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Mob: +92 300 3008585, Fax: +92 41 8815544

E-mail: editorpjn@gmail.com

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Effect of Chemical Properties of Milk Thistle Seed Oil on Serum Lipid Profile and Antioxidants Capacity in Rats Fed High Cholesterol and Cholesterol Free Diets

I.R. Dabbour[1], M.N. Ahmad[2], H.R. Takruri[2] and K.M. Al-Ismail[2]
[1]Department of Clinical Nutrition, Faculty of Applied Medical Science, Umm Al-Qura University, Makkah, Saudi Arabia
[2]Department of Nutrition and Food Technology, Faculty of Agriculture, University of Jordan, Amman-11942, Jordan

Abstract: This study aimed to examine the *in vivo* effect of chemical properties of cold-pressed Milk Thistle Seed Oil (MTSO) on serum lipid profile, lipid peroxidation product (MDA) and total antioxidant capacity (TAC).These examinations were carried out to investigate the long-term effect of feeding two levels of MTSO in experimental diets with and without cholesterol. Sixty-four adult male Sprague-Dawley rats were divided into eight groups. Four groups were on free cholesterol diets with either 4% MTSO, 8% MTSO, 4% control (soybean oil) or 8% control oil. The other 4 groups were fed 1% cholesterol with percentages of oils similar to the first four groups for 12 weeks. Regarding serum lipoproteins there were significant differences ($p<0.05$) in TC in 8% MTSO and 8% control cholesterol-supplemented diets when compared with 8%control cholesterol-free diets. Also LDL-C of cholesterol-supplemented diet groups were significantly higher ($p<0.05$) than cholesterol-free diet groups. An interesting result was the significantly high levels ($p<0.05$) of HDL-C in rats fed cholesterol-free diet with MTSO 4 and 8% when compared to cholesterol-supplemented diets. There was a significantly higher ($p<0.05$) HDL-C/LDL-C ratio for cholesterol-free diets than cholesterol-supplemented diets. Subsequently, high significant difference ($p<0.05$) was observed in AI values at 8% cholesterol-supplemented diet when compared with cholesterol-free diet. MDA showed clearly high levels at 8% control oil with or without cholesterol. Regarding the TAC, it was higher ($p<0.05$) in 4% cholesterol-free MTSO diet when compared to 8% cholesterol-free control diet and 4% cholesterol supplemented MTSO diet. Based on the results obtained, the % of oil and presence of cholesterol were the main factors affecting the lipid profile, calculated AI, lipid peroxidation and TAC. With respect to *in vivo* lipid peroxidation, MTSO was clearly superior to soybean oil.

Key words: Milk thistle seed oil-Lipid peroxidation product- Antioxidant capacity-Calculated AI-Lipoproteins

INTRODUCTION

Despite the successes in prevention of atherosclerosis, cardiovascular disease (CVD) is still responsible for the most common cause of death for both males and females worldwide (Houenschil *et al*., 2010). Mortality statistical data from Jordan Ministry of Health indicates that 38.2% of deaths in 2003 were attributed to CVD (Khader *et al*., 2010). Dyslipidemia, which is closely linked to the pathophysiology of CVD, is a key independent modifiable risk factor for CVD (Hammoudeh *et al*., 2008). Brown *et al*. (2009) and Khader *et al*. (2010) found that hypercholesterolemia and hyperglyceridemia are highly prevalent in Jordan with a pattern consistent with that of United States and other developed countries. Many studies showed that different dietary lipids can modulate plasma cholesterol level, depending on their fatty acid composition. High levels of dietary saturated fatty acids (SFA) or *trans* fatty acids are associated with the formation of atherosclerotic plaque. However, intakes of MUFA or oleic acid and PUFA of the n-3 series and n-6 series are associated with decreased risk of CVD (Moon *et al*., 2001; Cintra *et al*., 2006). Several clinical trials indicated that consumption of 13 to 21% dietary energy from PUFA decreased total plasma cholesterol by 13 to 15% and decreased CVD events by 25 to 43% (ADA, 2007).

It is known that oil seeds have been part of the human diet for a long time and their production has shown a remarkable increase in the last few decades (Tuberose *et al*., 2007; Parry *et al*., 2008). The potential use of these oils is gaining importance as nutraceuticals and phytoceuticals for improving human nutrition and health and for disease prevention (Bail *et al*., 2008; Garjani *et al*., 2009). Milk thistle (MT) seeds contain a relatively high amount of oil (20-26%). Extracted oil contains phytosterols, phenolic compounds and a high content of vitamin E, serving as a potential natural source of vitamin E (Parry *et al*., 2006; Fathi-Achachlouei and Azad mard-Damirchi, 2009; Dabbour *et al*., 2014). It has been reported that the extracted oil from MT seed contains fatty

Corresponding Author: I.R. Dabbour, Department of Clinical Nutrition, Faculty of Applied Medical Science, Umm Al-Qura University, Makkah, Saudi Arabia

acids such as linoleic acid, oleic acid, linolenic acid, palmitic acid, stearic acid and it has been suggested as being suitable as an edible oil (Abu-Rajouh and Takruri 2000; Dabbour *et al.*, 2014).

