

Article

Phenolic Compound Composition and Biological Activities of Fractionated Soybean Pod Extract

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Abstract: The objective of this study was to determine the potential of dry extracts from soybean pods as a source of bioactive compounds. The phenolic compound composition of the extract (E) and fraction (F1-F3) as well as their respective lipophilicity were analyzed by UPLC-ESI-QTOF-MS. The biological activity as measured by antioxidant and cytotoxic activity was also evaluated. The in vitro anticancer potential of the ethanolic extract and fraction of soybean pods was measured through MTT assay using human colorectal carcinoma (HTC-116) and prostate cancer (PC-3) cell lines. The F2 fraction, with medium lipophilicity, produced the most pronounced cytotoxic effect in PC-3 cells. Analysis of Pearson's correlation demonstrated a high relationship between the content of phenolic compounds, the antioxidant activity of the extract and fractions, and cytotoxic activity towards PC-3 cells. UPLC-ESI-QTOF-MS analysis of soybean pod extract resulted in putative identification of 50 polyphenols belonging to three different chemical families (phenolic acids, flavonoids, and other polyphenols), the first time such an analysis has been performed. Among the fractions with variable lipophilicity, fraction F2 was the most abundant in phenolic compounds. This was confirmed in the quantitative analysis, as a result of which the highest total content of phenolic compounds was found in the extract (E) and fraction F2. Soybean pods might be useful material for obtaining preparations with anti-radical and anti-cancer properties. They can also be an active food additive or a component in dietary supplements.

Keywords: soybean pods; antioxidant activity; cytotoxic activity; phenolic composition; UPLC-ESI-QTOF-MS; phenolic compounds; agricultural waste



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1. Introduction

The progress of science and technology has contributed to civilizational development, and to many previously unknown threats as well. These threats include diseases caused primarily by environmental and food pollution, improper nutrition, and a fast pace of life resulting in stress. Developed and developing countries face health problems with an extensive social range, such as obesity, diabetes, cardiovascular diseases, arterial hypertension, osteoporosis, and various cancers [1,2]. These diseases can be potentially counteracted by means of appropriate prevention, the many aspects of which are related to proper nutrition. A balanced diet is rich in certain substances and ingredients of natural origin that exert a beneficial influence on the functioning of the human organism [3]. Substances exhibiting positive properties in the treatment and prevention of civilizational diseases include polyphenolic compounds. These secondary plant metabolites are ingested with foods of plant origin, and are not synthesized by animals [4]. After ingestion, phenolic compounds are digested and absorbed in the gastrointestinal tract, and the presence of their metabolites has been confirmed in blood serum [5]. The activity of phenolic compounds is related to their chemical structure. They possess one or more aromatic ring with one or more

hydroxyl groups attached, with their presence and mutual location determining compound activity [6,7]. Phenolic compounds are classified based on the number and arrangement of their carbon atoms and may be divided into classes such as flavonoids, tannins, stilbenes, lignans, coumarins and phenolic acids. [8,9]. They occur in almost all anatomical parts of plants, but their type and concentration varies depending on the function performed in the plant and its species. An example of specific phenolic compounds to which phytoestrogenic properties are attributed are isoflavones. These compounds, together with their derivatives, constitute a significant group of polyphenols the presence of which has been confirmed in soybean seeds [7,10].

Soybean (*Glycine max* (L.) Merr.) is one of the main economically important crops worldwide due to its rich chemical composition, numerous health promoting properties, and multi-directional applications. Among legumes, soybean seeds constitute a valuable source of nutrients that are important to the human organism [1]. Following the WHO model, soybean seed protein is believed to be wholesome; therefore, it can be used as an animal protein substitute. It is also an excellent source of bioactive compounds offering unique health benefits, which are used in the prevention of such maladies as cardiovascular disease, obesity, type II diabetes, and hormone-dependent cancers [3,11].

For food purposes, soybean seed collected immediately before it reaches full ripeness is also used. Production of this type of soybean is linked with maintaining a considerable amount of leaves and often pods, which are generally treated as industrial waste, although they are sometimes used as animal feed or organic fertilizer [12,13]. They are a valuable source of bioactive compounds that can be used as functional additives replacing synthetic substances. Their nutraceutical value and functional properties have not yet been adequately studied, and knowledge about the active compounds of soybean pods and their potential usage remains limited. At the same time, due to the growing public awareness of the influence of foods on health, increasing attention is being paid to the development of new products with health-promoting properties or the enrichment of existing products with active ingredients.

For these reasons, the present study aims to evaluate and compare the chemical composition and biological activity of fractionated extracts from soybean pods in order to determine their potential as a source of bioactive compounds. The qualitative composition of the extract (E) and fraction (F1–F3) as well differences in their lipophilicity were evaluated by liquid chromatography quadrupole time-of-flight mass spectrometry (UPLC-ESI-QTOF-MS), while the chemical composition was determined by spectrophotometric methods. The biological activity, measured by antioxidant and cytotoxic activity, was also assessed.

