The Effect of Tocotrienol-Rich Fraction on the Expression of Glutathione S-Transferase Isoenzymes in Mice Liver

(Kesan Pecahan Kaya Tokotrienol kepada Ekspresi Isoenzim Glutation S-Transferase di dalam Hepar Tikus)

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ABSTRACT

Glutathione S-transferase isoenzymes (GSTs) catalyze the conjugation reaction between glutathione and electrophilic compounds. GSTs are involved in the detoxification of toxic and carcinogenic compounds, thus protecting the body from toxic injuries. Tocotrienols are part of the vitamin E family and is believed to possess potent antioxidant activity. The objective of this study was to determine the effect of increasing doses of tocotrienol rich fraction (TRF) supplementation on liver GSTs gene and protein expression. A total of 30 male ICR white mice were divided into five groups (n=6 for each group) and given treatment for 14 days through oral supplementation. Groups were divided as follows: - three groups administered with TRF at doses of 200, 500 and 1000 mg/kg, respectively, a positive control group administered with 100 mg/kg butylated hydroxyanisole (BHA) and a control group administered with only the vehicle (corn oil). At day 15, the mice were sacrificed and their livers isolated. Total RNA was extracted from the liver and quantitative real-time polymerase chain reaction (qPCR) assays were performed to analyze GSTs gene expression. Total liver protein was also extracted and the protein expression of GSTs was determined by Western blotting. The results showed that TRF oral supplementation caused a significant dose-dependent increase in liver GST isoenzymes gene and protein expression, compared to controls. In conclusion, TRF oral supplementation for 14 days resulted in increased gene and protein expression of GST isoenzymes in mice liver dose-dependently, with the highest expression seen in mice treated with 1000 mg/kg TRF.

Keywords: Glutathione S-transferase; isoenzymes; liver; tocotrienols; TRF

ABSTRAK

Glutation S-transferase (GST) isoenzim merupakan enzim yang memangkinkan tindak balas konjugasi antara glutation dan sebatian hidrofilik. GST terlibat dalam detoksifikasi sebatian toksik dan karsinogenik dan oleh itu melindungi badan daripada kecederaan toksik. Tokotrienol adalah sebahagian daripada keluarga besar vitamin E dan dipercayai mempunyai aktiviti antioksidan yang poten. Objektif kajian ini adalah untuk menentukan kesan pemberian dos secara meningkat fraksi kaya tokotrienol (TRF) terhadap pengekspresan gen dan protein beberapa isoenzim GST di hepar. Sebanyak 30 ekor mencit putih jantan ICR dibahagikan kepada lima kumpulan (n=6 untuk setiap kumpulan) dan dirawat secara suplementasi oral selama 14 hari. Kumpulan dibahagikan seperti berikut: - tiga kumpulan dirawat dengan pelbagai dos TRF (200, 500 dan 1000 mg/kg), kumpulan kawalan positif yang dirawat dengan 100 mg/kg butilated hidroksianisol (BHA) dan kumpulan kawalan yang hanya diberi bahan pelarut (minyak jagung). Pada hari ke-15, tikus telah dibunuh dan heparnya diasingkan. RNA jumlah diekstrak daripada hepar dan seterusnya asai reaksi rerantai polimerase kuantitatif (qPCR) telah dilakukan untuk menganalisis pengekspresan gen beberapa isoenzim GST. Protein jumlah hepar juga telah diekstrak dan pengekspresan protein beberapa isoenzim GST ditentukan dengan menggunakan kaedah 'Western blot'. Keputusan kajian menunjukkan bahawa suplementasi oral TRF menyebabkan peningkatan signifikan pengekspresan gen dan protein beberapa isoenzim GST yang bersandarkan dos, berbanding kumpulan kawalan. Kesimpulannya, suplementasi oral TRF selama 14 hari pada mencit berupaya menyebabkan peningkatan pengekspresan gen dan protein beberapa isoenzim GST yang bersandarkan dos dengan pengekspresan tertinggi diperhatikan pada mencit yang dirawat dengan 1000 mg/kg TRF.

Kata kunci: Glutation S-transferase; hepar; isoenzim; tokotrienol; TRF

INTRODUCTION

Eight different isoenzymes of vitamin E exist naturally - $\alpha, \beta, \gamma, \delta$ tocopherols and $\alpha, \beta, \gamma, \delta$ tocotrienols (Sylvester & Theriault 2003). The chromanol ring structure is present in both tocopherols and tocotrienols, which is responsible for their potent antioxidant activities. Tocotrienols differ from tocopherols in terms of the structure of their side chains (Das 2011). The phytyl side chains of the chromanol rings of tocotrienols are unsaturated, whereas the side chains of the chromanol rings of tocopherols are saturated (Sambanthamurthi et al. 2000). Another important difference lies in the observation that tocotrienols have greater antioxidant power compared to tocopherols (Inokuchi et al. 2003; Pruthi et al. 2001). Tocopherols are largely found in corn, olive, soybean, sesame, peanut and sunflower oils. Tocotrienols can be

found at lesser quantities in certain grains as barley, rice and wheat. Interestingly, the most widely available natural source that contains huge amounts of tocotrienols is palm oil (Atia & Abdullah 2013; Sen et al. 2010). The vitamin E found in palm oil is made up of 70% tocotrienols and 30% tocopherols (Sen et al. 2010; Sundram et al. 2003). A standardized tocotrienol-rich fraction (TRF) derived from palm oil consists mainly of α, β, γ and δ-tocotrienols. TRF is obtained from palm oil after it has undergone esterification, distillation, crystallization and chromatography (Sundram & Gapor 1992). TRF is the most common and cost-effective preparation of tocotrienols.