There are few data indicating capability of MT seed or its oil to modulate and positively affect lipoprotein metabolism and reduce the risk of CVD (Skottova *et al.*, 2003; Sobolova *et al.*, 2006). The health importance of MT seed oil was suggested to be due to the antioxidant activity of phytochemcals compounds against CVD and lipid peroxidation. These compounds include polyphenols, phytosterols, chlorophylls, squalene, tocopherols and carotenoids which represent a minor component in plant oils (Gorinstein *et al.*, 2003; Visavadiya and Narasimhacharge, 2008; Dabbour *et al.*, 2014). The USFA and phytochemical compounds also affect the pro-and antioxidative processes in the oils (Parry *et al.*, 2006; Bail *et al.*, 2008; Fathi-Achachlouei and Azadmard-Damirchi, 2009). The aims of the previous studies were to demonstrate a pharmaceutical effect of MT seed as based on the use of its extract, derivates, or whole seed powder. To the best of our knowledge, no studies were conducted to investigate the effect of chemical properties of MT seed oil on blood lipid profile. Information from controlled intervention trials in humans or animals regarding the *in vivo* effects of MT seed oil on the antioxidant capacity is not available. It is hypothesized that regular incorporation of cold-pressed MT seed oil in the diet will favorably affect blood lipid profile and antioxidant status in rats and the level of the oil and the cholesterol content of the diet may influence the extent of this effect. Therefore, we decided to investigate the effect of cold-pressed MT seed oil (MTSO) on *in vivo* serum lipid profile, total antioxidant capacity and lipid peroxidation product, namely malondialdehyde (MDA) in adult rats fed cholesterol-supplemented diets.

MATERIALS AND METHODS

Cold-pressed MTSO used in experimental diet mixtures was prepared and analyzed chemically by the way mentioned in our previous study (Dabbour *et al.*, 2014).

Preparation of experimental diet mixtures: Two experimental diet mixtures were prepared according to Reeves (1997) that have isonutrient content (isocaloric, isonitrogenous and others). Two levels of dietary oil were used in these diet mixtures: 4 and 8% MTSO. Two other diet mixtures (control) were prepared in the same manner but with soybean oil. Four other diet mixtures (2 containing MT seed oil and 2 containing soybean oil as control) were prepared in the same manner but 1% of cholesterol was added to these diets. The recommended fat content in normal diets of adult rats is 4% (Reeves, 1997). Table 1 shows the ingredient composition of the eight experimental diet mixtures. They were formulated by mixing the designated amounts of dietary oils, corn starch, casein, water-soluble vitamins, fat-soluble vitamin mix, mineral mix, DL-methionine and choline bitartrate in a stainless steel blender (Kenwood, Hampshire, England). The added amounts of fat (oils) were at the expense of carbohydrates energy in the diet mixtures (by replacing carbohydrate energy with added amounts of dietary oils). Cholesterol was added to diets at the expense of carbohydrate content. No additives, such as antioxidants were added. These diets provided 9.1 and 17.4% of energy from fat respectively. Experimental diet mixtures were freshly prepared once a week, well-packed and placed in dark jars and stored refrigerated at 4°C until needed during the feeding stage of the experimental animals.

Animal experimentation: The Animal Care Committee of Agricultural collage, Jordan University approved this study. Sprague-Dawley adult male rats (64 rats) with an average weight of 210 g were used. In the Animal Unit of the Department of Nutrition and Food Technology at Jordan University, rats were housed in plastic cages with stainless steel wire-mesh bottom (B. Holden and Crew 2001, North Kent Plsatic cages Ltd, England). They were maintained *ad libitum* on stock diet and tap water for 11 days prior to the start of the experiment for acclimatization. Environmental conditions were under control with a temperature of 22+2°C and 12 h light-dark cycle. Water was given in glass bottles with rubber stoppers.

At the beginning of the experimental feeding stage, animals were distributed randomly according to their weights into 8 groups (8 rats/group). Each experimental group of animals was assigned to one of the prepared diets for 12 weeks. Both diet and water were provided *ad libitum*. Animal weights and total food intake were measured once a week throughout the experiment. At the end of 12 weeks, rats were starved overnight and were anesthetized by chloroform. Blood samples were collected from the right ventricle of the heart of each rat using a medicinal syringe and transferred to plain tubes, centrifuged at 3200 rpm for 20 min (Clement, GS 150 centrifuge, Australia) to obtain serum. Sera were stored in Ependrof plastic tubes, duplicate for each rat, frozen at -20°C for later analyses of serum TC, HDL-C, LDL-C and TG, TAC and MDA. Weight gain in rats was calculated as follows:

Weight gain = Final weight of rat (g)-Initial weight of rat (g)

Food efficiency ratio (FER) was calculated from the weight gain and accumulative food intake at the end of 12 weeks according to the following equation:

FER = Body weight gain (g)/100 g food intake

Biochemical analysis

Analysis of lipid profile: The analysis of serum lipids were done in corporation with Al-Khalidi Medical Center Laboratories, Amman, Jordan. COBAS/Integra 400 plus analyzer (Roche Diagnostics GmbH, USA), a clinical chemistry photometric analyzer, was used for the analysis of lipid profile. Prior to analysis, calibration of the analyzer for total cholesterol (TC), high-density lipoprotein-cholesterol (HDL-C), low density lipoprotein-cholesterol (LDL-C) and triglycerides (TG) was done according to the manufacturer instructions. A quality control was run with the samples in each test. Fasting serum levels of TC, HDL-C, LDL-C and TG were determined by the enzymatic-colorimetric method using standard kit procedure (Roche, USA).

Total antioxidant capacity: Total antioxidant capacity (TAC) was measured using TAS kit which was purchased from Randox Laboratories Ltd. (Crumlin, United Kingdom), TAC test was carried on automated chemistry analyzer colorimetric Hitachi 911 (Tokyo, Japan). This analysis was done in corporation with Al-Khalidi Medical Center Laboratories, Amman, Jordan.

