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ORIGINAL PAPER

Fatty Acid Composition, Antioxidant Properties, and Antiproliferative Capacity of Selected Cold-Pressed Seed Flours

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Abstract Cold-pressed seed flours from pumpkin, parsley, mullein, cardamom, and milk thistle were examined for total oil, fatty acid profile of the oil, total phenolic content (TPC), scavenging activities against peroxyl (ORAC), hydroxyl (HOSC) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) (RDSC) radicals, and antiproliferative capacity against HT-29 human colon cancer cells. The cold-pressed parsley seed flour contained a very high concentration of total oil—17.6 g/100 g flour—with primarily C18:1 fatty acid at 86.2 g/100 g fatty acids. All other flour oils had relatively high levels of saturated fats, ranging from 39.0 to 62.9 g/100 g fatty acids. The tested seed flours demonstrated significant TPC and free radical scavenging activities. Milk thistle seed flour had the highest TPC value of 25.2 mg gallic acid equivalent per g flour (GAE mg/g) followed by that of parsley seed flour at 8.1 GAE mg/g. Milk thistle seed-flour extract also had significantly higher antioxidant activities than all other extracts against all tested radicals. The milk thistle seed-flour extract had an ORAC value of 1131 μmol trolox equivalents (TE) per g flour (TE μmol/g), a HOSC value of 893 TE μmol/g, and an RDSC value of 61 TE μmol/g. Also, ORAC, HOSC, and TPC values were significantly correlated ($P < 0.01$) under the experimental conditions. The cold-pressed milk thistle seed flour inhibited the proliferation of HT-29 cancer cells in a dose-dependent manner. Results from this study suggest that these cold-pressed seed flours may serve as natural sources of antioxidants and may be used to improve human health.

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Introduction

A number of studies have demonstrated that certain food components may reduce the risk of chronic diseases. Novel food ingredients rich in health beneficial components, including natural antioxidants, are in high demand for improving human health and life quality. This consumer desire has stimulated the discovery and development of such nutraceutical ingredients for food applications. It is widely accepted that an ideal nutraceutical preparation should have following characteristics: (1) health beneficial activities, (2) safety for long-term consumption, (3) cost effectiveness, (4) stability during storage, food formulation, and processing, and (5) no damage on sensory properties of the food products. Developing nutraceuticals from agricultural materials and the byproducts from agricultural and food processing may add value to the crop production and processing industries while benefitting human health.

Seed flour is a primary byproduct from edible seed oil production and is often discarded as waste. Our recent studies have shown that some cold-pressed edible seed flours may contain many health beneficial components such as natural antioxidants, tocopherols and carotenoids, and other beneficial properties [1] and components [2]. Seed flours of black raspberry, red raspberry, blueberry, pinot noir grape, and chardonnay grape have been shown to contain significant levels of phenolics and anthocyanins, tocopherols and carotenoids, α-linolenic acid (C18:3n-3),

antioxidant capacities, and antiproliferative activity against HT-29 human colon cancer cells [1, 2]. Alpha-linolenic acid may be converted in vivo to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), both of which may have protective effects against several chronic human diseases, including heart disease and cancer [3–6].

The antioxidant extracts of chardonnay grape seed flour and cranberry seed meal have also been found to exhibit potential in improving food quality, stability, and safety [7]. Both of these antioxidant extracts were able to enhance the shelf life of EPA (C20:5n-3), α-linolenic acid (C18:3n-3), and total n-3 fatty acids, to suppress total lipid peroxidation in fish oils, and to inhibit *Escherichia coli* under experimental conditions [7]. These data suggest that it is possible to develop natural nutraceutical preparations and food shelf-life enhancers from edible seed flours. These seed flour-based products may further add value to the oil seed production and processing industries, while improving food value and benefitting human health.