It has been well established that vitamin E possess strong antioxidant activity. It is also the most important lipid soluble antioxidant in humans (Nesaretnam et al. 1995). This is due to the presence of a phenolic group in the chromanol ring which neutralizes free radicals by converting them into more stable products. The potential beneficial effects of TRF towards health had been widely implicated through various animal studies. For example, a previous study had suggested that tocotrienols were significantly more efficient than tocopherols against lipid peroxidation and protein oxidation in rat brain mitochondria and liver microsomes (Budin et al. 2009). A recent study performed in rats indicated that TRF supplementation was able to protect the liver from oxidative damage due to subchronic fenitrothion treatment (Jayusman et al. 2017). The results of another recent study showed that long term TRF supplementation significantly increased the antioxidant enzymes activities in skeletal and heart muscles in aging mice (Karim et al. 2015). However, only a few studies have been conducted in order to observe the health benefit effects of TRF administration to humans. One such study in humans showed that TRF supplementation to individuals over 50 years of age resulted in improvement of plasma cholesterol levels, advanced glycosylation end products (AGEs) levels and antioxidant enzymes activities as well as reduction in protein damage, all of which might indicate a restoration of redox balance in these elderly individuals (Chin et al. 2011).

A previous research had tried to link the antioxidant activities of vitamin E with the induction of several phase II xenobiotic metabolizing enzymes involved in cancer chemoprevention (Mustacich et al. 2009). Most phase II xenobiotic metabolizing enzymes such as the glutathione S-transferase isoenzymes (GSTs) had been shown to be mainly regulated by nuclear factor (erythroid-derived 2)-like 2 (Nrf2) (Abdullah et al. 2012; Kitteringham et al. 2010) and are important for cellular defense by hastening the removal of free radicals and toxic by-products. As such, the GSTs are therefore postulated to play a protective role against cancer and other diseases (Aleksunes & Manautou 2007). GSTs are phase II detoxification enzymes that catalyze the conjugation of several endogenous and exogenous electrophilic compounds. In mammals, GSTs are divided into numerous cytosolic, mitochondrial and microsomal GST isoenzymes. The cytosolic isoenzymes are encoded by five related gene families (known as alpha (α), mu (μ), pi (π), sigma (σ) and theta (θ) GST), while the membrane-bound isoenzymes and microsomal GSTs are encoded by single genes and both have originated separately from the soluble GSTs (Hayes et al. 2005). GST upregulation is essential for the redox state of the cell because GST catalyzes the conjugation of the reduced glutathione, which is a major intracellular antioxidant (Cheng et al. 2011). Previous studies had indicated that tocotrienol administration in rats was able to modulate the activity of GST in rat liver (Iqbal et al. 2004; Rahmat et al. 1993). However, in these studies, only the GST enzyme activity was measured and no attempt was made to measure the gene and protein expression of different GST isoenzymes in rat liver after tocotrienol treatment. Furthermore, no study has been done in mice to investigate the effect of tocotrienol administration on the various GST isoenzymes in mice liver. Therefore, the objective of this study was to investigate the ability of three different doses of TRF to modulate GSTs at the gene and protein levels in the liver of ICR male white mice.

Studies performed on animal models suggested that GSTs are implicated in the prevention of chemically induced hepatotoxicity (Ajith et al. 2007; Gum et al. 2007). Increased expression of GSTs is suggested to confer increased resistance towards hepatotoxicity, while decreased GSTs expression is thought to increase the risk of hepatotoxicity (Morishita et al. 2006; Tanaka et al. 2007). In humans, the clinical importance of diminished endogenous GSTs expression and activity had been recognized for quite some time. It was discovered that individuals with homozygous glutathione S-transferase mu 1 (GSTM1) gene deletions, which resulted in decreased GSTM1 activity, appeared to be more susceptible to DNA damage and are more at risk of developing lung adenocarcinoma (Cantlay et al. 1994). It had also been suggested that decreased activity of glutathione S-transferase mu 1 (GSTM1) and glutathione S-transferase theta 1 (GSTT1) isoenzymes was associated with environmentally related cancers and alcoholic liver disease (Ladero et al. 2005; Martinez et al. 2006; Parl 2005). Human subjects with the double-null GSTM1 and GSTT1 genotype were found to be more susceptible to develop drug-induced liver injury (Lucena et al. 2008). The presence of abnormal variants of glutathione S-transferase pi 1 (GSTP1) genotypes was linked to decreased GSTP1 enzyme activity in certain individuals, which resulted in ineffective detoxification of carcinogens compared to individuals with wild-type GSTP1 genotype (Sundberg et al. 1998; Zimniak et al. 1994). Therefore, it would be interesting to find out whether dietary supplementation with TRF could increase liver GSTs expression dose-dependently, which could theoretically leads to reduced risk of experiencing drug/chemical-induced hepatotoxicity.