Lipid peroxidation assay: Thiobarbituric acid (TBA) reacts with lipoperoxidation aldehydes, such as malondialdehyde (MDA), as the most common method to assess the end products of lipid peroxidation in biological samples (Jentzsch *et al.*, 1996; Lapenna *et al.*, 2001). In particular, serum TBA test has been used clinically to investigate radical-mediated lipid peroxidation and oxidative stress in disease (Del Rio *et al.*, 2005; Apake *et al.*, 2007). The MDA analysis of the rats serum was carried out according to the method reported by Jentzsch *et al.* (1996) and Lapenna *et al.* (2001) with miner modification. Briefly, 0.5 mL of each serum sample was added to the screw-caped glass tube each containing a reaction mixture (1.0 mL) formed by equal parts of: 15% trichloroacetic acid TCA (7.5 g of TCA to 50 mL of deionized H_2O), 0.25 N HCl (25 mL of HCl to 100 mL of deionized H_2O), 0.375% TBA (BDH Laboratory, Supplies, England) (0.375 g of TBA to 100 mL of deionized H_2O), 2.5 mM BHT (0.0551 g of BHT to 100 mL of ethanol) and a 0.1 mL of 8.1% sodium lauryl sulphate solution (SDS) (dissolving 2.025 of SDS in 25 ml of deionized H_2O), all were mixed by vortex followed by 30 min heating at 95°C; pH value of the analytical reaction mixture was about 1.0. BHT was used to prevent lipid peroxidation during heating. After cooling to room temperature, the chromogen, (TBARS) was extracted with (1.5 mL) n-butanol. To facilitate phase separation, the test tubes were centrifuged at 3000 rpm for 10 min. The upper butanol phase was placed into cuvette and the absorption was read spectrophotometrically (Perkin Elmer lambda 25, 101 N8, USA) at 532 nm against a reaction mixture "blank' lacking serum but subjected to the entire procedure and extracted with n-butanol. To correct for background absorption, absorbance values at 572 nm were substracted from those at 532 nm, the latter representing the absorption maximum of the TBARS. MDA equivalents (TBARS) were calculated using the differences in absorption at the two wavelengths and quantification was made with the application of Beer's Law:

$$C = \frac{A}{\varepsilon x i}$$

where,

C = concentration (mol/L)

A = Absorbance

ε = Molar extinction coefficient for MDA/TBA-complex is 154000 (mol/L)

l = 1 cm light path length of cuvette

Statistical analysis: Statistical analysis of the measured and calculated data was performed using the statistical analysis system (SAS package, 2002). Standard error of the mean (SEM) were used to describe the study variables. Two and Three way analysis of Variance (ANOVA) was used to test mean differences among the eight groups for experimental rats. Tukey test was used to test any differences among means for which ANOVA indicated a significant level ($p<0.05$).

RESULTS

Body weight, accumulative food intake and FER: Table 2 shows initial and final body weight, body weight gain of rats fed two levels of MTSO and control diets with and without cholesterol for twelve weeks. Initial body weights of the group of rats assigned for different diets were essentially similar ($p>0.05$). Rats fed cholesterol-free diets and those fed cholesterol-supplemented diets with two levels of experimental MTSO and control diet exhibited similar ($p>0.05$) final body weights and body weight gain.

Table 3 shows accumulative food intake and FER of rats fed two levels of MTSO and control diets with and without cholesterol for twelve weeks. No significant differences ($p>0.05$) between these variables and experimental diets were observed.

Levels of blood lipids and lipoprotein: Table 4 shows the concentration of blood lipids and lipoprotein of rats fed two levels of MTSO and control diets with and without cholesterol for twelve weeks. With respect to serum levels of TC expressed as (mg/dL), there were significant differences ($p<0.05$) between rats fed cholesterol-supplemented diets with 8% MTSO and 8% control oil and cholesterol-free diets with 8% control oil. Also, cholesterol-supplemented diet with 8% control oil was significantly higher ($p<0.05$) than those with 4% MTSO added to cholesterol-free diets. Regarding HDL-C

Table 1: Ingredient composition of experimental diets (g/kg)[1-3]

Diet	Without cholesterol				With cholesterol			
Component (Fat %)	Control (4%)	Control (8%)	MTSO (4%)	MTSO (8%)	Control (4%)	Control (8%)	MTSO (4%)	MTSO (8%)
Corn starch	620.7	580.7	620.7	580.7	610.7	570.7	610.7	570.7
Sucrose	100	100	100	100	100	100	100	100
Cellulose	50	50	50	50	50	50	50	50
Casein	140	140	140	140	140	140	140	140
Soybean oil	36	76	-	-	76	76	-	-
MTSO	-	-	36	76	-	-	36	76
Mineral Mix.[1]	35	35	35	35	35	35	35	35
H_2O-soluble vitamin mix[2]	10	10	10	10	10	10	10	10
Fat-soluble vitamin mix[3]	5	5	5	5	5	5	5	5
DL-Methionine	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8
Choline Bitartrate	2.5	2.5	2.5	2.5	2.5	2.5	2.5	2.5
Cholesterol	-	-	-	-	10	10	10	10

