Separation and Purification Technology 236 (2020) 116217

Contents lists available at ScienceDirect

Separation and Purification Technology

journal homepage: www.elsevier.com/locate/seppur

Subcritical water extraction, identification, antioxidant and antiproliferative activity of polyphenols from lotus seedpod

Zheng Yan[a], Haihui Zhang[a], Courage Sedem Dzah[a], Jixian Zhang[a], Chunren Diao[a], Haile Ma[a,b], Yuqing Duan[a,b,*]

[a] School of Food and Biological Engineering, Jiangsu University, Zhenjiang 212013, China
[b] Institute of Food Physical Processing, Jiangsu University, Zhenjiang 212013, China

ARTICLE INFO

Keywords:
Subcritical water extraction
Polyphenols
Lotus seedpod
Identification
Activity

ABSTRACT

In this study, polyphenols from lotus seedpod were extracted by subcritical water. Based on single factor experiments, the extraction conditions were optimized by response surface methodology (RSM). Under optimal conditions [temperature of 140 °C, time of 20 min, liquid-solid ratio of 70 mL/g and 4‰ (W/V) $NaHSO_3$], the maximal yeild of lotus seedpod polyphenols (LSPP) was 178.32 mg of gallic acid equivalents (mg GAE) per gram dry weight (g DW), significantly higher than that of hot water extraction (HWE). This study further investigated antioxidant activity of LSPP. The results showed that, compared with HWE, LSPP extracted by SWE had better reducing power and ability to scavenge $DPPH^{\cdot}$, $ABTS^{*+}$ and NO_2^-, ($P < 0.05$). In addition, the antiproliferative ability on human hepatoma G2 (HepG2) cells of LSPP extracted by SWE was also evaluated to be significant ($P < 0.05$). Moreover, the antioxidant and antiproliferative activity of LSPP were found to be positively correlated with the polyphenols concentration. Detailed HPLC-ESI-MS^n analyses with literatures and contrasted with authentic standards allowed the identification of 8 compounds (proanthocyanidin dimer 1, proanthocyanidin dimer 2, catechin, cyanidin-3-*O*-glucoside, quercetin-3-*O*-glucuronoside, isoquercetin, kaempferol-3-*O*-glucuronide and isorhamnetin) in LSPP (SWE). This work can provide a reference for the utilization of subcritical water extraction in the field of natural product extraction and the development and utilization of lotus seedpod as bioactive materials.

1. Introduction

Many studies have evaluated the beneficial effects of polyphenols on the human body. Polyphenols have many good physiological activities, such as antibacterial, antioxidant, anti-inflammatory, cardiovascular protection, immunoregulation, neuroprotective and antitumor among others [47,57,68,83,87,89]. The health benefits derived from polyphenols make foods rich in these compounds a preference for consumers.

Lotus seedpod is the mature receptacle of lotus (*Nelumbo nucifera*), rich in a variety of healthful compounds, especially polyphenols [16,89]. Duan et al. [17] obtained procyanidins from the lotus seedpod using acetone–water, and found that lotus seedpod procyanidins have good anti-radiation effects. Gong et al. [25] extracted the proanthocyanidins from the lotus seedpod with Me_2CO/H_2O and purified by Sephadex LH-20 column chromatography. Although lotus seedpods contain valuable compounds, they are usually discarded as waste during the processing of lotus seeds. This makes polyphenol recovery a promising area for by-product valorization.

Traditional polyphenol extraction procedures such as maceretion and Soxhlet extraction, are known for their low efficiency, low yield and potential environmental hazards due to their high demand for organic solvents (methanol, diethyl ether, ethyl acetate, acetone and ethanol) [5]. In recent years, scholars around the world have developed a number of auxiliary extraction techniques to overcome the shortcomings of traditional extraction methods, such as microwave, microjet, ultrasonic, subcritical, supercritical, ultra-high pressure and so on [15,21,22,45,45,46,64,80,82]. Subcritical water extraction (SWE) is an eco-friendly technology that is increasingly used as an alternative to traditional extraction techniques [52]. SWE takes place at temperatures between the boiling point and critical point of water (100 °C and 374.1 °C), at pressures high enough to keep water in the liquid state [40]. Under ambient conditions, water is considered to be a polar solvent and it cannot be used to extract moderately polar and non-polar compounds. This drawback of water can be overcome by subcritical water, which causes the decrease of dielectric constant and makes

* Corresponding author at: No. 301, Xuefu Road, Jingkou District, Zhenjiang City, Jiangsu Province, China.
E-mail address: dyq101@ujs.edu.cn (Y. Duan).

https://doi.org/10.1016/j.seppur.2019.116217
Received 9 December 2018; Received in revised form 2 September 2019; Accepted 14 October 2019
Available online 15 October 2019
1383-5866/

Table 1
Design of single factor experiment.

