Preliminary exploration of phytochemical profiles, antioxidant and hepatoprotective activities of non-oil extracts of pumpkin seeds treated by four different processing methods

ZIJIAN SUN – FENGQIN QI – HUAN GAO – LIJUAN LIU

Summary

Nowadays, pumpkin seeds have been accepted as a popular snack food around the world due to their delicious taste and richness in nutrients. Because most studies on pumpkin seeds focused on the oil soluble ingredients at present, this paper evaluated the antioxidant activities of the non-oil soluble fractions of pumpkin seeds by 2,2-diphenyl-1-picrylhydrazyl radical-scavenging assay and ferric reducing power assay. Pumpkin seeds of various collection dates and origins were processed by four different methods and hepatoprotective activity against D-galactosamine-induced toxicity in human hepatoma HepG2 cells was evaluated in vitro. High performance liquid chromatographic analysis was performed and 11 compounds, represented by characteristic peaks, related closely to antioxidant activity were deduced by multiple correlation analysis. Total phenolic content, flavonoid content and nuclear magnetic resonance spectra were also determined. The results showed that raw pumpkin seeds with most phenolic constituents possessed the strongest antioxidant activity, effective concentration values providing 50 % of radical-scavenging capacity (EC_{50}) being 35.75 $\mu g \cdot ml^{-1}$, and the highest hepatoprotective activity, cell survival rate being 79.2 %, in comparison with baked pumpkin seeds, shelled pumpkin seeds, and shelled and baked pumpkin seeds. Shelling and baking negatively influenced the activities of pumpkin seeds.

Keywords

Semen Moschatae; biological activity; total flavonoid content; total phenolic content; phytochemical profiles

Pumpkin, a large genus of annual Cucurbitaceae, contains five cultivated varieties of Chinese pumpkin (*Cucurbita moschata*), Indian pumpkin (*C. maxima*), American squash (*C. pepo*), Mexico Pumpkin (*C. mixta*) and Black seed pumpkin (*C. ficifolia*) [1]. Pumpkin seeds (Semen Moschatae) which we studied, also known as pumpkin kernel, white melon seeds and golden melon seeds, have been widely accepted as a popular snack food around the world. Owing to their richness in nutrients and delicious taste, pumpkin seeds are considered to be a good source of nutrients for human health. Pumpkin seeds contain large amounts of proteins, polysaccharides, phenolic acids and polyunsaturated fatty acids [2–6]. Potassium, phosphorus, magnesium, phytosterols, carotenoids, tocopherol, zinc and squalene are abundant in edible pumpkin seeds as well [1, 7, 8].

Pumpkin seeds have been commonly used in traditional Chinese medicine for many years for safe vermifuge and galactagogue [1]. However, there is insufficient information on processing methods of pumpkin seeds before application, whether they should be used as raw, baked, shelled or unshelled. The oil-soluble extracts of pumpkin seeds were reported to possess several biological activities including repelling parasites, protecting the liver, lowering blood lipids, antioxidation, relieving hypertension, lowering bladder and urethral pressure, anticancer activity and alleviation of diabetes or arthritis through antioxidant activities [9–19].

Zijian Sun, Lijuan Liu, Department of Pharmaceutical Engineering, Heilongjiang University, Xuefu Road 74, 150080 Harbin, China.
Fengqin Qi, Experimental Teaching and Practical Training Center, Heilongjiang University of Chinese Medicine, Heping Road 24, 150040 Harbin, China.
Huan Gao, Department of Pharmacy, The First Hospital of Jilin University, Xinmin Street 71, 130021 Changchun, China.

Correspondence author:
Lijuan Liu, e-mail: liulijuan1972@163.com

A growing amount of evidence has shown that antioxidant activity is closely related to people's health and the treatment of some diseases. Antioxidants, in particular the naturally occurring ones, are widely used as dietary supplements in a hope of keeping health or preventing diseases. Many reports were published on the antioxidant activity of pumpkin seed oils [20–22]. However, the non-oil extracts of pumpkin seeds, which are usually used as feed or fertilizer, have been little studied. In order to continue our previous work [23], we evaluated the antioxidant activities of the non-oil soluble fractions of pumpkin seeds by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging assay and ferric reducing antioxidant power (FRAP) assay. Pumpkin seeds from different sources were processed by four different methods and hepatoprotective activity against D-galactosamine-induced toxicity in human hepatoma HepG2 cells was evaluated in vitro. The pumpkin seeds processed in various ways were raw pumpkin seeds (RPS), baked pumpkin seeds (BPS), shelled pumpkin seeds (SPS), and shelled and baked pumpkin seeds (SBPS). Total phenolic content (*TPC*), total flavonoid content (*TFC*) and nuclear magnetic resonance (NMR) spectra were determined. Moreover, high performance liquid chromatography (HPLC) analysis and multiple correlation analysis were performed to obtain phytochemical profiles.

MATERIALS AND METHODS

Reagents and materials

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical and 2,4,6-tripyridyl-*s*-triazine (TPTZ) were obtained from Dibo Biotechnology (Shanghai, China). Folin-Ciocalteu phenol reagent was obtained from Sigma-Aldrich (St. Louis, Missouri, USA). Gallic acid was obtained from Guangfu Fine Chemical Industry Research Institute (Tianjin, China). Acetonitrile and methanol of HPLC grade were obtained from Dikma Technology (Beijing, China). Ethanol was obtained from Fuyu Fine Chemical (Tianjin, China). Phosphoric acid and sodium carbonate anhydrous were obtained from Kermel Chemical Reagents (Tianjin, China). All other chemicals were of analytical grade without further purification.

Plant materials

Samples of pumpkin seeds (designated S1–S42) were collected from nine different regions of China and further authenticated as mature seeds of *Cucurbita moschata* Duch. The details of origin, collection date and processing methods are shown in Tab. 1. The pumpkin seeds were firstly selected to remove those that were damaged, then they were ground into powder with a grinder (CLF-06C, Zhejiang Chuangli Pharmaceutical Equipment Factory, Guangzhou, China) and passed through a 250 μm griddle. The samples of powder were stored in a refrigerator at 4 °C for further analysis and voucher specimens were deposited.

