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Mob: +92 300 3008585, Fax: +92 41 8815544
E-mail: editorpjn@gmail.com

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Chemical Characteristics and Antioxidant Content Properties of Cold Pressed Seed Oil of Wild Milk Thistle Plant Grown in Jordan

I.R. Dabbour[1], K.M. Al-Ismail[2], H.R. Takruri[2] and F.S. Azzeh[1]
[1]Department of Clinical Nutrition, Faculty of Applied Medical Science,
Umm Al-Qura University, Makkah, P.O. Box 715, 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 chemical characteristics and antioxidant content properties of cold-pressed milk thistle seed oil. Acidity and peroxide value were determined by chemical standard titration method and oxidative stability index was determined by Rancimate. Using GLC, fatty acid composition, phytosterols and squalene were measured. While, alpha-tocopherol content was determined by HPLC. The total phenolic content, radical-scavenging capacity and total antioxidant capacity were determined spectrophotometrically. The chemical characteristics of cold-pressed oil were as follows: acidity (0.64%), peroxide value (0.34 meq O_2/kg of oil), oxidative stability index (55.7 and 12.9 h at 80 and 100°C, respectively), fatty acid composition (19.5, 22.9 and 57.6% for SAT, MUFA and PUFA, respectively), phytosterol (2520 mg/kg), squalene (9.35 mg/kg), alpha-tocopherol content (237.4 mg/kg), total phenolic content (1.16 mg GAE/g oil), radical-scavenging capacity against stable DPPH radical (IC_{50} = 3.34 mg/mL) and total antioxidant capacity (2.29 mmol/L). These data suggest that cold-pressed milk thistle seed oil may serve as dietary source of high PUFA and MUFA, phytosterols, phenolic compounds and natural antioxidants and can be a remarkable candidate for use in healthy food preparations mixed with other vegetable oils or alone.

Key words: Antioxidant capacity-milk thistle seeds-phenolic content-phytosterols-squalen

INTRODUCTION

In the recent years, there has been a growing importance in the oils of non-traditional seeds such as the oils of flaxseeds, safflower seeds, pumpkin seeds, grape seeds, pomegranate seeds and milk thistle seeds (Abolfazl Fadavi *et al*., 2006; Bail *et al*., 2008; Houenschil *et al*., 2010; Shaker *et al*., 2010). Seeds oil, rich in factors beneficial to health, are of high demand because of consumers interest in disease prevention and health promotion through improved diets, including high content of monounsaturated fatty acids (MUFA'S), polyunsaturated fatty acids (PUFA'S), tocopherols, carotenoids and antioxidant phenolic compounds (Gorinstein *et al*., 2003; Garjani *et al*., 2009).

Milk thistle (*Silybum marianum*, L. Gaertn) is a wild plant from the *Compositeae* family. It is an annual plant, native to the Mediterranean area and North Africa (Tukan *et al*., 1998; Fathi-Achachlouei and Azadmard-Damirchi, 2009). It grows wild throughout Europe, some parts of the United States and Australia. It can be also cultivated (Barreto *et al*., 2003). One of the important issues about milk thistle plant is that it may be accepted as a safe and well tolerated herbal product, since no health hazards or side effects are known (Jacobs *et al*., 2002; Shaker *et al*., 2010). In Jordan, milk thistle (MT) is a common wild plant, which grows in many regions during the winter and spring seasons. People of the region, particularly in the rural areas, eat MT. The stems are consumed raw or cooked, the flower heads are cooked and the seeds are consumed raw or roasted (Abu-Rajouh and Takruri, 2000). It has been found in a number of studies that MT seeds contain a relatively high content of oil (20-26%) (Abu-Jadayil *et al*., 1999; Abu-Rajouh and Takruri, 2000; Parry *et al*., 2005, 2006; Fathi-Achachlouei and Azad mard-Damirchi, 2009). The main fatty acids in milk thistle seed oil (MTSO) were found to be linoleic acid, oleic acid, palmitic acid and stearic acid. Polyphenoles in MTseeds were found to have an antioxidant, anti-inflammatory, hypolipidaemic and anticarcinogenic properties (Jacobs *et al*., 2002; Skottova *et al*., 2003; Sobolova *et al*., 2006; Nyiredy *et al*., 2008; Shaker *et al*., 2010). One of these polyphenoles is silymarin, which protects the liver from toxic chemicals and drugs such as acetaminophen which can cause liver damage in high doses (Jacobs *et al*., 2002; Nyiredy *et al*., 2008).

Cold-pressing is a method that does not use organic solvents or heat to extract seed oil. In this process, neither the raw materials nor the oil are exposed to higher temperatures in accordance with the Codex Alimentarius for cold-pressed oils (FAO/WHO, 2005). Cold-pressing is belived to be a better technique for retaining beneficial value-added components in seed

Corresponding Author: I.R. Dabbour, Department of Clinical Nutrition, Faculty of Applied Medical Science, Umm Al-Qura University, Makkah, P.O. Box 715, Saudi Arabia

oils that might be lost by evaporation or chemically modified using conventional solvent extracting methods (Parker *et al*., 2003; Parry *et al*., 2006; Tuberose *et al*., 2007). Therefore, the shelf life of seeds oil extracted by cold-pressing is superior than conventional methods (Vujasinovic *et al*., 2010).

To the best of our knowledge, no studies were conducted to investigate the chemical characteristics of the cold-pressed oil of wild MT seeds grown in Jordan. Therefore, the main objectives of this study were to examine the chemical properties of the cold-pressed MTSO as well as antioxidant compounds including phytosterols, squalene, tocopherols and total phenols and its effect on *in vitro* radical scavenging capacity (DPPH•) and total antioxidant capacity (TAC).

MATERIALS AND METHODS

Collection and preparation of milk thistle seeds: MT seeds were obtained by threshing the dried mature flowers which were collected from different rural areas of Jordan during the 2010 season (April-May). The seeds were dried under the sun for a few days, cleaned from impurities by using gradated sieves and stored in airtight glass containers until oil extraction.

Proximate analysis of milk thistle seed: Moisture, crude fat, crude protein, ash and crude fiber were analyzed according to the official methods of the Association of Official Analytical Chemists (AOAC, 2000). Methods 976.05, 920.39, 976.05, 923.03 and 962.09, respectively and total carbohydrate were determined by difference. All determinations were done in triplicate.

