Mal J Nutr 22(2): 231 - 243, 2015

Potential of Mixed Tocotrienol Supplementation to Reduce Cholesterol and Cytokines Level in Adults with Metabolic Syndrome

Heng KS[1], Hejar AR[2] , Johnson Stanslas J[3], Ooi CP[3] & Loh SP[1,4]*

[1] *Department of Nutrition & Dietetics, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia*
[2] *Department of Community Health, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia*
[3] *Department of Medicine, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Malaysia*
[4] *Research Centre of Excellence, Nutrition and Non-communicable Diseases, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia, 43400 UPM Serdang, Selangor, Malaysia*

ABSTRACT

Introduction: Metabolic syndrome is associated with low-grade, chronic inflammation. Our study aimed to evaluate the effects of tocotrienols supplementation on cytokines and lipid profile in adults with metabolic syndrome. **Methods:** In a 16-week randomised, double-blind, placebo-controlled trial, 70 adults with metabolic syndrome aged 20-60 years were randomly assigned to a mixed tocotrienols group (n=35) that received 400mg/day of mixed tocotrienols or a placebo group (n=35) that received capsules containing soy bean oil. At baseline, week 8 and week 16, anthropometric, body composition and blood pressure measurements were conducted. At baseline and week 16 only, serum levels of total cholesterol (TC) and high density lipoprotein (HDL)-cholesterol, plasma levels of fasting plasma glucose (FPG), interleukin-6 (IL-6), tumouxr necrosis factor- α (TNF-α), leptin, adiponectin and high sensitivity C-reactive protein were also determined. Changes in dietary intake and physical activity level between baseline, week 8 and week 16 were also assessed. **Results:** In the tocotrienols group, significant reductions from baseline were found in diastolic blood pressure (p=0.001), TC (p=0.008), LDL-cholesterol (p=0.022), HDL-cholesterol (p<0.001), IL-6 (p=0.024) and TNF-α (p=0.013) at week sixteen. However, the changes in the tocotrienols group were not significantly different from those of the placebo group. **Conclusion:** The 16-week mixed tocotrienols supplementation exerted potential beneficial effects on cytokines and lipid profile in adults with metabolic syndrome. The results might have been confounded by the physiological effects produced by the soy bean oil in the placebo capsule.

Key words: Chronic inflammation, cytokines, lipid profile, metabolic syndrome, tocotrienols

* Correspondence: SP Loh; Email: *sploh@upm.edu.my*

INTRODUCTION

Metabolic syndrome is a clustering of metabolic abnormalities associated with cardiovascular disease and diabetes mellitus (Grundy *et al.*, 2005). Its development and progression are closely related to low-grade chronic inflammation. The production of inflammatory cytokines such as tumor necrosis factor-alpha (TNF-α), interleukin-6 (IL-6), leptin, plasminogen-activator inhibitor 1 and adiponectin from adipose tissue contributes to chronic inflammation (Espinola-Klein *et al.*, 2011; Andreozzi *et al.*, 2006; Kern *et al.*, 2001). These inflammatory cytokines have been targeted in many intervention studies in efforts to reduce chronic inflammation. In subjects with metabolic syndrome, lifestyle interventions, particularly dietary change were found to be consistently successful in improving the inflammatory condition independent of obesity (Beavers & Nickles, 2011). Furthermore, bioactive compounds from food such as isoflavone, anthocyanin, peptides and plant sterol were found to be protective against chronic inflammation (Rosa *et al.*, 2012). Supplementation of zinc and omega-3 fatty acids significantly reduced circulating C-reactive protein and interleukin-6 in human subjects (Pourteymour *et al.*, 2011; Kelly *et al.*, 2009)

Tocotrienols are one of the homologue series of vitamin E. It has a higher potency than its homologue, α-tocopherol due to its efficient penetration into tissues (Meydani, 1995) and high cellular uptake (Saito *et al.*, 2004). Its cholesterol lowering property is one of the most widely studied subjects in tocotrienols research. The cholesterol lowering effect of tocotrienols is comparable to the effect from lovastatin, an anti-cholesterol medication (Qureshi *et al.*, 2001). Findings from a few recent clinical trials (Chin *et al.*, 2011; Yuen *et al.*, 2011) showed that tocotrienols reduced total cholesterol, low density lipoprotein (LDL) cholesterol, advanced glycosylation end-product and protein carbonyl contents in human subjects. However, far too few studoes have been initiated on the potential of tocotrienols to combate chronic inflammation. Previous cell lines and animal studies (Qureshi *et al.*, 2011; Yam *et al.*, 2009; Wu *et al.*, 2008) have reported on the reduction of pro-inflammatory cytokines level following the treatment with tocotrienols. In an attempt to reproduce the findings in humans, the present study aimed to evaluate the effects of mixed palm tocotrienols supplementation on inflammatory cytokines and lipid profile in Universiti Putra Malaysia (UPM) staff with metabolic syndrome.