2. Materials and Methods

2.1. Plant Material

Glycine Max Merrill pods of the Aldana cultivar were obtained from Plant Breeding Stations. The experiment was carried out in the split-plot design, with three replications. The size of the plots was 12.5 m² (2.5 × 5 m) and the distance between plots was 1 m. The pods were collected in the R6 stage, when the pod cavity was completely filled but the seeds were completely green. The growth stages of the collected samples were determined according to pod number and seed maturity, as defined by Fehr et al. [14]. Soybean pods were obtained from 20 plant samples from which three extracts were prepared ($n = 3$) and analyzed in triplicate. Pods without seeds were frozen (−18 °C) and then lyophilized. Freeze-drying was carried out for 72 h using a freeze dryer (Free Zone 12 lyophilizer, Labconco Corporation, Kansas City, MO, USA) at −80 °C and 0.04 mbar.

2.2. Extract Preparation

Lyophilized pods (10 g) were subjected to extraction with 50 mL of an 80% ethanol solution (v/v). The extract was filtered at reduced pressure, and the supernatant was evaporated using a Büchi rotary evaporator (40 °C, 700 mPa). The dry residue was dissolved in 5 mL of redistilled water, frozen, and lyophilized in a Free Zone 12 apparatus by Labconco

(−80 °C, 0.04 mbar, 72 h). The lyophilizate was dissolved in 5 mL of the 80% aqueous ethanol solution and fractionated using Sep-Pak C18 cartridges. The hydrophilic fractions were eluted with water (F1), the intermediate lipophilic fraction was extracted with 40% methanol in water (F2), and the group of compounds with the highest hydrophobicity was eluted with 70% methanol in water (F3). The water fractions were frozen. Alcoholic fractions were evaporated using a vacuum evaporator. The dry residue was dissolved in 5 mL of redistilled water and frozen; subsequently, all fractions were lyophilized. The obtained fractions were subjected to spectrophotometric and chromatographic analyses. The scheme of the experiment is presented in Figure 1.

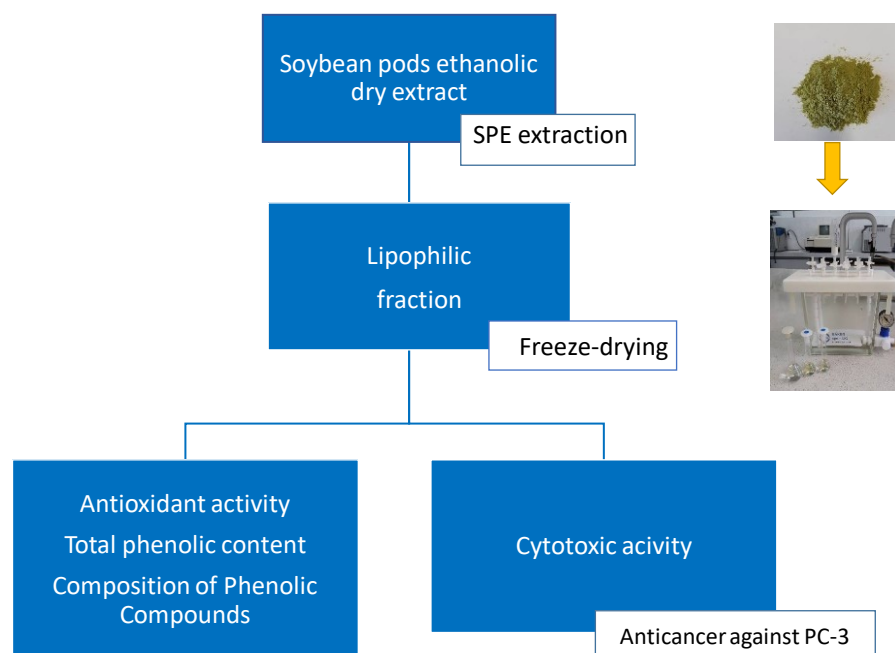


Figure 1. Scheme of the experimental procedure.

2.3. Determination of Polyphenol Content and Antioxidant Activity

Determination of total polyphenols with Folin–Ciocalteu reagent was performed in accordance with the methodology proposed by Mujic et al. [15]. To perform the analysis, an extract with a 1 mg/mL concentration (0.25 mL) was mixed with 2.0 mL of 10% Folin–Ciocalteu reagent (*v/v*). After one minute, 1.0 mL of a 7.5% sodium carbonate solution (*w/v*) was added. The samples were thoroughly mixed and incubated at room temperature for 120 min. Absorbance was read at 740 nm against water as a blank sample. Calculation of the content of phenolic compounds was performed based on the calibration curve prepared for gallic acid and expressed in mg gallic acid/g of extract.

The antioxidant activity of the extracts was determined with the DPPH radical (1,1-diphenyl-2-picrylhydrazyl) according to the method described by Brand-Williams et al. [16] and slightly modified by Mujic et al. [15]. For analysis, 3.6 mL of a DPPH solution (0.1 mM methanol solution) was added to 0.4 mL of the extract. The control sample was a mixture of 0.4 mL of methanol and 3.6 mL of DPPH. The absorbance was measured at 515 nm.

