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Cytotoxicity Assessment and Nutritional Profiling of Bio-Active Compounds Obtained from Food Waste

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Abstract: The purpose of the study was to evaluate the properties of by-products obtained from the oil industry, to identify the content of bioactive compounds and to test the safety of their reintroduction in the food industry. Three sunflower meals obtained after cold-pressing of whole (WSM), partially dehulled (PSM) and total dehulled (TSM) sunflower seeds were used. A higher protein, ash and fiber content was obtained for WSM, followed by PSM, and TSM meals. Conversely, the lipid content was higher in TSM and PSM, and lower in WSM meals. Sunflower meals are important sources of unsaturated fatty acids (more linoleic than oleic acid), the content ranging between 82.74 and 86.72%. Additionally, sunflower meals represent a significant source of compounds with antioxidant activity. TSM showed the highest concentration of total polyphenols and total flavonoids, while WSM the lowest. The values of antioxidant activity were higher for TSM compared to PSM and WSM. The weak cytotoxic activity at concentrations lower than 6.25 mg/mL, as well as cell viability which is not affected by the action of PSM and TSM but even increases in the case of WSM, give sunflower meals the potential to be added as ingredients in the production of functional foods.

Keywords: bio-active compounds; food waste; fatty acids; sunflower meal; cytotoxicity assays

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1. Introduction

Considering the challenges at the planetary level, such as climate change, represented in particular by severe drought, and their effect on agricultural production according to FAO [1], the food industry must save by-products resulting from food production and must capitalize them so that they represent a gain both for industry, by reducing waste, as well as for consumers by obtaining improved or reformulated food [2–4].

Food waste generated by the food industry is an organic waste which is having a major impact on environmental sector. This represents a major environmental problem, due to the fact that waste is not being utilized and ends up in municipal landfills [5]. Recently, greater importance has been given to the reuse of by-products from food wastes, especially those with the potential to contain bioactive compounds [6]. In the widely accepted meaning, a bioactive compound is a substance that has an effect on a living organism, even if it can be a positive or negative effect depending on the dose, chemical structure and bioavailability of the compound [7].

Sunflower seeds represent an important source in the production of vegetable oils, which are used for industrial applications. The production of sunflower oil generates by-products such as hulls, flakes, expelled cakes or extracted meal [8]. The main organic waste in the oil industry is represented by processed seed mass in the form of sunflower meal, which is already considered a high-potential by-product [3,8,9]. These by-products are a good source of non-lipophilic antioxidants. Therefore, the composition of sunflower meal depends on the efficiency of the dehulling process [10].

By-products of sunflower oil's production, meal and hulls are valuable sources of phenolic compounds that might be recovered and used as natural antioxidants [11]. The

content of phenolic compounds in meal may vary depending on the content of hulls in meal, sunflower variety and cultivation area. In many studies it has been demonstrated that sunflower meal has high antioxidant potential, which could be beneficial for further technological utilization [12].

In general, whole seeds, oils and oil cake by-products are rich sources of nutritional and bioactive compound such as peptides and fatty acids [13]. A standard sunflower seed contains linoleic acid (~65%) as major fatty acid [14]. In order to obtain a high quality cold-pressed sunflower oil, the dehulling process is an important step [14]. The sunflower meals resulting after seed cold-pressing are rich sources of unsaturated fatty acids, containing principally oleic and linoleic acids [15].

The aim of this study was to analyze the potential of sunflower meals to be used as by-products suitable for human consumption by re-introducing them as food ingredients. Chemical composition, fatty acid profile, bioactive compounds (total polyphenols and total flavonoids) and antioxidant activity, all of which were complemented by cytotoxicity tests, were evaluated.

2. Materials and Methods

2.1. Samples

For this study, three sunflower meals obtained after cold pressing of sunflower seeds (*Helianthus annuus* L.) were used. Meals had different dehulling percentages: whole seeds (undehulled) (WSM), partially dehulled (~50%) (PSM), and 100% dehulled (TSM). The meals were provided by a local oil producer.

The oilcakes were ground by using a sample mill (model SM-450C, MRC LTD, Holon, Israel) in order to be used for further analysis.

2.2. Reagents and Standards

Standards of F.A.M.E. Mix, C4-C24 (Bellefonte, PA, USA) and SRM®2377 (NIST certified, Gaithersburg, MD, USA) were used for fatty acid methyl esters (FAME) quantification. Solvents and reagents specially for chromatography were used for FAME preparation: 5.4 M methanolic solution of sodium hydroxide (Across, Fair Lawn, NJ, USA), 14% methanolic solution of boron trifluoride (Sigma Aldrich, Saint Louis, MO, USA), sodium chloride (Sigma Aldrich, St. Louis, MO, USA), methanol picograde and 2,2,4-trimethylpentane (isooctane) picograde (LGC Standards GmbH, Webel, Germany).