MATERIALS AND METHODS

MATERIALS

TRF (Gold Tri.E70) was purchased from Sime Darby Bioorganic (Kuala Lumpur, Malaysia) and contains α-tocopherol at 159.5 mg/g, α-tocotrienol at 205.1 mg/g, β-tocotrienol at 32.9 mg/g, γ-tocotrienol at 249.8 mg/g and δ-tocotrienol at 119 mg/g. TRIzol reagent was purchased from Life Technologies (Carlsbad, California, USA). iScript cDNA synthesis kit, iQ SYBR Green supermix (2X) kit and 48-well MJ Mini thermal cycler were purchased from Bio-Rad (Hercules, California, USA). Real-time PCR primers were purchased from Vivantis technologies (Oceanside, CA, USA). RIPA lysis buffer and goat anti-rabbit IgG peroxidase secondary antibody were purchased from Santa Cruz Biotechnology (USA). Chemiluminescence Western blotting detection reagents were purchased from Amersham (Uppsala, Sweden). Nitrocellulose membrane and Ponceau S solution were purchased from Sigma-Aldrich (Seelze, Germany). GSTA1 polyclonal primary antibody, GSTM polyclonal primary antibody, GSTP polyclonal primary antibody and β-actin rabbit polyclonal antibody were purchased from Abcam Biotechnology (Cambridge, UK). All other chemicals were purchased from Sigma-Aldrich unless otherwise stated.

ANIMAL HANDLING AND EXPERIMENTAL DESIGN

Male ICR white mice (25-30 g) were used in this study and were obtained from the Universiti Kebangsaan Malaysia Laboratory Animal Research Unit (LARU). The mice were kept in clean polypropylene cages in a ventilated room, with food and water available *ad libitum*. Food intake and body weight of mice were measured daily. Animals were treated with three different doses of TRF (dissolved in corn oil). The mice were divided into 5 groups. Mice in the first group ($n = 6$) was designated as the control group and were given only the vehicle, i.e. corn oil. Mice in the second group ($n = 6$) were treated with 200 mg/kg TRF followed by the third group ($n = 6$) treated with 500 mg/kg TRF and the fourth group ($n = 6$) treated with 1000 mg/kg TRF. Mice in the fifth group ($n = 6$), i.e. the positive control group, were treated with 100 mg/kg butylated hydroxyanisole (BHA). The doses of TRF chosen, as well as the time point and duration of TRF treatment, were based on previous studies done by Ima-Nirwana et al. (2011) and Oo et al. (1992). The justification for the use of BHA as the positive control and the dose of BHA chosen was based on a previous study done by Jaeschke and Wendel (1986). All treatments were administered via oral gavage for 14 consecutive days. At day 15, mice were sacrificed via cervical dislocation. Their livers were subsequently isolated, snapped frozen in liquid nitrogen and stored at -80°C until further use. All experimental procedures involving animals were approved by the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC) with the approval number: PP/FAR/2011/AZMAN/22-MARCH/361-MAY2011-MAY-2013.

RNA EXTRACTION

Total RNA from frozen liver tissues was isolated using TRIzol reagent, according to the manufacturer's instructions. Isopropyl alcohol (Sigma, USA) was added during each extraction step to precipitate the total RNA. The extracted total RNA pellet was then washed with 75% ethanol and dried before being dissolved in RNase-free water. Total RNA was stored at -80°C immediately after extraction. Concentration and purity of the extracted RNA were determined by NanoDrop spectrophotometer 2000c (Thermo Scientific, USA) at a wavelength of 260 nm (OD260). RNA with RNA integrity number (RIN) ranging from 7 to 10 and absorbance ratio of A260 to A280 ranging from 1.5 to 2.0 was used for cDNA synthesis.

REVERSE TRANSCRIPTION

Generation of cDNA from RNA was done using iScript cDNA synthesis kit (Bio-Rad, USA) according to the manufacturer's instructions. Briefly, a volume (containing 1 μg) of total RNA from each sample was added to a mixture of 4 μL of 5X iScript reaction mix, 1 μL of iScript reverse transcriptase and a suitable volume of nuclease-free water (the final reaction mix volume is 20 μL). The final reaction mix was kept at 25°C for 5 min, 42°C for 30 min and heated to 85°C for 5 min in a thermocycler (TC-412, Techne, Barloworld Scientific, UK). The cDNA was then used as a template for amplification by polymerase chain reaction (PCR).

QUANTIFICATION OF GSTS GENE EXPRESSION BY QUANTITATIVE REAL-TIME PCR

Quantitative real-time PCR was performed on the MiniOpticon cycler (Bio-Rad, USA) to study the expression of GSTs gene namely glutathione S-transferase alpha 1 (GSTA1), glutathione S-transferase alpha 3 (GSTA3), glutathione S-transferase mu 1 (GSTM1), glutathione S-transferase mu 3 (GSTM3) and glutathione S-transferase pi (GSTP). The total reaction volume used was 20 μL, consisting of 1 μL of 10 μM forward primer and 1 μL of 10 μM reverse primer (500 nM final concentration of each primer), 10 μL of iQ™ SYBR® Green Supermix (2X) (Bio-Rad, USA), 6 μL of nuclease-free water and 2 μL of cDNA. Both forward and reverse primers for the genes of interest in this study were designed according to previous studies (Kong et al. 2007; Xu et al. 2005) and synthesized by Vivantis Technologies (Oceanside, CA, USA). The primer sequences for the genes of interest are as shown in Table 1.