Milk thistle seeds oil extraction: Cold-pressed MTSO was obtained by pressing the cleaned and raw-dried seeds with a screw-press type unit (CLB-100, Sweden), run by a 4 KW electric motor and capacity of 10-20 kg/h. The squeezed oil was collected in a stainless steel vessel. Turbid substances resulting during pressing was removed by filtration then centrifugation at room temperature. The oil content of MT seed ranged from 26 to 27.5%. Filtered oil was filled into dark glass bottles and kept refrigerated at 4°C until the time of analysis.

Chemical characterization and antioxidant compounds of milk thistle seed oil

Oil quality analysis

Acidity and peroxide value: The acidity and peroxide value (PV) of MTSO were determined as described by American Oil Chemists Society, methods Ca 5a-40 and Cd 8b-90 respectively (AOCS, 1997). All measurements were taken in triplicate.

Oxidative stability index (OSI): OSI was determined by using a Rancimat instrument (Model 743; Metrohm Ltd., Switzerland). MTSO sample was exposed to accelerated oxidation conditions as following: 3 grams of oil were placed in a reaction vessel, temperature was set at 80 and 100°C in 2 separated channel and the air flow was 20 L/h. The OSI was measured as the hours for an oil sample to develop measurable rancidity by induction point determination. The OSI measurements were taken in triplicate.

Instrumental analysis

Fatty acid profile analysis: The fatty acids profile analysis of the MTSO was carried out according to the method reported by European Committee (2003) with minor modification. It was determined by conversion of oil to fatty acid methyl esters (FAMEs) followed by gas chromatography as follows.

Preparation of fatty acid methyl esters: About 50 g filtered oil was placed into a screw capped test tube, dissolved in 5 mL hexane (HPLC grade) and mixed by vortex mixer (V1 plus Biosan, Spain) for 60 seconds. A 200 µL of 2N potassium hydroxide (85%, GCC laboratory, UK) prepared in anhydrous methanol was added and mixed for another 30 sec. until solution became clear. Almost immediately after clearance, the solution became turbid due to the separation of glycerol, then 200 µL acetic acid was added to prevent the reformation of fatty acids.

Gas liquid chromatography analysis of the fatty acid methyl esters: The prepared FAMEs were analyzed on Shimadzu gas chromatograph (Model GC-2010, Shimadzu Inc., Koyoto, Japan) supplied with split injector port and flame ionization detector. A DB-23 capillary column (Teknokroma, Spain) (60 m x 0.25 mm internal diameter; film thickness was 0.15 µm and the active ingredients were 50% cyanopropyl phenyl and 50% diphenyl polysilosan cross linked and chemically bonded) was used. The oven analysis temperature started at 165°C for 8 min and was raised at a rate of 1°C/min to 185°C then left at 220°C for 9 min.

The injector temperature was 230°C and the detector temperature was 240°C. Flow rate of nitrogen (Carrier gas) was 1.2 mL/min and split ratio was 1:30. The FAMEs were identified from the chromatogram of a standard solution of FAMEs (Supelco 37 component FAME mixture No. 47885-4, USA). The chromatogram and the peak area were recorded by a digital integrator (Shimadzu C-R8A). The fatty acid composition was determined as a percent of total fatty acids. Measurements were taken in triplicates.

Phytosterols profile and squalene analysis: Phytosterols profile and squalene were determined in MTSO according to the method reported by European Committee (2003) and Fathi-Achachlouei and Azadmard-Damirchi (2009) with minor modification, as follows.

Cold saponification: Accurately, about 200 mg of filtered MTSO was weighed into a 15 mL screw capped test tube, 10 mL of 1N KOH (85% GCC Laboratories, UK) dissolved in methanol, 1ml of a squalan solution and 50 µg of 5 alpha-cholestane (Sigma, 98%, GC grade, Japan) as an internal standard were added to the sample. The mixture was shaken until the diminishing of dispersed oil particles, the sample was then placed in a dark place at room temperature for 22-24 h (Parks and Addis, 1986). The Saponified oil was transferred into a 100 mL separatory funnel and 10 mL of distilled water was added. Unsaponifiables were extracted 3 times with 10, 5 and 5 mL of diethyl ether (99% HPLC grade, GCC Laboratories, England).

Pooled diethyl ether extract was washed once with 0.5N KOH (85% GCC laboratories, UK) and 5 times with 5 mL distilled water. The ether was filtered using Whatman No.1 filter paper and dried over anhydrous sodium sulfate (Na_2SO_4) (GCC Laboratories, UK). The filter paper and the anhydrous sodium sulfate were washed twice with 5 mL diethyl ether (99% HPLC grade, GCC Laboratories, England). The solvent was concentrated using rotary evaporator (Heidolph, Germany) at 25°C and under vacuum to about 1 mL and then dried under ultra pure nitrogen stream after being transferred to a 5 mL vial.

Derivatization of the unsaponifiables: The trimethylsilyl derivatives (TMS) of the components of the unsaponifiable were obtained according to Giacometti (2001) as follows: a portion of 500 µL of silylation reagent, consisting of a mixture of pyridine (CBH, Nottingham, UK) /hexamethyl disilazane (Janssen, Belgium)/trimethylchlorosilane (Fluka, Switzerland), 9:3:1 (v/v/v) (prepared previously and stored at refrigerator) was added to the unsaponifiable matter, the vial was left for 40 min at ambient temperature. The pyridine was evaporated under a stream of extra pure nitrogen, then 4 mL of hexane (GC grade, lab scan, Dublin) was added and shacked vigorously. The solution was transferred into a 5 mL screw capped test tube and centrifuged at 4000 rpm for 5 min (Hettich EBA-20, Germany). The hexane layer was analyzed immediately by gas chromatography.