Single factor	Temperature (°C)	Time (min)	Liquid-solid ratio (mL/g)	$NaHSO_3$ addition amount (‰) (W/V)
Temperature	100, 120, 140, 160, 180.	10	30	1
Time	120	5, 10, 15, 20, 25.	30	1
Liquid-solid ratio	120	15	30, 40, 50, 60, 70.	1
$NaHSO_3$ addition amount	120	15	60	1, 2, 3, 4, 5, 6.

water suitable for the extraction of target compounds such as polyphenols [30,63]. In addition, subcritical water also has lower viscosity but higher diffusivity, which facilitates the diffusion into the plant matrix and the release of target compounds from the solid into liquid phase [74]. Some researches also have demonstrated that high pressure contributes to better extraction of the target compound [11,12]. In earlier studies, SWE has been used to extract many bioactive compounds, such as polysaccharides, protein, flavonoids, proanthocyanidins and polyphenols [19,23,39,44,66]. Compared with conventional extraction techniques, SWE has many advantages such as eco-friendly, high efficiency, cost-efficient and high yield [9,10,82], and shows a good application prospect.

Subcritical water extraction has been used to extract polyphenol compounds from many plant materials such as grape pomace [50], coriander (*Coriandrum sativum* L.) [85] and ginseng roots [90]. Interestingly, some studies have shown that the composition and biological activity of subcritical water extracts are different from those obtained by conventional techniques [20,44,45,48,90]. Švarc-Gajić et al. [73] found that the extract of the rhizome of ginger obtained by subcritical water has better antimicrobial (*Escherichia coli, Bacillus subtilis, Aspergillusniger, Klebsiella pneumonia, Staphylococcus aureus, Candida albicans, Proteus vulgaris and Proteus mirabilis*) and cytotoxic activity (murine fibroblast, human rhabdomyosarcoma and cervix carcinoma) than the extract obtained by boiling water at atmospheric pressure. Compared with classical extraction [water and three different ethanol/water solutions (30%, 50% and 70%)], *A. uva-ursi* herbal dust polyphenols obtained by SWE have higher total phenol and total flavonoid yields and better antioxidant ability [53]. Aliakbarian et al. [1] used three extraction techniques (subcritical water, ethanol extraction and conventional water) to extract phenolic compounds from grape pomace. Compared with traditional water extraction, subcritical water extracts have higher total phenolic content, total flavonoid content and superior anti-radical power. SWE achieves comparable levels of extraction compared to ethanol extraction, but is more efficient and environmentally friendly than ethanol extraction. Cvetanović et al. [9] compared the extracts of chamomile (*Matricaria chamomilla L*) obtained by four extraction techniques (ultrasonic assisted, subcritical water, microwave assisted and Soxhlet extraction), and the results showed that the extract of SWE has the highest yield, total phenolic content and antioxidant activity. However, according to previous literature, the use of subcritical water for the extraction of polyphenols from lotus seedpod has not been reported. In addition, there is a “poor” understanding of the effects of SWE conditions on the quality of polyphenols and the composition, antioxidant and antiproliferative ability.

Therefore, in order to develop a more effective extraction technique of polyphenols by subcritical water and better use lotus seedpod resources, we optimized the subcritical water extraction conditions of polyphenol from lotus seedpod, investigated the antioxidantive and antiproliferative ability of lotus seedpod polyphenols, and identified the composition of lotus seedpod polyphenols by HPLC-ESI-MS^n.

2. Materials and methods

2.1. Materials and chemicals

Fresh lotus seedpods were purchased from markets in Zhenjiang City (Jiangsu, China). The lotus seedpods were air-dried, milled, sieved through 100 meshes and adequately packaged, and then stored at −20 °C for further study [2,43,82].

AB-8 macroporous resin was purchased from Cangzhou Baoen Adsorption Material Technology Co., Ltd (Cangzhou, Hebei China). ABTS (2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), DPPH (2,2-diphenyl-1- picrylhydrazyl), ferric trichloride, potassium ferricyanide, Folin-Ciocalteu reagent, ethanol, sodium bisulfite, ferric trichloride, potassium ferricyanide and other reagents (analytical grade) were purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Dimethyl sulfoxide (DMSO) and methyl thiazolyl tetrazolium (MTT) were purchased from Sigma Company (USA). 1640 medium was purchased from Hyclone Company (USA). Catechin purchased from National Institutes for Food and Drug Control (Beijing, China). Gallic acid, quercetin-3-*O*-glucuronoside and isoquercetin (chromatographic grade) were purchased from Nanjing Spring & Autumn Biological Engineering Co., Ltd (Nanjing, China).