In addition, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), 2,20-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), ferric reducing antioxidant potential (FRAP), 2,4,6-tripyridyl-s-triazine (TPTZ), quercetin, gallic acid, and Trolox (6-hydroxy-2,5,7,8-tetrame thylchroman-2-carboxylic acid) were purchased from Sigma Chemical Co. (Buchs, Switzerland). Folin–Ciocalteu's phenol reagent was purchased from Merck (Darmstadt, Germany). All chemicals used were of analytical grade. Standard solutions were prepared with distilled deionized water.

2.3. Compositional Analysis

The characteristics of the sunflower meals were determined as follows: moisture content using the oven drying method [16], protein content by the Kjeldahl method, Nx6.25 (AOAC 979.09), fat content by Soxhlet method using the extraction with petroleum ether under reflux conditions (AOAC 963.15) and ash through gravimetric method by burning the sample at 550 °C in a furnace (AOAC 923.03) (AOAC, 2005) [17]. The crude fiber content was determined using a Fibretherm–Gerhardt instrument according to the SR EN ISO 6865:2002 [18].

2.4. Determination of Fatty Acid Composition

Fatty acids were converted to FAME by transesterification of the fat. The FAME content of the studied samples was determined according to the method described by Mihai et al. [19]. FAME quantification was determined using a gas chromatograph (Trace

GC Ultra) coupled with a mass spectrometer (TSQ Quantum XLS) from Thermo Fisher Scientific (San Jose, CA, USA). The positive electronic ionization mode (EI^+), and the "Selected Ion Monitoring- SIM" mode, by using 23 segments were used for FAME analyses. As stationary phase, a TR-FAME capillary column, with a stationary phase consisting of 70% cyanopropyl and 30% polysilphenylene-siloxane (60 m × 25 mm × 0.25 µm) from Thermo Fisher Scientific (USA) was used. As mobile phase, He of 99.9995% purity (5.0), with a constant flow of 1 mL/min was used. A volume of 0.5 µL of extract was injected at a temperature of 240 °C, in split mode, with a split ratio of 1:50 and a split flow rate of 50 mL/min. The analysis time for a sequence was 85.20 min. Xcalibur program was used for instrument control, data acquisition and processing.

The quantification was realized by using experimentally established correction factors which were determined from the calibration solutions of both standards, SRM®2377 and F.A.M.E. Mix C4-C24. By using this method, 40 FAME can be quantified of which 23 are common to both standards, 3 are SRM®2377 specific, and 14 are F.A.M.E. Mix C4-C24 specific. Based on the correction factors, FAME from the meal samples can be quantified as relative concentration expressed as mass percentages (%) of total identified FAME per 100 g fat and 100 g product. Mass percentages for FAME individually determined from the total FAME, saturated fatty acids (SFA), monounsaturated fatty acids (MUFA), polyunsaturated fatty acids (PUFA), ω-3, ω-6, and ω-9 content were calculated. Additionally, ω-6/ω-3 ratio was calculated.

2.5. Extraction Procedure

An amount of 0.2 g of sunflower meal was weighed and brought into 10 mL of methanol 80%. The extracts were obtained by extraction method involving a vortex equipment (Vortex Multi reax, Heidolph Intruments, Schwabach, Germany) for 3 h at room temperature. Then, the extracts were centrifuged for 30 min, at 4 °C, at 10,000 rpm to remove the secondary materials [20].

2.6. Determination of Phenol Content

Total phenol content (TPC) was determined by the Folin–Ciocalteu method [21]. A total of 500 µL of extract was mixed with 5 mL Folin–Ciocalteu reagent 1:15, and 500 µL of 20% sodium carbonate. After 20 min of incubation at room temperature, absorbance was measured at 752 nm using a Specord 210 UV-VIS spectrophotometer (Analytic Jena, Bremen, Germany). A standard curve was prepared by using different concentrations (0.005–0.175 mg/mL) of gallic acid in the same condition with samples (R^2 = 0.9999). Total phenolic content was expressed as mg gallic acid equivalent/100 g of meal (mg GAE/100 g).

2.7. Determination of Flavonoid Content

Total flavonoid content (TFC) was assessed through the $AlCl_3$ method described by Woisky and Salatino [22]. Briefly, 500 µL sample was mixed with 100 µL 10% $AlCl_3$, 100 µL sodium acetate and 2.8 mL methanol. Samples were then vortexed and incubated in the dark for 30 min. The absorbances were measured at 415 nm using a Specord 210 UV-VIS spectrophotometer (Analytic Jena, Germany). A standard curve was plotted by using different concentrations (0.02–0.30 mg/mL) of quercetin (R^2 = 0.9994). Total flavonoid content was expressed as mg quercetin equivalent/100 g of meal (mg QE/100 g).

2.8. Determination of Antioxidant Activity through DPPH Method

DPPH radical scavenging activity was determined based on the reduction in DPPH radical, according to Culetu et al. [23]. The reaction mixture consisted of 400 µL of sample and 6 mL of DPPH radical solution, which was incubated for 20 min in the dark. Then, the absorbance was measured at 517 nm using a Specord 210 UV-VIS spectrophotometer (Analytic Jena, Germany). Antioxidant activity was calculated using a calibration curve

(0.05–0.60 mmol/L) obtained with Trolox (R^2 = 0.9995). The results were expressed in mg Trolox/100 g of by-product.