METHODS

Two hundred and thirty four male and female staff of Universiti Putra Malaysia, aged 20-60 years, were screened for metabolic syndrome from February to March 2012. The diagnosis of metabolic syndrome was based on the criteria proposed by Alberti *et al.* (2009). At least three out of the following five components were required for the diagnosis: elevated waist circumference (≥90cm for men; ≥80cm for women), elevated triglycerides (≥1.7 mmol/L), reduced HDL-C (<1.0 mmol/L for men; <1.3 mmol/L for women), elevated blood pressure (systolic ≥ 130 and/or diastolic ≥ 85 mm Hg) and elevated fasting plasma glucose (≥ 5.6 mmol/L). However, individuals with diabetes, hypertension, cardiovascular disease or any other serious medical conditions were excluded. Individuals who were on medication that might affect any of the components of metabolic syndrome, as determined by physicians, or any form of supplements in the past three months, were not included. Smokers and pregnant women were also excluded. Informed consent was obtained from subjects who agreed to participate in the study. Ethical approval was obtained from the Medical Research Ethics Committee, Faculty of Medicine and Health Sciences, Universiti Putra Malaysia.

Sample size calculation

A sample size formula for two-group comparisons of means in a two-sided test (Machin & Campbell, 2005) was used to calculate the sample size in this study. A previous study of tocotrienols supplementation by Yuen *et al.* (2011) was used as a reference. The sample size per group was calculated as 28 persons. To allow for a drop-out rate of 25%, 35 subjects were recruited for each group. The total number of subjects recruited for the two groups was 70.

Randomisation and intervention

This is a randomised, double-blind , placebo-controlled trial. The seventy subjects recruited were randomly assigned to a mixed tocotrienols group or a placebo group by block randomisation with randomly selected block sizes using SAS Version 9.3 (SAS Institute Inc., NC, USA). The supplementation group received Tocovid Suprabio™, a mixed tocotrienols capsule derived from palm (manufactured by Hovid Sdn Bhd, Ipoh, Malaysia) for sixteen weeks. Each soft gelatin capsule of Tocovid Suprabio™ contains typically 200mg of mixed tocotrienols (61.52mg α-tocotrienol, 112.80mg γ-tocotrienol and 25.68mg δ-tocotrienol) and 61.1mg of α-tocopherol. During the sixteen weeks, the subjects in the supplementation group took 200mg tocotrienols capsules twice daily, morning and evening, after meals, with a total daily dosage of 400mg. The subjects in the placebo group received placebo capsules (manufactured by Hovid Sdn Bhd, Ipoh, Malaysia) containing approximately one gram of soy bean oil per capsule with weight, appearance and smell identical to the tocotrienols capsules. The subjects took one placebo capsule twice a day, morning and evening, after meals.

Subjects in both groups were instructed to maintain their habitual dietary intake patterns and lifestyle throughout the study period. No dietary or lifestyle advice was given to the subjects. They were also reminded not to take any form of other supplements during the study period. To maximise the compliance rate, the following measures were taken: three sessions of meeting were held with the subjects, concurrently with three assessment sessions: one at baseline, the second at week 8 and the third at week 16. The subjects were also followed-up through personal visits, short message system and emails. Each subject was given a participant's record card to record personal particulars, health assessment results, adverse events, any medication taken and contact details of researchers and physicians in case of emergency. The participants brought the record card to be reviewed during each meeting. A drug pill box with six compartments was given to each of the subjects. Participants filled each compartment with a capsule. The six compartments with six capsules were intended for a twice-a-day administration of the capsules for three days.

An adverse event was defined as an untoward medical occurrence, regardless of the relation to the treatment. If a subject reported any adverse events during the study, the details on time, length, frequency and severity of the symptoms were taken note and conveyed to the physicians to determine if the symptoms were clinically relevant to the supplementation. The subject was then called on a daily basis until the symptoms were resolved. If a prolonged, serious adverse event that was related to the supplementation occurred, the subjects would be withdrawn from the study.

The compliance of the subjects to the trial was assessed according to capsule counts. Remaining capsules in the bottles returned were counted in week 8 and week 16. Compliance was defined as the mean percentage of prescribed capsules absent from the returned capsule bottles. Only data from subjects with a compliance rate of at least 80% were included in the final analysis.