Cold-pressed parsley, milk thistle, mullein, cardamom, and roasted pumpkin seed oils are commercially produced and marketed as specialty edible oils [8]. Seed flours from these oil productions are treated as low-value wastes. As part of our continuing effort to study and develop value-added uses of byproducts from agricultural and food processing, we conducted this study with the aim of investigating cold-pressed roasted pumpkin, parsley, mullein, cardamom, and milk thistle seed flours for their total oil content, fatty acid profile of the flour oil, total phenolic content (TPC), antiproliferative effects against HT-29 human colon cancer cells, and antioxidant activities, including oxygen radical absorbance capacity (ORAC), hydroxyl radical scavenging capacity (HOSC), and relative 2,2-diphenyl-1-picrylhydrazyl ($DPPH^{\bullet}$) scavenging capacity (RDSC).

Materials and Methods

Materials

Cold-pressed roasted pumpkin (*Cucurbita pepo* L. cv. Triple Treat), parsley (*Petroselinum crispum*), mullein (*Verbascum thapsus*), cardamom (*Elettaria cardamomum*), and milk thistle (*Silybum marianum*) seed flours were obtained from Botanic Oil Innovations (Spooner, WI). Fatty acid methyl ester standards were purchased from NuChek Prep (Elysian, MN). Gallic acid, $DPPH^{\bullet}$, sodium acetate, potassium chloride, and trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma-Aldrich (St Louis, MO). 2,2′-Azobis (2-amino-propane) dihydrochloride (AAPH) was obtained from Wako Chemicals USA (Richmond, VA). Disposable cell culture ware was purchased from Corning Glass Works (Corning, NY). McCoy's 5A Media modified with L-glutamine, antibiotic/antimycotic, fetal bovine serum (FBS), and 0.25% trypsin with 0.9 mM EDTA were purchased from Invitrogen (Carlsbad, CA), and HT-29 human colorectal adenocarcinoma cancer cells were purchased from American Type Culture Collection (Rockville, MD). The ATPlite 1step Luminescence ATP Detection Assay System was purchased from PerkinElmer Life and Analytical Sciences (Waltham, MA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Sample Preparations

Oils in the cold-pressed seed flours were extracted with hexane using a Soxhlet apparatus and analyzed for total oil content and fatty acid profile. Cold-pressed seed flours were extracted with 50% acetone at a solvent–flour ratio of 10 mL per gram flour at ambient temperature. After centrifugation at 2000 *g* for 10 min, the supernatants were collected and subjected to examinations of TPC, and radical scavenging capacity against peroxyl (ORAC), hydroxyl (HOSC), and DPPH (RDSC) radicals. After removal of the solvent from a known volume of each 50% acetone extract, the solid residue was quantitatively redissolved in 50% DMSO at a final concentration of 500 mg flour equivalent per mL solvent and used for antiproliferative activity estimation. All samples were stored in the dark under nitrogen until analyzed.

Total Phenolic Content

The TPC of the seed-flour extracts was determined using Folin and Ciocalteu's (FC) reagent following a previously described method [8]. The FC reagent was freshly prepared, and the final reaction mixture contained 250 μL FC reagent, 750 μL 20% Na_2CO_3, 50 μL seed-flour extract or standard, and 3 mL H_2O. Absorbance was determined at 765 nm following 2 h of reaction at ambient temperature. Gallic acid was used as the standard. Measurements were taken in triplicate.

Fatty Acid Composition

Fatty acid methyl esters were prepared from the hexane-extracted oils using a previously described laboratory method [9]. Fatty acid compositions were analyzed using a Shimadzu GC-2010 with a flame ionization detector and a Shimadzu AOC-20i autosampler (Shimadzu, Columbia, MD). A Supelco 2380 column (30 m × 0.25 mm i.d.) with a 0.20-μm film thickness (Supelco, Bellefonte, PA) was used with helium as the carrier gas at a flow rate of 0.8 mL/

min. The injection volume was 1 μL at a split ratio of 10/1. Time and temperature ramps began with an initial oven temperature of 142 °C, increased 6 °C/min to 184 °C, held for 3 min, and then increased 6 °C/min to 244 °C [10]. Fatty acids were identified by comparing the gas chromatograph retention time of each peak with that of the authorized pure individual standard compounds, and they were quantified using the area under each fatty acid peak. Triplicate measurements were performed.