2.9. Determination of Antioxidant Activity through ABTS Method

ABTS assay was performed according to Re et al. [24] with slight modifications. The reaction mixture consisted in 200 µL of sample and 3 mL of $ABTS^+$ working solution. After 6 min of incubation at room temperature, the absorbances were measured at 730 nm using a Specord 210 UV-VIS spectrophotometer (Analytic Jena, Germany). The standard curve was linear between 0.025 and 0.25 mM Trolox (R^2 = 0.9994). The results were expressed in mg Trolox/100 g of sunflower meal.

2.10. Determination of Antioxidant Activity through FRAP Method

The determination of the antioxidant capacity of iron reduction was performed by the method described by Thaipong et al. [25]. The reaction mixture consisted in 450 µL of sample and 2700 mL FRAP solution. After incubation, the absorbance was read at 593 nm using a Specord 210 UV-VIS spectrophotometer (Analytic Jena, Germany). Antioxidant activity was calculated using a calibration curve between 0.25 and 100 mM Trolox (R^2 = 0.9996). The results were expressed in mg Trolox/100 g of by-product.

2.11. Cytotoxicity Evaluation by LDH and MTT

Due to the standardized system and cultivation characteristics, healthy cells, represented by standardized cultures from mouse fibroblasts L929 (ECACC—European Collection of Authenticated Cell Cultures), were selected as a test model for the evaluation of the cytotoxicity of sunflower meals.

The samples were prepared by aqueous extraction with an ultrasonic ice bath extraction technique. From each sample, 3 g were dissolved in 15 mL of PBS (Phosphate Buffered Saline—Life Technologies Europe, Paisley, UK) in a 50 mL vial. This mixture was placed in the ice-cooled ultrasonic bath at the extraction temperature of 35–40 °C and sonicated for 30 min at 20 Khz until the mixture was well homogenized [26]. The extracts obtained were then centrifuged for 10 min at 8000× g/rpm to remove insoluble material and then sterilized by filtration in 0.20 µm filter systems (Thermo Scientific Nalgene Rapid Flow Filters, Mexic City, Mexico) assisted by a vacuum pump. The extracts obtained were analyzed from an initial concentration of 100 mg/mL and then serially diluted to obtain the following concentrations: 50, 25, 12.5 and 6.25 mg/mL.

L929 cells were cultured in EMEM (Earle's Modified Eagle Medium, Life Technologies Corporation, New York, NY, USA) supplemented with 10% FBS (Fetal Bovine Serum—Life Technologies Europe, Paisley, UK) and 1% Pen/Strep (penicillin/streptomycin solution, 10 mg/mL—Life Technologies Corporation, New York, NY, USA) for 24 h at 37 °C, 95% humidity with 5% CO_2. After 24 h, cells were washed with PBS, harvested using trypsin (Life Technologies Europe, Paisley, UK) and counted using Trypan Blue (Life Technologies Corporation, New York, NY, USA) and a hemocytometer. The seeding density for the assay was optimized at 5×10^4 cells/mL.

The lactate dehydrogenase (LDH) assay involves the assessment of cell death by quantifying plasma membrane damage. LDH is a stable cytoplasmic enzyme present in all cells. It is rapidly released into the cell culture supernatant upon damage to the plasma membrane. First, NAD^+ is reduced to $NADH/H^+$ by LDH-catalyzed conversion of lactate to pyruvate. Second, the catalyst (diaphorase) transfers H/H^+ from $NADH/H^+$ to the tetrazolium salt INT which is reduced to formazan. An increase in the number of cells with a damaged or dead plasma membrane results in an increase in LDH enzyme activity in the culture supernatant. This increase in the amount of enzyme activity in the supernatant correlates directly with the amount of formazan formed. Therefore, the amount of color formed in the test is proportional to the number of cells lysed [27]. Cells seeded at a density of 5×10^4 cells/mL in a 96-well flat-bottom cell culture plate were treated with sunflower seeds extracts (sample concentrations: 100, 50, 25, 12.5 and 6.25 mg/mL) and

incubated for 24 h at 37 °C, 95% humidity with 5% CO_2. After 24 h of exposure to the tested compounds, the supernatant was extracted and moved to a new plate with 96 wells, then it is treated with the LDH reagent mix (Roche, Mannheim, Germany) for 15–30 min at room temperature, and then the LDH activity was measured using a spectrophotometric microplate reader (ELISA reader) at 492 nm with a reference wavelength of 630 nm [28].

