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Chemical composition and antioxidant activity of partially defatted milk thistle (*Silybum marianum* L.) seeds

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Milk thistle (*Silybum marianum* L.) is a herbaceous plant of the Asteraceae family that is widely grown in Bulgaria. It has been used in Egypt and Europe for over 2000 years as a medicinal plant for protecting, detoxifying and regenerating liver, gallbladder, spleen, and the entire body. The hepatoprotective effect of milk thistle is due to a mixture of flavonolignans contained in its seed, collectively called silymarin. Some producers of milk thistle process its seeds to obtain seed oil, then discarding the silymarin-rich residues. In our study, we investigated the chemical composition and antioxidant activity of partially defatted *S. marianum* seeds obtained after cold oil pressing. We found the milk thistle seed pressings to be a very rich source of silymarin – 3050 mg/100 g, expressed as silibinin. The total polyphenol content of seed pressings was 2796.4 mg/100 g, whereas their antioxidant activity measured by ORAC and HORAC methods was 1425.8 μmol TE/g and 192.6 μmol GAE/g, respectively. The investigated material was a very rich source of protein - 20.5 g/100 g and contained 10.8 g/100 g residual oil. The total carbohydrate content was 42.2 g/100 g embracing 39.7 g/100 g polysaccharides and 0.9 g/100 g reducing sugars. Free sugars in milk thistle seed pressings included fructose, glucose, galactose, rhamnose, xylose, and sucrose. Based on these results, it can be concluded that the residual pressings from *S. marianum* seeds after oil extraction are a rich source of protein, lipids, and carbohydrates. Moreover, they contain a substantial amount of silymarin and could be used as a hepatoprotective functional food or food supplement.

Keywords: Milk thistle (*Silybum marianum* L.); oil pressings; silymarin, chemical composition, antioxidant activity

INTRODUCTION

Milk thistle (*Silybum marianum*, Asteraceae) is an annual or biennial plant, native to the Mediterranean region, which grows in many warm and dry regions, mainly in Europe, Asia, and North America [1]. It has been used for centuries in Egypt and Europe as a medicinal plant for protecting, detoxifying and regenerating liver, gallbladder, spleen and the entire body. In recent years, several studies have indicated that milk thistle has antioxidant, antiatherosclerotic, antihypertensive, anti-obesity, anti-diabetic, anti-inflammatory, and anti-carcinogenic effects [2]. The hepatoprotective effect of *S. marianum* seeds is due to a mixture of flavonolignans collectively called silymarin [3]. It is mainly composed of silibinin, silychristin, silidianin, and isosilybin. Silymarin is found in the whole plant of milk thistle, including fruits, seeds, roots, stems, and leaves, but it is the most abundant in the seeds [4]. The anticancer activity of silymarin, as well as of silibinin was demonstrated against various cancer cells, such as breast, skin, colon, cervix, ovary, prostate, lung and hepatocellular cancers [5-8]. Silymarin has been shown to reduce the plasma levels of cholesterol, thus having a potential as a hypocholesterplaemic agent [9]. Apart from silymarin, milk thistle seeds contain a high amount of oil, exceeding 20%, as well as numerous beneficial components such as essential amino acids, carbohydrates, minerals, and phytochemicals with antioxidant or antimicrobial effects [10]. Due to the complex and expensive processing on the extraction and purification of silymarin, some producers of *S. marianum* use its seeds to obtain milk thistle oil, then discarding the silymarin-rich residues. The oil has a high concentration of unsaturated fatty acids, especially linoleic (omega-6) and oleic (omega-9) acids, which are beneficial to human health in preventing atherosclerosis, diabetes, and cancer [11]. Milk thistle seed oil has been recommended as a potential source of natural antioxidants, since it is rich in vitamin E [12]. Therefore, the present study aimed to investigate the chemical composition and antioxidant activity of partially defatted *S. marianum* seeds, obtained after cold oil pressing, in order to assess their potential to serve as a silymarin-rich food supplement.

MATERIALS AND METHODS

Chemicals

All solvents (HPLC grade) and reagents were purchased from Sigma-Aldrich (Steinheim, Germany).

Sugar standards (D-(+)-Fru, D-(+)-Glc, D-(+)-Gal, D-(+)-Xyl, Suc) were purchased from Sigma-

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Aldrich and Sigma Life Sci. L-Rhamnose (Alfa Aesar, Germany, 98+%) was purchased from VWR Prolabo.

