium).

Stimulation of RAW 264.7 cells

Adherent RAW 264.7 cells with 80-90% confluence were detached from plastic surface by a gentle scrapping. The suspension was then centrifuged at 1000 RPM for 10 minutes at 4°C. The concentration of cells was then adjusted to 1 x 10^6 cells/ml. An amount of 50 µl of diluted cell suspensions (1 x 10^6 cells/ml) in the DMEM was dispensed into all wells (this yielded a concentration of $5x10^4$ cells/well). RAW 264.7 cells were seeded in 96-well black microtiter plates with flat bottom (BD Falcon, UK) and incubated for 2 hours at 37°C, 5% CO_2 to allow for adherence. The media was then gently removed to discard unattached cells. Attached cells were then stimulated with an inducer containing recombinant mouse IFN-γ (400 U/ml) and LPS (20 µg/ml). Then, 50 µl from each concentrations of the serially diluted TRF were transferred into each wells containing stimulated RAW 264.7. Each well contained the same levels of FCS (10%) and ethanol (0.1%). Four control groups were prepared in the same culture plate which consists of Control 1 (cells in complete media), Control 2 (cells in complete media and inducer with 0.1% ethanol, Control 3 (cells in complete media with inducer only) and Control 4 (cells in complete media with 2 mM or 5 µg/ml (final concentration) quercetin, inducer and 0.1% ethanol). Cells were then incubated for 18 hours in a humidified incubator set at 37°C and 5% C_{O2}. Then, cells were prepared for cell cytotoxicity and micro-fluorometric kinetic assay.

Cell cytotoxicity

Cell cytotoxicity of RAW 264.7 cells with different concentrations of TRF was measured by the [3-(4, 5-Dimethylthiazol-2-yl)]-2, 5-diphenyltetrazolium bromide] metabolic assay or MTT assay (Mosmann, 1983). The [3-(4, 5-Dimethylthiazol-2-yl)] - 2, 5-diphenyltetrazolium bromide] is cleaved to formazan by the succinate-tetrazolium reductase system, which belongs to the mitochondrial respiratory chain and is active in living cells. Mitochondrial electron transport plays a major role in the cellular reduction of MTT and occurs mostly in the cytoplasm. Stimulated RAW 264.7 in each well was added with 20 µl of MTT at a concentration of 5 mg/ml in PBS and incubated for 4 hours in a CO_2 incubator at 37°C. Then, 170 µl of the medium was removed from each well before the addition of 100 µl of DMSO to solubilize the formazan crystal formed after the incubation with MTT. The plate was then incubated at room temperature for 30-50 minutes. The absorbance was measured at 550 nm using a microplate reader (Micro Quant, Biotek Instruments Inc, USA).

Micro-fluorometric kinetic assay (Dichlorofluorescein diacetate assay)

Intracellular oxidant stress was monitored by micro-fluometric kinetic assay (Wang and Joseph, 1999). Upon entry, DCFHDA is cleaved by cellular esterases to nonfluorescent 2',7'-dichlorofluorescein

(DCFH) and oxidized by radical oxygen species (ROS) to a fluorescent product dichlorofluorescein (DCF). Thus, the DCF fluorescence is directly proportional to the ROS productions. Stimulated RAW 264.7 cells were added with 10 µl of 10 mM 2',7'-dichlorofluorescein diacetate (DCFH/DA) into each well. The production of fluorescence intercellular was monitored kinetically for 30 minutes at 485 nm and 530 nm excitation and emission filters respectively by using a fluorescence plate reader. Data points were taken every 5 minutes over 30 minutes. Percentage of increase in production of fluorescence signal in each well was calculated by the following formula:

$(Ft_{30} - Ft_0) / Ft_0 \times 100$

Ft_{30} = fluorescence at 30 minutes and
Ft_0 = fluorescence at 0 minute (baseline)

Statistical analysis

Data were analysed using the SPSS statistical package version 12. Data are presented as mean ± SEM. The effects of different concentrations of TRF on oxidative stress were compared by ANOVA. Comparison between 2 different groups was analyzed by independent T-test. Significant level was set at $p<0.05$.