Oxygen Radical Absorbance Capacity

The ORAC values for the 50% acetone extracts of the seed flours were examined using a Victor^3 Multilabel Plate Reader (PerkinElmer, Turku, Finland) following a laboratory procedure previously described [11]. Fluorescein was used as the fluorescent probe, and all reagents were prepared in 75 mM phosphate buffer (pH 7.4) except for the seed-flour extracts and standards, which were prepared in 50% acetone. The final assay mixture contained 0.067 μM fluorescein, 53.6 mM AAPH, and 30 μL of solvent blank, antioxidant standard, or seed-flour extract. Fluorescence measurements were recorded every minute at an emission wavelength of 535 nm using an excitation wavelength of 485 nm. The ORAC values were calculated using relative area under the kinetic curve and expressed as micromole trolox equivalents (TE) per gram seed flour (TE μmol/g). Experiments were conducted in triplicate.

Hydroxyl Radical Scavenging Capacity

Hydroxyl radical scavenging capacities were investigated using a Victor^3 multilabel plate reader (PerkinElmer) according to a laboratory protocol described earlier [12]. Hydroxyl radicals were generated by a Fenton-like reaction using H_2O_2 and Fe^{3+}, and trolox was used to prepare the standard curve. Fluorescence measurements were determined at 485 nm excitation and 535 nm emission wavelength. The reaction mixture contained 170 μL 9.28×10^{-8} M fluorescein in 75 mM sodium phosphate buffer at pH 7.4, 30 μL solvent blank, standard or seed-flour extract, 40 μL 0.1990 M H_2O_2, and 3.43 mM $FeCl_3$. Results were calculated using relative area under the kinetic curve and expressed as μmol trolox equivalents (TE) per gram seed flour (TE μmol/g). All tests were conducted in triplicate.

Relative DPPH$^\bullet$ Scavenging Capacity

The RDSC values of the seed-flour extracts were obtained using the high throughput assay described by Cheng and others [13]. A Victor^3 Multilabel Plate Reader (PerkinElmer) was used for determination using 96-well plates. The reaction mixture contained 100 μL 0.2 mM DPPH$^\bullet$ and 100-μL of the standards, control, blank, or sample. Absorbance readings were determined at 515 nm. The standard curve was derived from the area under the curve from different concentrations of trolox. Measurements were conducted in triplicate.

Inhibition of HT-29 Cancer Cell Proliferation

HT-29 human colorectal adenocarcinoma cells characterized by Fogh and Trempe [14] were propagated in T-150 flasks containing McCoy's 5A media with 10% FBS and 1% antibiotic/antimycotic. The flasks were incubated at 37 °C in a humidified atmosphere with 5% CO_2 [2]. Cells were seeded at 2500 cells per well into 96-well plates and incubated for 24 h prior to treatment. The cells were then treated with the 50% DMSO solutions of seed-flour extracts at final concentrations of 3 and 6 mg seed-flour equivalents/mL media. Treatments and controls had final concentrations of DMSO in media of 6 μL/mL [2]. Cell proliferation was measured using an ATP Luminescence kit (PerkinElmer Life and Analytical Sciences) according to previously described protocol [15, 16]. Luminescence measurements were taken every 24 h for 4 days. All tests were conducted in triplicate. The cells were viewed and images were captured using a Nikon T2000-S microscope with a 3.1 megapixel camera at magnifications of 100× and 200×.

Statistical Analysis

Data were analyzed using SPSS for Windows ver. 10.0.5 (1999; SPSS, Chicago, IL). Data were reported as mean ± standard deviation ($n = 3$). Analysis of variance and Tukey's post hoc analysis were used to determine differences among means. The Pearson correlation coefficient was used to determine correlations among means. Significance was declared at $P < 0.05$. One lot of each cold-pressed seed flour was used in the present study, with the exception that two lots of the mullein seed flours were used to show the possible variations from lot to lot. Three analyses were performed for each cold-pressed flour sample, and the data were used for statistical analysis.