Extraction methods

A total of 100 g of pumpkin seeds were defatted by sonication with 1000 ml of petroleum ether. Then, the defatted residue was immersed in 10 times the volume of 95% ethanol for 12 h and then processed by ultrasonic-assisted extraction for 40 min. The extracted solution was filtered through an analytical filter paper and then vacuum-dried at 40 °C to give ethanol extract (4.57 g). A part of the ethanol extract (3.00 g) was applied to a D101 macroporous resin column (Guangfu Fine Chemical Industry Research Institute) and eluted with water and 30% ethanol. The 30% ethanol eluate fractions were combined and concentrated under reduced air pressure to give the sample extract for further analysis.

DPPH radical-scavenging capacity assay

The DPPH assay is one of the widely used antioxidant assay methods, and its results represent the ability to scavenge the stable free radical generated by DPPH. The scavenging capacities of the samples on DPPH free radical were measured according to the method described previously with slight modifications [24]. Briefly, 2 ml of various concentrations (0.01, 0.02, 0.04, 0.08, and 0.16 $mg \cdot ml^{-1}$) of sample extracts were added to 2 ml of freshly prepared 2×10^{-4} $mol \cdot l^{-1}$ DPPH radical-scavenger solution. The mixture was shaken vigorously and incubated in the dark at a room temperature for 30 min. The absorbance was measured at 517 nm by a UV-2700 spectrophotometer (Shimadzu, Kyoto, Japan). Ethanol was used as a blank reagent. The DPPH-scavenging capacity (AA_{DPPH}) was calculated according to Eq. 1 and expressed as a percentage of the radical DPPH inhibition with respect to the decrease in absorption of the control using the formula:

$$AA_{DPPH} = [1 - (A_2 - A_1)/A_0] \times 100 \quad (1)$$

where A_1 was the absorbance of sample without the DPPH solution, A_2 was the absorbance of the sample mixed with DPPH solution and A_0 was the absorbance of the DPPH solution without sample extract.

Tab. 1. Radical-scavenging capacities of pumpkin seeds treated by different processing methods.

Origin	Collecting date	Sample code	Processing method	EC_{50} [$\mu g \cdot ml^{-1}$]
Inner Mongolia	5 Jan 2018	S1	Raw	125.47 ± 0.40
		S2	Baking	445.80 ± 0.66
		S3	Shelling	471.60 ± 0.61
		S4	Shelling, baking	520.07 ± 2.97
	10 Jan 2018	S5	Raw	110.40 ± 0.80
		S6	Baking	374.00 ± 0.85
		S7	Shelling	485.70 ± 0.75
		S8	Shelling, baking	832.80 ± 0.89
	15 Jan 2018	S9	Raw	113.97 ± 0.21
		S10	Baking	403.57 ± 1.27
		S11	Shelling	491.70 ± 1.64
		S12	Shelling, baking	886.67 ± 4.40
	25 Feb 2018	S13	Raw	126.93 ± 1.14
		S14	Baking	436.50 ± 0.66
		S15	Shelling	500.73 ± 1.12
		S16	Shelling, baking	1 000.87 ± 4.80
Xinjiang	15 Jan 2018	S17	Raw	73.44 ± 2.23
		S18	Baking	201.53 ± 1.46
		S19	Shelling	451.67 ± 1.00
		S20	Shelling, baking	591.73 ± 1.69
	5 Mar 2018	S21	Raw	86.50 ± 1.58
		S22	Baking	220.50 ± 2.95
		S23	Shelling	466.00 ± 4.47
		S24	Shelling, baking	607.07 ± 2.62
Heilongjiang	20 Dec 2017	S25	Raw	99.22 ± 2.81
	5 Jan 2018	S26	Raw	90.04 ± 1.56
	20 Jan 2018	S27	Raw	94.36 ± 0.42
		S28	Baking	238.70 ± 0.75
Hebei	5 Jan 2017	S29	Raw	123.23 ± 1.19
	6 Jan 2017	S30	Raw	83.19 ± 0.97
	20 Dec 2017	S31	Raw	100.30 ± 0.78
		S32	Baking	333.20 ± 1.11
Jiangsu	5 Jan 2017	S33	Raw	85.91 ± 0.41
	10 Mar 2018	S34	Raw	69.37 ± 0.50
		S35	Baking	286.77 ± 1.25
Yunnan	10 Mar 2017	S36	Raw	180.63 ± 2.12
Zhejiang	15 Jan 2017	S37	Raw	48.79 ± 1.20
Shandong	15 Jan 2017	S38	Raw	74.26 ± 1.13
		S39	Baking	159.60 ± 2.19
Gansu	15 Jan 2017	S40	Raw	35.75 ± 0.55
Xinjiang	5 Jan 2017	S41	Raw	55.43 ± 0.84
Inner Mongolia	20 Dec 2016	S42	Raw	100.91 ± 1.29

EC_{50} – effective concentration providing 50% of radical-scavenging capacity, values are means ± standard deviation of triplicate assays and the mean difference is significant at the 0.05 level.

Effective concentration values providing 50% of AA_{DPPH} (EC_{50}) were obtained from linear regression analysis and expressed as micrograms per millilitre of extracts. All tests were repeated in triplicate.

Ferric reducing antioxidant power assay

The FRAP assay was performed according to Daud et al. [25] with minor modifications. The FRAP reagent containing 2 ml of a 10 mmol·l^{-1} TPTZ solution in 40 mmol·l^{-1} HCl, 2 ml of 20 mmol·l^{-1} $FeCl_3 \cdot 6H_2O$ solution and 20 ml of 300 mmol·l^{-1} acetate buffer solution (pH 3.6). A volume of 6 ml of the FRAP solution was mixed with 0.2 ml of sample (1 mg·ml^{-1}) and 0.6 ml of distilled water. The mixture was shaken evenly and then kept at 37 °C for 10 min. Absorbance was monitored at 593 nm by a UV-2700 spectrophotometer. The FRAP value (AA_{FRAP}) was expressed in moles of $FeSO_4$ equivalents per kilogram of extracts.

Determination of total phenolic content

Total phenol content (*TPC*) of extracts was determined by Folin-Ciocalteu colorimetric assay method with some modifications and gallic acid was used as the standard [26]. Briefly, 0.5 ml of pumpkin seeds extract (0.5 mg·ml^{-1}) was added to 2.5 ml of Folin-Ciocalteu solution (0.1 mg·ml^{-1}). The solution was shaken well, allowed to stand for 4 min and then 2 ml of Na_2CO_3 (40 mg·ml^{-1}) was added. After 2 h of reaction at room temperature in the dark, the absorbance at 760 nm was determined. *TPC* of sample was expressed as grams of gallic acid equivalents (GAE) per kilogram of dry weight. All tests were repeated three times.