Mineral mix composed of the following: 357 g $CaCO_3$, 250 g KH_2 PO_4, 59.11 g K_2HPO_4, 74 g NaCL, 46 g K_2SO_4, 24 g MgO, 6.06 g $C_6H_5O_7F$. $3H_2O$, 3.79 g $ZnSO_4$, 1.45 g $Na_2SiO_3.9$ H_2O, 0.92 g $MnSO_4$, 0.68 g $CuSO_4$, 0.28 g CrK $(SO_4)_2$, 0.0815 g H_3BO_3, 0.0635 g NaF, 0.071 g Ni $(NO_3)_2$, 0.0174 g Licl, 0.0103 g Na_2 SeO_4, 0.010 g KIO_3, 0.0069 g VCl_3, 0.006 g MOO_3. Mixture weight was continued to 1 kg by sucrose (Reeves, 1997)

1 kg H_2O-soluble vitamin mix composed of the following: 0.6 g thiamin hydrochloride, 0.6 g riboflavin, 1.6 g calcium pantothenate, 3 g nicotinic acid, 0.02 g biotin, 0.2 g folic acid. 0.7 g pyridoxine hydrochloride, 0.0025 g cyanocobalamin. Mixture weight was contained to 1kg by sucrose (Reeves, 1997)

Fat soluble vitamin mix composed of the following: 0.088g vitamin A (all-trans-retinyl palimtate, 3 g vitamin E (α-tocopherol acetate), 0.03 g vitamin K_1 (phylloquinone) 0.001 g vitamin D_2 (Ergocalciferol). All were dissolved in 200 mL soybean oil or MT seed oil (Reeves, 1997)

Table 2: Initial and final body weights, body weight gain of rats fed two levels of MTSO and control diets with and without cholesterol for 12 weeks[1-2]

Experimental diets	Body indexes	Initial body weight (g)	Final body weight (g)	Body weight gain (g)
Cholesterol free diets	MTSO (4%)	212.5±6.7[a]	373.3±12.9[a]	160.7±11.7[a]
	Control (4%)	215.1±7.2[a]	373.5±14.0[a]	158.4±12.6[a]
	MTSO (8%)	211.0±7.9[a]	367.7±15.4[a]	156.6±13.8[a]
	Control (8%)	212.7±7.2[a]	384.1±14.0[a]	171.4±12.7[a]
Cholesterol supplemented diets	MTSO (4%)	214.2±7.2[a]	387.1±14.0[a]	172.9±12.7[a]
	Control (4%)	212.9±7.2[a]	374.2±14.0[a]	161.3±12.7[a]
	MTSO (8%)	215.0±7.2[a]	408.1±14.0[a]	193.1±12.7[a]
	Control (8%)	214.1±7.2[a]	414.9±14.0[a]	200.9±12.7[a]

Values are given as mean±SEM for 8 rats

Values with different superscripts within the same column are significantly different ($p<0.05$)

Table 3: Accumulative food intake and food efficiency ratio of rats fed two levels of MTSO and control diets with and without cholesterol for 12 weeks[1-2]

Experimentals diets	Variables	Accumulative food intake (g)	Food efficiency ratio (FER)
Cholesterol free diets	MTSO (4%)	1571.9±46.6[a]	10.23±0.61[a]
	Control (4%)	1495.2±50.3[a]	10.58±0.65[a]
	MTSO (8%)	1480.4±55.2[a]	10.56±0.72[a]
	Control (8%)	1567.9±50.3[a]	10.90±0.65[a]
Cholesterol supplemented diets	MTSO (4%)	1554.4±50.3[a]	11.10±0.65[a]
	Control (4%)	1549.7±50.3[a]	10.35±0.65[a]
	MTSO (8%)	1613.0±50.3[a]	11.95±0.65[a]
	Control (8%)	1540.8±50.3[a]	12.98±0.65[a]

Values are given as means±SEM for 8 rats

Food efficiency ratio is expressed as g body weight gain/100 g food intake

levels, no significant differences ($p>0.05$) were detected between rats fed two levels of MTSO and control oil with cholesterol supplemented diets (Table 4). However, rats fed diets containing 4 and 8% MTSO exhibited significantly higher ($p<0.05$) levels of HDL-C as compared with rats fed 8% control oil within cholesterol free diet groups. Higher significant ($p<0.05$) differences in HDL-C concentration were also observed between rats fed cholesterol free diets and rats fed cholesterol diets.

On the other hand, serum levels of LDL-C of rats fed the cholesterol free diets were significantly lower ($p<0.05$) than those fed the cholesterol-supplemented diets (Table 4). Regarding TG (Table 4), rats fed the cholesterol free diet containing 4% MTSO exhibited significantly lower ($p<0.05$) levels in comparison with those fed 4% control oil diet. Also, the latter diet group was significantly higher ($p>0.05$) than other rat groups fed cholesterol-supplemented diet and cholesterol-free diet.

Table 5 shows indices of blood lipids and lipoprotein of rats fed two levels of MTSO and control diets with and without cholesterol. High significant differences ($p<0.05$) in HDL-C/LDL ratio between rats fed cholesterol-free diet and those fed cholesterol supplemented diets were indicated. Rats fed the diet containing 8% MTSO and 8% control diet with cholesterol had significantly higher ($p<0.05$) TC/TG ratio than all cholesterol-free diets (Table 5).