Results and Discussion

Total Phenolic Content

Total phenolic content was investigated because phenolic compounds may be a major contributor to the overall antioxidant activities of selected botanical materials. The

TPCs of the tested seed flours ranged from about 1.6 to 25.2 mg gallic acid equivalents (GAE) per gram seed flour (GAE mg/g) (Table 1). The milk thistle seed flour had the highest TPC value followed, in decreasing order, by parsley, mullein, cardamom, and roasted pumpkin seed flours. This order differed to that observed for the corresponding cold-pressed seed oils with 100% methanol as the extraction solvent [8]. However, similar to our results for seed flours, the milk thistle seed oil had the highest TPC (3.07 GAE mg/g) and roasted pumpkin seed oil had the lowest TPC (0.98 GAE mg/g) under the experimental conditions [8]. In 2006, Kosinska and Karamac [17] examined the TPC of roasted pumpkin seeds and the defatted seeds. The defatted roasted pumpkin seeds had a TPC value of 0.91 mg catechin equivalents/g using 80% MeOH as the extracting solvent, with a material–solvent ratio of 1:8 (w/v) and an extraction temperature of 70 °C [17]. This TPC value is hard to compare with what observed in our study because of the different solvent and phenolic standard. The TPC of the two mullein samples were significantly different from each other (Table 1), suggesting a possible variation between flour samples due to the seed genotype, crop growing conditions, and/or seed storage conditions.

Fatty Acid Composition

Oil content and fatty acid (FA) profile of the cold-pressed seed flours were examined. The highest total oil content was detected in the parsley seed flour, 17.6 g/100 g, and the lowest was detected in cardamom seed flour, 0.7 g/100 g (Table 1). All of the tested seed flours had relatively high levels of C18:1 FA ranging from 36.5 to 86.2 g/100 g total FA in the mullein2 and parsley seed flours, respectively (Table 2). The oil of the cold-pressed parsley seed flour was highly unsaturated, containing about 96% unsaturated FA, while the other seed flours had relatively high levels of total saturated FA, primarily as stearic and palmitic acids (Table 2). The FA compositions of these cold-pressed seed flours are different from those of their corresponding cold-pressed seed oils that were obtained from the same seeds [8].

Oxygen Radical Absorbing Capacity

Antioxidant activities of the seed-flour extracts were estimated using the ORAC assay. The ORAC assay measures the scavenging capacity of antioxidants against peroxyl radical, a physiologically relevant free radical. All of the seed-flour extracts demonstrated radical scavenging activities against the peroxyl radical. The ORAC values of the cold-pressed seed flours ranged from 35 to 1131 TE μmol/g for the cardamom and milk thistle seed flours, respectively (Table 3). The ORAC values of the two extracts of mullein seed flour were significantly different from each other, with Mullein1 having an ORAC value of 127.3 TE μmol/g and Mullein2, a value of 98.2 TE μmol/g.

This relative order of ORAC values of the seed flours is very different to that observed with the corresponding cold-pressed seed oils that were extracted with 100% MeOH [8]. For the latter, the highest ORAC value, 1098 TE μmol/g oil, was detected in the cold-pressed parsley seed oil, followed by cold-pressed cardamom and milk thistle seed oils at 941 and 125 TE μmol/g oil, respectively [8]. The ORAC value of mullein seed oil was 26.9 TE μmol/g, which was much lower than that of cardamom seed oil but was over 20-fold higher than that of the roasted pumpkin seed oil (1.1 TE μmol/g) [8]. Interestingly, in this study, the cold-pressed milk thistle seed flour had the strongest ORAC value, 1130.7 TE μmol/g, which was much higher than that observed in the cold-pressed parsley and cardamom seed flours (Table 3). The cold-pressed cardamom seed flour

Table 1 Phenolic and oil contents of tested cold-pressed edible seed flours

Plant origin of seed flours analyzed[a]	TPC (GAE mg/g)[b]	Total oil (g/100 g)[c]
Pumpkin	1.58 ± 0.02 e	12.3
Parsley	8.11 ± 0.23 b	17.6
Mullein1	4.73 ± 0.02 c	12.4
Mullein2	4.09 ± 0.06 d	11.0
Cardamom	1.85 ± 0.09 e	0.7
Milk Thistle	25.22 ± 0.59 a	7.5

Values with different letters are significantly different ($P < 0.05$)

[a] Pumpkin, parsley, mullein, cardamom, and milk thistle represent the cold-pressed roasted pumpkin, parsley, mullein, cardamom, and milk thistle seed flours. Mullein1 and Mullein2 are two different samples from the same variety