Gas chromatographic analysis: The TMS derivatives of the unsaponifiables were analyzed on Shimadzu gas chromatograph (model GC-2010, Shimadzu Inc., Koyoto, Japan) supplied with split-split injector port and flame ionization detector, an RTX-65 TG (Restek, USA) CP-SIL 8CB capillary column (30 m x 0.25 mm internal diameter, film thickness was 0.25 µm and the active ingredients were 35% diphenyl 65% dimethly polysiloxan was used. Oven analysis temperature was 265°C and holding time for analysis was 55 min. The injector temperature was 280°C and the detector temperature was 290°C, flow rate of nitrogen (carrier gas) was 1.11 mL/min and split ratio was 1:30. Squalene and phytosterols profile peaks were identified by comparison with the retention times of reference standard of squalane and 5 alpha-Cholestan. The concentration of the analyte was calculated using the internal standard technique of squalane and 5 alpha-Cholestane. The chromatogram and the peak area were recorded by a digital integrator (Shimadzu C-R8A). The samples were analyzed in triplicates and the results reported are the means of these.

Total phenols analysis: The phenol content of MTSO was determined following the method of Parry *et al.* (2006) with minor modifications.

Extraction and sample preparation: MTSO was extracted with 100% methanol (MeOH) and evaluated for total phenolic contents (TPC) and radical scavenging capacity ($DPPH^{\bullet}$). Two grams of oil were extracted 3 times by 6ml of MeOH by vortexing for 5 min and centrifugation at 6000 rpm for 5 min. The supernatant was collected and combined then brought to 20 mL final volume of extraction solvent (MeOH). Oil extract was kept in the dark under N_2 until analyzed.

Total phenolic contents: The TPC of the MTSO was determined using Folin-Ciocalteu (FC, reagent) colorimetric method, based on the reaction of the reagent with the functional hydroxyl groups of phenols. Briefly, 1 mL from the oil extract was placed into a 10 mL volumetric flask. Five milliliter distilled water and 0.25 mL (2N) FC reagent (Sigma-Aldrich, USA) were added and mixed well for 3 min. Two mL of Na_2CO_3 (10%) were added and the flask was filled with distilled water up to the mark, mixed and allowed to stand for 60 min. A calibration curve was prepared using a pure phenolic compound (gallic acid) as a standard, from which serial dilutions were prepared. The absorbance of the blue color formed was measured at 725 nm in a Perkin Elmer, UV/Vis Spectrophotometer (Perkin Elmer Lambda, 25, 101 NB, USA) against a blank sample. The TPC was expressed as gallic acid equivalents (GAE) in mg/g of oil. Triplicate measurements were taken.

Vitamin E (alpha-tocopherol): The alpha-tocopherol of the MTSO sample was analyzed by HPLC according to the method described by Gimeno *et al.* (2000) as follows.

Sample and standard preparation: Stock standard solution of alpha-tocopherol acetate (98%, Sigma, USA) was prepared in ethanol, stored at-18°C in dark bottles. Working standard solution was prepared from the stock standard solution. To prevent the loss of vitamin E (alpha-tocopherol) from the MTSO sample, amber-coloured material was used.

One mL of oil sample was diluted in 10 mL of hexane (1:10). Thereafter, 200 µL was transferred to a screw-capped tube, where 600 µL of methanol and 200 µL of the internal standard solution (300 µg/mL of alpha-tocopherol acetate in ethanol) were added. After being vortex-mixed and centrifuged at 3000 rpm for 5 min, the sample was filtered through a 0.45 µm pore size filter and an aliquot of the overlay was directly injected into the HPLC.

HPLC analysis and quantification: Determination of alpha-tocopherol in oil sample by HPLC was carried out using a Perkin Elmer series 200 (USA) with a PE-1050 pump and 7125 injector model with a final volume loop of 500 µL. The detector was a PE-1040M photodiode-array detection system. The column was a C-18 (25 cm x 4.4 mm internal diameter, 5 µm particle size) (Perkin Elmer, USA).

The injection volume of sample was 50 µL. The mobile phase was methanol: water (96:4 v/v) (HPLC grade, GCC laboratories, England) and the elution was performed at a flow-rate of 1 mL/min. The column was kept at 45°C. Detection was performed at 292 nm. To determine the alpha-tocopherol in the sample, the working standard solution was analyzed together with the sample and peak-area ratios were used for calculations following the internal standard method. The sample was analyzed in triplicates and the results reported are the mean of these.

Radical ($DPPH^{\bullet}$) scavenging capacity assay: This method evaluates the radical scavenging capacity of antioxidant compounds by its reaction with the stable radical $DPPH^{\bullet}$. The assay was carried out in methyl alcohol and the result expressed as IC_{50}; which represent the antioxidant concentration of the extract in mg/ml necessary to scavenge or inhibit the initial $DPPH^{\bullet}$ concentration by 50%. $DPPH^{\bullet}$ scavenging capacity of the MTSO was determined according to the procedure reported by Parry *et al*. (2006) and Al-Ismail *et al*. (2007) as follows.

Extraction and sample preparation: The extraction and sample preparation method is the same mentioned in (2.4.2.3).

$DPPH^{\bullet}$ assay: Different aliquots of a methanolic solution of the MTSO extract were added to 0.2 mL of $DPPH^{\bullet}$ (ICN Biomedicals Inc. USA) solution (50 mg in 100 mL of methanol) and the volume adjusted (made up) to a final volume of 4 mL with methanol. Six different concentrations (100, 300, 600, 1000, 1200, 1500 µL) were used for assay. The blank sample of 0.2 mL of $DPPH^{\bullet}$ solution and the volume adjusted to 4 mL with methanol was used. After a 45 min incubation in darkness and at ambient temperature, the absorbance was measured at 515 nm in a spectrophotometer (UV/Vis double beam) (Labomed, INC. USA) against a blank. The inhibition percentage (I%) values were calculated from absorbance of the blank and of the sample. The inhibitory concentration (IC_{50}) value of oil extract was calculated from the dose-response curve by plotting oil extract concentrations against I%. Triplicate measurements were taken.

Total antioxidant capacity (TAC): TAC of MTSO was measured using the trolox equivalent antioxidant capacity (TEAC) assay. Total antioxidant status (TAS) kit was purchased from Randox Laboratories Ltd. (Crumlin, United Kingdom) and was used to measure TAC of MTSO manually on spectrophotometer at 600 nm (UV/Vis double beam, Labomed, Inc., USA).

All results in the following tables were expressed as Mean±SD.

RESULTS

Proximate analysis of milk thistle seeds: Table 1 demonstrates proximate analysis of MT seeds. Moisture and protein content were 4.61 and 17.64%, respectively. Fat represented the major component in MT seeds (26.9%), while ash and fiber contents were 5.10 and 25.32%, respectively.