Milk thistle seeds

Partially defatted milk thistle seeds, obtained after cold pressing, were supplied by Phytoviv Ltd. (Veliko Tarnovo, Bulgaria).

Extraction of carbohydrates

The extraction of carbohydrates was performed according to Denev *et al.* [13] with some modifications. Briefly, approximately one gram of partially defatted *S. marianum* seeds was accurately weighed and subjected to extraction with 30 mL of distilled water for 1 h at 30°C on a shaking thermostatic water-bath (NÜVE, Turkey). After that, the samples were centrifuged (6000×g) and the supernatants were used for HPLC analysis of sugars.

Extraction of silymarin and other phenolic compounds

Extraction of silymarin and other phenolic compounds was performed according to the procedure described for milk thistle seeds in the British pharmacopeia, 2013 [14]. Briefly, 5 g of partially defatted milk thistle seeds were placed in a continuous-extraction Soxhlet apparatus and 100 mL of light petroleum was added. Extraction was performed at 70°C in a water-bath for 8 h. The solid residue was separated and dried at room temperature. Petroleum ether was evaporated *in vacuo* and the residual lipid extract was weighed in order to determine the lipid content of the partially defatted seeds.

The defatted seeds were placed in a continuous-extraction Soxhlet apparatus with 100 mL of methanol and extracted at 60°C in a water-bath for 5 h. The methanolic extract was reduced *in vacuo* to a volume of about 30 mL, filtered into a 50 mL volumetric flask, by rinsing the extraction flask and the filter, and diluted to 50 mL with methanol. Five mL of this solution was further diluted to 50 mL with methanol and used for the determination of silymarin, total polyphenols, ORAC and HORAC analyses.

Determination of protein content

The protein content of milk thistle seeds was evaluated by the micro-Kjeldahl method [15]. Acetylacetone-formaldehyde colorimetric method, using ammonium sulfate as a standard, was used for the determination of nitrogen expressed as ammonia content of the digested sample [16]. The results were calculated using 6.25 as a conversion factor.

Determination of total carbohydrate content

The total carbohydrate content of *S. marianum* seeds was determined by the phenol-sulfuric acid method, using glucose for the calibration curve construction [17]. Firstly, the defatted plant material was incubated at 50 °C for 1 h with 70% (v/v) aqueous ethanol and then the solids were separated by centrifugation (14,000×g, 10 min, 5 °C). The same procedure was repeated 3 times. Finally, the residue was washed 2 times with acetone at room temperature and vacuum-dried to obtain alcohol-insoluble solids (AIS). Before analysis, the AIS samples were solubilized with 72% (w/w) H_2SO_4 for 1 h at 30 °C, followed by a hydrolysis step with 1 M H_2SO_4 for 3 h at 100 °C [18]. The obtained hydrolyzates were used for the determination of total carbohydrate content. The absorbance was measured at 490 nm.

Determination of reducing sugar content

The reducing sugar content of the samples was estimated by the method of Miller with the 3,5-dinitrosalicylic acid reagent, using glucose for the calibration curve construction [19]. The extraction of soluble sugars was performed according to Denev *et al*. [18]. In brief, approximately 1 g of the defatted milk thistle seeds were weighed and subjected to extraction with 30 mL of distilled water for 1 h at 30 °C on a magnetic stirrer. Before analysis, the mixture was centrifuged (6000×g) and the supernatant was used after an appropriate dilution for analysis of reducing sugars.

Total polyphenol compound analysis

The total polyphenols were determined according to the method of Singleton & Rossi with the Folin-Ciocalteu's reagent [20]. Gallic acid was used for the calibration curve and results were expressed as gallic acid equivalents (GAE) per 100 g dry weight (DW).

High-Performance Liquid Chromatography (HPLC) analysis of free sugars

The HPLC determination of free sugars was performed on an HPLC system Agilent 1220 (Agilent Technology, USA), with a binary pump and a refractive index detector (Agilent Technology, USA). The column was Zorbax Carbohydrate (150 × 4.6 mm, 5 μm, Agilent), connected to a guard column Zorbax Reliance Cartridge (Agilent), and as eluent was used 80% acetonitrile in water at a flow rate of 1.0 mL/min and temperature 25°C. The quantification of free sugars was done by comparison of areas of the peaks with those of the corresponding standards. Results were expressed as mg/100 g DW.