[b] TPC is the total phenolic content in the respective seed flours and is measured as milligrams of gallic acid equivalents per gram seed flour (GAE mg/g)

[c] Total oil is expressed as g oil per 100 g seed flour (g/100 g)

Table 2 Fatty acid profiles of the cold-pressed seed flour oils

Fatty acids[a]	Pumpkin	Parsley	Mullein1	Mullein2	Cardamom	Milk thistle
12:0	0.2 ± 0.0	t	ND	ND	ND	ND
14:0	0.2 ± 0.1	ND	t	0.1 ± 0.0	1.0 ± 0.1	0.5 ± 0.4
16:0	23.4 ± 0.0	2.8 ± 0.0	21.0 ± 0.0	28.1 ± 0.0	47.6 ± 0.1	27.4 ± 0.1
16:1	0.1 ± 0.0	0.1 ± 0.0	0.4 ± 0.0	0.3 ± 0.0	1.5 ± 0.0	0.2 ± 0.0
18:0	20.7 ± 0.0	1.0 ± 0.0	15.6 ± 0.0	30.8 ± 0.0	6.6 ± 0.0	17.7 ± 0.1
18:1	53.1 ± 0.0	86.2 ± 0.0	42.5 ± 0.1	36.5 ± 0.1	40.6 ± 0.0	37.6 ± 0.2
18:2	2.3 ± 0.1	9.4 ± 0.0	17.3 ± 0.0	0.6 ± 0.0	0.7 ± 0.0	4.6 ± 0.0
18:3	ND	0.4 ± 0.0	ND	ND	ND	ND
20:0	ND	0.1 ± 0.0	2.3 ± 0.0	3.1 ± 0.0	1.0 ± 0.04	9.3 ± 0.0
20:1	ND	t	1.0 ± 0.0	0.5 ± 0.0	0.9 ± 0.02	2.8 ± 0.2
SFA	44.5	3.9	39.0	62.1	56.2	54.8
MUFA	53.2	86.3	43.8	37.3	43.1	40.6
PUFA	2.3	9.8	17.3	0.6	0.7	4.6

ND, Not detected; t, trace

Data are expressed as mean ± SD ($n = 3$). The units are g/100g FA

Pumpkin, parsley, mullein, cardamom, and milk thistle represent the cold-pressed roasted pumpkin, parsley, mullein, cardamom, and milk thistle seed flour oils. Mullein1 and Mullein2 are two different samples of the same variety

[a] SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids

Table 3 Antioxidant activities of selected cold-pressed edible seed flours

Plant origin of seed flours analyzed[a]	ORAC (TE μmol/g)	HOSC (TE μmol/g)	RDSC (TE μmol/g)
Pumpkin	37.6 ± 0.4 e	22.2 ± 2.1 d	2.2 ± 0.0 e
Parsley	390.0 ± 15.9 b	311.5 ± 21.0 b	18.1 ± 1.5 d
Mullein1	127.3 ± 10.1 c	74.3 ± 3.6 c	24.0 ± 1.3 b
Mullein2	98.2 ± 4.1 d	75.3 ± 3.4 c	21.2 ± 1.3 c
Cardamom	35.3 ± 2.1 e	22.6 ± 2.0 d	19.5 ± 0.6 c,d
Milk Thistle	1130.7 ± 31.3 a	893.0 ± 62.4 a	61.1 ± 0.3 a

Values in the same column with different letters are significantly different ($P < 0.05$)

ORAC, Oxygen radical absorbance capacity; HOSC, hydroxyl radical scavenging capacity; RDSC, relative DPPH$^{\bullet}$ scavenging capacity. All are expressed as Trolox equivalents (TE) in micromoles per gram seed flour (TE μmol/g)

[a] Pumpkin, parsley, mullein, cardamom, and milk thistle represent the cold-pressed roasted pumpkin, parsley, mullein, cardamom, and milk thistle seed flours

had the lowest ORAC value among the tested seed flours under the experimental conditions.