Quality indicators of cold-pressed milk thistle seed oil: The basic quality indicators of cold-pressed MTSO are presented in Table 2. The tested oil had an acidity of 0.636% and peroxide value of 0.340 meq O_2/kg of oil. The OSI value of the cold-pressed MT seed oil had an induction time of 55.7 and 12.88 hours at 80 and 100°C, respectively.

Fatty acid profile in cold-pressed milk seed oil: The fatty acids detected in the cold-pressed MTSO were shown in Table 3. The cold-pressed MTSO contained a relatively high level of total unsaturated fatty acids that reached about 80.5% from total fatty acids. Linoleic acid was the most prevalent fatty acid in cold-pressed MTSO (56.79 g/100 g oil). Also, it contained significant concentration of oleic acid (22.03 g/100 g oil) and had low concentration of linolenic acid (0.67 g/100 g oil). On the other hand, saturated fatty acids (SFA) reached about 19.53% of the total fatty acids. Figure 1 shows the GLC chromatogram of fatty acids profile of cold-pressed MTSO.

Table 1: Proximate analysis of MT seed on dry matter basis

Component	MT seeds (%)
Moisture	4.61±0.96
Protein	17.64±1.12
Fat	26.90±1.10
Fiber	25.32±1.12
Ash	5.10±1.15
NFE	20.43±1.10

Results are given as Mean±SD (n = 3)
NFE: Nitrogen Free Extract

Table 2: Acidity, peroxide value and oxidative stability index of the oil extracted from cold pressed MT seeds

		Oxidative stability index(OSI) (h)	
Acidity (Linoleic acid) (%)	Peroxide value (mEq O_2 /Kg)	80°C	100°C
0.636±0.02	0.340±0.07	55.70±0.18	12.88±0.43

Data were expressed as mean±SD (n= 3)

Table 3: Fatty acid composition of oil extracted from cold-pressed MT seeds

Fatty acids composition		(g/100 g)
C14 : 0	Myristic acid	0.08±0.001
C16 : 0	Palmitic acid	8.61±0.007
C16 : 1	Palmitoleic acid	0.10±0.001
C17 : 0	Heptadecanoic acid	0.09±0.001
C17 : 1	Heptadecenoic Acid	0.03±0.007
C18 : 0	Stearic acid	5.24±0.001
C18 : 1	Oleic acid	22.03±0.007
C18 : 2	Linoleic acid	56.79±0.03
C18 : 3	Linolenic acid	0.76±0.02
C20 : 0	Arachidic acid	2.88±0.007
C20 : 1	Eicosenoic acid	0.76±0.02
C22 : 0	Behenic acid	2.04±0.007
C24 : 0	Lignoceric acid	0.58±0.007
	SFA*	19.53±0.03
	MUFA*	22.92±0.007
	PUFA*	57.55±0.02

Data were expressed as mean±SD (n = 3)
*SFA: Saturated fatty acids
MUFA: Monounsaturated fatty acids
PUFA: Polyunsaturated fatty acids

Table 4: Phytosterols composition and squalene content in the oil extracted from cold pressed MT seedss

Sterols	(mg/kg)
Cholesterol	381.7±2.62
Brassicasterol	ND*
24-methylene-cholesterol	5.80±0.183
Campesterol	167.8±2.12
Campestanol	5.54±0.177
Stigmasterol	210.2±6.40
Delta-7-campesterol	52.42±1.43
Delta-5,23-stigmastadienol	ND*
Clerosterol	16.38±0.183
β-sitosterol	1136.3±25.5
Sitostanol	42.10±0.190
Delta-5-avenasterol	14.90±0.00
Delta-5-24-stigmastadienol	38.00±0.707
Delta-7-stigmastenol	400.9±16.20
Delta-7-avenasterol	32.50±1.13
Erythrodiol	11.30±0.141
Uvaol	4.03±0.177
Total phytosterol	25.20±0.135
Squalene content (mg/kg)	9.35±0.362

Data were expressed as means±SD (n = 3)
*ND: not detected

Phytosterol composition and squalene content in the cold-pressed MTSO: Fifteen phytosterols were determined in oil sample (Table 4). β-sitosterol was the predominant (1136.3 mg/kg of oil) followed by delta-7-stigmasterol (400.9 mg /kg of oil), cholesterol (381.7 mg/kg of oil), stigmasterol (210:2 mg/kg of oil) and campesterol (167.8 mg/kg of oil). Subsequently, the total phytosterols concentration was (2520 mg/kg of oil). The squalene concentration in the cold-pressed MTSO was (9.35 mg/kg of oil) as shown in Table 4. GLC chromatograms of phytosterols and squalene are given in Fig. 2 and 3, respectively.

Table 5: Total phenolic content, vitamin E (as alpha-tocopherol), radical (DPPH) scavenging capacity and total antioxidant capacity in the oil extracted from cold-pressed MTSO[1]

Test	Values
Total phenolic content (mg GAE/g oil)	1.16±0.008
Vitamin E (as alpha-tocopherol) (mg/kg)	237.4±5.23
Radical (DPPH) scavenging activity (expressed as IC50) (mg/mL)	3.34±0.987
Total antioxidant capacity(TAC) (mmol/L)	2.29±0.112

Data were expressed as means±SD (n = 3)

Antioxidant compounds and capacity of cold-pressed milk thistle seed oil: TPC, which plays a dominant role in the antioxidant potential of seed oils, was 1.16 mg gallic acid equivalent/g oil (GAE mg/g) as shown in Table 5. In addition, this table shows that the concentration of alpha-tocopherol was 237.4 mg/kg of oil. HPLC chromatogram of alpha-tocopherol of cold-pressed MTSO is given in Fig. 4.

To further confirm the antioxidant properties of MTSO components, the methanol extracts were evaluated for their capacity to directly react with and quench DPPH$^{\bullet}$, as well as the TAC estimation was performed. As shown in Table 5, cold-pressed MTSO extract exhibited DPPH$^{\bullet}$ scavenging activity under the experiment conditions. After a 45 min of antioxidant-DPPH$^{\bullet}$ reactions, cold-pressed MTSO extract was able to quench 50% of DPPH$^{\bullet}$ in the reaction mixture at IC_{50} = 3.34 mg/mL. On the other hand, the TAC concentration was 2.29mmol/L Table 5.