The thermocycling conditions were initiated at 95°C for 30 s, followed by 40 PCR cycles of denaturation at 95°C for 15 s and annealing/extension at 60°C for 30 s. At the end of each cycle, a melting curve (dissociation stage) analysis was performed in order to determine the specificity of the primers and the purity of the final PCR product. All measurements were performed in triplicate and no-template controls (NTC) were incorporated onto the same set of PCR tubes to test for contamination by any assay

TABLE 1. Primer sequence for GSTA1, GSTA3, GSTM1, GSTM3, GSTP and GAPDH

Gene	Primer sequence	Reference
GSTA1	F: 5'AGAATGGAGTGCATCAGGTGGTGGCTC 3' R: 5'- GGCAGGCAAGTAACGGTTTTTGGT-3'	Xu et al. (2005)
GSTA3	F: 5'- GAGATCGACGGGATGAAACTGGTG-3' R: 5'- GCGCTTTCAGGAGAGGGAAGTTGT-3'	Xu et al. (2005)
GSTM1	F: 5'- AGCACCCTGGCCTTCTGCACT-3' R: 5'- TTCGCAGAAACGGGCTGTGAG-3'	Xu et al. (2005)
GSTM3	F: 5'- TGATTAGGCCCCTGCCATGCT-3' R: 5'- TTGGGTCTGGGCACCAATGAA-3'	Xu et al. (2005)
GSTP	F: 5'-TTTGGGGGCTTTATGGGAAAAACCA-3' R: 5'-ACATAGGCAGAGAGCAGGGGGAAG- 3'	Xu et al. (2005)
GADPH	F: 5'-GTGGAGTCTACTGGTGTCTTCA-3' R: 5'-TTGCTGACAATCTTGAGTGAGT-3'	Kong et al. (2007)

reagents. Threshold cycles were determined for each gene and quantification of templates was performed according to the relative standard curve method. The relative gene expression (ΔΔCt) technique, as defined in the Applied Biosystems User Bulletin No. 2 (Livak & Schmittgen 2001), was used to analyse the real-time PCR data. In short, the expression level of each target gene was given as relative amount normalized against GAPDH standard controls.

TOTAL PROTEIN EXTRACTION FOR WESTERN BLOTTING

Liver tissue samples (0.1 g) were homogenized in 0.5 mL RIPA lysis buffer (which contained 10 μL phenylmethylsulfonyl fluoride (PMSF), 10 μL sodium orthovanadate and 10 μL protease inhibitor cocktail solution per 1 mL of 1X RIPA lysis buffer). After centrifugation (13,000 × g for 30 min at 4°C), the supernatants were collected and their protein concentrations were determined using the Lowry method, with bovine serum albumin was used as standard (Lowry et al. 1951).

WESTERN BLOTTING

Standard Western blotting procedure was used for the immunodetection of proteins. Briefly, 100 μg of liver protein was separated using 15% sodium dodecyl sulfate-polyacrylamide gel (SDS-PAGE) electrophoresis. The proteins in the gel were then transferred to a nitrocellulose membrane. The membrane was then incubated for 20 min at room temperature in a blocking solution (150 mM NaCl, 3 mM KCl, 25 mM Tris, 0.1% (v/v) tween-20 and 10% non-fat milk powder (pH7.4). After blocking, the membrane was incubated with the following antibodies: primary polyclonal rabbit anti-mouse GSTA, primary polyclonal rabbit anti-mouse GSTM1, primary polyclonal rabbit anti-mouse GSTP and primary polyclonal rabbit anti-mouse actin for 1 h at room temperature. Subsequently, incubation with a peroxidase-conjugated goat anti-rabbit IgG secondary antibody was carried out for another hour at room temperature. Protein bands were visualized using the enhanced chemiluminescence method according to the manufacturer's instructions (Amersham, Uppsala, Sweden). The intensity of the protein bands were quantified, relative to the signals obtained for actin, using ImageJ software.

STATISTICAL ANALYSIS

Data are presented as mean ± standard error of the mean (SEM). Significant differences between mean values of multiple groups were determined using one-way ANOVA and Student's t-test. Statistical analysis was conducted using the SPSS software version 22. The result was considered statistically significant when $p<0.05$.

RESULTS

BODY WEIGHT AND FOOD INTAKE AFTER TRF TREATMENT

In order to investigate whether different doses of palm TRF affect body weight and food intake in the control and experimental mice, body weight of mice in each group were recorded and food intake of each mouse was individually measured every two days till the end of the treatment period. Table 2 shows that in palm TRF-treated groups, body weight and food consumption were only slightly inhibited by the administration of 1000 mg/kg TRF but they were not inhibited by the administration of 500 and 200 mg/kg TRF. However, the difference in body weight and food consumption after 14 days was not statistically significant for all groups. Administration of BHA alone did not induce any significant alterations in those parameters and the results did not differ significantly from those of the control and TRF-treated groups.

LIVER GSTA1, GSTA3, GSTM1, GSTM3 AND GSTP GENE EXPRESSION

To examine the effect of TRF administration on GSTs gene expression, mice were fed with different doses of

TABLE 2. Body weight and food intake of control and treated mice

Groups	Body weight (bw)		Food intake
	Day 1 (g)	Day 14 (g)	(g/bw/day)
Control	28.67 ± 2.13	33.33 ± 2.27	4.50 ± 1.11
T200	27.67 ± 1.96	32.33 ± 2.11	4.37 ± 1.85
T500	28.33 ± 1.25	30.33 ± 1.84	4.17 ± 1.92
T1000	31.67 ± 1.17	30.33 ± 2.18	2.09 ± 1.16
BHA	30.00 ± 1.32	30.67 ± 1.43	4.93 ± 1.23

T200, T500, T1000: Groups of mice treated with oral TRF at a daily dose of 200, 500 and 1000 mg/kg body weight, respectively. Control: control mice, BHA: positive control group that was given butylated hydroxyanisole (100 mg/kg). Values are given as mean ± SEM (n = 6 for each group). No statistical significance was found between control and treated groups (ANOVA).