Hydroxyl Radical Scavenging Capacity

The hydroxyl radical is one of the most abundant and reactive oxygen species in biological systems and is capable of attacking DNA, membrane lipids, and proteins, potentially leading to degenerative chronic diseases such as cancer, atherosclerosis, and autoimmune disorders, among many others. Hydroxyl radical scavenging capacity was observed in all tested seed-flour extracts (Table 3). The cold-pressed milk thistle seed flour had the highest HOSC value of 893 TE μmol/g followed, in descending order, by cold-pressed parsley, mullein, and cardamom and roasted pumpkin seed flours. No difference was observed between the cold-pressed roasted pumpkin seed flour and cardamom seed flour in terms of their ORAC, HOSC, and TPC values, although they were different in their total fat contents. The HOSC, TPC, and ORAC values were significantly correlated among themselves with $r = 0.990$ ($P < 0.01$) for HOSC and TPC, $r = 0.995$ ($P < 0.01$) for HOSC and ORAC, and $r = 0.995$ ($P < 0.01$) for ORAC and TPC (Table 3).

Relative DPPH$^{\bullet}$ Scavenging Capacity

Relative DPPH$^{\bullet}$ scavenging capacity was also evaluated for these cold-pressed seed-flour extracts. This assay

examines the free radical scavenging activity of the flour extracts against the DPPH radical. Values are reported as TE in micromoles per g flour (TE μmol/g). The milk thistle seed flour extract had a RDSC value of 61.1 TE μmol/g, and similar to the other antioxidant tests, had a significantly higher antioxidant activity than all other samples (Table 3). The mullein1 extract had a significantly higher RDSC value than mullein2, which was consistent with the results of the ORAC and TPC assays, but no difference was seen against the hydroxyl radical. In previous studies, water-soluble extracts of cardamom seed and whole parsley were examined for their IC_{50} against DPPH radicals [18]. The cardamom seed extract demonstrated an IC_{50} value of approximately 7.8 mg/mL while the IC_{50} of the whole parsley extract was about 12 mg/mL [18]. In the previous study of the corresponding cold-pressed seed oil extracts, the cold-pressed parsley seed oil extract had the highest DPPH radical scavenging activity and milk thistle seed oil extract had the lowest activity [8]. A lower $DPPH^{\bullet}$ scavenging activity was also seen in the cardamom seed oil extract, which may reflect the effects of the antioxidant/radical system on antioxidant activity estimation.

Inhibition of HT-29 Cell Proliferation

Antiproliferative effects against HT-29 human colon cancer cells were determined for all of the seed-flour extracts tested at 3 and 6 mg flour equivalents/mL media final concentrations. The results after 48 and 96 h of treatment at 6 mg/mL are shown in Fig. 1. The milk thistle seed-flour extract at both 3 and 6 mg flour equivalents/mL significantly inhibited HT-29 cell growth—in a dose-dependent manner—compared to the control at both 48 h (Fig. 2) and 96 h of treatment (Fig. 1). The parsley seed flour extract showed inhibition only at 6 mg flour equivalents/mL after 48 h, while all other tested seed-flour extracts had no inhibitory effect against HT-29 cells under the experimental conditions (Fig. 1). A water control was included to show the effects of DMSO on antiproliferation (results not shown). Over the 4 days of the study, the DMSO control averaged 92.2% cell proliferation compared to the water control and was not significantly lower at any of the experimental time points. Differences in the morphology of the HT-29 cells treated with pumpkin and milk thistle seed flour extracts at 6 mg per mL media are depicted in Fig. 3, along with that of the control sample containing only DMSO. Cell proliferation in the pumpkin flour extract-treated cells was higher than that in the control at 48 and 96 h of treatment (Fig. 1); however, the difference was not significant. The granulated appearance and blebbing of the plasma membrane of the milk thistle-treated cells are indicative of cell death. Also, the cells in the milk thistle extract detached from the bottom of the well and were found as floating spheres; this gave them the appearance of being smaller than those cells in the other solutions, which were attached and spread out. The antiproliferative effect of the milk thistle seed-flour extract on HT-29 cells is consistent with the results of previous investigations on