Determination of total flavonoid content

The total flavonoid content (*TFC*) was measured by the method based on flavonoid-aluminum complex formation [27], which was slightly modified. Briefly, 2 ml of pumpkin seeds extract (9 mg·ml^{-1}) were mixed with 3 ml of ethanol and 0.3 ml of $NaNO_2$ (50 g·l^{-1}) for 6 min. After that, 0.3 ml of $Al(NO_3)_3$ (100 g·l^{-1}) was added to react for another 6 min, followed by addition of 4 ml of NaOH (40 g·l^{-1}). Distilled water was added to get a total volume of 10 ml. The solution was mixed thoroughly and kept at room temperature for 15 min. The absorbance was determined at 510 nm. The levels of *TFC* were calculated in accordance with the calibration curves of rutin and expressed as grams of rutin per kilogram of dry weight of sample [28].

HPLC

The HPLC analysis was carried out using a Hitachi L-2000 system (Hitachi, Tokyo, Japan) equipped with a L-2130 dual pump and UV detector (Techcomp Scientific Instruments, Suzhou, China). Separation was carried out on an Hypersil ODS C_{18} column (250 mm × 4.6 mm, 5 μm; Thermo Fisher Scientific, Waltham, Massachusetts, USA). Before the analysis, all samples were dissolved in methanol and filtered through a membrane filter (pore size 0.45 μm). The flow rate was 0.8 ml·min^{-1} and the detection wavelength was 264 nm. The mobile phase solvent A was methanol : acetonitrile (1 : 1), and the solvent B was phosphoric acid (3.4 g·l^{-1}) in water. The gradient elution program was: 1–11 % A in 0–15 min, 11–15 % A in 15–22 min, 15–19 % A in 22–43 min, 19–20 % A in 43–46 min, 20–22 % A in 46–50 min, 22–24 % A in 50–55 min, 24–45 % A in 55–60 min, 45–50 % A in 60–65 min and 50–74 % A in 65–75 min.

NMR spectrometry

A Bruker AM-600 spectrometer (Bruker, Billerica, Massachusetts, USA) was used to measure the ^1H and ^{13}C NMR spectra. Trimethylsilane (TMS) was used as the internal reference, and the chemical shift δ was expressed in parts per million (ppm).

Hepatoprotective assay

Hepatoprotective assays were accomplished on a Synergy 2 multimode microplate reader (BioTek, Winoosk, Vermont, USA). 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) was obtained from Goodtime Bio-Technology (Wuhan, China). The cytotoxicity against human hepatoma HepG2 cells was measured by the method of Mosmann [29]. Each cell suspension of 1.2×10^4 cells in 100 μl of high-glucose Dulbecco's modified eagle medium (DMEM) medium containing 10% fetal bovine serum (FBS), penicillin (100 μg·ml^{-1}) and streptomycin (100 μg·ml^{-1}) was placed in a 96-well microplate and incubated at 37 °C in a humidified atmosphere of 5% CO_2 for 24 h. The test samples (2–8 μg·ml^{-1}) and bicyclol as positive control (10 μmol·l^{-1}, Beijing Union Pharmaceutical Factory, Beijing, China) were added into the wells and cultured for 2 h. The incubated cells were exposed to 35 mmol·l^{-1} D-galactosamine (Dalian Meilun Biotechnology, Dalian, China) for 24 h. Then, 100 μl of 0.5 mg·ml^{-1} MTT was added to each well after the withdrawal of the culture medium and incubated for additional 2.5 h. Supernatant was removed and formazan was dissolved in 150 μl of dimethyl sulfoxide (DMSO) by shaking for 10 min. Optical density (*OD*) of the formazan

solution was measured on a microplate reader at 490 nm [30].

Statistical analysis

All experiments were repeated three times and the results were expressed as mean ± standard deviation (*SD*). All data were tested with ANOVA and Tukey's test (SPSS 18.0 software, SPSS, Chicago, Illinois, USA) to determine significant differences ($P < 0.05$).

RESULTS AND DISCUSSION

Antioxidant activity

The radical-scavenging capacities of pumpkin seeds that underwent four different ways of processing were determined and the results for 42 batches of samples are presented in Tab. 1, where they are expressed as EC_{50} values. The lower EC_{50} values represent the stronger antioxidant activity of the extract. The results highlighted that the antioxidant activity decreased with the trend S1 > S2 > S3 > S4. That is, raw pumpkin seeds (batch S1) exhibited the best AA_{DPPH} with EC_{50} of 125.47 $\mu g \cdot ml^{-1}$, which was approximately equivalent to the oil-part of pumpkin seeds as previously reported by JIAO et al. [31]. Comparing batch S2 (EC_{50} of 445.80 $\mu g \cdot ml^{-1}$) to S1, AA_{DPPH} of pumpkin seeds was significantly reduced by 72 % after cooking. Comparing batch S3 (EC_{50} of 471.60 $\mu g \cdot ml^{-1}$) to S1, the EC_{50} value was considerably reduced by 73 % after the shell was removed. It indicated that the methods of baking and shelling will lead to the loss of antioxidant activity. However, differences were not obvious between batches S2 and S4 (EC_{50} of 445.80 $\mu g \cdot ml^{-1}$ and 520.07 $\mu g \cdot ml^{-1}$, respectively). It demonstrated that husks were not important factors for baked pumpkin seeds to affect antioxidant activity.

In order to confirm the results that raw pumpkin seeds had the best antioxidant activity, we collected five batches of raw pumpkin seeds from Inner Mongolia and Xinjiang of China, and treated them in four different ways for each batch, resulting in a total of 20 batches of samples (S5–S24). The radical-scavenging capacity was evaluated by DPPH assay as mentioned above.

RPS (batches S5, S9, S13, S17 and S21) showed the best radical-scavenging capacities that were approximately 4–7 times higher than SPS (batches S7, S11, S15, S19 and S23). It was observed that dehulling caused lowering of the antioxidant activity. Meanwhile, SBPS (batches S8, S12, S16, S20 and S24) exhibited the weakest antioxidant activity with EC_{50} values approximately of one-fifth to one-fourteenth of RPS (batches S5, S9, S13, S17, S21), indicating that baking and shelling were both disadvantageous regarding antioxidant activity.