The antioxidant activity was also determined with the ABTS^{•+} radical (2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) in line with the methodology described by Re et al. [17]. ABTS^{•+} was generated via reaction of an ABTS aqueous solution (7 mM) with potassium persulfate (2.45 mM) in the dark for 12–16 h. The initial solution was diluted until absorbance reached 0.7 (±0.02) at 734 nm. The reaction mixture contained a 3 mL ABTS^{•+} solution and a 0.1 mL extract. A solution with ethanol instead of the samples was used as a control. The absorbance was measured at 515 nm after 10 min at room temperature.

Antioxidant activity was assessed in a series of solutions obtained from dry extracts with a concentration range from 0.01 to 10 mg of extract/mL. Based on the linear relationship between the ability to neutralize the DPPH radical and the ABTS cation radical and the concentration of the extract, the IC_{50} [mg/mL] value was calculated, which determines the concentration of the extract needed to neutralize 50% of radicals.

2.4. Composition of Phenolic Compounds

UPLC-QTOF Mass Spectrometry. Phenolic characterization of the fractionated pod extract was conducted using Agilent 1260 Infinity II (G7116A) coupled to Agilent 1260 Infinity II Autosampler (G7129A) and Agilent 6530 accurate-mass Q-TOF mass spectrometer with Jet Stream Ionization source (Agilent Technologies, Santa Clara, CA, USA). Chromatography was performed using a Zorbax C-18 column (2.1 mm \times 50 mm, 1.8 μ m) (Agilent Technologies, Santa Clara, CA, USA). Mobile phase A was 0.1% (*v/v*) formic acid in acetonitrile, and mobile phase B was 0.1% (*v/v*) formic acid in water. The program of the gradient elution was set as follows: 5–45% A (0 to 5 min); 45% A (5 to 10 min); 45–95% A (10 to 15 min); 95–5% A (15 to 16 min); 5% A (16 to 18 min). Filtration of the samples was performed with a syringe coupled with the 0.22 μ m syringe filter before the filtrates were transferred into HPLC vials. The injection volume of each sample was set to 5 μ L, the flow rate was set to 0.4 mL/min, and column temperature was set as 30 $^{\circ}$ C.

QTOF conditions were as follows: capillary voltage 3500 V, nitrogen gas temperature 300 $^{\circ}$ C at a flow rate of 5 L/min, shield gas temperature 300 $^{\circ}$ C at a flow rate of 8 L/min, and a nebulizer pressure of 35 psi. Signals in the *m/z* 100–2000 range with a scan time of 1.0 s were recorded in positive (ESI +) ionisation mode. Data processing was performed with Agilent MassHunter Qualitative Analysis software.

2.5. Cytotoxic Activity

2.5.1. Cell Lines

The experiments were conducted on two adherent cancer cell lines: human colorectal carcinoma (HCT116) and prostate adenocarcinoma (PC-3). The cells of the PC-3 line carry a homozygous mutation on the PTEN and TP53 genes. Furthermore, PC-3 cells are insensitive to androgens, glucocorticosteroids, and epidermal growth hormone. The HCT116 cell line carries a mutation on codon 13 of the RAS proto-oncogene. L929 cell line mouse fibroblasts from subcutaneous adipose tissue were used as a normal cell model, as these are recommended for research on the cytotoxicity of chemical compounds [18]. The HCT116, PC-3, and L929 cell lines were obtained from the American Type Culture Collection (ATCC, Rockville, MD, USA). The PC-3 cells were cultured in DMEM-F12 medium, while RPMI-1640 medium was used for the HCT116 cells. The normal cells were cultured as a monolayer in IMDM medium. All media were supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The incubation process was conducted at 37 $^{\circ}$ C, 95% humidity, and a 5%CO₂ concentration. All stages of the study were conducted in chambers with laminar airflow in sterile conditions. Exponential growth of the cells was maintained by regular passaging using 0.025% trypsin/EDTA twice a week. All cells were routinely checked for mycoplasma contamination.

2.5.2. MTT Cytotoxicity Test

Assessment of the *in vitro* cytotoxic activity of the fractionated pod extract was accomplished using the MTT(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide) assay for cancer and normal cell lines, following the method described by Mosmann [19]. The method assumes that MTT yellow water-soluble tetrazolium salt is reduced to blue water-insoluble MTT formazan by the mitochondrial succinate dehydrogenase of living cells. Formazan is impermeable for cell membranes, and thus accumulates in healthy cells [20]. Following the change in the environment and dissolution of crystals in an organic solvent, a colored solution is formed, the coloration intensity of which is spectrophotometrically measured and is directly proportionate to the count of live cells. This

facilitates assessment of their survival rate, and thus the assessment of the action of cytotoxic compounds [21]. In the case of damaged or dead cells, formazan is generated in lower amounts or is not formed at all [22].