DISCUSSION

Proximate analysis of milk thistle seeds: As seen in Table 1, fat represented the major component in MT seeds (26.90%). This result is within the range found by Fathi-Achachlonei and Azadmard-Damirchi (2009), which was 25-31%. The proximate analysis of MT seeds agreed with those reported by Abu-Rajouh and Takruri (2000) and that reported by Abu-Jadayil *et al.* (1999).

Quality indicators of cold-pressed milk thistle seed oil: It is obvious that the highest quality of MTSO was obtained by the technique of cold-pressing of seeds. This has been proven by basic chemical analysis of acidity% and PV as quality parameters of cold-pressed MTSO (Table 2). The acidity% and PV of cold-pressed MTSO were 0.636% and 0.340 meq O_2/kg of oil, respectively. These values were lower than

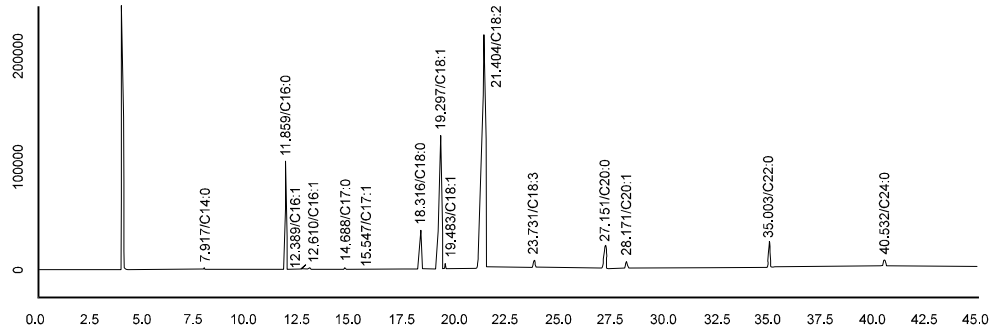

Fig. 1: Gas chromatogram of fatty acids profile of cold-pressed MTSO

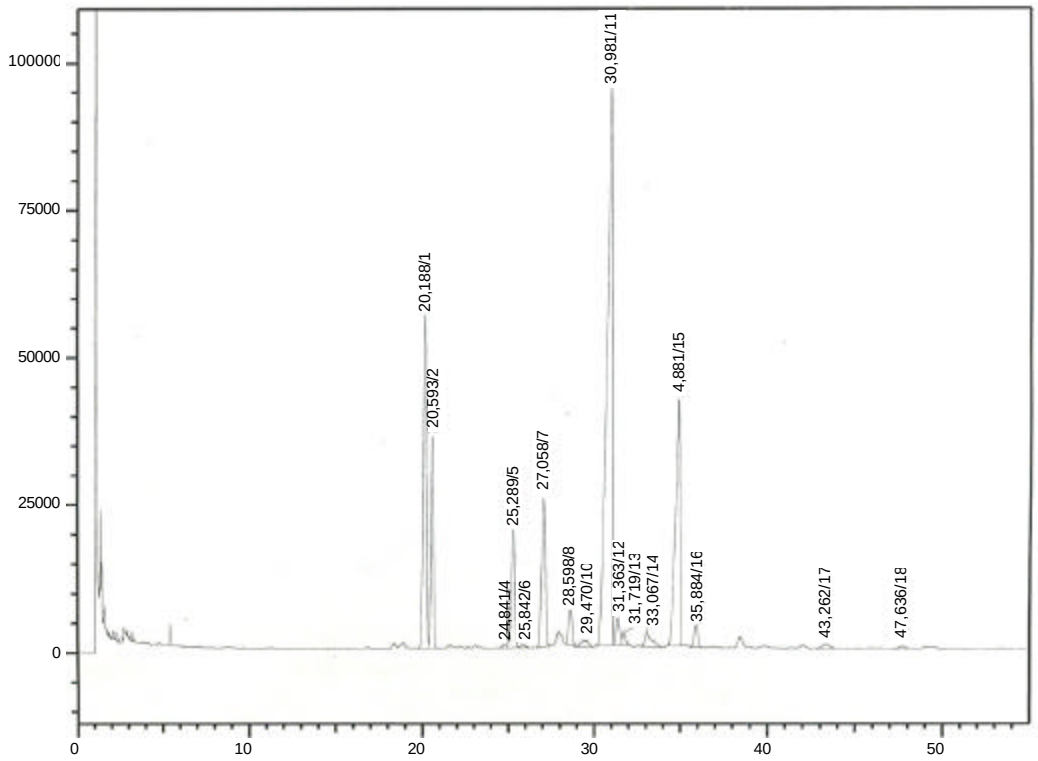

Fig. 2: Gas chromatogram of phytosterols of cold-pressed MTSO (Peaks identification: 1: Cholesterol, 2: IS Cholestanol (internal standard), 4: 24-methylene-cholesterol, 5: Campesterol, 6: Campestanol, 7: Stigmasterol, 8: Delta-7-campesterol, 10: Clerosterol, 11: β-sitosterol, 12: Sitostanol, 13: Delta-5-avenasterol, 14: Delta-5-24-stigmastadienol, 15: Delta-7-stigmastenol, 16: Delta-7-avenasterol, 17: Erythrodiol, 18: Uvaol)

the maximum values permitted for cold-pressed and virgin oils prescribed by the Codex Standards for vegetable oils (0.8% acidity and 15 mEq O_2/kg oil as PV) (Joint FAO/WHO, 2005).

The very low acidity% (<1) and PV (<15 mEq O_2/kg oil) in cold-pressed oils indicate that there was no chemical or enzymatic hydrolysis of glycerides and also indicate the freshness of the sample and the good quality of oil (Arena *et al*., 2007; Vujasinovic *et al*., 2010). These results are in agreement with data of cold-pressed seed oil reported in the literature. Arena *et al*. (2007) reported that the acidity% and PV of the cold-pressed pistachio nut oil were 0.66% and 5.7mEq/kg, respectively. Also, values of 0.6-0.9% and 1.5-6.5 mEq/kg, respectively were reported for five varieties of cold-pressed oils (Nyam *et al*., 2009). The lower acidity of these seed oils indicate that they are edible and could have a long shelf life (Arena *et al*., 2007; Nyam *et al*., 2009).