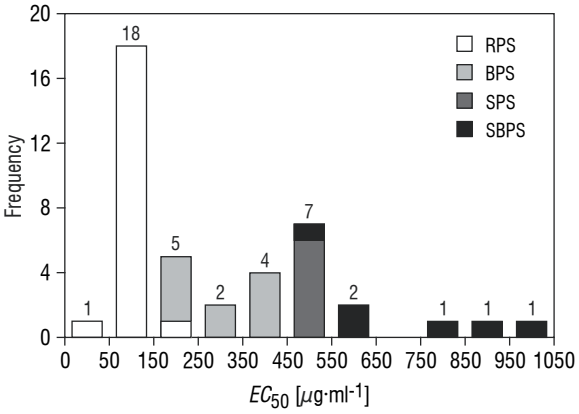

Fig. 1. Histogram with the frequency of EC_{50} values interval of pumpkin seeds.

Number of pumpkin seeds batches was 42.
RPS – raw pumpkin seeds, BPS – baked pumpkin seeds, SPS – shelled pumpkin seeds, SBPS – shelled and baked pumpkin seeds.

To further affirm the reliability of the results, we collected other 18 batches of pumpkin seeds (S25–S42) adding up to 42 batches of samples in total. That was 20 batches of RPS, 10 batches of BPS, 6 batches of SPS and 6 batches of SBPS (Tab. 1). To clearly reveal the distribution type and characteristics of the data, a histogram with the frequency of samples falling into the EC_{50} values interval was constructed (Fig. 1). It was observed that 19 batches of RPS fell into the interval of 0–150 $\mu g \cdot ml^{-1}$ of EC_{50} values. There were 10 batches of BPS and 1 batch of RPS between 150–450 $\mu g \cdot ml^{-1}$ of EC_{50} values. Six batches of SPS and 1 batch of SBPS fell into the interval of 450–550 $\mu g \cdot ml^{-1}$ and 5 batches of SBPS were between 550–1050 $\mu g \cdot ml^{-1}$ of EC_{50} values. The trend confirmed that RPS had strongest antioxidant activity among the samples of differently treated pumpkin seeds.

FRAP assay measures the Fe^{3+} to Fe^{2+} reducing potential of antioxidants under acidic conditions [32]. It is one of the simplest method for determining total antioxidant activity of medicinal plants [33]. As shown in Tab. 2, the reducing power of batch S1 (41.24 $mol \cdot kg^{-1}$) was approximately 2–3 times higher than S2 (20.68 $mol \cdot kg^{-1}$), S3 (16.47 $mol \cdot kg^{-1}$) and S4 (13.71 $mol \cdot kg^{-1}$), indicating that shells play an important role in the antioxidant activity of pumpkin seeds. The results were consistent with those determined by DPPH test.

Tab. 2. Ferric reducing power, total phenolic content and total flavonoid content of pumpkin seeds.

Sample code	AA_{FRAP} [mol·kg^{-1}]	*TPC* [g·kg^{-1}]	*TFC* [g·kg^{-1}]
S1	41.24 ± 0.45	1.50 ± 0.02	36.61 ± 0.25
S2	20.68 ± 0.32	0.53 ± 0.01	15.31 ± 0.11
S3	16.47 ± 0.26	0.52 ± 0.01	22.83 ± 0.11
S4	13.71 ± 0.21	0.41 ± 0.00	65.30 ± 0.11

Values are given as mean ± standard deviation of triplicate assays and the mean difference is significant at the 0.05 level.
S1 – raw pumpkin seeds, S2 – baked pumpkin seeds, S3 – shelled pumpkin seeds, S4 – shelled and baked pumpkin seeds.
AA_{FRAP} – antioxidant activity determined by ferric reducing antioxidant power assay (expressed as moles of $FeSO_4$), *TPC* – total phenolic content (expressed as grams of gallic acid equivalents), *TFC* – total flavonoid content (expressed as grams of rutin).

Total phenolic and flavonoid contents

Tab. 2 shows *TPC* and *TFC* of S1 to S4 samples, which differed significantly ($p < 0.05$) depending on the different processing methods. GAE values decreased in the order of S1 (1.50 g·kg^{-1}) > S2 (0.53 g·kg^{-1}) > S3 (0.52 g·kg^{-1}) > S4 (0.41 g·kg^{-1}). Batch S1 of RPS had the highest *TPC* value and was comparable with the oil-extract of pumpkin seeds previously reported by Veronezi and Jorge [34]. Moreover, *TPC* of RPS was approximately 3 times higher than of other samples, which demonstrated that *TPC* decreased sharply with the treatment of baking or shelling. Regarding results of *TFC* shown in Tab. 2, SBPS had the highest *TFC* of 65.30 g·kg^{-1}, followed by RPS (36.61 g·kg^{-1}) and SPS (22.83 g·kg^{-1}), while BPS had the lowest *TFC* of 15.31 g·kg^{-1}.

Correlation analysis

As is clear from Tab. 2, the highest *TPC* pertained to RPS (batch S1), which had the strongest antioxidant activity. It could be pointed out that there is a potential correlation between *TPC* and antioxidant activity.

Thus, Pearson correlation analysis was performed and a significant negative correlation ($P < 0.01$) was found between EC_{50} values (AA_{DPPH}) and *TPC*. Moreover, *TPC* positively correlated with AA_{FRAP} values ($P < 0.05$). The correlation coefficients were –0.997 and 0.988, respectively. It means that the higher *TPC*, the stronger the antioxidant capacity of pumpkin seeds. This is in accordance with the trend discussed previously by Xanthopoulou et al. [35].

In contrast, the correlation between EC_{50} values and *TFC* ($R = 0.120$) as well as AA_{FRAP} values and *TFC* ($R = –0.154$) was weak and had no statistical meaning. It indicated that flavonoids were not the main components in pumpkin seeds contributing to antioxidant activity, while the phenolic compounds were.