The tested fractionated pods extracts were dissolved in a culture medium to obtain the required concentration. The tested cell lines were seeded on a 96-well plate (Nunc, Roskilde, Denmark) at a concentration of $6-8 \times 10^3$ cells per well and were subjected to 24 h incubation. After this time, the growth medium was replaced with a medium containing the fractionated extract of the pods in concentrations ranging from 10 to 200 $\mu\text{g}/\text{mL}$. The cells were incubated with the extract for 72 h (temp. 37 °C). The metabolic activity of live cells subjected to the effects of the potentially cytotoxic compounds contained in the extract was determined with the MTT test by adding 0.02 mL of a fresh MTT solution at a 5 mg/mL concentration to each well. This system was incubated for an additional 4 h at 37 °C. After this time, the purple crystals formed in the cells after MTT reduction were dissolved by adding 0.1 mL of DMSO/well. Absorbance was measured at wavelength $\lambda = 595$ nm on a PowerWave XS spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). The control sample comprised the HCT116, PC-3, and L929 cell lines with no addition of the tested plant extracts. DMSO was used as a blank. The cell survival rate was expressed as the IC_{50} value [$\mu\text{g}/\text{mL}$], which was the concentration of the tested extract/fraction required to reduce cell survival to 50% in comparison with the series free of the tested extracts/fractions. GraphPad Prism Version 7.0 (GraphPad Prism Software Inc., San Diego, CA, USA) software was used to estimate these values.

2.6. Statistical Analysis

Unless stated otherwise, all values represent means \pm SD (standard deviation) of three independent experiments. Analysis of variance was carried out by the one-way ANOVA system using STATGRAPHIC Centurion software, version XVI (Statgraphic Technologies, Inc., Reston, VA, USA), and the significance of the differences was determined on the basis of Tukey's test, with significance assumed at $p < 0.05$. In order to determine the relationship between the tested parameters, the Pearson linear correlation coefficient was determined using Microsoft Office Excel 2013 software from Microsoft Corporation (Redmond, WA, USA).

3. Results and Discussion

Qualitative analysis of the phenolic compounds from fractionated soybean pod extracts was conducted using UPLC-ESI-QTOF-MS in positive ionization mode. Putative identification of compounds present in the extracts and fractions with variable lipophilicity was made on the basis of their m/z value and MS spectral data obtained with Agilent MassHunter data acquisition software, Personal Compound Database and Library (PCDL) software and available online databases (MassBank, PubChem), as shown in Table 1. In total, 50 different phenolic compounds were tentatively characterized in soybean pod extract. These compounds can be grouped into three different chemical families: phenolic acids, flavonoids and other polyphenols, as mentioned in Table 1. In the available literature there are no reported results on the qualitative composition of polyphenols in soybean pods; this is the first investigation to analyze polyphenolic composition in agricultural soybean waste.

The largest number of compounds detected in the soybean pod extracts and fractions with variable lipophilicity were from the class of flavonoids. Identified subgroups of flavonoids included isoflavonoids, flavanols, flavones, flavonols and chalconids. Most of the detected flavonoids were of glycoside forms. One of the most important groups of phenolic compounds present in the studied soybean pod extracts and fractions are isoflavones, which have well documented health-promoting properties. They can be divided into three groups in soybeans on the basis of their aglycone moiety, namely daidzein, genistein and glycitein. In each group, four different forms are present: malonylglucoside, acetylglucoside, glucoside (daidzin, genistin, glycitin) and aglycone [10,23]. Aglycones

and glucosides were previously identified as the major group of phenolic compounds in soybean pods [24,25]. In the present study, all 12 isoflavones were tentatively identified. Among the isoflavonoids, we also identified 2'-hydroxyformononetin. This compound has been previously described in soybean leaves [26].

Another group of polyphenols identified in soybean pod extract was flavones. Among these, apigenin, luteolin and their derivatives were identified, which is in accordance with previous research [27,28]. Boue et al. [27] were the first to identify these compounds in soybean pods. In this flavonoid subclass we also tentatively identified chrysin (5,7-dihydroxyflavon), which was also previously described in soybean leaves [26,29]. The flavonols quercetrin, quercetin 3-*O*-glucoside, dihydroquercetin 3-*O*-rhamnoside, dihydroquercetin (taxifolin) and the flavanone naringenin were also tentatively identified in the studied soybean pod extracts. Quercetin derivatives were previously reported by Romani et al. [24] in soybean pod extract. The naringenin was synthesized from *p*-coumaroyl-CoA, which is derived from *p*-coumaric acid. This compound has been considered as a central intermediate in the production of different flavonoids including apigenin and genistein [8]. This fact could explain the presence of these flavonoids in the extract.

The flavanols (+)-catechin/(-)-epicatechin and 3'-*O*-methylcatechin and the chalconoids phloridzin and 3-hydroxyphloridzin were found in soybean pod extract for the first time and putatively identified. These compounds have already been found in soybean seeds [23,30,31], but never in the pods.

The last group of phenolic compounds tentatively identified in the studied extracts were phenolic acids. The presence of ferulic acid, *p*-coumaric acid and *p*-hydroxybenzoic acid were previously reported in the extracts of soybean seeds and flour [30,32]. Among the phenolic acids we also tentatively identified derivatives of *p*-coumaric acid (3-*p*-coumaroylquinic acid), ferulic acid (3-feruloquinic acid and ferulic acid 4-*O*-glucoside) and caffeic acid (3,5-*O*-dicafeoylquinic acid, caffeoylmalic acid and dihydrocaffeic acid 3-*O*-glucuronide). The presence of caffeic acids derivatives in soybean pods was previously confirmed by Romani et al. [24]. The derivatives of ferulic and *p*-coumaric acid has been described for the first time in soybean pods.