Results

Ferric thiocyanate method

Figure 1(a) shows the absorbance values of different concentrations of palm oil derived tocotrienol rich fraction (TRF) measured by FTC method. TRF ranging from 0.1 to 20,000 µg/ml was measured using this method with both positive and negative controls included. Quercetin and BHT (4000 µg/ml) were used as positive controls while solvent (instead of TRF) was used as a negative control. Lower absorbance value indicates less formation of peroxides and the higher antioxidant activity. BHT and Quercetin have been shown to inhibit the peroxides formation as indicated by low absorbance reading (0.003 ± 0.0 and 0.1 ± 0.005 respectively). Different concentrations of TRF resulted in a significant inhibition ($p<0.0001$) of peroxide production. The 'U' shape trend was observed from the results with TRF at the moderate concentrations ranging from 10-100 µg/ml) showing the lowest absorbance readings of 0.050 ± 0.001 and 0.050 ± 0.002 respectively. The highest TRF concentrations (20,000 µg/ml) that was used in this assay had higher absorbance value compared to TRF at concentrations of 10 - 100 µg/ml and the lowest concentration of TRF (0.1 µg/ml) used by this assay had the highest absorbance reading (1.00). Percentage (%) inhibition of different concentrations

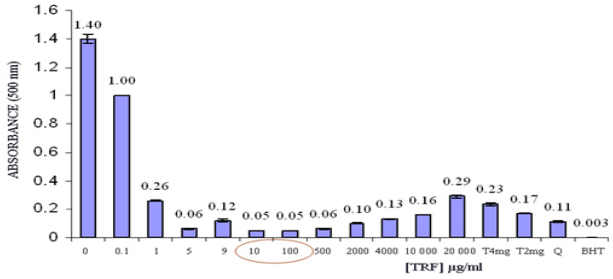

Figure 1(a). Absorbance values of different concentrations of TRF (0.1 – 20 000 µg/ml) as measured by FTC method. BHT and Quercetin (4000 µg/ml) as the positive controls. FTC - Ferric Thiocyanate; BHT - Butylated Hydroxytoluene; Q - Quercetin; TRF - Tocotrienol Rich Fraction. Data are expressed as mean ± SEM. ANOVA (0.1 – 20 000 µg/ml) was $p<0.0001$.

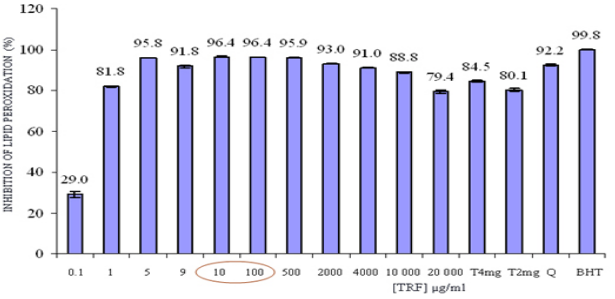

Figure 1(b). Percentage (%) inhibition of different concentrations of TRF (0.1 – 20 000 µg/ml) in FTC method. FTC - Ferric Thiocyanate; BHT - Butylated Hydroxytoluene; Q - Quercetin. Data are expressed as mean ± SEM. ANOVA (0.1 – 20 000 µg/ml) was $p<0.0001$.

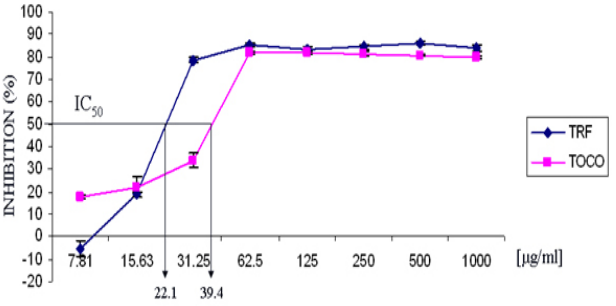

Figure 2(a). Percentage (%) inhibition of different concentrations of TRF and α- tocopherol in DPPH free radical scavenging assay. TRF - Tocotrienol Rich Fraction; TOCO - Tocopherol; DPPH - 1,1-diphenyl-2-picrylhydrazyl. Data are expressed as mean ± SEM.

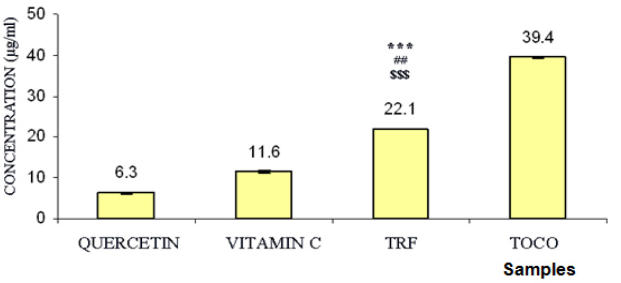

Figure 2(b). The IC_{50} comparison of TRF with quercetin, vitamin C and tocopherol in DPPH free radical scavenging activity assay. TRF - Tocotrienol Rich Fraction; TOCO - Tocopherol; DPPH - 1,1-diphenyl-2-picrylhydrazyl.Data are expressed as mean ± SEM. ***$p <$ 0.0001 compared to quercetin, ## $p<0.005$ compared to vitamin C, $$$ $p<0.0001$ compared to tocopherol.