Atherogenic index (AI) of the eight experimental diet groups was calculated as (TC-HDL-C)/HDL-C (Martin-Carron *et al*., 1999). It was observed that there were no significant differences ($p>0.05$) among rats fed cholesterol-free diets (Table 5). However, rats fed the cholesterol-supplemented diet with 8% MTSO and 8%

Table 4: Concentration of blood lipids and lipoproteins of rats fed two levels of MTSO and control diets with and without cholesterol for 12 weeks$^{(1\text{-}2)}$

		Blood lipids and lipoproteins (mg/dL)			
Experimental diets		Total cholesterol	High density lipoprotein cholesterol	Low density lipoprotein cholesterol	Triglycerides
Cholesterol free diets	MTSO (4%)	66.5±7.0^{bc}	75.3±3.9^{a}	16.6±5.3^{c}	54.6±8.3^{b}
	Control (4%)	86.8±7.6^{abc}	72.4±4.3^{ab}	18.2±5.7^{c}	106.0±9.0^{a}
	MTSO (8%)	89.0±8.3^{abc}	76.4±4.6^{a}	18.2±6.3^{c}	75.6±9.8^{ab}
	Control (8%)	59.8±7.6^{c}	60.1±4.2^{b}	15.0±5.7^{c}	60.7±9.0^{b}
Cholesterol supplemented diets	MTSO (4%)	79.0±7.6^{abc}	48.3±4.2^{c}	49.4±5.7^{ab}	52.8±9.0^{b}
	Control (4%)	71.2±7.6^{abc}	43.0±4.2^{c}	40.5±5.7^{b}	58.8±9.0^{b}
	MTSO (8%)	97.7±7.6^{ab}	52.5±4.2^{c}	66.7±5.7^{a}	44.0±9.0^{b}
	Control (8%)	104.8±7.6^{a}	42.2±4.2^{c}	71.6±5.7^{a}	50.2±9.0^{b}

Values are given as mean±SEM for 8 rats

(2) Values with different superscript within the same column are significantly different ($p<0.05$)

Table 5: Indices of blood lipids and lipoproteins of rats fed two levels of MTSO and control diets with and without cholesterol for 12 weeks$^{(1\text{-}3)}$

Experimental diets		HDL-C/LDL-C ratio	TC/TG ratio$^{(4)}$	Atherogenic index (AI)
Cholesterol free diets	MTSO (4%)	4.64±0.20^{a}	1.30±0.25^{bc}	0.15±0.13^{c}
	Control (4%)	4.07±0.23^{a}	0.89±0.27^{c}	0.19±0.14^{c}
	MTSO (8%)	4.47±0.24^{a}	1.15 ±0.29^{bc}	0.15±0.16^{c}
	Control (8%)	3.73±0.22^{a}	1.11±0.27^{c}	0.07±0.14^{c}
Cholesterol supplemented diets	MTSO (4%)	1.17±0.22^{b}	1.47±0.27^{abc}	0.67±0.14^{bc}
	Control (4%)	1.17±0.22^{b}	1.27±0.27^{abc}	0.66±0.14^{bc}
	MTSO (8%)	0.85±0.22^{b}	2.48±0.27^{a}	1.00±0.14^{a}
	Control (8%)	0.62±0.22^{b}	2.40±0.27^{a}	1.51±0.14^{a}

Values are given as mean±SEM for 8 rats

(2) Values with different superscript within the same column are significantly different ($p<0.05$)

(3) Atherogenic index = (TC-HDL-C) X $HDL\text{-}C^{-1}$ (Martin-Carron *et al.*, 1999)

(4) An indicator of atherogenicity

Table 6: Total Antioxidant capacity and malondialdehyde levels of rats fed two levels of MTSO and control diets with and without cholesterol for a period of 12 weeks$^{(1\text{-}2)}$

Experimental diets		Total Antioxidant capacity (mmol/L)	Malondialdehyde (nmol/mL)
Cholesterol free diets	MTSO (4%)	1.41±0.041^{a}	6.51±0.78^{c}
	Control (4%)	1.27±0.044^{ab}	7.94±0.85^{bc}
	MTSO (8%)	1.32±0.048^{ab}	6.88±0.93^{c}
	Control (8%)	1.15±0.044^{b}	12.10±0.85^{a}
Cholesterol supplemented diets	MTSO (4%)	1.21±0.044^{b}	8.21±0.85^{bc}
	Control (4%)	1.25±0.044^{ab}	9.56±0.88^{b}
	MTSO (8%)	1.23±0.044^{ab}	7.27±0.85^{c}
	Control (8%)	1.26±0.044^{ab}	12.18±0.85^{a}

Values are given as mean±SEM for 8 rats

(2) Values with different superscript within the same column are significantly different ($p<0.05$)

control had the highest AI among all cholesterol-containing and cholesterol-free diet groups.

Total antioxidant capacity: Table 6 shows total antioxidant capacity (TAC) of rats fed two levels of MTSO and control diets with and without cholesterol for twelve weeks. Rats fed the diet containing 4% MTSO without cholesterol had significantly higher ($p<0.05$) TAC than those on 8% control oil diet without cholesterol and those on 4% MTSO diet with cholesterol.

Malondialdehyde: When serum levels of malondialdehyde (MDA) were investigated (Table 6), rats fed the cholesterol-free diet containing 8% control oil exhibited significantly higher ($p<0.05$) levels than those fed other cholesterol-free diets. MDA levels were significantly higher ($p<0.05$) in rats fed 8% control oil of the cholesterol-supplemented diet when compared with rats fed 4% control oil, 4% MTSO and 8% MTSO with cholesterol and rats on 4% MTSO, 4% control oil and 8% MTSO of cholesterol free diets.

DISCUSSION

To achieve better results, the American Institute of Nutrition Rodent Diets (AIN-93), was adopted in the present study. The AIN-93 diet proved to give a better balance of essential nutrients for long-term studies (Reeves, 1997). For example, soy bean oil was used as an adequate source of essential fatty acid, linoleic and linolenic acid. Whether for soybean oil in control group or MTSO, the amount of oil in the present study was added to reach the maintenance requirements of adult rats (4% of the diet) according to Reeves (1997). Additional two groups of soybean oil and MTSO with oil of 8% were added to exert the effect of the tested oil on blood lipids, TAC and MDA.