The tetrazolium salt assay (MTT) is used to measure cellular metabolic activity as an indicator of cell viability, proliferation, and cytotoxicity. This colorimetric assay is based on the reduction of yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells [29]. This non-radioactive colorimetric analysis system using MTT was first described by Mosmann [30] and improved in the following years by several other investigators [31]. Viable cells contain NAD(P)H-dependent oxidoreductase enzymes that reduce MTT to formazan. Insoluble formazan crystals are dissolved using a solubilizing solution and the resulting colored solution is quantified by measuring the absorbance at 500–600 nanometers using a spectrophotometric microplate reader (ELISA). The darker the solution, the greater the number of viable, metabolically active cells.

Cells seeded at a density of 5×10^4 cells/mL in a 96-well flat-bottom cell culture plate were treated with sunflower storum extracts (sample concentrations: 100, 50, 25, 12.5, and 6.25 mg/mL) and incubated for 24 h at 37 °C, 95% humidity with 5% CO_2. After 24 h of exposure to the test compounds, cells were washed with PBS to remove spent medium and any trace of FBS (as it may interfere with the MTT reagent), then incubated for 4 h with MTT reagent (Roche, Mannheim, Germany) at 37 °C, 95% humidity with 5% CO_2. After incubation, cells were treated with MTT solvent (Roche, Mannheim, Germany) for 15 min at room temperature. Absorbance was measured using a spectrophotometric microplate reader (ELISA reader) at OD = 570 nm [28].

2.12. Statistical Analyzes

All samples were analyzed in duplicate and results were expressed as mean ± standard deviation (SD). Results were statistically analyzed by using Minitab statistical software version 20. One-way analysis of variance (one-way ANOVA) followed by Tukey's test was used to evaluate the statistical significance between samples. The chosen level of significance was set at $p < 0.05$.

3. Results and Discussions

3.1. Chemical Composition of Sunflower Meals

The chemical composition of sunflower meals is presented in Table 1.

Table 1. Chemical composition of sunflower meals.

Sample	Moisture (%)	Protein (% d.m.)	Fat (% d.m.)	Ash (% d.m.)	Crude Fiber (% d.m.)
WSM	7.15 ± 0.01 [a]	30.91 ± 0.27 [a]	15.12 ± 0.05 [a]	5.44 ± 0.02 [a]	24.56 ± 0.23 [a]
PSM	4.96 ± 0.01 [b]	24.18 ± 0.09 [b]	44.75 ± 0.12 [b]	3.61 ± 0.01 [b]	19.53 ± 0.52 [b]
TSM	2.43 ± 0.01 [c]	22.24 ± 0.52 [c]	57.11 ± 0.07 [c]	3.21 ± 0.01 [c]	11.24 ± 0.12 [c]

Values followed by different letters ([a–c]) are statistically different at $p < 0.05$%.

The moisture content of sunflower meals decreased with the increase of dehulling process, ranging between 2.43% and 7.15%.

The WSM meal has a higher protein, ash and fiber content, but a lower fat content compared to the PSM and TSM meals. Thus, the protein content of the WSM meal is approximately 1.4-fold higher than that of the PSM and TSM meals (24.2% and 22.2%, respectively).

According to Kaur and Ghoshal [32] the protein content in sunflower seeds varies between 30 and 50% and, depending on the efficiency of the fat extraction and dehulling

process, this content can reach a percentage of up to 66%. Similarly, in our study the protein content of sunflower meal WSM obtained from whole seeds was 30.91%.

Other researchers reported similar results for the compositional analysis of different types of sunflower meals. For example, for the partially dehulled meal, de Oliveira Filho and Egea [33] reported a value of 3.1% moisture, 19.7% protein, 53.0% lipid, 1.0% ash content, and 4.5% crude fiber content. Petraru et al. [34] found the following values for the sunflower seeds: 7.88% moisture, 7.82% protein, 8.81% lipids and 2.45% ash content. Referring to 100% dehulled sunflower seeds, the authors reported the following values: 4.60% moisture, 23.73% protein, 32.50% lipid, and 3.31% ash content.

3.2. Fatty Acids Profile of Sunflower Meals

The fatty acid composition of the three types of cold-pressed sunflower meals is presented in Table 2.

Table 2. Fatty acid methyl esters content of meal samples analyzed.