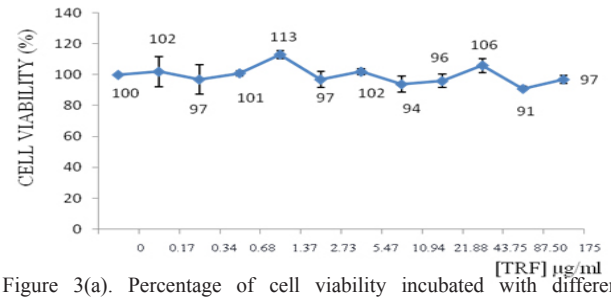

Figure 3(a). Percentage of cell viability incubated with different concentrations of TRF (0 – 175 µg/ml). TRF - Tocotrienol Rich Fraction. Data are expressed as mean ± SEM.

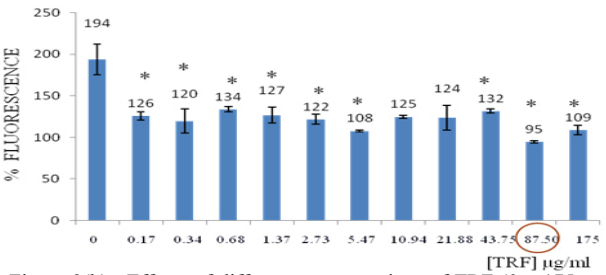

Figure 3(b). Effects of different concentrations of TRF (0 – 175 µg/ml) on the percentage increase of DCF fluorescence in RAW 264.7. TRF - Tocotrienol Rich Fraction; DCF – dichlorofluorescein. Data are expressed as mean ± SEM. * p<0.05 compared to negative control (0 µg/ml).

of TRF as compared with the negative control is illustrated by Figure 1(b). BHT and quercetin resulted in the inhibition resulting in 99.8 ± 0.3% and 92.2 ± 0.4% of peroxide formation respectively. TRF at 20,000 µg/ml and 0.1 µg/ml concentrations reduced production of peroxides by 79.4 ± 0.8% and 29.0 ± 1.4% respectively. TRF at 10 and 100 µg/ml concentrations have the highest inhibitory effects (96.4 ± 0.2% and 96.3 ± 0.2%).

DPPH radical scavenging method

The radical scavenging activity of different concentrations of TRF was determined from the percentage of absorbance reduction at 517 nm due to TRF scavenging of stable DPPH free radical. Initially, the percentage inhibition increased with increasing TRF concentration, up to 31.3 µg/ml, after which the effect started to plateau off. The optimal TRF concentration was observed at 62.5 µg/ml with inhibition of 85.2 ± 0.8%, Figure 2(a). Similar trend was shown by α-tocopherol where the optimal tocopherol concentrations were observed at 62.5 µg/ml with 81.6 ± 0.6% inhibition. Figure 2 (b) shows the IC_{50} (inhibition concentration 50%, the concentration that gave 50% inhibition as compared to the untreated) comparison of TRF with quercetin, vitamin C and tocopherol. The IC_{50} of TRF at 22.1 ± 0.01 µg/ml, was higher than that for quercetin (6.3 ± 0.3 µg/ml, p<0.0001) and vitamin C (11.6 ± 0.3 µg/ml, p<0.005), but lower than α-tocopherol (39.4 ± 0.2 µg/ml, p<0.0001). This indicates that TRF is a more potent antioxidant than tocopherol but is less potent compared to quercetin and vitamin C.

Effects of TRF on cell viability

Figure 3 (a) shows the RAW 264.7 cell viability assays performed with different concentrations of TRF. The cell viability was unaffected by TRF concentration up to 175 µg/ml with the percentage of viability was higher than 90 %. Therefore, the inhibition could not be explained by detachment of the HUVECs from the culture plates. Hence, TRF concentration up to 175 µg/ml was used for the DCFHDA assay.