Data collection

Socio-demographic, clinical and biochemical assessment

At baseline, a self-administered socio-demographic questionnaire was used to collect information on the age, ethnicity, education level and monthly income of the subjects. At baseline, week 8 and week 16, weight was measured to the nearest 0.1kg in light clothing without shoes using a digital weighing scale (Tanita, Japan). Height was measured to the nearest 0.1cm with height measuring rod (SECA, Germany). Body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Waist circumference (WC) was measured at the high point of the iliac crest at minimal respiration to the nearest 0.1cm at the end of normal expiration, using a measuring tape. Hip circumference was measured from the widest part of the hip. Waist-hip-ratio (WHR) was calculated by dividing the waist circumference and the hip circumference. All anthropometric measurements were done in duplicate and the average of two readings was used for analysis. Percentage of body fat was assessed using TBF-300 Body Composition Analyzer (Tanita, Japan). The percentage of body fat was measured with participants being barefoot and with an empty bladder. Blood pressure was measured in duplicate at 2-minute intervals using IA2 Omron Automatic Blood Pressure Monitor (Omron, Japan) with the participants rested and seated. Elevated blood pressure was defined as either elevated systolic BP or diastolic BP alone or a combination of both.

At baseline and week 16, 10 ml of a 12-hour overnight fasted blood samples were collected. The concentrations of serum total cholesterol (TC), serum HDL-cholesterol and fasting plasma glucose (FPG) were determined using commercial kits (Abbot Laboratories, IL, USA) on an automated analyser (Advia® 2400 Chemistry System, Siemens Healthcare Diagnostics, NY, USA). The LDL cholesterol was then determined by Friedewald's formula as follows: LDL cholesterol = total cholesterol - [HDL cholesterol + (0.46 X triglycerides)]. Plasma levels of IL-6, TNF-α and leptin was determined by a multiplex cytokine assay using a customised Luminex-based multiplex immunoassay kits (Procarta Cytokine Assay Kit: Affymetrix Panomics, CA, USA) according to the manufacturer's protocol. The intra-assay values were 6.6% for IL-6 (n = 20), 3.0% for TNF-α (n = 15) and 6.6% for leptin (n = 20). Adiponectin level in plasma samples was determined by using a commercial high sensitivity human adiponectin enzyme immunoassay (EIA) kit (SPI Bio, France). The intra- and inter-assay coefficients of variance were 4.4% (n=8) and 5.7% (n=9), respectively. The C-reactive protein (CRP) level in the plasma samples was determined by using a commercial high sensitivity CRP enzyme immunoassay kit (IBL International, Hamburg, Germany). The intra- and inter-assay coefficients of variance were 5.1% (n=10) and 6.1% (n=24), respectively. All assays were run in duplicate.

Dietary and physical activity assessment

Dietary intake and physical activity level of the subjects were assessed at baseline, week 8 and week sixteen. A 2-day, 24-hour dietary recall questionnaire was used to collect detailed information on all foods and beverages consumed by the subjects in two separate days. Details about the name of foods or drinks, portion size and any ingredients added to the food or drinks were obtained and tabulated. Data were analysed using the Nutritionist Pro software, version 2.2 (Axxya Systems Nutritionist Pro, TX, USA). To assess the physical activity level, a short version of the International Physical Activity Questionnaire (IPAQ) in Malay was used. The questionnaire comprised nine items on three types of physical activity intensity, namely walking, moderate intensity activity and vigorous intensity activity. Subjects were interviewed and requested

to recall their frequency and duration of physical activity performed according to intensity for the past seven days. The data was then used to calculate MET-min per week, a value of metabolic equivalent.

Data analysis

Data was analysed using Statistical Package for Social Sciences, version 22 (IBM, New York, USA). For normally distributed variables, interval or ratio variable values were presented as mean ± standard deviation (SD). A paired sample *t*-test and an independent sample *t*-test were used to determine within-group and between-group differences in variables measured at two time points. For variables measured at three time points, a General Linear Model (GLM) Repeated Measures ANOVA was used. For variables that violated the normality assumption, interval or ratio values were presented as median ± interquartile range. Wilcoxon signed ranks test and Mann-Whitney U test were used to determine within-group and between-group differences in variables measured at two time points. A Friedman test was used to determine differences over three time points. Chi-square was used to examine the association between categorical variables while Pearson correlation was used to determine the association between two continuous variables. A statistical probability level of $p<0.05$ (two-sided) was considered as significant.