HPLC analysis of silymarin

HPLC analysis of silymarin was performed according to the procedure described in the British pharmacopeia, 2013, for milk thistle seeds [14] on a HPLC system Agilent 1220 (Agilent Technology, USA), with a binary pump and UV-Vis detector (Agilent Technology, USA). Separation was performed on an Agilent TC-C18 column (5 μm, 4.6 mm × 250 mm) at 25ºC and a wavelength of 288 nm was used. The following mobile phases were used: (A) phosphoric acid, methanol, water (0.5:35:65 V/V/V) and (B) 100% acetonitrile at a flow rate of 0.8 ml/min. The gradient elution started with 0 % A, between 0 min and 28 min linearly increased to 100% B, the same ratio was maintained until 35 min, and then until 36 min A linearly increased to 100%. Silicristin, silidianin, silibinin A, silibinin B, isosilibinin A and isosilibinin B were identified by comparison with the chromatogram obtained with *S. marianum* standardized dry extract CRS. The percentage content of silymarin, calculated as silibinin, was determined using the following expression:

$$\frac{(A1 + A2 + A3 + A4 + A5 + A6) \times m_1 \times p \times 1000}{(A7 + A8) \times m_2 \times (100 - d)}$$

where:

A1 = area of the peak due to silicristin in the chromatogram;

A2 = area of the peak due to silidianin in the chromatogram;

A3 = area of the peak due to silibinin A in the chromatogram;

A4 = area of the peak due to silibinin B in the chromatogram;

A5 = area of the peak due to isosilibinin A in the chromatogram;

A6 = area of the peak due to isosilibinin B in the chromatogram;

A7 = area of the peak due to silibinin A in the chromatogram;

A8 = area of the peak due to silibinin B in the chromatogram;

m_1 = mass of milk thistle standardized dry extract CRS used to prepare the reference solution, in grams;

m_2 = mass of the drug to be examined, in grams;

p = combined percent content of silibinin A and silibinin B in milk thistle standardized dry extract CRS;

d = percent loss on drying of the drug.

Oxygen Radical Absorbance Capacity (ORAC) assay

ORAC was measured according to the method of Ou *et al.* [21] with some modifications, described in details by Denev *et al*. [22]. Solutions of AAPH, fluorescein (FL), and Trolox were prepared in phosphate buffer (75 mM, pH 7.4). Samples were diluted in the phosphate buffer as well. Reaction mixture (total volume 200 μL) contained FL (170 μL, final concentration 5.36×10^{-8} mol/L), AAPH (20 μL, final concentration 51.51 mM), and sample – 10 μL. The FL solution and sample were incubated at 37°C for 20 min directly in a microplate reader, and AAPH (dissolved in buffer at 37°C) was added. The mixture was incubated for 30 s before the initial fluorescence was measured. After that, the fluorescence readings were taken at the end of every cycle (1 min) after shaking. For the blank, 10 μL of phosphate buffer was used instead of the extract. The antioxidant activity was expressed in micromole Trolox equivalents (μmol TE) per liter of extract. Trolox solutions (6.25; 12.5; 25 and 50 μmol/L) were used for defining the standard curve. ORAC and HORAC analyses were carried out using a FLUOstar OPTIMA plate reader (BMG Labtech, Germany), an excitation wavelength of 485 nm, and an emission wavelength of 520 nm were used. The results were expressed in micromole Trolox equivalents (μmol TE) per g DW.

Hydroxyl Radical Averting Capacity (HORAC) assay

The HORAC assay measures the metal-chelating activity of antioxidants in the conditions of Fenton-like reactions employing a Co(II) complex and hence, the protecting ability against the formation of hydroxyl radicals [23]. Hydrogen peroxide solution of 0.55 M was prepared in distilled water. 4.6 mM Co(II) solution was prepared as follows: 15.7 mg of $CoF_2.4H_2O$ and 20 mg of picolinic acid were dissolved in 20 mL of distilled water. Fluorescein - 170 μL (60 nM final concentration) and 10 μL of the sample were incubated at 37°C for 10 min directly in the FLUOstar plate reader. After incubation,10 μL of H_2O_2 (27.5 mM final concentration) and 10 μL of Co(II) (230 μM final concentration) solutions were added. The initial fluorescence was measured, after which the readings were taken every minute after shaking. For the blank sample, phosphate buffer solution was used and 100, 200, 600, 800 and 1000 μM gallic acid solutions (in phosphate buffer 75 mM, pH = 7.4) were used for building the standard curve.