TRF (200, 500, 1000 mg/kg) in the presence of vehicle-treated control mice (fed corn oil) and a positive control group administered BHA (100 mg/kg) for 14 days. After 14 days of treatment, the mice were sacrificed and the gene expression of various isoenzymes of GST in the liver was measured using quantitative real-time PCR (qPCR). As shown in Figure 1, TRF at concentrations of 200, 500, and 1000 mg/kg caused a significant dose-dependent increase in the fold change of GSTA1 gene expression levels (2.48 ± 0.39-fold ($p<0.05$), 3.13 ± 0.50-fold ($p<0.05$) and 4.40 ± 0.36-fold ($p<0.001$), respectively, as compared to controls). Mice treated with BHA (100 mg/kg) showed the highest significant increase in GSTA1 gene expression levels (7.46 ± 0.37-fold), compared to control mice ($p<0.0001$) and mice treated with 200, 500 and 1000 mg/kg TRF ($p<0.01$).

As shown in Figure 1, TRF at concentrations of 200, 500 and 1000 mg/kg caused a significant dose-dependent increase in the fold change of GSTA3 gene expression levels (1.53 ± 0.03-fold ($p<0.001$), 2.71 ± 0.26-fold ($p<0.01$) and 3.93 ± 0.17-fold ($p<0.0001$), respectively, as compared to controls). Mice treated with BHA (100 mg/kg) showed the highest significant increase in GSTA3 gene expression levels (6.71 ± 0.22-fold), compared to control mice ($p<0.0001$) and mice treated with 200, 500 and 1000 mg/kg TRF ($p<0.001$).

As shown in Figure 1, TRF at concentrations of 200, 500 and 1000 mg/kg caused a significant dose-dependent increase in the fold change of GSTM1 gene expression levels (2.04 ± 0.12-fold ($p<0.001$), 2.40 ± 0.15-fold ($p<0.001$) and 3.23 ± 0.12-fold ($p < 0.0001$), respectively, as compared to controls). Mice treated with BHA (100 mg/kg) showed the highest significant increase in GSTM1 gene expression levels (7.33 ± 0.31-fold), compared to control mice ($p<0.0001$) and mice treated with 200, 500 and 1000 mg/kg TRF ($p<0.001$).

As shown in Figure 1, TRF at concentrations of 200, 500 and 1000 mg/kg caused a significant dose-dependent increase in the fold change of GSTM3 gene expression levels (1.40 ± 0.03-fold ($p < 0.05$), 2.36 ± 0.12-fold ($p < 0.001$) and 3.23 ± 0.09-fold ($p < 0.0001$), respectively, as compared to controls). Mice treated with BHA (100 mg/kg) showed the highest significant increase in GSTM3 gene expression levels (6.23 ± 0.23-fold), compared to
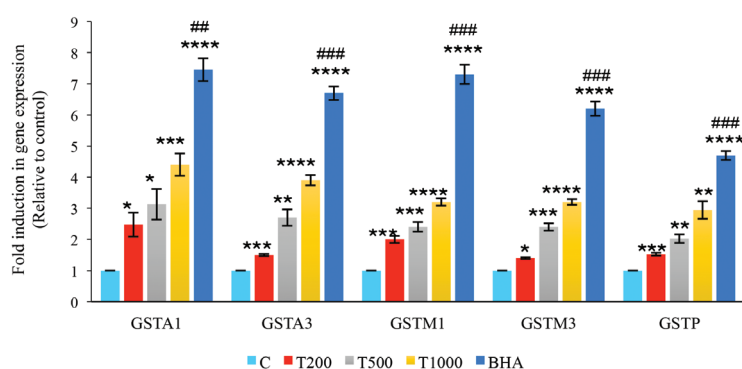

FIGURE 1. Effect of different doses of TRF on GSTA1, GSTA3, GSTM1, GSTM3 and GSTP gene expressions in the livers of mice. GAPDH served as the reference gene. Mice were treated with 200, 500 and 1000 mg/kg TRF for 14 days. Their livers were then harvested and the gene expression levels were determined using qPCR. Triplicate reactions were performed for each experiment and the values presented are the means of three independent experiments. Data is presented as mean ± SEM. T200: TRF at a dose of 200 mg/kg; T500: TRF at a dose of 500 mg/kg; T1000: TRF at a dose of 1000 mg/kg; C: control mice; BHA: positive control group given butylated hydroxyanisole (100 mg/kg). *Significant difference from control group ($p<0.05$). **Significant difference from control group ($p<0.01$). ***Significant difference from control group ($p<0.001$). ****Significant difference from control group ($p<0.0001$). ##Significant difference from T1000, T500 and T200 groups ($p<0.01$). ###Significant difference from T1000, T500 and T200 groups ($p<0.001$)

control mice ($p < 0.0001$) and mice treated with 200, 500 and 1000 mg/kg TRF ($p<0.001$).