RESULTS

Screening, recruitment and drop-out

Upon screening, 164 of staff members were excluded, leaving a total of seventy subjects eligible for the study. The seventy subjects were randomised into the tocotrienols group (n=35) and the placebo group (n=35). Only one subject (2.9%) dropped out from the tocotrienols group due to being on anti-inflammatory medication during the study. Two subjects (5.7%) from the placebo group dropped out due to pregnancy and withdrawal from taking capsules, respectively. The compliance rate for the tocotrienols group and the placebo group were 85.3% and 84.8%, respectively. Twenty nine subjects (82.9%) from the tocotrienols group and twenty eight subjects (80.0%) from the placebo group were finally included in data analysis.

Baseline characteristics of the subjects

In the tocotrienols group, 13 subjects (44.8%) were men and 16 (55.2%) were women. In the placebo group, 7 (25.0%) and 21 (75.0%) were men and women, respectively. No statistically significant difference was found in the proportion of men and women between the two groups. The mean age was 40.5 ± 9.0 in the tocotrienols group and 42.0 ± 10.0 in the placebo group. At baseline, no significant difference was found in any of the study parameters between the two groups (Table 1).

Changes in obesity measures and blood pressure

No significant change was found in BMI, WC, WHR, percentage of body fat and systolic blood pressure between baseline, week 8 and week 16 in either the tocotrienols or the placebo group. A significant reduction in diastolic blood pressure between week 16 and baseline was found in the tocotrienols group (p=0.002). However, no significant difference between groups was detected (Table 2).

Changes in lipid profile and cytokines

In the tocotrienols group, significant reductions from baseline were found in TC (p=0.008), LDL-cholesterol (p=0.022), HDL-cholesterol ($p<0.001$), IL-6 (p=0.024) and TNF-α (p=0.013) at week sixteen. A significant increase in TC/HDL ratio (p=0.005) was observed. Across the same time points, a significant reduction was found in the placebo group for TC (p=0.003), LDL-cholesterol (p=0.033), HDL-cholesterol (p=0.002) and adiponectin

Table 1. Baseline data of the subjects

Parameter	*Tocotrienols (n=29)*†	*Placebo (n=28)*†	*p*§
BMI (kg/m²)‡	30.2 ± 4.7	31.7 ± 5.0	0.096¶
WC (cm)‡	100.6 ± 11.9	105.3 ± 16.5	0.084¶
WHR	0.94 ± 0.06	0.94 ± 0.05	0.389
Body fat (%)	35.5 ± 5.5	38.3 ± 6.4	0.097
SBP (mmHg)‡	136.5± 9.0	133.1 ± 7.04	0.719¶
DBP (mmHg)	85.4 ± 8.2	83.8 ± 7.0	0.577
TG (mmol/L)	2.07 ± 0.78	1.76 ± 0.81	0.186
TC (mmol/L)	5.82 ± 0.76	5.57 ± 0.86	0.246
LDL-C (mmol/L)	3.75 ± 0.67	3.67 ± 0.74	0.688
HDL-C (mmol/L)	1.11 ± 0.19	1.09 ± 0.21	0.684
FPG (mmol/dL)‡	4.85 ± 1.35	5.10 ± 1.03	0.640¶
IL-6 (pg/ml)‡	3.24 ± 1.45	3.66 ± 2.76	0.513
TNF-α (pg/ml)‡	2.27 ± 1.12	2.50 ± 1.98	0.798¶
CRP (mg/L)	4.87 ± 2.93	5.95 ± 2.60	0.115
Adiponectin (µg/ml)	5.59 ± 2.11	5.18 ± 1.84	0.464
Leptin (ng/ml)	2.95 ± 1.65	3.67 ± 1.85	0.164

BMI = body mass index; WC = waist circumference; WHR = waist hip ratio; SBP = systolic blood pressure; DBP = diastolic blood pressure; TG = triglycerides; TC = total cholesterol; LDL-C = Low density lipoprotein cholesterol; HDL-C = High density lipoprotein cholesterol; FPG = Fasting plasma glucose; IL-6 = Interleukin-6; TNF-α = Tumor necrosis factor-α; CRP = C-reactive protein

† Values are means ± SD unless indicated otherwise.

‡ Values are median ± interquartile range.

§ No significant difference between groups by independent-samples t-test unless indicated otherwise.

¶ No significant difference between groups by Mann-Whitney U test.

(p=0.049) while a significant increase in FPG (p=0.019) was observed (Table 3). However, changes in variables in the tocotrienols group were not significantly different from the placebo group.

Changes in dietary intake and physical activity level

No significant change was found in caloric intake, percentage of carbohydrates, protein and fat and MET-min in the tocotrienols and placebo group between baseline, week 8 and week 16 (Table 4).