FAME Name	FAME Codification	FAME Composition (Mean ± SD), %		
		WSM	PSM	TSM
myristic	C14:0	0.18 ± 0.02 ^{a}	0.21 ± 0.03 ^{a}	0.17 ± 0.01 ^{a}
pentadecanoic	C15:0	0.05 ± 0.00 ^{a}	0.04 ± 0.01 ^{a}	0.03 ± 0.00 ^{a}
palmitic	C16:0	9.63 ± 0.34 ^{a}	7.22 ± 0.59 ^{a}	7.28 ± 0.96 ^{a}
palmitoleic	C16:1n7	0.19 ± 0.02 ^{a}	0.14 ± 0.02 ^{a}	0.17 ± 0.01 ^{a}
heptadecanoic	C17:0	0.09 ± 0.00 ^{a}	0.06 ± 0.01 ^{b}	0.06 ± 0.00 ^{b}
stearic	C18:0	4.83 ± 0.02 ^{a}	3.75 ± 0.43 ^{a}	2.53 ± 0.24 ^{b}
oleic	C18:1n9	28.32 ± 1.49 ^{a}	35.46 ± 3.10 ^{a}	38.77 ± 3.65 ^{a}
***cis* vaccenic**	C18:1n11	0.95 ± 0.19 ^{a}	0.58 ± 0.07 ^{a}	0.71 ± 0.08 ^{a}
linoleic (LA)	C18:2n6	52.88 ± 0.37 ^{a}	50.19 ± 4.24 ^{a}	45.76 ± 4.30 ^{a}
α-linolenic (ALA)	C18:3n3	0.23 ± 0.06 ^{a}	0.13 ± 0.03 ^{a}	0.16 ± 0.01 ^{a}
arachidic	C20:0	0.60 ± 0.09 ^{a}	0.56 ± 0.14 ^{a}	0.73 ± 0.03 ^{a}
gondolic	C20:1n9	0.16 ± 0.03 ^{a}	0.21 ± 0.06 ^{a}	0.28 ± 0.01 ^{a}
heneicosanoic	C21:0	0.02 ± 0.00	-	-
behenic	C22:0	1.28 ± 0.22 ^{b}	0.57 ± 0.02 ^{c}	2.47 ± 0.04 ^{a}
tricosanoic	C23:0	0.10 ± 0.03	-	-
lignoceric	C24:0	0.49 ± 0.11 ^{a}	0.87 ± 0.13 ^{a}	0.88 ± 0.03 ^{a}
SFA, % fat		17.26 ± 0.83 ^{a}	13.28 ± 1.00 ^{b}	14.15 ± 0.60 ^{a,b}
MUFA, % fat		29.62 ± 1.26 ^{a}	36.40 ± 3.20 ^{a}	39.93 ± 3.71 ^{a}
PUFA, % fat		53.12 ± 0.43 ^{a}	50.32 ± 4.21 ^{a}	45.92 ± 4.31 ^{a}
ω-3, % fat		0.23 ± 0.06 ^{a}	0.13 ± 0.03 ^{a}	0.16 ± 0.01 ^{a}
ω-6, % fat		52.88 ± 0.37 ^{a}	50.19 ± 4.24 ^{a}	45.76 ± 4.30 ^{a}
ω-9, % fat		28.48 ± 1.47 ^{a}	35.67 ± 3.16 ^{a}	39.03 ± 3.64 ^{a}
ω-6/ω-3 ratio		229.91	386.08	286.00
Fat (g/100 g product)		14.04 ± 0.05 ^{c}	42.53 ± 0.11 ^{b}	55.73 ± 0.06 ^{a}
SFA, % product		2.42	5.65	7.89
MUFA, % product		4.16	15.48	22.25
PUFA, % product		7.46	21.40	25.59
ω-3, % product		0.03	0.05	0.09
ω-6, % product		7.42	21.35	25.50
ω-9, % product		4.00	15.17	21.76

Values followed by different letters ($^{a–c}$) are statistically different at $p < 0.05$%.

Meal samples had a high content of palmitic (C16:0) and stearic (C18:0) acids. WSM was characterized by the highest content of palmitic acid (9.63%). The meal samples obtained from dehulled seeds had a similar palmitic acid content of 7.22–7.27%. The content of stearic acid decreased significantly from 4.83% to 2.53% with increasing the degree of hulling of sunflower seeds. In addition, meal samples showed a content of behenic acid (C22:0) significantly different ($p < 0.05$), ranging between 0.57% for PSM, and 2.47% for TSM.

The SFA content of analyzed meals varied significantly between 13.28% and 17.26%. The highest SFA content was determined in the meal obtained after cold-pressing of whole seeds. For meals obtained from dehulled sunflower seeds, the SFA content ranged between 13.28% and 14.15%.

Sunflower is one of the most cultivated oil crops in Romania, and during its processing in order to obtain the oil, a high amount of cakes are generated, which are rich in oleic acid. Codex Alimentarius [35] classified sunflower oil based on the oleic acid content into three categories: traditional sunflower oil (14.0–39.4%), medium oleic sunflower oil (43.1–71.8%), and sunflower oil with a high oleic acid content (75.0–90.7%). According to the results, the sunflower meals analyzed in this study were obtained after pressing sunflower seeds with normal oleic acid content, below 39.4% of total fatty acids. With the increase in the dehulling degree of sunflower seeds, the oleic acid content increased in the analyzed meals. The sunflower meal obtained after whole seeds cold-pressing had an oleic acid content of 28.32%. The meal obtained from partially and 100% dehulled seeds, had an oleic acid content of 35.46% and 38.75%, respectively. This fatty acid is known for having a beneficial effect on total blood cholesterol and LDL cholesterol [36] and thus can prevent heart diseases.

Sunflower meals had a cis-vaccenic acid content of 0.58–0.95%. Additionally, these meals contained palmitoleic (C16:1n7) and gondoic (C20:1n9) acids in a percentage below 0.3%.