As shown in Figure 1, TRF at concentrations of 200, 500 and 1000 mg/kg caused a significant dose-dependent increase in the fold change of GSTP gene expression levels (1.52 ± 0.04-fold ($p<0.001$), 2.03 ± 0.14-fold ($p<0.01$) and 2.95 ± 0.28-fold ($p<0.01$), respectively, as compared to controls). Mice treated with BHA (100 mg/kg) showed the highest significant increase in GSTP gene expression levels (4.70 ± 0.14-fold), compared to control mice ($p<0.0001$) and mice treated with 200, 500 and 1000 mg/kg TRF ($p<0.001$).

LIVER GSTA, GSTM1 AND GSTP PROTEIN EXPRESSION

Administration of TRF at concentrations of 200, 500 and 1000 mg/kg to mice for 14 days through oral gavage significantly increased liver GSTA protein (26 kd) expression levels by 1.80 ± 0.23-fold ($p<0.05$), 2.29 ± 0.16-fold ($p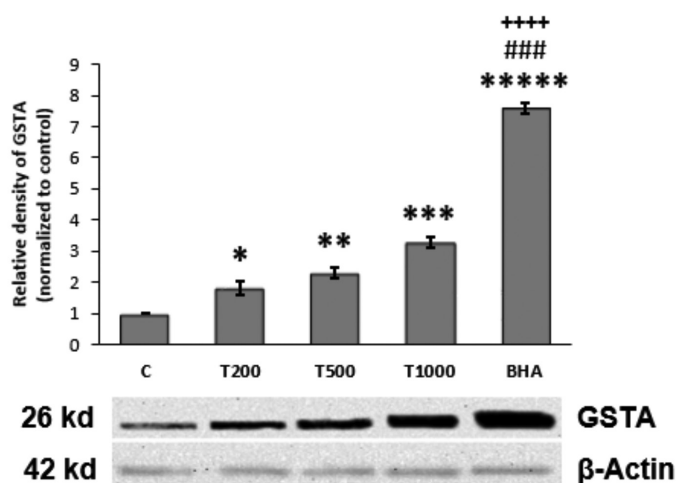<0.01$) and 3.27 ± 0.17-fold ($p<0.001$), respectively, compared to controls. However, mice treated orally with BHA (100 mg/kg) for 14 days showed the highest significant increase in GSTA protein expression levels (7.58 ± 0.19-fold), compared to control mice ($p<0.00001$) and mice treated with 200 ($p<0.0001$), 500 ($p<0.0001$) and 1000 mg/kg TRF ($p<0.001$) (Figure 2).

Administration of TRF at concentrations of 200, 500 and 1000 mg/kg to mice for 14 days through oral gavage significantly increased liver GSTM1 protein (26 kd) expression levels by 2.78 ± 0.21-fold ($p<0.01$), 2.92 ± 0.26-fold ($p<0.01$) and 3.85 ± 0.37-fold ($p<0.01$), respectively,

compared to controls. Then, mice treated orally with BHA (100 mg/kg) for 14 days showed the highest significant increase in GSTM1 protein expression levels (5.01 ± 0.41-fold), compared to control mice ($p<0.01$) and mice treated with 200, 500 and 1000 mg/kg TRF ($p<0.05$) (Figure 3).

Administration of TRF at concentrations of 200, 500 and 1000 mg/kg to mice for 14 days through oral gavage significantly increased liver GSTP protein (26 kd) expression levels by 1.56 ± 0.05-fold ($p<0.001$), 2.28 ± 0.14-fold ($p<0.01$) and 2.94 ± 0.17-fold ($p<0.001$), respectively, compared to controls. After 14 days, mice treated orally with BHA (100 mg/kg) for 14 days also showed the highest significant increase in GSTP protein expression levels (4.50 ± 0.19-fold), compared to control mice ($p<0.001$) and mice treated with 200 ($p<0.001$), 500 ($p<0.01$) and 1000 mg/kg TRF ($p<0.01$) (Figure 4).

The observed effect of TRF on GSTA, GSTM1 and GSTP protein expression levels is in agreement with the observed effect on gene expression levels, in which both are enhanced in dose-dependent manner following administration of TRF. Meanwhile, the highest significant expression of GSTs gene and protein were observed when the mice were administered with BHA at the dose of 100 mg/kg, as indicated by the results of statistical analysis between the BHA and TRF groups presented in the figures and paragraphs.
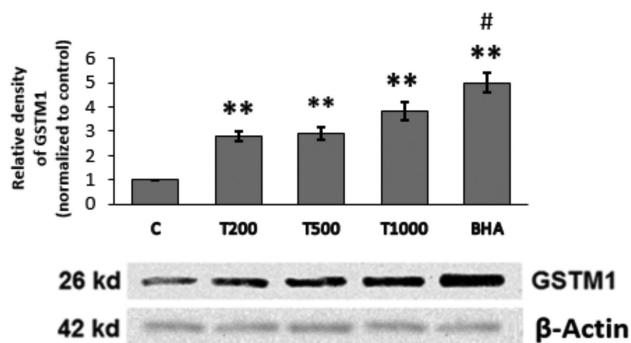
DISCUSSION

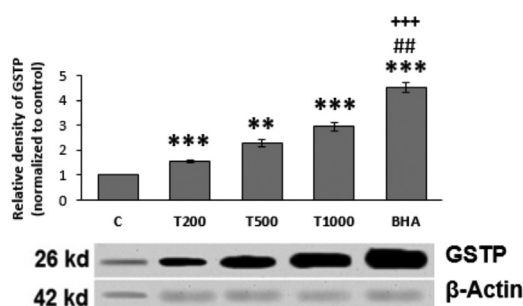The liver is the main organ responsible for the metabolism of most drugs and chemicals. Drug-metabolizing enzymes in