Adverse events

No serious adverse event was reported in both groups. Mild complaints in the tocotrienols group were dry mouth (5 cases or 17.2%), increased appetite (4 cases or 13.8%), headache and skin itchiness (2 cases or 6.9%, respectively) and increased body heat, dizziness and constipation (1 cases or 3.4%, respectively). Subjects in the placebo group experienced dry mouth (3 cases or 10.3%), skin itchiness (2 cases or 6.9%) and increased appetite, headache and increased body heat (1 cases or 3.4%, respectively). No subject withdrew from the study due to an adverse event.

DISCUSSION

To the best of our knowledge, this is the first study showing the potential benefit of mixed palm tocotrienols supplementation in reducing circulating IL-6 and TNF-α in subjects with metabolic syndrome. In the tocotrienols group, a significant reduction of 13.9% (p=0.024) and 10.6% (p=0.013) from baseline was found at week 16. Although the magnitude of changes was

Table 2. Obesity measures and blood pressure at baseline and week 16

Parameters	*Tocotrienols (n=29)*				*Placebo (n=28)*			
	Baseline†	*8 weeks*†	*16 weeks*†	*p*	*Baseline*†	*8 weeks*†	*16 weeks*†	*p*
BMI (kg/m²)‡	30.2 ± 4.7	30.0 ± 5.3	30.0 ± 4.8	0.991	31.8 ± 5.2	31.6 ± 4.1	32.0 ± 4.5	0.120
WC (cm)‡	100.6 ± 11.9	99.5 ± 12.5	100.5 ± 12.4	0.156	105.5 ± 15.9	103.0 ± 12.1	105.0 ± 13.5	0.181
WHR	0.94 ± 0.06	0.94 ± 0.06	0.93 ± 0.06	0.526	0.95 ± 0.05	0.95 ± 0.05	0.96 ± 0.05	0.204
Body fat (%)	35.5 ± 5.5	36.0 ± 5.7	35.6 ± 5.8	0.238				
	38.3 ± 6.4	38.7 ± 6.3	38.1 ± 6.9	0.403				
SBP (mmHg)‡	136.5 ± 9.0	132.0 ± 16.0	130.0 ± 16.0	0.057	138.0 ± 12.0	135.0 ± 17.5	132.0 ± 18.0	0.165
DBP (mmHg)	85.4 ± 8.2	82.5 ± 9.1	80.8 ± 8.8§	0.001	83.8 ± 7.0	82.8 ± 7.5	80.5 ± 7.8	0.096

BMI = body mass index; WHR = waist hip ratio; † Values are means (SD) unless indicated otherwise; ‡ Values are median (interquartile range); § Significantly different from baseline by repeated measures ANOVA.

Table 3. Metabolic parameters and inflammatory markers at baseline and week 16

Parameters	*Tocotrienols (n=29)*				*Placebo (n=28)*			*Treatment effect*		
	Baseline†	*16 weeks*†	*Change*	*p*	*Baseline*†	*16 weeks*†	*Change*	*p*	*Differences*	*p*
TC (mmol/L)	5.82 ± 0.76	5.55 ± 0.78¶	-0.27	0.008	5.57 ± 0.86	5.23 ± 1.02¶	-0.34	0.003	+0.07	0.635
TG (mmol/L)	2.07 ± 0.78	2.11 ± 1.01	+0.04	0.751	1.76 ± 0.81	1.69 ± 0.85	-0.07	0.479	+0.11	0.691
LDL-C (mmol/L)	3.75 ± 0.67	3.55 ± 0.70¶	-0.20	0.022	3.67 ± 0.74	3.49 ± 0.90¶	-0.18	0.033	-0.02	0.812
HDL-C (mmol/L)	1.11 ± 0.19	1.01 ± 0.17¶	-0.10	<0.001	1.09 ± 0.21	1.00 ± 0.17¶	-0.09	0.002	-0.01	0.765
LDL/HDL	3.47 ± 0.80	3.61 ± 0.94	+0.14	0.088	3.45 ± 0.76	3.53 ± 0.70	+0.07	0.429	0.07	0.581
TC/HDL	5.33 ± 0.96	5.61 ± 1.25	+0.28	0.005	5.23 ± 0.97	5.44 ± 0.96	+0.21	0.068	0.07	0.628
FPG (mmol/dL)‡	4.85 ± 1.35	4.80 ± 0.80	-0.05	0.480	5.10 ± 1.03	5.25 ± 1.15§	+0.15	0.013	-0.33††	0.028
IL-6 (pg/ml)‡	3.24 ± 1.45	2.79 ± 1.22§	-0.45	0.024	3.66 ± 2.76	2.90 ± 1.46	-0.76	0.056	+0.31	0.342
TNF-α (pg/ml)‡	2.27 ± 1.12	2.03 ± 0.80§	-0.24	0.013	2.50 ± 1.98	2.05 ± 0.93	-0.45	0.121	+0.21	0.768
CRP (mg/L)	4.87 ± 2.93	4.93 ± 3.24	+0.06	0.908	5.95 ± 2.60	6.70 ± 3.58	+0.75	0.167	-0.70	0.337
Adiponectin (µg/ml)	5.59 ± 2.11	5.31 ± 1.92	-0.28	0.254	5.18 ± 1.84	4.68 ± 1.79††	-0.51	0.049	+0.22	0.526
Leptin (ng/ml)	2.95 ± 1.65	3.11 ± 2.19	+0.16	0.473	3.67 ± 1.85	3.72 ± 1.84	+0.05	0.810	+0.11	0.712