The MUFA content of the analyzed samples ranged between 29.62% and 39.93%. This content was influenced by the dehulling degree of sunflower seeds, increasing with the increase of dehulling percentage.

In terms of individual PUFA, meals were characterized by a high content of linoleic acid and a lower content of linolenic acid. These acids are considered essential fatty acids as it cannot be synthetized by the human body, playing an important role in a good balanced diet. Linoleic acid is the most known essential ω-6 fatty acid, while linolenic acid is the most known essential ω-3 fatty acid.

According to Codex Alimentarius [35] the sunflower seed oil has a linoleic acid content ranging between 48.3% and 74.0%. The linoleic acid content of meal samples was in accordance with the one reported by Codex Alimentarius [35], ranging between 45.73% and 52.88%. The content was lower than the one reported by Mihai et al. [37] and Romanić and Lužaić [14]. The dehulled process decreased the linoleic acid content of sunflower meals. The linolenic acid content of the samples varied between 0.13% and 0.23%. The linoleic acid content of meal samples makes it an important dietary source of this essential fatty acid.

Meal samples analyzed are rich in PUFA, the content ranging between 45.92% and 53.11%, the lowest content being determined for the TSM, and the highest being recorded for the WSM sample.

The valorization of these by-products by incorporation in food products can help increase the content of oleic and linoleic acid in the human consumption. Sunflower meals can be used as food ingredients in cereal-based products in order to enhance the nutritional value of end products [38–40]. In a study conducted by Škrbić and Filipčev [41] it was shown that by incorporating sunflower seeds in bread formulation, the fatty acid profile of bread samples was improved. The content of unsaturated fatty acids in white and wholegrain breads increased with the increase in additional sunflower seeds.

3.3. Amount of Phenolic Compounds and Flavonoid Compounds

The amount of total phenolic compounds and total flavonoids in the sunflower meal extracts were determined by the Folin-Ciocalteau test, respectively, by the $AlCl_3$ method. The results of these colorimetric methods are presented in Table 3.

Table 3. Phenolic content and flavonoid content in the sunflower meals analyzed.

Sample	TPC (mg GAE/100 g)	TFC (mg QE/100 g)
WSM	1686.34 ± 1.03 [c]	249.85 ± 1.06 [c]
PSM	2185.53 ± 1.14 [b]	260.15 ± 1.07 [b]
TSM	2496.26 ± 1.39 [a]	284.64 ± 1.09 [a]

Values followed by different letters ([a–c]) are statistically different at $p < 0.05\%$.

Following the analyzes carried out, it was observed that the by-products obtained from the vegetable oil industry contain significant amounts of phenolic compounds (Table 3). The analyzed extracts showed a content of TPC ranging between 1686.34 and 2496.26 mg GAE/100 g of meal. The TSM sample showed the highest amount of TPC. The lowest level was obtained by extracting the WSM. The concentration of polyphenols in each type of sunflower meal is significantly different ($p < 0.05$). It is known that sunflower seeds are rich in phenolic compounds, the TPC ranging between 1000 and 4200 mg/100 g [10]. Žilić et al. [42] investigated the TPC of three sunflower genotypes. They found that the level of polyphenols content varied between 1468 and 1824 mg GAE/100 g for sunflower seeds, and between 1628 and 2013 mg GAE/100 g for sunflower kernel, respectively. Chlorogenic and caffeic acids represented 70% of phenolic compounds in sunflower flour [10]. Matthäus et al. [43] analyzed different sunflower extracts. The content of phenolic compounds decreased with decreasing solvent polarity: 70% methanol (1610 mg GAE/100 g), 70% acetone (470 mg GAE/100 g), 70% ethylacetate (270 mg GAE/100 g). Water extraction proved to be the most effective (3880 mg GAE/100 g).

The concentration of TFC in the analyzed sunflower meals ranged between 249.85 and 284.64 mg QE/100 g. The flavonoid content represents 14.81% of total polyphenols for meal WSM, 11.90% for PSM and 11.40% for TSM. The level of flavonoids in sunflower by-products is significantly different ($p < 0.05$). Matthäus et al. [43] analyzed the content of total flavonoids in sunflower and depending on the extraction solvent, the level of flavonoids varied between 145 and 1203 mg catechin/100 g.

The variation in total polyphenolic content and TFC could be due to differences in variety, climate, harvest period, and other factors that affect the nutritional quality of plants [44]. Furthermore, the content of TPC and TFC depend on the extraction solvent.

3.4. Comparison of the Antioxidant Activity of Selected By-Products

The response of antioxidants to different radicals sources may be different. Therefore, no method accurately reflects the mechanism of action of all radical sources or all antioxidant compounds in a complex system [45]. The estimation of antioxidant content using different methods is important to obtain the overall antioxidant potential of any food matrix. The total antioxidant activity of the sunflower meals was estimated using three methods: DPPH, ABTS and FRAP. The measurements of DPPH, ABTS and FRAP values for the analyzed sunflower meals are presented in Table 4.

Table 4. Antioxidant activity of sunflower meal extracts.