The area under the curves was calculated as it was done for the ORAC. The results were expressed in micromole gallic acid equivalents (μmol GAE) per gram DW.

Statistical analysis

The processing was repeated two times and the analyses were performed in duplicate or triplicate. The results were expressed as mean values ± standard deviations. One-way analysis of variance (ANOVA) and Student's t-test were used to evaluate the differences of the mean between groups. P values less than 0.05 were considered to be significant.

RESULTS AND DISCUSSION

Protein, lipid and carbohydrate contents of partially defatted milk thistle seeds

The total amount of protein, lipids and carbohydrates in partially defatted milk thistle seeds was 73.5%, as shown in Table 1.

Table 1. Protein, lipid, carbohydrate, polysaccharide and reducing sugar contents of partially defatted milk thistle seeds

	Partially defatted milk thistle seeds
Total lipids, g/100 g	10.8
Total protein, g/100 g	20.5
Total carbohydrate, g/100 g	42.2
Reducing sugars, g/100 g	0.9
Polysaccharides, g/100 g	39.7

The total carbohydrate content was 42.2 g/100 g, embracing 39.7 g/100 g polysaccharides. Although *S. marianum* seeds are a rich source of carbohydrates, the amount of reducing sugars is very low - 0.9 g/100 g, indicating the presence of a substantial quantity of polysaccharides. It has been found that milk thistle residues contain different bioactive heteropolysaccharides, which express *in vitro* antioxidant, hemolysis inhibitory, *α*-amylase and *α*-glucosidase inhibitory activities [24]. The investigated material was a very rich source of protein - 20.5 g/100 g and it also contained 10.8 g/100 g residual oil. Compared with the data found in literature, the partially defatted milk thistle seed used in this work contained less oil, due to the preliminary cold pressing. Dabbour *et al.* reported that oil content of *S. marianum* seeds grown in Jordan is 26.90 g/100 g [25], whereas Fathi-Achachlouei and Azadmard-Damirchi reported oil content from 26 to 31 g/100 g in four varieties of *S. marianum* planted in Iran [26]. Additionally, Wallace *et al.* and Abenavoli *et al.* found that the plant seeds contained 15 – 30 % lipids and about 30 % protein [27, 28].

It was of interest to investigate the individual sugars present in the studied raw material. The results from the HPLC analysis are shown in Table 2. The predominant free sugar in milk thistle seeds was sucrose (1643.3 mg/100 g), but fructose, glucose, galactose, rhamnose, and xylose were also found. Quantitative data on the free sugar composition in *S. marianum* seeds were not found in the available literature.

Table 2. Free sugar content (mg/100 g) and composition of partially defatted milk thistle seeds

	Partially defatted milk thistle seeds
Fructose	113.9
Glucose	168.2
Galactose	143.9
Rhamnose	162.5
Xylose	134.2
Sucrose	1643.3

However, Barnes *et al.* have reviewed that the seeds contain arabinose, rhamnose, xylose and glucose [29]. Furthermore, Ghafor *et al.* determined high carbohydrate content in the stems, especially fructose [30].

Polyphenol and silymarin contents, and antioxidant activity of partially defatted milk thistle seeds

The total polyphenol and silymarin contents of the studied partially defatted milk thistle seeds are shown in Figure 1.

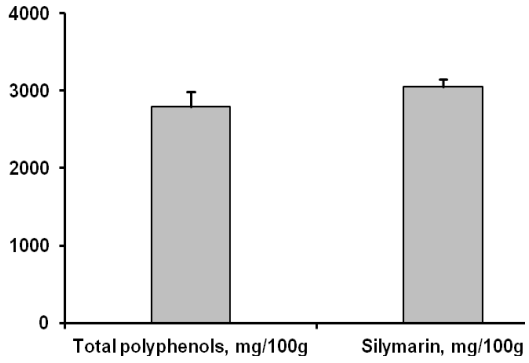

Figure 1. Silymarin and total polyphenol contents of partially defatted milk thistle seeds