TC = total cholesterol; LDL-C = Low density lipoprotein cholesterol; HDL-C = High density lipoprotein cholesterol; FPG = Fasting plasma glucose; IL-6 = Interleukin-6; TNF-α = Tumor necrosis factor-α; CRP = C-reactive protein; † Values are mean ± SD unless indicated otherwise; ‡ Values are median ± interquartile range; § Significant differences between baseline and 16 weeks (P<0.05) by Wilcoxon signed ranks test; ¶ Significant differences between baseline and 16 weeks (P<0.05) by paired-samples *t* test; †† Significant differences between tocotrienols and placebo groups by Mann-Whitney U test.

Table 4. Dietary intake and physical activity level at baseline, week 8 and week 16

	Tocotrienols (n=29)†	*p‡*	*Placebo (n=28)†*	*p‡*
Energy (kcal)				
Baseline	2115.4 (615.2)	0.150	2694.7 (603.0)	0.168
Week 8	1925.6 (628.7)		2015.7 (558.7)	
Week 16	1930.9 (597.8)		2196.8 (803.8)	
Carbohydrate (%)				
Baseline	53.6 ± 6.7	0.980	50.5 ± 6.9	0.224
Week 8	54.0 ± 9.5		49.4 ± 10.5	
Week 16	53.8 ± 7.5		49.0 ± 6.9	
Protein (%)				
Baseline	17.2 ± 3.6	0.511	18.1 ± 4.0	0.789
Week 8	16.2 ± 3.6		17.1 ± 4.3	
Week 16	16.8 ± 3.2		18.3 ± 3.6	
Fat (%)				
Baseline	29.1 ± 4.8	0.993	31.4 ± 5.3	0.286
Week 8	29.8 ± 7.3		33.4 ± 8.7	
Week 16	29.3 ± 5.2		32.6 ± 5.5	
Physical activity level (MET-min/week)				
Baseline	3461.7 ± 4289.1		2382.4 ± 2689.7	0.627
Week 8	3539.8 ± 3735.1	0.443	2574.3 ± 3975.0	
Week 16	3023.7 ± 3538.0		2139.3 ± 2163.8	

† Values are means ± SD.
‡ p value of differences three time points by Repeated Measures ANOVA

not significantly different from that of the placebo group, the findings indicate the potential anti-inflammatory activity of tocotrienols in low-grade chronic inflammation, a phenomenon commonly observed in metabolic syndrome (Marsland *et al.*, 2010; Lee *et al.*, 2007). In an earlier human study, 400mg/day tocotrienols supplementation produced a reduction in circulating IL-6 after 56 days in healthy women who received a tetanus toxoid vaccine challenge (Mahalingam *et al.*, 2010). However, the inflammatory state in the study was chemically-induced. Its higher baseline IL-6 level (approximately 90 pg/ml vs 3.24 pg/ml in the present study), does not allow for comparisons with the present study. Individuals with metabolic syndrome tend to have higher circulating IL-6 and TNF-α as they have higher amounts of adipose tissue. Adipocytes produce monocyte chemo-attractant protein-1 (MCP-1), a cytokine which recruits macrophages to the adipose tissue. The infiltrated macrophages secrete TNF-α which promotes the production of various cytokines from adipocytes (Olefsky & Glass, 2010). It has been demonstrated that macrophages constitute an amazing proportion of 60% of total adipose tissue cell content in obese individuals, which is significantly higher than only up to10% in non-obese (Weisberg *et al.*, 2006). Higher systemic cytokines level in obese subjects than non-obese (Bullo *et al.*, 2012). However, in the present study, the reduction in IL-6 and TNF-α in the tocotrienols group did not correlate to a reduction in waist circumference or percentage of body fat. The effects of the palm tocotrienols on body weight and body fat were negligible and consistent with other studies (Chin *et al.*, 2011; Yuen *et al.*, 2011). In a human monocytic cell