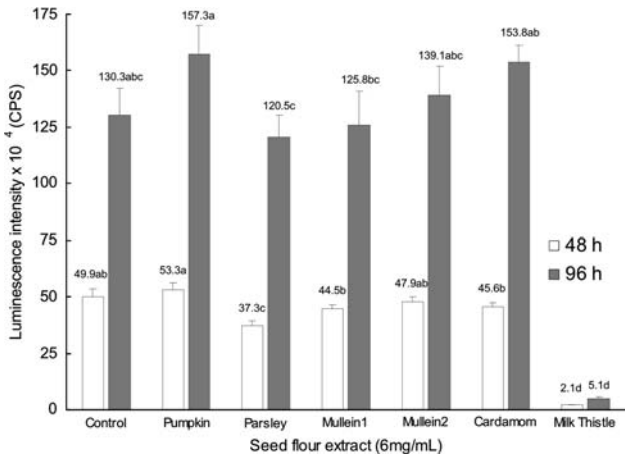

Fig. 1 Antiproliferative activity against HT-29 cells. Pumpkin, parsley, mullein, cardamom, and milk thistle represent the cold-pressed roasted pumpkin, parsley, mullein, cardamom, and milk thistle seed flour extracts. *CPS* Luminescence counts per second. Cells were treated with seed-flour extracts at a final concentration of 6 mg seed flour equivalents/mL and counted following 48 and 96 h of treatment. Relative cell growth was determined by ATP luminescence, which was directly correlated to cell number. *Vertical bars* represent standard deviation ($n = 3$). *Different letters* on same *colored bars* represent significant differences ($P < 0.05$)

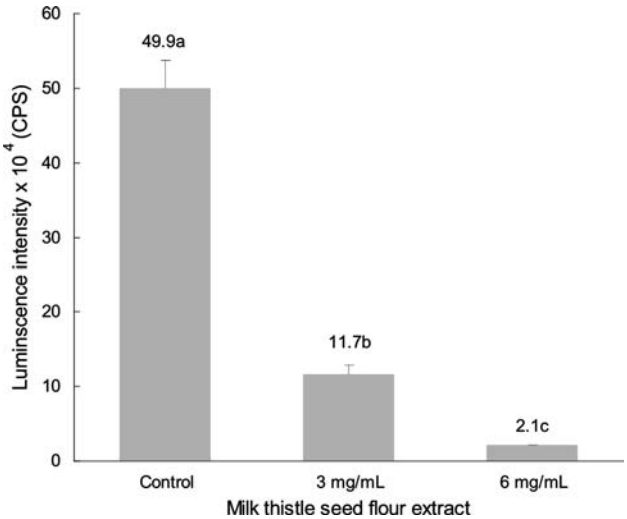

Fig. 2 Relative growth of HT-29 cells treated with milk thistle seed-flour extract. Cells were treated with milk thistle seed-flour extract at final concentrations of 3 and 6 mg flour equivalents/mL media. *CPS* Luminescence counts per second. Relative cell growth was determined following 48 h of treatment. *Vertical bars* represent standard deviation ($n = 3$). *Different letters* represent significant difference ($P < 0.0001$)