The fractionation of the ethanol extract allowed separation of most of the compounds present in the ethanol extract. For this reason, each of the fractions had a different qualitative (Table 1) and quantitative composition, which was confirmed by the spectrophotometric analyses (Table 2). In the present study, 10, 30 and 22 phenolic compounds were found in F1, F2 and F3 fractions, respectively. Hydrophilic compounds dominated in the F1 fraction, which is characteristic for this type of extraction as it is effective in removing sugars and organic acids. The F2 fraction contained mainly isoflavones, however their acetyl derivatives were found in the F3 fraction where the presence of apigenin and its derivatives has also been confirmed.

Table 1. Compounds identified in soybean pod extracts by UPLC-ESI-QTOF-MS, recorded in positive (ESI+) ionisation mode.

Time Retention (min.)	Tentative Identification	Polyphenol Classes	Molecular Formula	Mw Observed	Mw Theoretical	Error (ppm)	<i>m/z</i>	E	F1	F2	F3
0.288	3-feruloquinic acid	Phenolic acid	C ₁₇ H ₂₀ O ₉	368.1111	368.1107	1.09	369.1187	+	+	-	-
0.393	Dihydrocaffeic acid 3- <i>O</i> -glucuronide	Phenolic acid	C ₁₅ H ₁₈ O ₁₀	358.0909	358.09	2.64	359.1001	+	+	-	-
0.538	<i>p</i> -hydroxybenzoic acid	Phenolic acid	C ₇ H ₆ O ₃	138.0310	138.0317	-4.7	139.0383	+	+	-	-
0.588	<i>p</i> -Coumaric acid	Phenolic acid	C ₉ H ₈ O ₃	164.047	164.0473	-1.5	165.0545	+	+	+	+
0.638	Ferulic acid	Phenolic acid	C ₁₀ H ₁₀ O ₄	194.0578	194.0579	-0.57	195.0650	+	+	-	-
0.654	Ferulic acid 4- <i>O</i> -glucoside	Phenolic acid	C ₁₆ H ₂₀ O ₉	356.1101	356.1107	-1.81	357.1092	+	+	-	-
0.771	Caffeoylmalic acid	Phenolic acid	C ₁₃ H ₁₂ O ₈	296.0546	296.0532	4.53	297.0618	+	+	-	-
1.686	Quercetin 3- <i>O</i> -galactoside	Flavonols	C ₂₁ H ₂₀ O ₁₂	464.0951	464.0955	-0.9	465.1025	+	+	-	-
3.154	Dihydroquercetin (Taxifolin)	Flavonols	C ₁₅ H ₁₂ O ₇	304.0587	304.0583	1.19	305.0657	+	-	+	-
3.321	Daidzein	Isoflavonoids	C ₁₅ H ₁₀ O ₄	254.0580	254.0579	0.26	255.0652	+	-	+	-
3.321	Naringenin	Flavanones	C ₁₅ H ₁₂ O ₅	272.069	272.0685	1.81	273.0761	+	-	+	-
3.487	4-hydroxycoumarin	Other polyphenols	C ₉ H ₆ O ₃	162.0326	162.0317	4.83	163.0399	+	-	+	-
3.571	Dihydrosinapic acid	Phenolic acid	C ₁₁ H ₁₄ O ₅	226.0846	226.0841	1.98	227.0917	+	-	+	-
3.820	Phloridzin	Chalconoids	C ₂₁ H ₂₄ O ₁₀	436.1371	436.1369	0.24	437.1443	+	-	+	-
3.837	3-hydroxyphloridzin	Chalconoids	C ₂₁ H ₂₄ O ₁₁	452.1296	452.1319	-5.0	453.1366	+	-	+	-
3.837	(+)-Catechin/(-)-Epicatechin	Flavanols	C ₁₅ H ₁₄ O ₆	290.0788	290.079	-0.78	291.0860	+	-	+	-
3.837	Coumarin	Other polyphenols	C ₉ H ₆ O ₂	146.0365	146.0368	-1.61	147.0438	+	-	+	+
3.904	Dihydroquercetin 3- <i>O</i> -rhamnoside	Flavonols	C ₂₁ H ₂₂ O ₁₁	450.1188	450.1162	4.79	451.1274	+	-	+	-
3.904	Genistein	Isoflavonoids	C ₁₅ H ₁₀ O ₅	270.0531	270.0528	0.88	271.0603	+	-	+	-
3.970	Apigenin 6,8-di- <i>C</i> -glucoside	Flavones	C ₂₇ H ₃₀ O ₁₅	594.1570	594.1585	-2.48	595.1662	+	-	+	+
4.017	Myricetrin	Flavonols	C ₂₁ H ₂₀ O ₁₂	464.0955	464.0955	0.02	465.1031	+	-	+	-
4.037	3- <i>p</i> -Coumaroylquinic acid	Phenolic acid	C ₁₆ H ₁₈ O ₈	338.1014	338.1002	3.57	339.1087	+	-	+	-
4.103	Daidzin	Isoflavonoids	C ₂₁ H ₂₀ O ₉	416.1112	416.1107	1.21	417.1185	+	-	+	-
4.137	Luteolin	Flavones	C ₁₅ H ₁₀ O ₆	286.0480	286.04770	0.75	287.0551	+	-	+	+
4.187	Quercitin	Flavonols	C ₁₅ H ₁₀ O ₇	302.0434	302.0427	2.43	303.0505	+	-	+	+
4.220	Glycitein	Isoflavonoids	C ₁₆ H ₁₂ O ₅	284.0697	284.685	4.21	285.0768	+	-	+	-
4.253	Myricetin 3- <i>O</i> -rutinoside	Flavonols	C ₂₇ H ₃₀ O ₁₇	626.1488	626.1483	0.84	627.1558	+	-	+	+
4.386	Quercetin 3- <i>O</i> -rutinoside (Rutin)	Flavonols	C ₂₇ H ₃₀ O ₁₆	610.1578	610.1534	4.29	611.1646	+	-	+	+