study (Wu *et al.*, 2008), changes in various cytokines including TNF-α, IL-4 and IL-8, and the production of nitric oxide synthase (NOS) and cyclooxygenase (COX)-1 and -2, and the expression of nuclear factor-κB (NF-κB) were studied. Tocotrienols extracted from palm oil were found to suppress the production of cytokines via the inhibition on iNOS and COX-2 production and the expression of NF-κB in the cell. The findings were supported by another study (Yam *et al.*, 2009) which looked into the effect of individual tocotrienol isomers on pro-inflammatory markers in the RAW264.7 macrophages cell line. The production of IL-6 and TNF-α in the cell line was also remarkably reduced following the treatment of each tocotrienol isomers (α-, γ- and δ-tocotrienol) and tocotrienol-rich fraction extracted from palm oil. The expression of COX-2 was down-regulated mostly by δ-tocotrienols, followed by α-tocotrienol, γ-tocotrienol and tocotrienol-rich fraction. Interestingly, δ-tocotrienol was also shown to be the best suppressor of IL-6 and TNF-α. Qureshi *et al.*, in 2011 found that δ-tocotrienol also reduced serum TNF- α levels by 83% in chicken, which was comparable to the use of other anti-inflammatory agents including quercetin and dexamethasone. In our study, the amount of δ-tocotrienol in the capsule was approximately 13% of the total mixed tocotrienols, being the least among the three tocotrienols isomers. Therefore, a formulation with higher δ-tocotrienol may produce better anti-inflammatory results. Besides, a moderate, significant correlation ($r=0.68$, $p<0.001$) was found between changes in IL-6 and changes in TNF-α when controlling for changes in caloric intake and MET-min, indicating that both IL-6 and TNF-α were reduced concurrently following the tocotrienols supplementation. Both IL-6 and TNF-α promote serine phosphorylation of IRS-1 and suppress the expression of glucose transporter-4 leading to impaired insulin signalling and insulin resistance at the cellular level (Kershaw & Flier, 2004; Hotamisligil, 2001). Therefore, further studies should include insulin resistance measures in tocotrienols supplementation study.

The present study also showed the potential cholesterol lowering effects of the mixed palm tocotrienols. In a previous study using an identical mixed palm tocotrienols formulation (Yuen *et al.*, 2011), a lower tocotrienols dosage (300mg/day) produced a significant reduction in TC (8.9%) and LDL (12.8%) cholesterol at week twenty. In the present study, a higher tocotrienols dosage at 400mg/day produced a reduction in TC (4.6%) and LDL (5.3%) in just four months. Higher dosage might have contributed to the effects in a shorter period. However, the magnitude of reduction was lesser, most likely due to the fact that all the subjects in the previous study were hypercholesterolemic with higher baseline TC (6.91±0.11 mmol/L vs 5.82±0.76 mmol/L in the present study) and LDL (4.82±0.17 mmol/L vs 3.75±0.67 mmol/L in the present study). Of note, in both studies, the subjects were on habitual diet without any diet modification. Consumption of healthy foods has been recommended as an alternative strategy for treating metabolic syndrome (Fappa et al., 2012). A low fat diet with 100 mg/day of the tocotrienols supplement was the more effective in lowering cholesterol level in hypercholesterolemic subjects compared to low fat diet or supplementation alone (Qureshi *et al.*, 2002). Therefore, tocotrienols supplementation that is coupled with dietary modification deserves further investigation.

Tocotrienols reduce cholesterol level by inhibiting 3-hydroxy-3-methyl-glutaryl-CoA (HMG-CoA) reductase activity via stimulating the degradation of the enzyme (Parker *et al.*, 1993). γ-tocotrienol and δ-tocotrienol stimulated the degradation of HMG-CoA reductase through ubiquitination (Song & DeBose-Boyd, 2006). Among all the tocotrienols

isomers, γ-tocotrienol and δ-tocotrienol have shown greater capacity in lowering cholesterol (Song & DeBose-Boyd, 2006; Yu *et al.*, 2006) due to the number and position of methyl groups in the chromanol ring. The tocotrienols capsules used in the current study have highest proportion of γ-tocotrienol (approximately 56%). Also, a self-emulsifying formulation of tocotrienols that was reported to produce a better and more consistent absorption (Yuen *et al.*, 2011) was used in the present study. These might have contributed to the cholesterol lowering results.