HPLC analysis

In order to better understand the correlation between the antioxidant activity and the constituents of pumpkin seeds, a reverse-phase HPLC analysis was performed under the optimized conditions. Firstly, peaks which appeared in all chromatograms of the samples treated with different processing methods were assigned as common peaks, 25 peaks being clearly recognized in total (Fig. 2). Then, multiple correlation analysis, a statistical analysis method that aims to study the correlation between two groups of variables, was applied to evaluate the composition-efficacy relationship. The partial correlation coefficient of the EC_{50} values and the peak area of 25 characteristic peaks was calculated (not shown). The results demonstrated that peaks 6, 11, 12, 14, 15, 17, 19, 21, 22, 23 and 25 with a negative partial correlation coefficient greater than 0.78 were closely re-

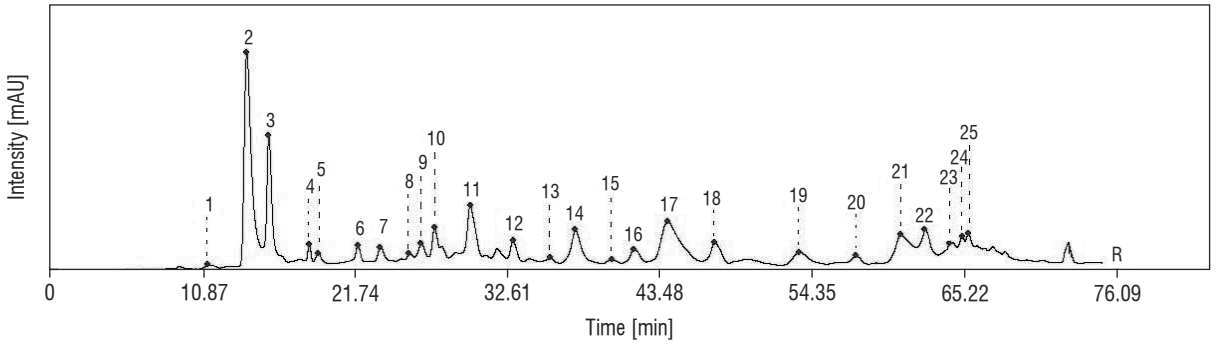

Fig. 2. Mean chromatogram showing 25 common peaks in batches S1–S4 of pumpkin seeds.

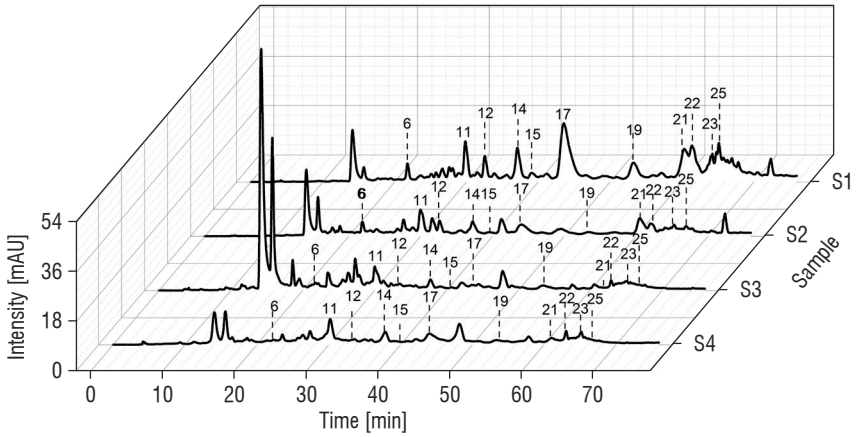

Fig. 3. Eleven chromatographic peaks of compounds connected with antioxidant activity of pumpkin seeds as identified by multiple correlation analysis.

S1 – raw pumpkin seeds, S2 – baked pumpkin seeds, S3 – shelled pumpkin seeds, S4 – shelled and baked pumpkin seeds.

lated to the antioxidant activity of the samples. These 11 peaks might be the potential antioxidant components that contribute to the antioxidant activity of pumpkin seeds (Fig. 3). In order to obtain more information on the active peaks in pumpkin seeds treated by different processing methods, we drew a histogram chart of the increase and decrease of peak areas of the eleven peaks (Fig. 4). It was observed that the peak areas of the eleven peaks of sample S1 were larger than those of S2, S3 and S4. Obviously, after pumpkin seeds were shelled and baked, most of these peak areas decreased, and correspondingly, the antioxidant activity weakened. So, we can come to the conclusion that processing methods of shelling and baking will negatively influence the antioxidant activity of pumpkin seeds. However, details of the chromatographic profiles as well as identity and antioxidant activity of the separated compounds need to be further studied.

NMR spectrometric analysis

Since RPS exhibited the strongest antioxidant activities and SBPS showed the weakest ones, ^{1}H-NMR and ^{13}C-NMR spectra were determined to compare their chemical profiles. In ^{1}H-NMR spectrum, there were signals ranging from δ of 6.0 ppm to 8.5 ppm assigned to benzene rings as shown in Fig. 5A and Fig. 5B. The signals in Fig. 5B were weaker, which indicated that SBPS contained lower amounts of phenolic constituents. Observing the ^{13}C-NMR spectrum (Fig. 5C), anomeric carbons of saccharide did not appear from δ of 90 ppm to 110 ppm, which suggested that the phenolic constituents of RPS contained no glycosides and were probably present as aglycons. Moreover, five signals of ester carbonyl (δ of 167.8 ppm, 168.9 ppm, 168.9 ppm, 169.4 ppm and 169.6 ppm) were found in the range of chemical shifts of δ of 160–175 ppm. It corresponded to carbonyl group (δ of 167.8 ppm, 167.8 ppm, 167.7 ppm, 167.8 ppm and 167.8 ppm) of five phenolic glycosides isolated from pumpkin seeds in our previous study [23]. It means that RPS might contain the analogues of those five compounds without sugar units. The obvious signals ranging from δ of 90 ppm to 110 ppm in Fig. 5D revealed the presence of anomeric carbons, indicating that SBPS might contain glycosides. Since the antioxidant activity of RPS was stronger than that of SBPS, it is speculated that saccharide weakened

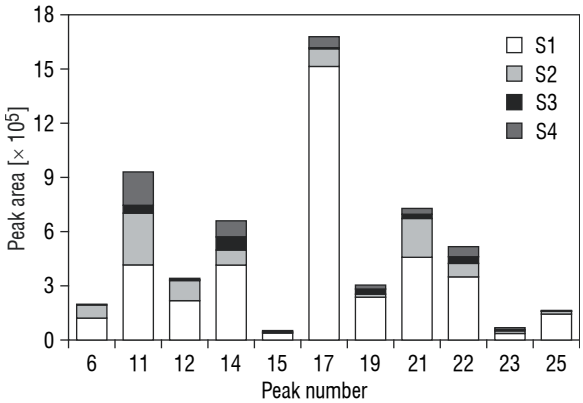

Fig. 4. Areas of the eleven chromatographic peaks of compounds connected with antioxidant activity of pumpkin seeds.