*Significant difference from control group ($p<0.05$). **Significant difference from control group ($p<0.01$). ***Significant difference from control group ($p<0.001$). *****Significant difference from control group ($p<0.00001$). ###Significant difference from T1000 group ($p<0.001$). ++++Significant difference from T500 and T200 groups ($p<0.0001$)

FIGURE 2. Effect of different doses of TRF on GSTA liver protein expression. Mice were treated with 200, 500 and 1000 mg/kg TRF for 14 days. Their livers were then harvested and GSTA protein expression levels were determined by Western blotting. The intensity of protein bands were quantified relative to the signals obtained for actin using ImageJ software, and was normalized to control. The graph represents the average optical density ($\pm$ S.E.M.) of bands from three different experiments. T200: TRF at a dose of 200 mg/kg; T500: TRF at a dose of 500 mg/kg; T1000: TRF at a dose of 1000 mg/kg; C: control mice; BHA: positive control group given butylated hydroxyanisole (100 mg/kg)

**Significant difference from control group ($p<0.01$). #Significant difference from T1000, T500 and T200 groups ($p<0.05$)

FIGURE 3. Effect of different doses of TRF on GSTM1 liver protein expression. Mice were treated with 200, 500 and 1000 mg/kg TRF for 14 days. Their livers were then harvested and GSTM1 protein expression levels were determined by Western blotting. The intensity of protein bands were quantified relative to the signals obtained for actin using ImageJ software, and was normalized to control. The graph represents the average optical density (± S.E.M.) of bands from three different experiments. T200: TRF at a dose of 200 mg/kg; T500: TRF at a dose of 500 mg/kg; T1000: TRF at a dose of 1000 mg/kg; C: control mice; BHA: positive control group given butylated hydroxyanisole (100 mg/kg)

**Significant difference from control group ($p<0.01$). ***Significant difference from control group ($p<0.001$). ##Significant difference from T1000 and T500 groups ($p<0.01$). +++Significant difference from T200 group ($p<0.001$)

FIGURE 4. Effect of different doses of TRF on GSTP liver protein expression. Mice were treated with 200, 500 and 1000 mg/kg TRF for 14 days. Their livers were then harvested and GSTP protein expression levels were determined by Western blotting. The intensity of protein bands were quantified relative to the signals obtained for actin using ImageJ software, and was normalized to control. The graph represents the average optical density (± S.E.M.) of bands from three different experiments. T200: TRF at a dose of 200 mg/kg; T500: TRF at a dose of 500 mg/kg; T1000: TRF at a dose of 1000 mg/kg; C: control mice; BHA: positive control group given butylated hydroxyanisole (100 mg/kg)

the liver are involved in the metabolism, biotransformation and detoxification of drugs, toxic chemicals and their derivatives. Phase I enzymes i.e. the cytochrome P450s are found in the hepatocyte microsomes and catalyze various xenobiotic biotransformations (Almazroo et al. 2017). Phase II enzymes such as the GSTs catalyze the detoxification of xenobiotics including carcinogenic chemicals. GSTs are involved in phase II metabolism and play important roles in protecting cells, organelles and macromolecules from various harmful entities (Hayes et al. 2005). GSTs stimulate nucleophilic attack via reduced glutathione (GSH) on nonpolar compounds containing an electrophilic carbon, making them less reactive and more hydrophilic (Cui et al. 2010; Knight et al. 2008). Tissues expressing high levels of GSTs (and other cytoprotective enzymes) are normally protected from damage by oxidants, harmful chemicals and xenobiotics (Bostwick et al. 2007). Increasing the expression of these enzymes and their activities in vital organs such as the liver (which is subjected daily to oxidative stress and chemical insults) would definitely improve the liver detoxification potential. The present study demonstrated the dose-dependent effects of TRF (200, 500 and 1000 mg/kg) on several GSTs (GSTA1, GSTA3, GSTM1, GSTM3 and GSTP) gene and protein expression levels, by means of qPCR and Western blot analysis, in the liver tissues of mice. The results obtained clearly showed that TRF positively induced the gene and protein expression levels of various GST isoenzymes in a dose-dependent manner, with the highest expression seen in mice treated with 1000 mg/kg. Our results also showed

that the liver GSTA gene expression showed the highest fold of increments amongst the GSTs (followed by GSTM and GSTP) after mice were administered with three different doses of TRF (200, 500 and 1000 mg/kg). However, the GSTM isoenzyme was the highest expressed at the protein level compared to other GSTs, after the administration of TRF to mice. Therefore it could be assumed that different isoenzymes of GST showed differential responses to TRF administration.

Our findings showed that GSTA1, GSTA3, GSTM1, GSTM3 and GSTP expressions were significantly induced by all doses of TRF administered, with the highest level observed following TRF administration at a dose of 1000 mg/kg, followed by 500 and 200 mg/kg, respectively. However, the highest observed level of GSTs expression induced by TRF was still below the expression levels induced by BHA treatment, as indicated by the statistical analysis performed. Since BHA is the classical inducer of phase II enzymes, it could be assumed that the increased antioxidant activities observed after tocotrienol treatment in previous animals studies might be partly mediated through the increased expression of phase II genes and proteins (Adam et al. 1996; Hsieh & Wu 2008; Lee et al. 2009; Newaz & Nawal 1999).