OSI measures the volatile secondary products of the free radical mediated lipid peroxidation and gives an

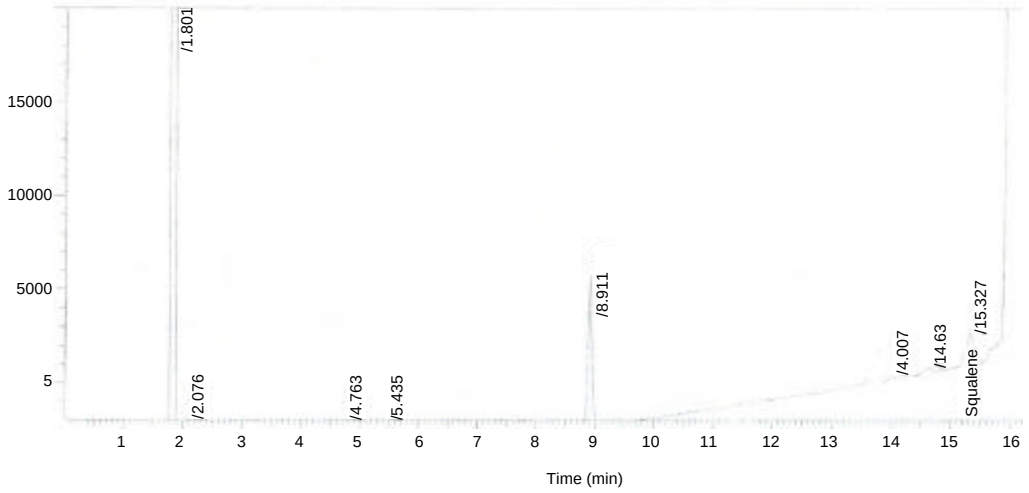

Fig. 3: Gas chromatogram of sequalene of cold-pressed MTSO (magnified 10 times)

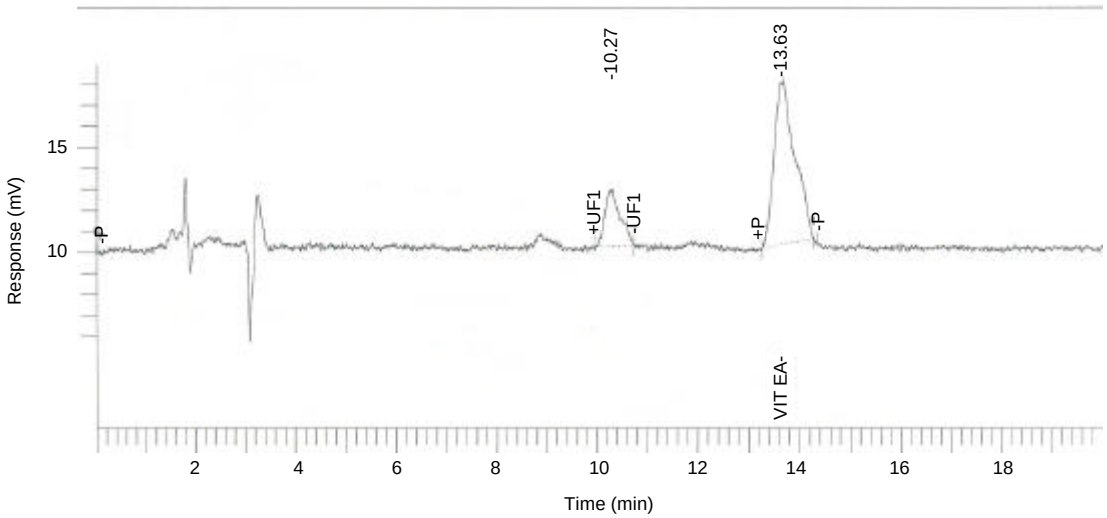

Fig. 4: HPLC chromatogram of alpha-tocopherol of cold-pressed MTSO

indication of the shelf life. Greater OSI value is associated with a longer shelf life (Luttrodt *et al*., 2010). As seen in Table 2, OSI value gave a longer shelf life for cold-pressed MTSO at 80°C, while the OSI of cold pressed MTSO at 100°C gave the shorter shelf life, reflecting a 4.5-fold difference in their OSI. Thus, there is an inverse relation between the temperature used in OSI test and hours of induction period of oxidation.

At 80°C, the OSI value in the present study (55.7 h) was higher than that of both commercial soy bean oils (47 h) and MTSO (14 h), while it was lower than that of corn oils (66.8 h) as reported by Parry *et al.* (2006). The difference in these values between the same type of oil (cold-pressed MTSO) may be due to different experimental conditions like an air flow of 7 L/h in Parry study, while it was 20 L/h in this study. Also, these differences may be due to geographical, climatic differences of areas where the MT seeds had been grown and influence of chemical components in MT seeds that affects the quality of oil.

Luttrodt *et al.* (2010) suggested that the level of unsaturated fatty acids present in the oils plays a determining role in their oxidative stability, with higher unsaturation associated with the lower OSI. Also, they suggested that other factors such as the contents of polyphenolics and other naturally-occurring antioxidants in the oil such as alpha-tocopherol contribute to oxidative stability.

Fatty acid profile in cold-pressed milk thistle seed oil: Interestingly, the number of fatty acids detected in the present study (13 fatty acids) (Fig. 1) was more than those detected by two recent studies, Parry *et al.* (2006) (8 fatty acids) and Fathi-Achachlouei and Azadmard-Damirchi (2009) (9 fatty acids) in MTSO.

The primary fatty acid in the cold-pressed MTSO was linoleic acid followed by oleic acid and palmitic acid (Table 3). These results agreed, with slight differences with those reported by Abu-Rajouh and Takruri (2000), Parry *et al.* (2006). These slight differences may be due to geographical and climatic differences of areas where the MT seeds had been grown and the method of oil extraction.