Body weight, accumulative food intake and FER: The results of the present long-term MTSO feeding study (12 weeks) show no significant change in final body weights, body weight gain of rats feed two levels of MTSO compared with control diet groups (Table 2). These results were consistent with those of many animal studies on different seed oils (Moon *et al.*, 2001; Gorinstein *et al.*, 2003; Cintra *et al.*, 2006; Makni *et al.*, 2009; Visavadiya and Narasimacharya, 2008). An

obvious increase in liver size (hepatomegaly) was particularly observed in groups fed cholesterol supplemented diet in the present study (data not shown). It appears that the high-cholesterol diet caused an increase of liver weight. This may be due to accumulation of lipids in liver because no difference in body weight was observed across rats in different groups. This was in accordance with the finding of Ha *et al.* (2005) who studied the effect of rice bran oil on lipid profile in Sprague-Dawley rats fed high cholesterol diet (1%) for 4 weeks. On the other hand, Visavadiya and Narasimacharya (2008) did not find any significant differences in liver weight when feeding sesame seed powder with 0.5% cholesterol for 4-weeks which is lower than that used in the present diet (1%). Accumulative food intake and food efficiency ratio (FER) of rats fed two levels of MTSO were insignificantly different with and without cholesterol (Table 3). This is consistent with the results obtained by Ha *et al.* (2005) who have reported insignificant changes in FER in rats fed rice-bran oil diet with and without cholesterol. This was also confirmed by Cintra *et al.* (2006) who found no significant difference in FER when studying four types of seed oils in rats.

Levels of blood lipids and lipoproteins: The results of the present study show a high significant difference in total cholesterol (TC) in 8% MTSO and 8% control cholesterol-supplemented diets when compared to 8% control cholesterol-free diet. Also LDL-C of cholesterol-supplemented diet groups were significantly higher than cholesterol-free diet groups. An interesting result in the present study is the high levels of HDL-C in rats fed cholesterol free diet with MTSO 4% and MTSO 8% when compared to cholesterol-supplemented diets. Present results are in accordance with those obtained by Ha *et al.* (2005) who found high and significant differences in the total cholesterol in high cholesterol control diet and rice bran oil supplemented with cholesterol when compared to control groups. Regarding HDL-C they found that feeding rice bran oil with add cholesterol to rats resulted in high levels of HDL-C when compared with cholesterol control group but not higher than cholesterol free-control group which in turn resulted in significantly higher levels of HDL-C than other groups. Similar findings were also observed by Gorinstein *et al.* (2003) when feeding oil-supplemented diets with or without cholesterol to rats. They found that sunflower and rape seed oil with cholesterol-supplemented diets and cholesterol control diet resulted in higher serum total cholesterol and LDL-C when compared with sunflower seed oil or rape seed oil diets. In the same study HDL-C levels in cholesterol-containing diets did not differ from those in control group. Gurr *et al.* (2002) explained that the lipoprotein pattern of rats is of the HDL type; that is, HDL is the dominant lipoprotein class. The function of this large HDL-C is poorly understood and highly variable between species. On the contrary, safflower seed extract diets supplemented with cholesterol resulted in decreased TC when compared with cholesterol-containing control group (Moon *et al.*, 2001). The authors explained that total phenolic content and low saturated fatty acid content in safflower extract were most potent in lowering the plasma cholesterol-concentrations. Also, Moon and Co-workers (2001) came with the same results of Gorinstein *et al.* (2003) with respect to HDL-C with no significant differences in safflower seed extract cholesterol diets compared with cholesterol containing group. While the HDL-C/TC ratio was higher for safflower seed extract cholesterol diet than in control group due to decrease in TC.

Similar results were obtained by Makni *et al.* (2009) who found that flax and pumpkin seed mixture diet supplemented with cholesterol resulted in lower plasma TC and LDL-C when compared with cholesterol-supplemented control diet. Whereas for HDL-C, levels were higher in mixed seeds cholesterol supplemented diets when compared with cholesterol control group. However, results were not similar in all of the studies. The discrepancy might be attributed to the protocols and composition of diet mixtures.

Our results regarding high blood TC and LDL-C at 8% control and MTSO cholesterol-supplemented diets, compared to 8% control cholesterol free diet suggest cholesterol and oil level dose effects. To elucidate the (%) of fat, Reeves (1997) recommended that fat% in maintenance diet for adult rats should be at 4% of the diet, giving (a polyunsaturated: saturated fatty acid) ratio of at least 2:1 for soybean oil. This ratio is of great influence on tissue lipids and eicosanoids production. Fatty acid pattern of cold-pressed MTSO in our previous study was found to resemble that of soybean oil (control oil) and subsequently reaching the ratio mentioned above. Soybean oil content of saturated fatty acid (SFA) is 15.9% and PUFA content is 61.5% while for MTSO, SFA is 19.5% and PUFA is 57.5% (Whent *et al.*, 2010; Dabbour *et al.*, 2014). The SFA'S, palmitic acid (C16:0) and stearic acid (C18:0), are found at 8.6, 5.2, 10.9 and 4.9% in MTSO and soybean oil respectively. The relatively high content of both fatty acids in MTSO and soybean oil may give rise to high blood TC and LDL-C as reported by Salter *et al.* (1998); Billett *et al.* (2000); Kritchevsky and Chen (2005) and Cintra *et al.* (2006). They have clarified that the fatty acids composition of dietary fat is important in determining plasma lipoprotein and cholesterol concentration and thus an interactive effect of palmitic acid and stearic acid as SFA in diet trigger high level of cholesterol in blood. They also found that supplementing experimental diet with different levels of high cholesterol exaggerated blood cholesterol levels and platelet aggregation in rats, which in turn becomes more hypercholesterolaemic. Kritchevsky and