In our study, we used animal (mice) model and we looked specifically at the effect of TRF in the liver. Other studies which utilized *in vitro* techniques (mostly cell culture) have reported similar results. A previous *in vitro* study had investigated the effect of TRF on several cancer-related gene expression using the estrogen-dependent (MCF-7) and estrogen-independent (MDA-MB-231) human breast cancer cell lines (Nesaretnam et al. 2004). They found that supplementation of TRF (8 μg/mL for 72 h) modulated significantly 46 out of 1200 genes in MDA-MB-231 cells. In MCF-7 cells, TRF administration was associated with a lower number of affected genes. Out of all the genes affected, TRF administration significantly elevated the mRNA level of GST homologue in MDA-MB-231 but not in MCF-7 cells (Nesaretnam et al. 2004). In Oxidative Stress-Induced *Caenorhabditis elegans* worms, TRF had been found to induce the expression of GSTP1 protein (Aan et al. 2015). In another *in vitro* study involving human retinal pigment epithelial cells, pretreatment with α-tocopherol inhibited ROS generation, increased Nrf2 expression and induced many phase II enzymes such as GST (Feng et al. 2010).

In an *in vivo* study using rabbits, vitamin E supplementation induced up-regulation of GST expression in rabbit aortic tissues, which was correlated with elevated Nrf2 expression. These results suggested that vitamin E may afford protection against oxidative stress in atherogenesis via an Nrf2 redox-regulated pathway in the rabbit aorta (Bozaykut et al. 2014). Additionally, treatment with 0.1% γ-tocopherol-enriched mixed tocopherol diet (γ-TmT) for a period of 24 weeks upregulated the expression of GSTM1 gene and protein levels and inhibited tumour development in the prostate of the murine prostate cancer model (TRAMP) mice (Barve et al. 2009).

In our study, TRF was able to increases hepatic GSTs levels in wild type mice. The antioxidant activities of tocotrienols (which are the major components of TRF) have been linked to Nrf2 activation in an *in vitro* study (Hsieh et al. 2010). However, the exact mechanism by which tocotrienol/TRF induce GSTs protein expression in mice liver is still not fully understood. Since GSTs are phase II enzymes and phase II enzymes are regulated by Nrf2 (Aleksunes & Manautou 2007), it is suggested that tocotrienols (either directly or indirectly through their by-products) are able to dissociate the Nrf2/Keap1 complex, allowing Nrf2 to translocate to the nucleus and increase the expression of phase II enzymes (including the GSTs) in the liver cells. Further studies are needed to confirm this mechanism.

The activity of GSTs enzymes was significantly increased during diethylnitrosamine (DEN)/2-acetylaminofluorene (AAF)-induced hepatocarcinogenesis in rats. However, TRF treatment to DEN/AAF-treated rats substantially decreased the activity of GSTs enzymes (Iqbal et al. 2004; Makpol et al. 1997). Similarly, TRF maintained low levels of GST activities in liver and mammary glands of DMBA-treated rats (Iqbal et al. 2003). In these cases, it is postulated that increased GSTs activities was able to prevent further organ/tissue damage caused by those carcinogens. Administration of TRF was thought to attenuate lipid peroxidation due to its free radical scavenging properties and halted the process of organ/tissue damage and therefore less GSTs enzyme activities was needed to counter the redox imbalance in those organs/tissues, thus leading to substantially decreased GSTs activity. However, the gene and protein expression of GSTs was not measured in those studies and their expression might still be up-regulated even though their activities had decreased (feedback mechanism).

On the other hand, pretreatment of LPS, turpentine or zymosan-stressed hamsters with tocotrienol enriched diet resulted in a significant increase of GST activities in the liver and kidney (Khan et al. 2011). In another study, supplementation of vitamin E restored the activity of GST in the kidneys of tenofovir-treated Wistar albino rats (Adaramoye et al. 2012). However, in light of the knowledge that tocotrienols are the major chain-breaking antioxidants, it might be suggested that tocotrienols are able to counter the oxidative stress condition caused by the damage to biological membranes by free radicals, resulting in the production of protein carbonyl adducts. It is thought that these protein carbonyl adducts (or other by-products produced during tocotrienol-related counter-attack on free radicals) are able to dissociate the Nrf2/Keap1 complex, allowing Nrf2 to translocate to the nucleus and eventually activates a diverse cytoprotective genes (Bruno & Traber 2006; Gao et al. 2007). However, extensive biochemical assays are needed to confirm such interaction.

CONCLUSION

As discussed, increased expression of GSTs in the liver could potentially protect the liver from oxidative and

chemicals insults. Excessive oxidative and chemical insults to the liver could potentially cause acute/chronic liver damage and carcinogenesis. In order to prevent such ailments to occur, it would be wise to take some chemopreventive measure. In this respect, increasing the expression of various isoforms of GSTs by pharmacological means, offers a potential new therapeutic target for liver degenerative diseases. In our study, TRF had been shown to be able to increase the expression of various isoenzymes of GST in a dose-dependent manner. Therefore, the recommended therapeutic dose of TRF which is effective in the prevention or treatment of liver diseases in humans should be aggressively investigated. Consuming palm oil TRF in the form of supplements could be an affordable chemoprotective measure in the long run. Further studies are needed to conclusively support this strategy.

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