Percentage increase of DCF fluorescence in RAW 264.7 cells (DCFHDA assay)

Figure 3 (b) shows the effects of different concentration of TRF (0–175 µg/ml) on percentage increase of DCF fluorescence in RAW 264.7 cells stimulated with LPS and IFN-γ. Fluorescence reading was taken for 30 minutes after the addition of DCFH-DA (10 mM) into each well and the percentage increase was calculated for each samples [(Fluorescence at time 30 min – Fluorescence at time 0 min)/ Fluorescence reading at 0 min x 100]. The lower the percentage increases of DCF fluorescence production, the lower the ROS production. Throughout all TRF concentrations, percentage increase of DCF fluorescence production was lower than negative control or 0 (RAW 264.7 cells with the presence of LPS + IFNγ only). Lowest percentage increase of DCF fluorescence production was at 87.5 µg/ml TRF concentration compared to negative control (95 ± 1% vs 194 ± 19%, p< 0.05).

Discussions

In view of the important role of oxidative stress in atherogenesis, the antioxidant properties of vitamin E have stirred great research interest (Berliner *et al*., 1995; Ross, 1999). It is predicted that antioxidants should provide therapeutic benefits in human atherosclerotic vascular disease (Azzi *et a*l., 1998). There is a link between oxidative stress with inflammation and endothelial dysfunction in atherogenesis (Brand *et al*., 1996; Collins, 1993). The oxidation theory suggests that modified lipoproteins within the arterial wall are essential to atherogenesis, by attracting and damaging the endothelium, attracting monocytes, and promoting foam cell formation. Accumulation of cytotoxic ox- LDL in the vascular wall may lead to the production of oxygen derived free radicals and causes inactivation of endothelium

derived nitric oxide, leading to endothelial dysfunction (Keaney *et al*., 1994).

Tocotrienol rich fraction (TRF) derived from palm oil is more effective in terms of inhibition of oxidative damage to lipids and protein compared to α-tocopherol alone. Furthermore, it prevents lipid peroxidation process 40-60 times higher than alpha-tocopherol as shows by an *in vitro* study (Serbinova *et al*., 1991). Although previous studies have shown that tocotrienol has more potent anti-oxidant activity than tocopherol, the optimal concentration of TRF as an antioxidant has not been ascertained. The present *in vitro* study has clearly shown that palm oil derived TRF at concentrations between 10-100 µg/ml (equivalent 15 – 147 µM) consistently exhibits optimal antioxidant activity in all three *in vitro* assays (Ferric thiocyanate, DPPH and DCFHDA).

Ferric thiocyanate (FTC) assay is a method to study the ability of any substance against peroxidation of linoleic acid emulsion by the inhibition of peroxide which was produced during the process (Lee *et al*., 2004). Although the inhibition of lipid peroxidation activity by palm oil derived TRF is already known, the optimal TRF concentration that lead to the highest inhibition of peroxidation is not well reported. This study found that TRF at the concentrations from 0.1 to 20,000 µg/ml expressed inhibition of lipid peroxidation and was optimal at the concentrations between 10 – 100 µg/ml, as has been shown by the FTC method. TRF extracted from rice bran oil has also been shown to have active inhibitory effects against peroxidation of linoleic acid emulsion when a similar FTC method was used (Kim, 2005). However the percentage of lipid peroxidation inhibition reported by TRF derived from the rice bran is much lower compared to TRF derived from palm oil used in this study. The highest percentage of inhibition reported in that study was 74.5%, shown by TRF at concentrations of 160 µg/ml. In the present study however, the highest inhibition of lipid peroxidation was at 96.4% which was due to the use of the lower concentration of TRF (100 and 10 µg/ml). It is possible that this finding is attributed to the higher lipid peroxidation inhibition capacity of palm oil derived TRF compared to the TRF extracted from rice bran.

The experimental model of scavenging stable radicals is widely used as a method to evaluate antioxidant activities in a relatively short time (Gulcin *et al*., 2006). DPPH is usually used as a substrate to evaluate the anti-oxidative activity of antioxidants (Kim, 2005). Absorbance of DPPH radical is decreased in the presence of antioxidants. Results of the present study indicate that TRF derived from palm oil acts as an active free radical scavenger. The DPPH assay has shown that the optimal free radical scavenging activity of TRF was at 62.5 µg/ml with 85.2% inhibition. However, DPPH free radical scavenging activity plateau with increasing TRF concentrations, up to a concentrations of 1000 µg/ml. Kim (2005) has reported on the active DPPH free radical scavenging activity by TRF derived from rice bran. In that study, optimal concentration of TRF extracted from rice bran oil was higher compared to TRF extracted from palm oil (160.0 vs. 62.5 µg/ml). This suggests that, palm oil derived TRF is more active as a free radical scavenger as shown by the lower optimal concentration compared to rice bran derived TRF.