Chen (2001) conducted a study about serum and liver lipids in rats fed various mixtures of corn and palm oils (high level of palmitic acid) with and without cholesterol. They found that introduction of the lowest level of palm oil raised serum cholesterol levels significantly and they continued to rise as more palm oil was introduced into the diet containing cholesterol. They concluded that palmitic acid becomes hypercholesterolemic only in cholesterol containing diets. However, they demonstrated the noncholesterolemic effect of palmitic acid in rats fed cholesterol-free diets. Moreover, in certain rats, hamster and monkey experiments a generalized cholesterolemic effect of saturated fat occurred only if some cholesterol was present in the diet (Gilani *et al*., 2002). Dietary cholesterol appears to down regulate the LDL-C receptor activity, which would decrease LDL removal and thereby elevate circulating levels of cholesterol.

Billet *et al*. (2000) and Cintra *et al*. (2006) suggested an interactive effect between different dietary SFA and dietary cholesterol on lipoprotein metabolism through exerting a specific modulation of the expression of the LDL receptor and apolipoprotein B-genes and subsequently increase of the atherogenicity. These findings are in agreement with the results of present study with respect to TC, LDL-C, HDL-C, HDL-C/LDL-C ratio and atherogenic index (AI) as affected by cholesterol and type of oil and (%) of oil (Table 4 and 5). To support present study results regarding higher TC and lower HDL-C as atherogenic factors, AI was calculated. There was an obvious and significant higher AI values for cholesterol-supplemented diets at 8% of oil.

It is worth mentioning in the present study that despite the relatively good content of PUFA (57.5%), phyto-sterols (2520 mg/kg) mainly β-sitosterol (46% of total phytoseroles), α-tocopherols (237.4 mg/kg) and TPC (1.16 mg/g oil) in MTSO, its effect on blood lipoprotein did not differ, when comparing 4% MTSO with 4% control group and 8% MTSO with 8% control group. This could be due to similar chemical composition of soybean oil used in control diet and examined MTSO (Whent *et al*., 2010; Dabbour *et al*., 2014). Presence of bioactive components (phytochemical compounds) in MTSO and control oil (soybean oil) and lower concentration of SFA at 4% in comparison with 8% that could lead to inhibition of HMG-CoA reductase and thus reduction of blood lipid indicators. However, it is well-known that HMG-CoA reductase inhibitors drugs (lovastatine) do not have a hypocholesterolemic effect in rodents (rats) yet do in hamsters, rabbits and humans; this might be due to differences in lipoprotein metabolism among animal species as reported by Bok *et al*. (1999). They mentioned that despite these differences rats can be used as an animal model to study cholesterol metabolism if experimental conditions are appropriately controlled. Doses of HMG-CoA reductase inhibitors have to be very high to exert a hypocholesterolemic response in rats. Bok *et al*. (1999) found that tangerine peel extract and a mixture of two citrus flavonoids (naringin and hesperidin) when supplemented with cholesterol (1%) lowered plasma lipid profile of Sprague-Dawely rats significantly when compared cholesterol control group. They explained that highly concentrated citrus bioflavonoids have inhibited the measured HMG-CoA reductase and thus have decreased plasma lipid levels.

In the present study increasing the % of tested MTSO and control soybean oil to 8% as hypothesized to decrease serum lipids, did not result in lowering serum lipids. On the contrary values were significantly higher than 4% of oil diets supplemented with cholesterol. The expected positive effect of different phytochemical compounds was not observed at 8% oil this may be due to higher SFA concentration in 8% oil which in turn may hinder the effect of different phytochemical compounds in lowering blood lipids.

With respect to blood TG concentration in present study, no significant variations were found in MTSO diets when compared with control diets whether with or without cholesterol. Although the reasons for these observations are unclear. One of the possibilities is that the relatively high saturated fatty acid content of MTSO and soybean oil in diet groups could have influenced the TG levels as SFA are known substrates for TG synthesis (Harvey *et al*., 2005). This was clarified by Visavadiya and Narasimhacherya (2008) who failed to find any significant variation in blood TG in experimental diets using sesame seeds oil and powder which is high in SFA content. These results are consistent except for the 4% cholesterol-free control diet group which tend to have higher TG value ($p<0.05$) when compared to all diet groups. A result that may be explained by the presence of higher carbohydrate content in expense of fat in 4% fat groups when compared with 8% fat-containing groups (Table 1). This was in accordance with result found by Cintra *et al*. (2006) who found that animal feed normal diet had higher levels of TG than did animals fed high fat diet.