The polyphenol content of the investigated material was 2796 mg GAE/100 g and its silymarin content was significant - 3050 mg/100 g. As already mentioned, the hepatoprotective properties of milk thistle are due to its silymarin content, and therefore the studied milk thistle oil pressings (or by-product) could be used as a hepatoprotective functional food or food supplement. It is known that silymarin content and composition strongly depend on *S.*

marianum cultivar, geographical location of cultivation (soil physical properties, weather), and agronomic conditions (time of sowing, fertilizing, irrigation, time of harvest, maturity of seeds) [31-33]. The current study showed that silymarin content is slightly higher than the results proposed by Stancheva *et al.* [34]. However, considerably high content of silymarin has been reported for fruits of *S. marianum* by Hasanloo *et al.* [35]. Hasanloo *et al.* [36] obtained a higher silymarin content in cell cultures of *S. marianum* kept in dark. Our results for the total polyphenol content in the partially defatted milk thistle were comparable to those reported by Mhamdi *et al*. (29 mg GAE/g DW) [37]. On the contrary, Lucini *et al*. [38] indicated a considerably lower total phenolic content in *S. marianum* (3.6 mg GAE/g DW). This difference might be due to both genotypic and environmental factors, extraction conditions and further analysis. Mhamdi *et al*. [37] have also observed that *S. marianum* seed extract contains different phenolic acids and flavoligans. Eight phenolic compounds have been successfully identified in the seeds, and the major were silybin A (12.3%), silybin B (17.6%), isosilybin A (21.9%), isosilybin B (12.8%), silychristin (7.9%) and silydianin (7.5%) [37]. These compounds are known as some of the most active phytochemicals and are largely responsible for the claimed benefit of the silymarin complex.

The antioxidant properties of the studied residual pressings from *S. marianum* seeds were assayed by ORAC and HORAC methods and to the best of our knowledge, this is the first report on the evaluation of ORAC and HORAC antioxidant activity of milk thistle seeds. ORAC method measures the ability of an antioxidant to scavenge peroxyl radicals, whereas HORAC method is an indicator for its metal-chelating properties, thus reflecting its ability to act as a preventive antioxidant [22]. The high amount of silymarin resulted in a very high ORAC value - 1426 μmol TE/g, whereas HORAC result was lower - 192 μmol GAE/g (Figure 2).

Flavonolignans have shown radical scavenging properties and protective effects against the damage of lipid membranes [39] and oxidation of low-density lipoproteins [40]. The antioxidant effect is due to the modulation of pathways such as cell growth, apoptosis, and differentiation [41]. Silymarin can scavenge free radicals, it also has been found to increase the production of glutathione in hepatocytes and the activity of superoxide dismutase in erythrocytes [42]. In another *in vitro* study, free radical scavenging activity and antioxidant properties of silymarin (>200 μM) were shown by four different assays [43].

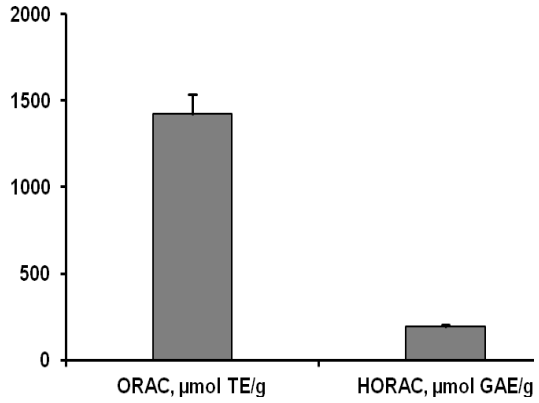

Figure 2. ORAC and HORAC antioxidant activity of partially defatted milk thistle seeds

It is worth mentioning that the free radical scavenging activity of pure individual compounds of silymarin has been reported to vary considerably, with silydianin and silychristin being 2-10-fold more active than silibinin and on a dry mass basis, silymarin is shown to be about 8 times more potent than silibinin as a free radical scavenger [44]. In our study, silymarin ability to scavenge peroxyl radicals was demonstrated by the high ORAC value of the investigated partially defatted milk thistle seeds.

CONCLUSION

Based on our results, it can be concluded that residual pressings from milk thistle seeds, after oil extraction, are a rich source of protein, lipids, and carbohydrates. Additionally, seed pressings contain a substantial amount of silymarin and could be used as a hepatoprotective functional food or food supplement.

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