Sample	DPPH	ABTS	FRAP
WSM	709.48 ± 4.39 [b]	1320.12 ± 8.21 [c]	4417.77 ± 10.85 [c]
PSM	716.37 ± 5.75 [b]	1554.15 ± 8.89 [b]	6294.92 ± 12.31 [b]
TSM	736.40 ± 3.99 [a]	1597.60 ± 7.96 [a]	6505.87 ± 11.23 [a]

all results were reported as mg Trolox/100 g. Values followed by different letters ([a–c]) are statistically different at $p < 0.05\%$.

The antioxidant activity values measured by the DPPH method for the by-products analyzed ranged between 709.49 and 736.40 mg Trolox/100 g. TSM sample showed the highest antioxidant activity, while the DPPH value for WSM sample was the lowest. The

antioxidant activity of TSM using DPPH assay is significantly different ($p < 0.05$) that the one of WSM and PSM samples.

Numerous studies have demonstrated that sunflower meal has high antioxidant capacity, presenting potential benefits in further technological uses [12]. Grasso et al. [40] investigated the antioxidant activity of defatted sunflower seed meal and reported a value of 450 mg Trolox/100 g, lower than the one reported in the present study. Due to the different contribution of the concentration of phenolic compounds on the antioxidant activity, in the present research, a correlation between the investigated compounds was performed (Table 5). The results showed a strong positive correlation (0.9178) of TPC with DPPH. A strong correlation (0.9991) was also recorded between DPPH and TFC.

Table 5. Correlation coefficients between the content of TPC and TFC with DPPH, ABTS and FRAP.

	DPPH	ABTS	FRAP
TPC	0.9178	0.9499	0.9559
TFC	0.9991	0.7760	0.7759

ABTS values for the analyzed sunflower meals varied between 1320.12 and 1597.60 mg Trolox/100 g as follows: WSM (1320.12 mg Trolox/100 g), PSM (1554.15 mg Trolox/100 g) and TSM (1597.60 mg Trolox/100 g). All analyzed samples are significantly different ($p < 0.05$). Karamać et al. [46] analyzed sunflower seed fractions in order to determine antioxidant activity by ABTS assay. The results varied between 2500 and 55250 mg Trolox/100 g.

The correlation coefficients between TPC and TFC with the ABTS method are presented in Table 5. The correlation coefficients are 0.9499 and 0.7760, respectively.

With respect to the values of the antioxidant activity of the sunflower meals determined by FRAP method, they were between 4417.77 and 6505.87 mg Trolox/100 g. The highest FRAP value was presented by the TSM meal, while the WSM sample recorded the lowest antioxidant activity. FRAP results of sunflower meals are significantly different ($p < 0.05$). A high correlation factor (0.9559) was observed between TPC and FRAP (Table 5). Moreover, the correlation coefficient between TFC and FRAP was calculated (0.7759).

According to the literature, the antioxidant activity is mostly dependent on the content of total polyphenols [47–50]. Pietta [51] have also attributed the antioxidative effects of many plants to the presence of flavonoids, phenolic acids, and phenolic diterpenes. Antioxidant activities of plant extracts could also be due to the presence of other secondary metabolites such as volatile oils, carotenoids, vitamins and so on [52], or even to the synergistic action of various classes of phytochemicals present in the plant. Therefore, for the by-products analyzed, it is observed that the level of polyphenolic compounds contributes strongly to the value of the antioxidant activity, while the concentration of flavonoids contributes to a lesser extent to the total antioxidant activity, presenting a lower correlation factor compared to the one obtained for the content of total polyphenols.

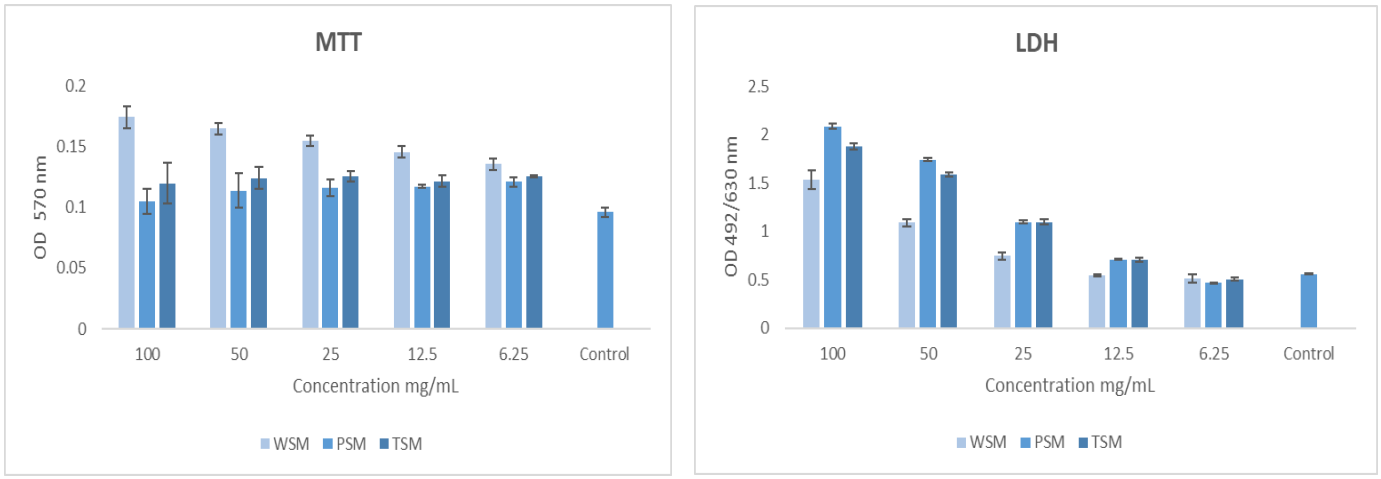
Therefore, it is evident that is a strong relationship between TPC and TFC with the antioxidant activity determined through DPPH, ABTS and FRAP of the three analyzed sunflower meals. In the present study, the secondary products analyzed showed high values of antioxidant activity by the FRAP method. This may be due to the chemical structures present in the sunflower meals that can function as ligands.