TRF extracted from palm oil was suggested to have more potent antioxidant activity due to the presence of α-tocotrienol in the crude extract. In contrast, rice bran TRF contain negligible amount or no δ-tocotrienol at all compared to TRF extracted from palm oil. Recently, δ-tocotrienol has been suggested as the best tocotrienol isomers in terms of lipid peroxidation inhibition compared to the other isomers (Palozza *et al*., 2006). δ-tocotrienol is also the best isomers that reduced the ROS formation induced by hydroperoxide in cultured fibroblast (Palozza *et al*., 2006). In that study, it has been suggested that the potent antioxidant activity is due to the methylation of α-tocotrienol chromane ring which allows the greater cellular uptake by the cell membranes. TRF is found to be more potent free radical scavenger than α-tocopherol as shown by the lower IC_{50} value. However, when compared to quercetin and vitamin C, TRF showed less potent free radical scavenging activity.

Formation of intracellular ROS in the macrophage can be measured by DCFHDA assay. The monocyte/macrophage is thought to play a major role in the pathogenesis of atherosclerosis. Therefore, the modulation of its activation processes may be used as a potential therapeutic target (Li *et al*., 2001). Evidence obtained from models of atherosclerosis has shown that a reduction of monocyte recruitment to the site of the atheroma may reduce the disease progression (Gosling *et al*., 1999). The formation of the fatty streak represents an early phase in the pathogenesis of atherosclerosis, which is initiated by the adherence and migration of circulating monocytes into the subendothelial space. The cells enter a differentiation program in which the resulting macrophage accumulates oxidized low-density lipoprotein (LDL) to form the characteristic foam cell. Macrophage oxidation of LDL is thought to be dependent on the superoxide produced from NADPH

oxidase (Poolman *et al*., 2005).

Measurement of oxidation of DCFH has gained a lot of interest as a method of estimating cellular ROS production. This method is convenient, as acetmethoxy ester of DCFHDA easily penetrates cellular membranes. DCFHDA is then hydrolysed inside the macrophage to become essentially non-permanent DCFH, which is non-fluorescent. However, in the present of ROS, DCFH is oxidized to become fluorescence DCF (Wang and Joseph, 1999). Different concentrations of TRF ranging from 3 x 10^{-5} to 175 µg/ml inhibited intracellular ROS formation in the macrophage and the lowest production of ROS has been shown by supplementation of TRF at a concentration of 87.5 µg/ml. This finding is important since the increase of macrophage accumulation in the intima will provide an increase of ROS production which will then contribute to the increase of the oxidation of LDL into atherogenic ox-LDL. The presence of tocotrienol may reduce the production of ROS by macrophages and thus decreases ox-LDL formation.

The present study showed that, in the DPPH assay, 62.5 µg/ml was the optimal concentration where it posseses the highest free radical scavenging activity. In the DCFHDA assay, the optimal concentration of TRF to cause the lowest ROS production was at 88 µg/ml. The results from these two assays are consistent with the findings from the FTC method which reported optimal TRF concentration within the range of 10-100 µg/ml.

The potent antioxidant activity of TRF reported by the present study is in agreement with other previous studies, although different antioxidant assays was used. Tocotrienol was reported to have a potent antioxidant activity against lipid peroxidation (Kamat and Devasagayam, 1995). More recently, TRF has been shown to have a direct cardioprotective role through its potent antioxidant activity, in experiments performed by using isolated rat hearts (Das *et al*., 2005). In addition to that experiment, palm oil derived TRF also has been reported to prevent damage of rat bone by inhibition of the radical formation (Ahmad *et al*., 2005).

Conclusions

Palm oil derived tocotrienol rich fraction (TRF) as compared to α-Tocopherol exhibits more potent antioxidant and free radical scavenging activities. In addition, unequivocal endorsement from 3 different in vitro assays showed that TRF is a potent antioxidant, with optimal concentrations between 10-100 µg/ml (equivalent to 15-47 µM). These suggest that appropriate dose of TRF need to be ascertained, which may lead to beneficial effects in the prevention and inhibition of progression of atherosclerosis.

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