Table 1. Cont.

Time Retention (min.)	Tentative Identification	Polyphenol Classes	Molecular Formula	Mw Observed	Mw Theoretical	Error (ppm)	m/z	E	F1	F2	F3
4.432	3-O-methyl quercetin	Flavonols	C ₁₆ H ₁₂ O ₇	316.0606	316.0583	4.36	317.0677	+	-	-	+
4.565	Apigenin 7-O-apiosylglucoside	Flavones	C ₂₆ H ₂₈ O ₁₄	564.1483	564.1479	0.72	565.1556	+	-	-	+
4.603	Genistin	Isoflavonoids	C ₂₁ H ₂₀ O ₁₀	432.1078	432.1056	5.0	433.1147	+	-	+	-
4.615	Apigenin 7-O-glucoside	Flavones	C ₂₁ H ₂₀ O ₁₀	432.1061	432.1056	0.98	433.1134	+	-	-	+
4.615	Luteolin 7-O-glucoside	Flavonols	C ₂₁ H ₂₀ O ₁₁	448.1013	448.1006	1.65	449.1083	+	-	+	+
4.736	6''-O-malonylaidzin	Isoflavonoids	C ₂₄ H ₂₂ O ₁₂	502.1121	502.1111	1.97	503.1195	+	-	+	-
4.783	Qercetin 3-O-glucoside	Flavonols	C ₂₁ H ₂₀ O ₁₂	464.0957	464.0955	0.45	465.1043	+	-	-	+
5.015	6'-O-acetyloaidzin	Isoflavonoids	C ₂₃ H ₂₂ O ₁₀	458.1223	458.1213	2.17	459.1294	+	-	-	+
5.181	Glycitin	Isoflavonoids	C ₂₂ H ₂₂ O ₁₀	446.1204	446.1213	-1.97	447.1296	+	-	+	-
5.302	6'-O-malonylgenistin	Isoflavonoids	C ₂₄ H ₂₀ O ₁₃	516.0909	516.0904	0.96	517.0977	+	-	+	-
5.331	Chrysin	Flavones	C ₁₅ H ₁₀ O ₄	254.0582	254.0579	1.14	255.0654	+	-	-	+
5.416	Kaempferol 3-O-arabinoside	Flavonols	C ₂₀ H ₁₈ O ₁₀	418.0909	418.090	-0.22	419.0985	+	+	+	+
5.448	2'-hydroxyformononetin	Isoflavonoids	C ₁₆ H ₁₂ O ₅	284.06888	284.0685	1.24	285.0761	+	-	-	+
5.602	6''-O-malonylglycitin	Isoflavonoids	C ₂₅ H ₂₄ O ₁₃	532.1230	532.1217	2.39	533.1296	+	-	+	-
5.781	6'-O-acetylogenistin	Isoflavonoids	C ₂₃ H ₂₂ O ₁₁	474.1118	474.1162	3.78	475.1250	+	-	-	+
5.801	3,5-O-Dicaffeoylqunic acid	Phenolic acid	C ₂₅ H ₂₄ O ₁₂	516.1272	516.1268	0.82	517.1344	+	-	+	-
6.230	Isotrifoliol	Other polyphenols	C ₁₆ H ₁₀ O ₆	298.0491	298.0477	4.57	299.0564	+	-	-	+
6.280	Apigenin	Flavones	C ₁₅ H ₁₀ O ₅	270.0534	270.0528	2.08	271.0606	+	-	-	+
6.313	Coumestrol	Other polyphenols	C ₁₅ H ₈ O ₅	268.0379	268.0372	2.83	269.450	+	-	-	+
6.946	Formononetin	Isoflavonoids	C ₁₆ H ₁₂ O ₄	268.0740	268.0736	1.78	269.0813	+	-	-	+
7.911	3'-O-methylcatechin	Flavanols	C ₁₆ H ₁₆ O ₆	304.0932	304.0947	-4.95	305.1004	+	-	-	+
14.377	Methyl cinnamate	Other polyphenols	C ₁₀ H ₁₀ O ₂	162.0682	162.0681	-0.23	163.0755	+	+	+	+

E—80% ethanolic extract, F1—water fraction, F2—40% methanol-water fraction, F3—70% methanol fraction.