Lipid peroxidation and total antioxidant capacity in blood: MDA is a breakdown product of spontaneous fragmentation of peroxides form from polyunsaturated fatty acids (PUFA) mainly from the oxidation of cell membranes (Niki, 2010). In the present study, the measurement of lipid peroxidation in serum, expressed as MDA (nmol/mL), showed clearly high levels of MDA in 8% cholesterol-free control diet when compared with other cholesterol-free diets. Additionally the 8% cholesterol supplemented control diet was higher than that of 8% cholesterol supplement MTSO diet ($p<0.05$) (Table 6). This shows that higher level of MDA was in 8%

soybean control oil with or without cholesterol. This is because soybean oil contains high amount of unsaturated fatty acids and lower SFA and is more susceptible to oxidation when compared with MTSO which is lower in oxidation. This was explained by Aguilera *et al.* (2004) and Nevin and Rajamohan (2008), who found that feeding oil rich in PUFA and has low antioxidant activity results in their accumulation of MDA in cell membranes and increased the oxidative stress, since PUFA's are highly susceptible to peroxidation compared to MUFA and SFA. This may give a reason for the higher MDA formation in the serum of rats fed soybean control oil which contain low α-tocopherol level and high PUFA's. Moreover, Niki (2010) stated that MDA concentration in serum are inversely correlated to the proportion of PUFA mainly linoleic and linolenic acid in serum lipoprotein lipids. His findings suggested that other factors such as the availability of antioxidants, polyphenols, unsaponifiable compounds (such as phytosterols and squalene) may be of greater influence on intravascular lipid peroxidation.

In our previous study, MTSO rich in linoleic acid (56.8% n-6), minor amount of linolenic acid (0.76% n-3), good amounts of MUFA (22%) and relatively high amounts of SFA (19.5%) is compared to control oil (soybean oil) (Dabbour *et al.*, 2014). Soybean oil is rich in linolenic acid (7.5%), linoleic acid (54%), MUFA (22.7%) and SFA (15.9%) (Whent *et al.*, 2010). We cannot ignore the presence of phytochemical compounds in the experimental MTSO which is nearly similar to control oil (soybean oil) except for α-tocopherol, where MTSO has higher (237.4 ppm) than soybean oil (92.1 ppm). Baba *et al.* (2000) showed that sesame oil contains small amounts of tocopherols but has good amounts of other phenols which makes it a unique oil because of its superior oxidative stability. Olive oil has the biophenol which was shown to improve *in vivo* an antioxidant defenses (Aguilera *et al.*, 2004). On the other hand, canola oil, although rich in oleic acid (62.5%), has low content of saturates and of tocopherols which are removed during the refining process and its high content of α-linolenic (n3) (≈10%) may render it susceptible to lipid peroxidation (Baba *et al.*, 2000). In one study, Fang *et al.* (2004) found that genistein (an isoflavonoid from soy) raised the serum total antioxidant capacity TAC and decreased the MDA. In the present study, when taking into consideration the effect of high cholesterol diet, there were high lipid peroxidation of MDA (12.2 nmol/mL) for 8% soybean control oil diet group with cholesterol. The MDA levels were exaggerated due to the effect of both cholesterol and oil type since soybean oil is high in PUFA. These results corresponded with the observations that cholesterol supplemented diet leads to a decrease in blood antioxidant activity and increase in MDA (Gorinstein *et al.*, 2003). This effect was focused on by Gokkusu *et al.* (2004) who found that high cholesterol diets supplemented with polyunsaturated fats seem to have a tendency to exaggerate lipid peroxidation in serum, as well as to disturb the balance between cholesterol and phospholipid in blood. Recently, Nevin and Rajamohan (2008) studied the effects of feeding virgin coconut oil (VCO) of low PUFA on *in vitro* oxidation of LDL in comparison with copra oil of high SFA and sunflower oil of high PUFA in cholesterol fed rats. VCO was found to prevent the oxidation of LDL from oxidants. The properties of VCO may be attributed to the presence of biologically active unsaponifiable phytochemical components (α-tocopherols, polyphenols and phytosterols) which are more active than other tested oil.

Regarding the total antioxidant status, TAC was higher in 4% cholesterol free MTSO diet when compared to 8% cholesterol free control diet (soy oil) and 4% cholesterol supplemented MTSO diet. It seems the TAC obtained here is attributed to the presence of phenolic compounds, phytosterols mainly β-sitosterol (46%) and α-tocopherol, a potent antioxidant, in MTSO. The result of serum TAC in MTSO group was on going with those of *in vitro* TAC values of MTSO determined in our previous study (Dabbour *et al.*, 2014). The TAC value of MTSO oil was 2.29 mmol/L which is considered high when compared to TAC of other oils. TAC values of soybean oil is 1.75 mmol/L, olive oil 1.29 mmol/L, flaxseed oil 1.01 mmol/L and sunflower oil 1.1 mmol/L as reported by Tuberose *et al.* (2007). TAC value of corn oil (2.3 mmol/L) was similar to that of MTSO (Cintra *et al.*, 2006). Bartosz (2003) pointed that the long term dietary intervention may not result in significant changes of serum TAC as compared with control groups. He has reported that oral supplementation of ascorbic acid for 4 weeks did not affect TAC significantly when compared to control group. He has also reported the same effect after 30 days of feeding low-birth-weight infants with a formula containing w6 and w3 long chain PUFA as compared with a group fed human milk. The author explained that there is a possible contribution of polyphenols present in blood plasma at the concentrations of 0.2-2 mM. However, this may explain the lack of significant differences in TAC values in different diet groups of the present study.

Conclusions: Under the conditions of this study, it seems that the negative effects of the presence of cholesterol in the rat diets were counterbalanced by the positive effects of MUFA, PUFA and antioxidant content in oils added in these diets. Therefore, it must be emphasized that the expected improvement in lipid metabolism and TAC was not observed in the groups of rats fed cholesterol-containing diet. Consequently, the MTSO and soybean oil (control) had the same effect on these variables. On the other hand, the results of the investigation *in vivo* lipid peroxidation support previous

our suggestions that oil with higher *in vitro* antioxidant capacity (MTS) is biologically more active than oil with lower antioxidant capacity (soybean).

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