S1 – raw pumpkin seeds, S2 – baked pumpkin seeds, S3 – shelled pumpkin seeds, S4 – shelled and baked pumpkin seeds.

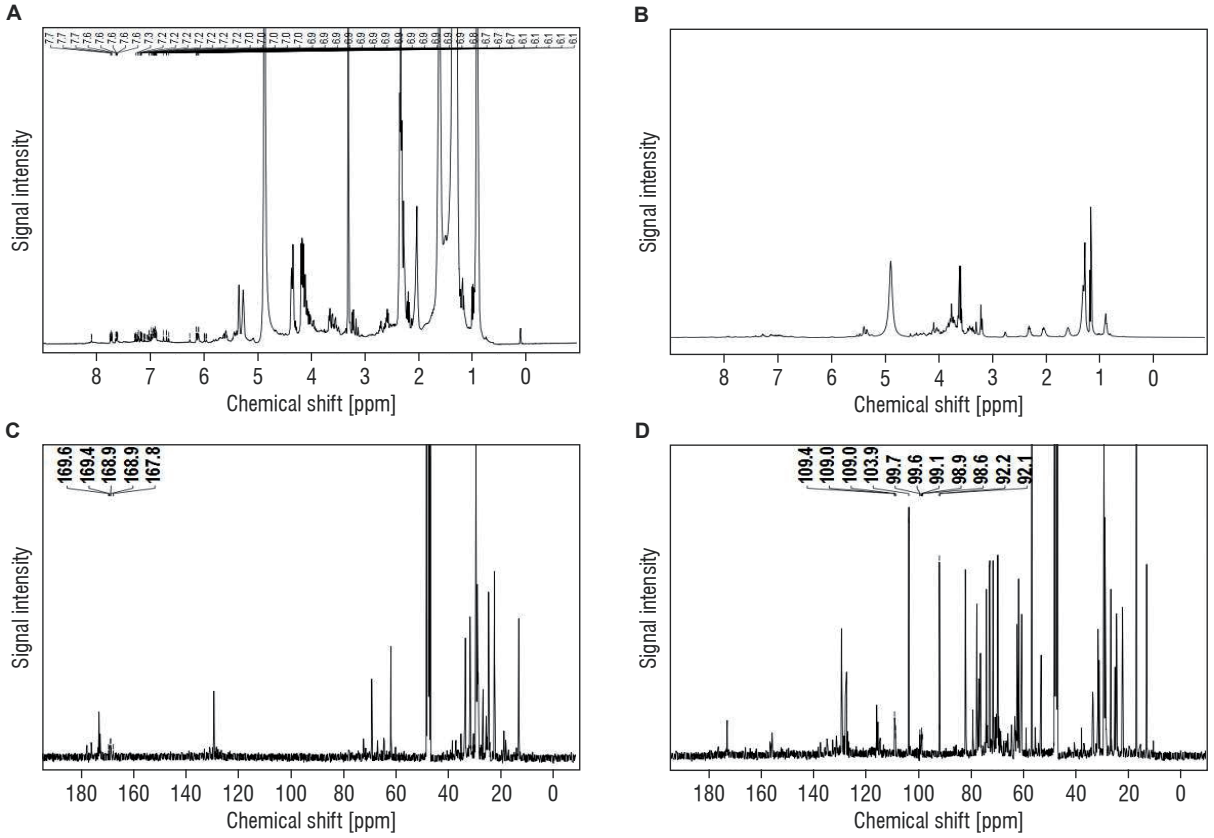

Fig. 5. ^{1}H– and ^{13}C–nuclear magnetic resonance spectra of pumpkin seeds.

A – ^{1}H-NMR spectrum of raw pumpkin seeds, B – ^{1}H-NMR spectrum of shelled and baked pumpkin seeds, C – ^{13}C-NMR spectrum of raw pumpkin seeds, D – ^{13}C-NMR spectrum of shelled and baked pumpkin seeds.

Tab. 3. In vitro hepatoprotective activities of pumpkin seeds.

Sample	Cell survival rate [%]
Normal cells	100
D-Galactosamine	58.1 ± 8.7^{c}
Bicyclol (positive control)	81.1 ± 5.9^{B}
S1	79.2 ± 9.8^{B}
S2	69.6 ± 12.4^{b}
S3	67.9 ± 11.7^{b}
S4	75.4 ± 9.9^{aA}

Cell survival rate is expressed as percentage of normal cells (cells without adding any pumpkin seeds sample). Values represent mean ± standard deviation (n = 3).
Lowercase letters in superscript indicate statistical significance in comparison with D-galactosamine group (a – $p < 0.05$, b – $p < 0.01$, c – $p < 0.001$).
Uppercase letters in superscript indicate statistical significance in comparison with normal cells (A – $p < 0.05$, B – $p < 0.01$).
S1 – raw pumpkin seeds, S2 – baked pumpkin seeds, S3 – shelled pumpkin seeds, S4 – shelled and baked pumpkin seeds.

the antioxidant activity and aglycons may play an important role in the enhancement of antioxidant activity.

Hepatoprotective activities

Makni et al. [10] reported that a mixture of flax and pumpkin seeds had hepatoprotective effects, which were probably mediated by unsaturated fatty acids present in the seed mixture. Since the hepatoprotective activities of non-oil extracts of pumpkin seeds were not previously reported, we evaluated in vitro hepatoprotective activities against D-galactosamine-induced toxicity in human hepatoma HepG2 cells of the samples processed by four different methods. As shown in Tab. 3, RPS (batch S1) exhibited the significant hepatoprotective activity (79.2 % of cell survival rate) at the test concentration comparable to that of the positive control (81.1 % of cell survival rate). Since RPS had the highest content of phenolic components and showed the strongest antioxidant activities, it suggested that phenols are important bioactive constituents contributing to the phar-

macological effectiveness of pumpkin seeds. At the same time, the hepatoprotective activities of pumpkin seeds may be closely related to their potent antioxidant effects.

CONCLUSIONS

This study focused on the phytochemical profiles, antioxidant and hepatoprotective activities of non-oil extracts of pumpkin seeds, being based on the comparison and evaluation of four different processing methods. Raw pumpkin seeds with the highest content of potentially antioxidant compounds, as determined by HPLC, had the highest *TPC* and exhibited the strongest antioxidant and hepatoprotective activities. In contrast, shelled or baked pumpkin seeds showed the weakest antioxidant activity. The results also indicated that cooking and dehulling decreased *TPC* and the content of potentially antioxidant compounds. Therefore, it could be suggested that consumers should prefer raw pumpkin seeds when using them for health care or treatment. This paper provides the knowledge useful for utilization of the non-oil part of pumpkin seeds and could be a reference for consumers' daily application or treatment of diseases.