Table 2. Total phenolic content and antioxidant and cytotoxic activity of 80% ethanolic extract (E), water (F1), 40% methanol-water (F2), and 70% methanol (F3) fractions obtained from soybean pods.

	Total Phenolic Content ¹	Antioxidant Activity ²		Cytotoxic Activity ³		
		DPPH	ABTS	HCT116	PC-3	L929
E	24.649 ^a ± 0.618	427.3 ^c ± 4.1	73.1 ^d ± 1.1	116 ^b ± 3.7	54 ^b ± 3.1	70 ^c ± 2.6
F1	0.016 ^d ± 0.019	2668.7 ^a ± 10.3	956.5 ^a ± 8.3	102 ^c ± 3.4	56 ^b ± 2.1	56 ^d ± 2.1
F2	14.510 ^b ± 0.097	320.2 ^d ± 3.5	154.7 ^c ± 2.4	156 ^a ± 3.3	66 ^a ± 2.8	112 ^a ± 2.8
F3	6.028 ^c ± 0.214	510.6 ^b ± 5.2	320.2 ^b ± 4.7	120 ^b ± 3.1	70 ^a ± 2.9	80 ^b ± 3.3
5-fluorouracil ⁴	n.a. ⁵	n.a.	n.a.	33 ^d ± 3.2	23 ^c ± 2.58	8 ^e ± 1.1

Means with the different letters (a,b,c,d) in the column are significantly different ($p < 0.05$). Data represent the mean ± SD of three independent measurements. ¹ (mg gallic acid/g dry extract); ^{2,3} IC₅₀ (µg/mL); ⁴ 5-fluorouracil (µM)-positive control; ⁵ n.a.—not applicable.

The initial ethanol extract contained all identified phenolic compounds; among the fractions with variable lipophilicity, the F2 fraction was the most abundant in these compounds. This was directly reflected in the quantitative analysis, as a result of which the highest total content of phenolic compounds was found in extract (E) and in the F2 fraction (24.649 and 14.510 mg GAE/g, respectively). The water fraction (F1) contained the lowest amount of phenolic compounds, which was confirmed in qualitative (LC-QTOF-MS) analyses (Table 1). The present results are consistent with those obtained by Tan et al. [33], who also noted the lowest content of phenolic compounds in the aqueous fraction obtained from soybean seed extract.

The ethanol extracts from the soybean pods and their fractions both constitute a multi-component composition of active compounds. The variable concentrations and interactions of these compounds determine their biological activity, measured by antioxidant and cytotoxicity activity (Table 2).

All analyzed fractions and their produced extracts exhibited an ability to neutralize DPPH• and ABTS•+, depending on the concentration (Table 2). The F2 fraction with the highest content of phenolic compounds exhibited the most pronounced antiradical activity determined for DPPH• and ABTS•+ (IC₅₀ 320.2 and 154.7 µg/mL, respectively). The value of the Pearson correlation coefficient ($r = -0.99$ for the method with DPPH• and ABTS•+; Table 3) suggests a strong correlation between the content of phenolic compounds and the antioxidant activity expressed as the IC₅₀ value. These results are in agreement with the available literature [31,33,34].

Table 3. Pearson's correlation coefficient.

	DPPH	ABTS	TPC
TPC	-0.99188	-0.99941	-
Cytotoxic activity (PC-3)	0.97829	0.949829	-0.94401

One of the main current directions in pharmacology and oncology is the search for new anticancer drugs that do not damage normal cells as much as the widely used chemotherapy drugs, or not at all. Therefore, the research on natural compounds that can be used as an adjunct in the treatment of certain cancers is of great importance. The results presented below constitute the first stage of this type of analysis.

The fractions with variable lipophilicity isolated from the ethanol extract were tested for their cytotoxic activity against human colorectal carcinoma (HCT116) and prostate adenocarcinoma (PC-3) cells. The L929 cell line, i.e., mouse fibroblasts from subcutaneous adipose tissue, was used as a normal cell model, as recommended for research associated with the cytotoxicity of chemical compounds and the comparison of other results. The cytotoxic effects of the investigated samples depended on the type of cancer cells, and did not exceed 160 µg/mL (Table 1). It was shown that the PC-3 cell line was the most sensitive to the effect of the soybean pod extracts and fractions with variable lipophilicity.