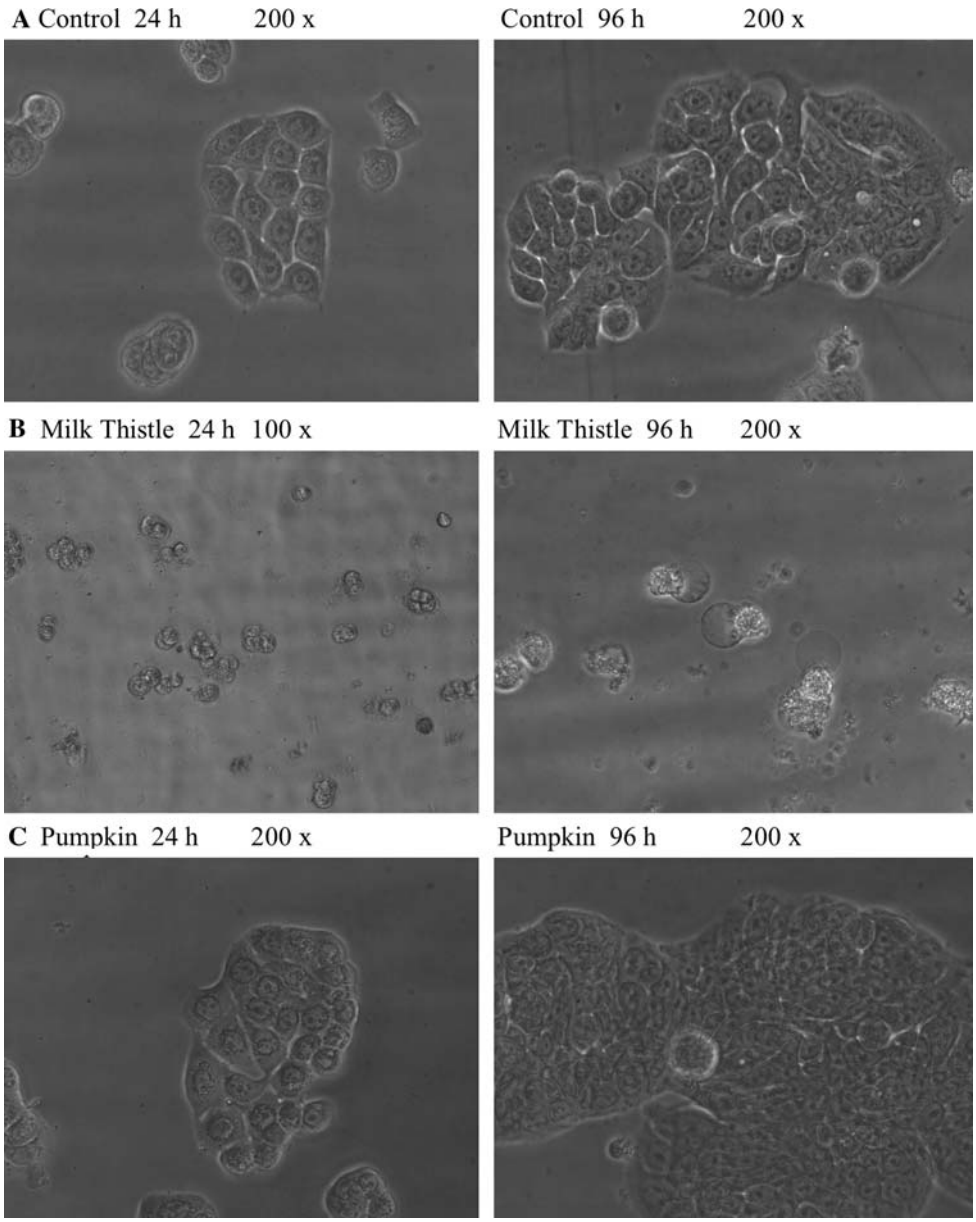

Fig. 3 HT-29 cell morphology at treatment 24 and 96 h at 200× magnification. Milk thistle at 24 h is 100× magnification. **a** Control cells with no treatment, **b** cells treated with 6 mg/mL milk thistle seed flour extract, **c** cells treated with 6 mg/mL pumpkin seed flour extract. All wells contained 6 μL dimethyl sulfoxide per milliliter media

specific bioactive components, including silymarins in milk thistle. Silymarins, which are polyphenolic antioxidant compounds, have been reported to have antiproliferative effects against several different cancer cell lines at the molecular level [19–32]. Antiproliferative effects of milk thistle seed components have been demonstrated in prostate [19–23], bladder [24–26]; colon [27, 28], lung [29–31], and breast [32] cancer cells by several mechanisms, including cell cycle arrest, decreases in cyclin-dependent kinases and cyclins, and induction of apoptosis. However, one recent study on mammary carcinogenesis in rat and mouse models demonstrated increases of mammary tumors with silymarin treatment in comparison to the control [33]. Additional research to characterize the antiproliferative components in the milk thistle seed flour is needed. Further animal studies are also required to evaluate the potential utilization of milk thistle seed-flour components in cancer treatment and prevention.

In summary, the results of the study reported here demonstrate that the cold-pressed parsley, milk thistle, and mullein seed flours from the seed oil production contain significant levels of natural antioxidants and may serve as edible sources of antioxidants. Milk thistle seed flour is very rich in antioxidants and contains antiproliferative components. Further research of these seed flours may lead to value-added use of these byproducts in improving human health while enhancing the profitability of seed production and seed oil industries.

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