REFERENCES

1. Caili, F. – Huan, S. – Quanhong, L.: A review on pharmacological activities and utilization technologies of pumpkin. Plant Foods for Human Nutrition, *61*, 2006, pp. 70–77. DOI: 10.1007/s11130-006-0016-6.
2. Fan, S. H. – Hu, Y. N. – Li, C. – Liu, Y. R.: Optimization of preparation of antioxidative peptides from pumpkin seeds using response surface method. PloS One, *9,* 2014, e92335. DOI: 10.1371/journal.pone.0092335.
3. Wang, L. – Liu, F. C. – Wang, A. X. – Yu, Z. Y. – Xu, Y. Q. – Yang, Y.: Purification, characterization and bioactivity determination of a novel polysaccharide from pumpkin (*Cucurbita moschata*) seeds. Food Hydrocolloids, *66*, 2017, pp. 357–364. DOI: 10.1016/j.foodhyd.2016.12.003.
4. Košťálová, Z. – Hromádková, Z. – Ebringerová, A.: Isolation and characterization of pectic polysaccharides from the seeded fruit of oil pumpkin (*Cucurbita pepo* L. var. *Styriaca*). Industrial Crops and Products, *31*, 2010, pp. 370–377. DOI: 10.1016/j.indcrop.2009.12.007.
5. Krimer-Malešević, V. – Mađarev-Popović, S. – Vaštag, Ž. – Radulović, L. – Peričin, D: Phenolic acids in pumpkin (*Cucurbita pepo* L.) seeds. In: Preedy, V. R. – Patel, V. B. – Watson, R. R. (Eds.): Nuts and seeds in health and disease prevention. Amsterdam : Academic Press, 2011, pp. 925–932. ISBN: 978-0-12-375688-6. DOI: 10.1016/B978-0-12-375688-6.10109-4.
6. Applequist, W. L. – Avula, B. – Schaneberg, B. T. – Wang, Y. H. – Khan, I. A.: Comparative fatty acid content of seeds of four *Cucurbita* species grown in a common (shared) garden. Journal of Food Composition and Analysis, *19*, 2006, pp. 606–611. DOI: 10.1016/j.jfca.2006.01.001.
7. Glew, R. H. – Glew, R. S. – Chuang, L. T. – Huang, Y. S. – Millson, M. – Constans, D. – Vanderjagt, D. J.: Amino acid, mineral and fatty acid content of pumpkin seeds (*Cucurbita* spp.) and *Cyperus esculentus* nuts in the Republic of Niger. Plant Foods for Human Nutrition, *61*, 2006, pp. 49–54. DOI: 10.1007/s11130-006-0010-z.
8. Qi, H. J. – Yang, Y. – Wang, L. B. – Xu, Y. Q. – Qu, S. P.: Determination of bioactive ingredients in five pumpkin seeds cultivars. Advanced Materials Research, *554–556*, 2012, pp. 985–989. DOI: 10.4028/www.scientific.net/amr.554-556.985.
9. Aziz, A. R. A. – AbouLaila, M. R. – Aziz, M. – Omar, M. A. – Sultan, K.: In vitro and in vivo anthelmintic activity of pumpkin seeds and pomegranate peels extracts against *Ascaridia galli*. Beni-Suef University Journal of Basic and Applied Sciences, *7*, 2018, pp. 231–234. DOI: 10.1016/j.bjbas.2018.02.003.
10. Makni, M. – Fetoui, H. – Gargouri, N. K. – Garoui, E. M. – Jaber, H. – Makni, J. – Boudawara, T. – Zeghal, N.: Hypolipidemic and hepatoprotective effects of flax and pumpkin seed mixture rich in ω-3 and ω-6 fatty acids in hypercholesterolemic rats. Food and Chemical Toxicology, *46*, 2008, pp. 3714–3720. DOI: 10.1016/j.fct.2008.09.057.
11. Nkosi, C. Z. – Opoku, A. R. – Terblanche S. E.: In vitro antioxidative activity of pumpkin seed (*Cucurbita pepo*) protein isolate and its in vivo effect on alanine transaminase and aspartate transaminase in acetaminophen induced liver injury in low protein fed rats. Phytotherapy Research, *20*, 2006, pp. 780–783. DOI: 10.1002/ptr.1958.
12. Gossell-Williams, M. – Hyde, C. – Hunter, T. – Simms-Stewart, D. – Fletcher, H. – McGrowder, D. – Walters, C. A.: Improvement in HDL cholesterol in postmenopausal women supplemented with pumpkin seed oil: pilot study. Climacteric, *14*, 2011, pp. 558–564. DOI: 10.3109/13697137.2011.563882.
13. Rezig, L. – Chouaibi, M. – Msaada, K. – Hamdi, S.: Chemical composition and profile characterization of pumpkin (*Cucurbita maxima*) seed oil. Industrial Crops and Products, *37*, 2012, pp. 82–87. DOI: 10.1016/j.indcrop.2011.12.004.
14. Zuhair, H. A. – Abd El-Fattah, A. A. – El-Sayed, M. I.: Pumpkin-seed oil modulates the effect of felodipine and captopril in spontaneously hypertensive rats. Pharmacological Research, *41*, 2000, pp. 555–563. DOI: 10.1006/phrs.1999.0622.
15. Nishimura, M. – Ohkawara, T. – Sato, T. – Takeda, H. – Nishihira, J.: Pumpkin seed oil extracted from *Cucurbita maxima* improves urinary disorder in human overactive bladder. Journal of Traditional and Complementary Medicine, *4*, 2014, pp. 72–74.