A significantly lower degree of cytotoxicity was noted in the experimental series with the use of normal cells from cell line L929. The F2 fraction with medium lipophilicity produced the most pronounced cytotoxic effect in the PC-3 cells ($IC_{50} = 66 \mu\text{g/mL}$), which was two times higher than in the case of the L929 cells ($IC_{50} = 112 \mu\text{g/mL}$); the 80% ethanol extract was very effective as well ($IC_{50} = 54 \mu\text{g/mL}$). The aqueous fraction (F1) did not exhibit PC3-specific cytotoxicity ($IC_{50} = 56 \mu\text{g/mL}$ for PC-3 and L929). The analysis of Pearson's correlation (Table 3) demonstrated a high relationship between the content of phenolic compounds and the values of IC_{50} which reflects the antioxidant activity of the extracts and fractions and the cytotoxic activity towards the PC-3 cells.

The colorectal carcinoma cells showed slight sensitivity to the action of the pod extract and the fraction with variable lipophilicity. This is illustrated by the IC_{50} values from 102 to 156 $\mu\text{g/mL}$, which markedly exceed the values obtained for L929 cells. A higher degree of cytotoxicity was noted in the experimental series using normal cells (IC_{50} from 56 to 112 $\mu\text{g/mL}$).

One of the preliminary tests necessary to determine the proper action and safety of a potential drug are cytotoxicity studies using *in vitro* cell cultures. Although the conventional *in vitro* methods used to determine cytotoxicity have lower specificity or ability to evaluate the mechanism of action than animal models, they are a widely used and useful tool for screening new compounds. In the case of anti-tumor activity, limited information is available in the literature on soybean pod extract. Lee et al. [35] assessed the effect of gamma radiation on the content of bioactive compounds and the anticancer properties of pod extract. They observed reduced viability of colorectal cancer cells (HCT116) and breast cancer cells (MCF7) exposed to the irradiated pod extract, by 64 and 84%, respectively, in comparison with non-irradiated extract. The authors also noted an increase in the content of polyphenols in the extract exposed to gamma radiation and suggested that these compounds may be responsible for the anticancer effect of the pod extract. The results of the present study also indicate a strong correlation between the content of phenolic compounds and the cytotoxic effect of the extracts and fractions with variable lipophilicity. In an *in vitro* study, Kwak and Ju [36] noted an inhibitory effect of ethanol extract from soybean leaves on the growth and formation of independent colonies of HCT116 colon cancer and H1299 lung cancer cells. The H1299 tumor cells were slightly more susceptible to the soybean leaf extract than the HCT116 cells ($IC_{50} = 285$ and $170 \mu\text{g/mL}$, respectively). The results of this study and the authors cited above indicate different sensitivity of cancer cells to the extracts, with colon cancer cells being less sensitive to the applied factors (Table 1) [35,36].

In the literature, some results of *in vitro* and *in vivo* tests involving animals and humans are available which indicate that isoflavones are the main bioactive compounds in soybeans which show anti-tumor activity. The results of the studies by Kumar et al. [37] showed that a solution of three phytoestrogens (genistein, biochanin A and quercetin) with the same concentration ($8.33 \mu\text{mol/L}$) showed a stronger inhibitory effect on prostate cell growth than single phytoestrogens ($25 \mu\text{mol/L}$) or a mixture of only two ($12.5 \mu\text{mol/L}$). These results suggest a synergistic effect of phytoestrogens in terms of their physiological concentrations in the prevention of prostate cancer. *In vitro* studies by Hsu et al. [38] show that soybean extract containing isoflavones at the concentration of $25 \mu\text{mol/L}$ induced a significantly higher percentage of prostate cancer cells to undergo apoptosis than genistein or daidzein alone at a concentration of $25 \mu\text{mol/L}$. Moreover, these authors observed no significant changes in cell cycle arrest or apoptosis in non-cancerous (BPH-1) cells exposed to soybean extract. This suggests a specific action of soybean extract against neoplastic cells. On the other hand, genistein and daidzein induced apoptosis in BPH-1 cells, which suggests that individual isoflavones may exhibit cytotoxicity in non-neoplastic cells. The mentioned authors emphasize that food products containing a combination of active substances may be more effective and safer as chemotherapeutic agents than individual compounds. In the presented study, the F2 fraction showed the highest cytotoxic effect against prostate cancer cells. The results of the qualitative analysis showed the presence of isoflavones, which confirms the cited reports on the cytotoxic effect of isoflavones on prostate cancer cells.

The results of the *in vitro* tests indicated that the presence of the initial ethanolic extract and the water–methanol fraction (F2) in the culture medium led to inhibition of the growth of prostate cancer cells. This suggests the potential use of soybean pods, which are considered a by-product of soybean production, as a functional food with chemopreventive properties. However, these results require further *in vivo* verification on animal models, followed by clinical studies.

4. Conclusions

Soybean pods are a form of agricultural waste that could represent a source of bioactive compounds for functional foods or the cosmetic industry. However, the literature and data concerning the metabolites of soybean pods are scarce. To the authors' best knowledge, the current study is the first report on the metabolite profile of soybean pods. The UPLC-ESI-QTOF-MS technique was successfully applied for separation and tentative identification of 50 phenolic compounds in soybean pod extracts.

These preliminary data suggested that soybean pods and their byproducts could be used as a source of bioactive phenolic compounds with potential as antioxidants and anti-cancer agents, and could therefore be useful in the food, pharmaceutical and nutraceutical industries. Their use for these purposes should be further investigated.

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