The tocotrienols group showed a reduction of 9.0% ($p<0.00$) in HDL cholesterol level at the end of the study which might have contributed to an increase in TC/HDL ratio (p=0.005). This is in contrast with previous studies that showed a significant increase in HDL cholesterol (Chin *et al.*, 2011) and insignificant changes (Yuen *et al.*, 2011; Baliarsingh, Beg & Ahmad, 2005; Mustad *et al.*, 2002) following tocotrienols supplementation. In the present study, surprisingly the placebo group also reported a significant reduction of 9.0% in HDL cholesterol (p=0.002). As no significant change was shown in dietary intake and physical activity level which might affect the cholesterol metabolism, the discrepancy is puzzling. However, more time points of HDL cholesterol measurements in the course of the study might give a better view of changes in trend. In the study by Chin *et al.* (2011), a marginal reduction of 2.9% in HDL cholesterol was recorded in the third month after tocotrienols supplementation and a significant increase was only observed at the sixth month. Of note, the tocotrienols group recorded a 5.4% significant reduction (p=0.002) in diastolic blood pressure and a 4.8% non-significant reduction in systolic blood pressure at week sixteen. However, no significant difference with the placebo group was found. The reason might be that all subjects recruited had nearly normal blood pressure. The baseline mean systolic and diastolic blood pressure in this study was 136.5 mmHg and 85.4 mmHg, respectively.

Over time, although the tocotrienols group recorded significant improvements in some cytokines, lipid markers and blood pressure, no significant treatment effect or difference from placebo group was shown. One main reason was that unexpectedly the placebo group also experienced some improvement concurrently. The placebo group showed significant reduction over the sixteen weeks for TC (p=0.003) and LDL cholesterol (p=0.033) and reduction in IL-6 and TNF-α although the change did not reach statistical significance. It is difficult to explain this increase as in the placebo group, no significant change was found in the dietary intake and physical activity level over the study period. In previous studies, the soy bean oil from the same manufacturer (Hovid Sdn Bhd, Ipoh, Malaysia) also did not produce any significant change when used as placebo (Yuen *et al.*, 2011; Mahalingam *et al.*, 2010). The filler of each placebo capsule was approximately one gram of soy bean oil. When it was taken twice a day at a total dosage of two grams, the soy bean oil that contained polyunsaturated fatty acids might have produced some cholesterol lowering and anti-inflammatory effects. Soybean oil contains more than 40% of myrsitic acid with other unsaturated fatty acids such as oleic acids and linoleic acids (Sodamade, Oyedepo & Bolaji, 2013) and has been found to exert some physiochemical effects in humans (Holguin *et al.*, 2005) and in animals (Hassan & Abdel-Wahhab, 2012; Furman, Volkova & Aviram, 2006). To avoid this confounder, the filler of the placebo capsules should be formed by the excipient of the tocotrienols capsules, making it comparable between the two. A previous study (Chin *et al.*, 2011) used palm superolein oil as filler of the placebo capsules while the treatment capsules were

tocotrienols suspended in an identical type of oil. No significant change in any of the study variables was reported in the study.

The supplementation capsule conained α-tocopherol which might have interfered with the absorption and utilisation of tocotrienols isomers. Antagonising effects of α-tocopherol on the cholesterol lowering activity of tocotrienols was observed in an animal study (Qureshi *et al.*, 1996). Future studies to test the effects of tocotrienols should use pure tocotrienols instead of a mixed tocotrienols and tocopherol.

Few mild adverse events were reported in the tocotrienols group. However, it was observed that no adverse event was severe enough for withdrawal from the study. Compliance with the supplementation was satisfactory, indicating that the mixed palm tocotrienols supplement was well tolerated by the subjects with metabolic syndrome.

There are some limitations to our study. The sample size was relatively small which might have reduced the power to detect between group differences. As the measurement of biochemical parameters was carried out at two time points (baseline and week 16), it limited the observation of a trend. The study was conducted in an ethnically homogenous population in a geographically limited location in the university campus. Therefore, the results from this study cannot be generalised to a larger population.

The study suggests that the mixed palm tocotrienols supplementation exert potential beneficial effects in chronic inflammation and lipid profiles in adults with metabolic syndrome. However, the results were most likely confounded by the physiological effects produced by the soy bean oil in the placebo capsules. Further studies are needed to clarify this by selecting a more appropriate placebo capsule content. Longer prospective studies with clinical end-points are required to evaluate whether the improvements following tooctrienols supplementation contribute to a risk reduction of diabetes and cardiovascular disease.

ACKNOWLEDGEMENTS

The study was funded under the Research University Grants, Higher Education Ministry of Malaysia (RUGS 9370600). We thank Hovid Sdn Bhd, Ipoh, Malaysia for the provision of study capsules and technical assistance.

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