When compared with other seed oils, cold-pressed MTSO contained about 19.5 g of SFA/100 g total fatty acids, which is much lower than that of 30.8 g/100 g total fatty acids in the cold-pressed cardamom seed oil and comparable to that of 17.3 and 20.0 g/100 g total fatty acids found in the cold-pressed grape seed oil and sunflower seed oil, respectively (Parry *et al.*, 2006; Bail *et al.*, 2008). This saturated fat level was higher than that of 7.4-9.7 g/100 g total fatty acids in the cold-pressed Parsley, onion, hemp, Mullein and cranberry seed oils (Parker *et al.*, 2003; Parry *et al.*, 2006).

Cold-pressed MTSO contained significant level of MUFA's (about 22.9 g/100 g total fatty acids), which is comparable to the cold-pressed hemp, cranberry, blue berry, onion, flax seed, sunflower and black cumin seed oils, but was much lower than that of 81 and 82% in the cold-pressed carrot and parsley seed oils (Parker *et al.*, 2003; Parry *et al.*, 2005; 2006; Lutterodt *et al.*, 2010). Cold-pressed MTSO had a PUFA content of 57.6 g/100 g total fatty acids. This PUFA content was comparable to that in the cold-pressed black cumin (58.7 g/100 g), sunflower (57.2 g/100 g) and cotton (56.6 g/100 g) seed oils, but lower than that in the cold-pressed red raspberry, marion-berry, hemp, boysenberry and mullein seed oils with a PUFA content of 75-89 g/100 g total fatty acids (Parker *et al.*, 2003; Aguilera *et al.*, 2004.; Parry *et al.*, 2005, 2006; Tuberose *et al.*, 2007; Lutterodt *et al.*, 2010). As a result, the fatty acid profile of cold-pressed MTSO in the present study seems to be similar to that of sunflower seed oil and black cumin seed oil.

Phytosterol composition and squalene content in the cold-pressed milk thistle seed oil: Phytosterols are minor components of all vegetable oils and form a major proportion of the unsaponfiables (Roberfroid, 2000; Lagarda *et al.*, 2006; Nyam *et al.*, 2009). Phytosterols are important from a health point of view because they contribute to the lowering of serum cholesterol levels in humans (Fathi-Achachlouei and Azadmard-Damirchi, 2009). Phytosterols have been classified as 4-demethylsterols of the cholestane series, all of which have double bonds at the C-5 position of the ring. In plants, more than 200 different types of phytosterols have been reported the most abundant of which being β-sitosterol, campesterol and stigmasterol (Roberfroid, 2000; Lagarda *et al.*, 2006).

Six sterols in MTSO were determined by Fathi-Achachlouei and Azadmard-Damirchi (2009) at four varieties of MT grown in Iran (2 genetically modified foreign varieties and 2 native varieties). However, these authors mentioned that there were a few unknown sterols peaks which need further study for identification. In the present study, 15 sterols were determined (Fig. 2); in addition to the 6 sterols determined by them we could detect and for the first time, extra 9 sterols. Results show that the total sterols in cold-pressed MTSO had a higher content (2520 mg/kg of oil) compared with four varieties of MTSO (refined solvent-extracted) grown in Iran (1800-2200 µg/g) and with seed oil (refined solvent-extracted) of wild growing MT in Turkey (2261 mg/kg) (Deraz and Bayram, 1995). Though there was a slight difference in the values obtained, β-sitosterol was the predominant followed by delta-7-stigmasterol, which in turn is in agreement with the results of four varieties of MTSO grown in Iran and with wild growing MT in Turkey. The high content of β-sitosterol in seed oils (including MTSO) is an additional advantage of these seeds oils. The difference in total sterols content and values of each sterol between the present study and those studies may be due to the refining process and the method of oil extraction that affect the quality of oil and subsequently the chemical components, including phytosterol content in oil (Lagarda *et al.*, 2006).

Squalene is an important hydrocarbon present in unsaponifiable matter in most oils, mainly olive oil with a reported range of 2500 to 9250 ppm. It is a precursor of phytosterols in vegetable oils (Tuberose *et al.*, 2007; Bail *et al.*, 2008). Antioxidant activity of squalene is demonstrated against PUFA (Dessi *et al.*, 2002) and it is secondary to that of phenols and tocopherols (Tuberose *et al.*, 2007; Nyam *et al.*, 2009). In general, there is a scarcity of information regarding the content of squalene in seed oils. To our knowledge, the present study is the first to provide squalene analysis of MTSO.

Table 4 indicates that the squalene level in MTSO is relatively low (9.35 mg/kg) in comparison with data of other seed oils, where Tuberose *et al.* (2007) reported a squalene content in sunflower, rapeseed and pumpkin seed oils of 170.5, 437.4 and 3529.9 mg/kg, respectively; a higher content was found in olive oil (5990 mg/kg). Also, they mentioned that squalene was not detected in flax seeds, grape seeds and soybean oils. Maguire *et al.* (2004) also reported in another study that soybean oil and walnut oil had 220 µg/g and 9.4 µg/g of squalene, respectively. Nyam *et al.* (2009) reported that the amount of squalene varies from 36.9 to 1603.2 mg/kg in Kenaf and Kalahari melon seed oil, respectively and reached the highest amount in pumpkin seed oil (5907.0 mg/kg).

The decrease in squalene level with ripening of seed oils could be due to an internal mechanism in the seeds that squalene is a biochemical precursor of phytosterol in vegetable oils which in turn is affected by the degree of seed ripening levels before harvesting. That is if

seeds are premature or at the beginning of ripening, the level of squalene increases gradually till being fully ripen (proper harvesting date) then the level gradually decrease due to its involvement in phytosterol synthesis. Rodrigues-Estrada (2000) found that oil samples extracted from olives harvested at different ripening levels, had a varying level of squalene which was practically absent in the oils obtained from mature olives.

Antioxidant compounds and capacity of cold-pressed milk thistle seed oil: alpha-tocopherol and polyphenols in cold-pressed MTSO were identified as possibly being the main compounds responsible for their free radical scavenging capacity in vegetables oils and foods as well as in the body (Bozan and Temelli, 2008; Nyam *et al*., 2009). In the present study, the result obtained for the amount of alpha-tocopherol content (237.4 mg/kg of cold-pressed MTSO) was within the range of previously published results reported for four varieties of MTSO grown in Iran with values of 187.4 to 465.1 mg/kg (Fathi-Achachlouei and Azadmard-Damirchi, 2009). This wide range of alpyha-tocopherol in these varieties may be due to different farming processes. The CN-seed and Budakalaszi varieties produced by irrigated farming generally had lower amount of alpha-tocopherol, compared with other varieties, Babak castle and khoreslo, produced by dry farming which had higher amount of alpha-tocopherol. It is worth mentioning that the MT studied in the present study grows wild not cultivated. On the other hand, Parry *et al*. (2006) found that cold-pressed MTSO had a lower amount of alpha-tocopherol (156.3 mg/kg oil) compared with the present study.