DOI: 10.4103/2225-4110.124355.
16. Medjakovic, S. – Hobiger, S. – Ardjomand-Woelkart, K. – Bucar, F. – Jungbauer, A.: Pumpkin seed extract: cell growth inhibition of hyperplastic and cancer cells, independent of steroid hormone receptors. Fitoterapia, *110*, 2016, pp. 150–156. DOI: 10.1016/j.fitote.2016.03.010.
17. Bharti, S. K. – Kumar, A. – Sharma, N. K. – Prakash, O. – Jaiswal, S. K. – Krishnan, S. – Gupta, A. K. – Kumar, A.: Tocopherol from seeds of *Cucurbita pepo* against diabetes: validation by in vivo experiments supported by computational docking. Journal of the Formosan Medical Association, *112*, 2013, pp. 676–690. DOI: 10.1016/j.jfma.2013.08.003.
18. Makni, M. – Fetoui, H. – Gargouri, N. K. – Garoui, E. M. – Zeghal, N.: Antidiabetic effect of flax and pumpkin seed mixture powder: effect on hyperlipidemia and antioxidant status in alloxan diabetic rats. Journal of Diabetes and its Complications, *25*, 2011, pp. 339–345. DOI: 10.1016/j.jdiacomp.2010.09.001.
19. Fahim, A. T. – Fattah, A. A. A.-E. – Agha, A. M. – Gad, M. Z.: Effect of pumpkin-seed oil on the level of free radical scavengers induced during adjuvant-arthritis in rats. Pharmacological Research, *31*, 1995, pp. 73–79. DOI: 10.1016/1043-6618(95)80051-4.
20. Nawirska-Olszańska, A. – Kita, A. – Biesiada, A. – Sokół-Łętowska, A. – Kucharska, A. Z.: Characteristics of antioxidant activity and composition of pumpkin seed oils in 12 cultivars. Food Chemistry, *139*, 2013, pp. 155–161. DOI: 10.1016/j.foodchem.2013.02.009.
21. Can-Cauich, C. A. – Sauri-Duch, E. – Moo-Huchin, V. M. – Betancur-Ancona, D. – Cuevas-Glory, L. F.: Effect of extraction method and specie on the content of bioactive compounds and antioxidant activity of pumpkin oil from Yucatan, Mexico. Food Chemistry, *285*, 2019, pp. 186–193. DOI: 10.1016/j.foodchem.2019.01.153.
22. Potočnik, T. – Cizej, M. R. – Košir, I. J.: Influence of seed roasting on pumpkin seed oil tocopherols, phenolics and antiradical activity. Journal of Food Composition and Analysis, *69*, 2018, pp. 7–12. DOI: 10.1016/j.jfca.2018.01.020.
23. Koike, K. – Li, W. – Liu, L. J.– Hata, E. – Nikaido, T.: New phenolic glycosides from the seeds of *Cucurbita moschata*. Chemical and Pharmaceutical Bulletin, *53*, 2005, pp. 225–228. DOI: 10.1248/cpb.53.225.
24. Li, S. Q. – Shah, N. P.: Effects of various heat treatments on phenolic profiles and antioxidant activities of *Pleurotus eryngii* extracts. Journal of Food Science, *78*, 2013, pp. C1122–C1129. DOI: 10.1111/1750-3841.12189.
25. Daud, M. N. H. – Fatanah, D. N. – Abdullah, N. – Ahmad, R.: Evaluation of antioxidant potential of *Artocarpus heterophyllus* L. J33 variety fruit waste from different extraction methods and identification of phenolic constituents by LCMS. Food Chemistry, *232*, 2017, pp. 621–632. DOI: 10.1016/j.foodchem.2017.04.018.
26. Carvalho, I. S. – Cavaco, T. – Brodelius, M.: Phenolic composition and antioxidant capacity of six *Artemisia* species. Industrial Crops and Products, *33*, 2011, pp. 382–388. DOI: 10.1016/j.indcrop.2010.11.005.
27. Al-Matani, S. K. – Al-Wahaibi, R. N. S. – Hossain, M. A.: Total flavonoids content and antimicrobial activity of crude extract from leaves of *Ficus sycomorus* native to Sultanate of Oman. Karbala International Journal of Modern Science, *1*, 2015, pp. 166–171. DOI: 10.1016/j.kijoms.2015.11.007.
28. Sharma, K. – Assefa, A. D. – Kim, S. – Ko, E. Y. – Lee, E. T. – Parka, S. W.: Evaluation of total phenolics, flavonoids and antioxidant activity of 18 Korean onion cultivars: a comparative study. Journal of the Science of Food and Agriculture, *94*, 2014, pp. 1521–1529. DOI: 10.1002/jsfa.6450.
29. Mosmann, T.: Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. Journal of Immunological Methods, *65*, 1983, pp. 55–63. DOI: 10.1016/0022-1759(83)90303-4.
30. Sun, Y. J. – Zhang, H. M. – Cheng, M. – Cao, S. J. – Qiao, M. – Zhang, B. L. – Ding, L. Q. – Qiu, F.: New hepatoprotective isoflavone glucosides from *Pueraria lobata* (Willd.) Ohwi. Natural Product Research, *33*, 2019, pp. 3485–3492. DOI: 10.1080/14786419.2018.1484461.
31. Jiao, J. – Li, Z. G. – Gai, Q. Y. – Li, X. J. – Wei, F. Y. – Fu, Y. J. – Ma, W.: Microwave-assisted aqueous enzymatic extraction of oil from pumpkin seeds and evaluation of its physicochemical properties, fatty acid compositions and antioxidant activities. Food Chemistry, *147*, 2014, pp. 17–24. DOI: 10.1016/j.foodchem.2013.09.079.
32. Bolling, B. W. – Dolnikowski, G. – Blumberg, J. B. – Chen, C. Y. O.: Polyphenol content and antioxidant activity of California almonds depend on cultivar and harvest year. Food Chemistry, *122*, 2010, pp. 819–825. DOI: 10.1016/j.foodchem.2010.03.068.
33. Jones, A. – Acquaviva, A. – Dennis, G. R. – Shalliker, R. A. – Soliven, A.: Bioactive screening of complex tea samples using the ferric reducing antioxidant power assay incorporating reaction flow HPLC columns for post column derivatisations. Microchemical Journal, *138*, 2018, pp. 197–202. DOI: 10.1016/j.microc.2018.01.005.
34. Veronezi, C. M. – Jorge, N.: Bioactive compounds in lipid fractions of pumpkin (*Cucurbita* sp.) seeds for use in food. Journal of Food Science, *77*, 2012, pp. C653–C657. DOI: 10.1111/j.1750-3841.2012.02736.x.
35. Xanthopoulou, M. N. – Nomikos, T. – Fragopoulou, E. – Antonopoulou, S.: Antioxidant and lipoxygenase inhibitory activities of pumpkin seed extracts. Food Research International, *42*, 2009, pp. 641–646. DOI: 10.1016/j.foodres.2009.02.003.

Received 16 January 2020; 1st revised 23 February 2020; accepted 27 February 2020; published online 5 March 2020.