3.5. Cytotoxicity Assessment by LDH and MTT

The LDH and MTT assays are popular and widely-used colorimetric assays for in vitro evaluation of cytotoxicity providing beneficial aspects such as reliability, rapidity and important data on cellular metabolic activity. The evaluation of the toxicity of a substance that is intended to be exposed to living organisms is carried out by in vitro evaluation of the dose response [53].

LDH assay provides information about cell membrane damage after cell treatment, completing the overall picture given by the MTT assay in order to draw conclusions on the potential mechanism of action [30].

The sunflower meal samples were analyzed from a starting concentration of 100 mg/mL, continuing with 50, 25, 12.5, and 6.25 mg/mL as the scientific literature indicates [26]. The results obtained show that this concentration provokes the biggest cellular viability reduction in the case of PSM and TSM extracts (up to 20% viability loss) and the loss of viability tends to decrease and stabilize at lower concentrations close to the values obtained by the control, as seen in Figure 1.



(**a**) (**b**)

Figure 1. (**a**) Assessment of cytotoxicity using MTT; (**b**) assessment of cytotoxicity using LDH assay. Control is represented by L929 cells in culture media with PBS (samples solvent).

The greatest percentage of viability was obtained for the WSM extracts at all tested concentrations, in this case the WSM extracts have beneficial effects, acting as a nutrient compound for cells.

The results obtained by the LDH assay underline the action of the tested extracts on the integrity of the cell membrane, especially of the PSM and TSM samples which at concentrations above 12.5 mg/mL expressed a strong cytotoxic effect. All analyzed samples showed membrane damage above 6.25 mg/mL. The weakest cytotoxic effect was obtained by the WSM sample, confirming the results of the MTT test. This result demonstrates that the tested samples have an impact on the metabolic activities of cells and affect their integrity as described by Mason and Rathmell [54], causing damage to cell membranes and such effects can be attributed to the presence of anti-nutritional compounds in the composition of sunflower meals [55].

4. Conclusions

In the present study, the chemical composition, fatty acid composition, phenolic content, the level of total flavonoids and the antioxidant activity, together with the cytotoxic activity of three sunflower meals with different dehulling percentages were determined. The protein, ash and fiber content decreased with the increase of dehulling process. The protein content of the meal samples ranged between 22.24% and 30.91%, while the ash content varied between 3.21% and 5.44%. The meal samples had a crude fiber content varying between 11.24% and 24.56%. The richest source of crude fiber was observed in the case of WSM. The fat content increased with the dehulling process, ranging between 15.12 and 57.11%.

Sunflower meals analyzed had a high content of PUFA (45.92–53.12%) and MUFA (29.62–39.93%), and a low content of SFA (13.28–17.26%). The linoleic acid content ranged between 45.76 and 52.88%, decreasing with the dehulling process.

The obtained results confirmed that sunflower meals represent a significant source of compounds with antioxidant activity. The TSM showed the highest concentration of TPC and TFC, while the WSM had the lowest contents in these compounds. The values of antioxidant activities were higher in the case of TSM compared to PSM and WSM meals. Strong correlations were observed between TPC and antioxidant activity ($r > 0.90$) and between the level of TFC and antioxidant activity ($r > 0.75$) in all analyzed samples. Thus, in this study, the level of compounds with phenolic structure and the concentration of TFC contribute to the increase of antioxidant activity.

Taking into account the analysis of the weak cytotoxic activity at concentrations lower than 6.25 mg/mL for all extracts from sunflower meals, as well as the effect on cell viability which is close to the control value, we can say that sunflower meals can represent a source of bio-active compounds as long as they are used as secondary ingredients and considering the presence of possible anti-nutritional factors in their composition. Considering the results obtained, we can conclude that the analyzed sunflower meals have the potential to become sources of bioactive compounds with the aim of being used in the development of new foods, the improvement of the current ones as well as reformulations.

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