When compared with other oils, alpha-tocopherol amount (237.4 mg/kg of cold-pressed MTSO) was higher than or comparable with those reported for commercial extra virgin olive, peanut, corn and sunflower seed oils (164-578 mg/kg) and higher than soybean oil (92 mg/kg) (Cabrini *et al*., 2001), but much lower than that of wheat germ oil (1330 mg/kg oil) (Wagner *et al*., 2004). They are also higher than those detected in cold-pressed blueberry, red raspberry, marionberry and boysenberry seed oils (21-150 mg/kg oil) (Parry *et al*., 2005).

The TPC value of the cold-pressed MTSO (1.16 mg GAE/g oil) was lower than cold-pressed MTSO (3.0 mg GAE/g oil) in the study of Parry and Collegues (2006). Also, it was lower than that of 1.73-2.0 mg GAE/g oil for the cold-pressed red raspberry, blue berry and boysenberry seed oils and that of 1.8-3.4mg GAE/g oil for the cold-pressed parsley, onion, cardamom and mullein seed oils (Parry *et al*., 2005; 2006). The TPC value was greater than of 0.38-0.79mg GAE/g detected in eight Maryland-grown soft wheat grain samples (Moore *et al*., 2005) and that of 0.08-0.70 mg GAE/g oil for the peanut, hazelnut, almond, walnut and pistachio nut oils (Arranz *et al*., 2008). On the other hand, the TPC value was comparable to that of 0.99 and 1.31mg GAE/g oil for the cold-pressed roasted pumpkin and black cumin seed oils, respectively (Parry *et al*., 2006; Lutterodt *et al*., 2010). The differences in TPC may be explained by the different extraction solvents which were used during sample preparation in these studies. 100% methanol is a preferred solvent for antioxidant extraction compared with 50% acetone or 70-80% (methanol:water) solution. 100% methanol is an excellent solubilization of lipophilic antioxidants. The effects of solvent extraction on antioxidant compounds estimation were observed and discussed in details in the study of Parry and Collegues (2006). On the other hand, Gorinstein *et al*. (2003) and Tuberose *et al*. (2007) explained that these differences were due to genetic factors variation, growing conditions such as soil and temperature, post-harvesting treatments including the mechanical grinding during oil processing and storage may significantly alter the chemical composition of selected botanical materials.

To assess the antioxidant capacity, the two most common radical scavenging assays DPPH and ABTS radical were chosen because they are widespread methods used and based on an electron transfer and involves reduction of colored oxidants (Apake *et al*., 2007; Niki, 2010).

The cold-pressed MTSO methanolic extract exhibited $DPPH^{\bullet}$ scavenging capacity at IC_{50} = 3.34 mg/mL indicating a higher scavenging capacity than the result obtained by Parry *et al*. (2006); who got an IC_{50} of 6.7 mg/ml as they were comparing MTSO with other seed oil methanolic extracts. Parsley seed oil exhibited the strongest $DPPH^{\bullet}$ scavenging capacity and IC_{50} of 0.91 mg/ml, onion seed oil extract had the next strongest $DPPH^{\bullet}$ scavenging capacity (IC_{50} of 2.3 mg/mL), followed by Cardmom and roasted pumpkin seed oil extract with IC_{50} = 5.8 and 6.4 mg/mL, respectively.

In other studies, water-soluble extracts of cardamom seed and whole parsley were examined for their IC_{50} against DPPH radical. The cardamom seed extract demonstrated an IC_{50} = 7.8 mg/mL, while the IC_{50} of the whole parsley extract was 12 mg/mL (Hinneburg *et al*., 2006). This difference may be due to the fact that methanolic extract had higher phenolic compounds as a result of extraction of both non polar and semi polar soluble phenolic acids.

When comparing all previously mentioned extracts with ascorbic acid, BHA, BHT and gallic acid as references potent antioxidants for DPPH radical scavenging capacity, the IC_{50} values were 0.09, 0.09, 0.21 and 0.03 mg/mL, respectively (Garjani *et al*., 2009; Lutterodt *et al*., 2010).

Regarding the TAC (ABTS assay), the cold-pressed MTSO had a higher TAC value (2.29 mmol/L) compared with other methanolic extracted seed oils such as flaxseed, grape, peanut, pumpkin, rapeseed and

sunflower seed which had TAC values of 1.01, 1.43, 0.45, 0.95, 0.80 and 1.11 mmol/L, respectively. On the other hand, the TAC value in the present study was comparable with maize and soybean oil (2.3 and 1.85 mmol/L, respectively) (Tuberose *et al*., 2007; Bail *et al*, 2008).There was a difference between values obtained from the two used assays. This is because the TAC (ABTS assay) is based on the generation of a blue/green ABTS•+, which is applicable to both hydrophilic and lipophilic antioxidant systems; whereas DPPH assay uses a radical dissolved in organic media and is, therefore, applicable to hydrophobic systems. This finding is in agreement with what was explained by Floegel *et al*. (2011).

Conclusions: Under the environment of this study, fat represented the major component in MT seeds. Cold-pressing technique resulted in high quality MTSO with low acidity and peroxide value as quality parameters. Cold-pressed MTSO could have a long shelf-life with greater OSI value at 80 C. These may be due to the ratio of USFA:SFA, amount of polyphenolic compounds, alpha-tocopherols and other natural-occurring antioxidant in the MTSO. The main fatty acids were found to be linoleic acid, oleic acid, palmitic acid and stearic acid. Linolenic acid was found in minor amount. Cold-pressed MTSO had high content of phytosterols (mainly β-sitosterol) and alpha-tocopherol which are considered important from a nutritional and functional point of view. However, squalene was found in a relatively low level. Cold-pressed MTSO was found to have a high *in vitro* free radical scavenging capacity as indicated by TAC due to the presence of polyphenolic compounds and alpha-tocopherol.

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