2.2. Apparatus

Subcritical water extractor (Zhenjiang Dantu Huanqiu Accessory Factory, China); UV-1601 UV–Vis spectrophotometer (Beijing Rayleigh Analytical Instruments, China); ALPHAI-4/2-4 freeze dryer (CHRIST, German); HPLC-MS system (Thermo LXQ, American); Infinite PRO TWIN 200 multi-functional microplate reader (Tecan, Switzerland)

2.3. Single factor extraction experiments

This study explored the effect of four factors (Temperature, time, liquid-solid ratio and $NaHSO_3$ addition) on the yield of LSPP. Single factor experimental design was shown in Table 1. Single factor experiments were used as basis for possible extraction ranges for RSM.

2.4. Experimental design for the RSM.

According to the results of the single factor experiment, a reasonable parameter range was selected for RSM. The Box-Behnken experimental design was followed for the optimization of the four process variables at 3 levels with 29 runs (Table 2).

2.5. Hot water extraction (HWE)

Hot water extraction condition: the extraction temperature was 100 °C, the extraction time was 20 min, the liquid-solid ratio was 70 mL/g and the $NaHSO_3$ addition amount was 4‰ (W/V) [45,82].

Table 2
Factors and levels of subcritical water extraction parameters.

Level	X_1	X_2	X_3	X_4
	Temperature (°C)	Time (min)	liquid-solid ratio (mL/g)	$NaHSO_3$ addition amount (‰) (W/V)
−1	100	10	50	4
0	120	15	60	5
1	140	20	70	6

2.6. Total polyphenol content (TPC) determination

TPC was measured using the Folin-Ciocalteu method described by Singleton and Rossi [71], (slightly modified). 4 mL of Folin-Ciocalteu solution (10-fold dilution) was added to 1 mL of the sample solution, followed by the addition of sodium carbonate solution (5 mL, 7.5%). After standing for 2 h (25 °C), the absorbance (765 nm) was measured. Gallic acid was used as the standard. Result was expressed as milligram of gallic acid equivalents (mg GAE) per gram dry weight (g DW).

2.7. Total flavonoid content (TFC) determination

The total flavonoid content was measured as described by Jia et al. [33] and Jiang et al. [34] with minor modification. 4 mL of distilled water was added to 1 mL of sample solution, followed by the addition of $NaNO_2$ (5%, 0.3 mL). After standing at 25 °C for 6 min, $AlCl_3$ (10%, 0.3 mL) was added. After 6 min, the mixture was mixed with NaOH (4%, 4 mL). Finally, 0.4 mL of distilled water was added to the mixture and the absorbance was measured at 510 nm. Rutin was used as a standard and results were expressed as milligram rutin equivalent (mg RE) per gram dry weight (g DW).

2.8. Preparation of LSPP

The insoluble impurities in the extract prepared by SWE or HWE were removed by centrifugation (4000 r/min, 8 min) and suction filtration. After that, the extract was further purified using an AB-8 macroporous resin adsorption chromatography column. After the sample was loaded, it was washed with distilled water to remove impurities such as proteins and sugars. Then, it was eluted with 5 column volumes (Flow rate: 2 mL/min) of ethanol solution (70%, V/V), and the eluate was collected. The eluate was concentrated by reducing pressure and freeze-dried by vacuum. Finally, LSPP (extracted by SWE and HWE, respectively) was obtained [43,82].

2.9. Antioxidant activity analysis

2.9.1. Reducing power (RP) measurement

The ability of the extracts to reduce Fe^{3+} was assayed by the method of Hafsi et al. [29] and Oyaizu [55]. Briefly, 1 mL of sample solution was mixed with 2.5 mL of phosphate buffer (0.2 mol/L, pH 6.6) and 2.5 mL of $K_3Fe(CN)_6$. After incubation at 5 °C for 25 min, 2.5 mL of trichloroacetic acid (10%) was added and the mixture was centrifuged at 3000 r/min for 10 min. Finally, 2.5 mL of the upper layer was mixed with 2.5 mL of distilled water and 0.5 mL of an aqueous $FeCl_3$ solution (0.1%). The absorbance was measured at 700 nm. Higher absorbance indicates higher reducing power.

2.9.2. DPPH free radical-scavenging activity

The $DPPH^{\cdot}$ scavenging capacity of LSPP was measured according to the method of Blois [4] with minor modification. Briefly, 2 mL of sample solution was mixed with 2 mL of 200 μmol/L $DPPH^{\cdot}$ solution. The reaction mixture was incubated for 30 min at room temperature in the dark. The absorbance of the resulting solution was measured with a spectrophotometer at 517 nm. Control was prepared as above without any extract. The free radical scavenging capacity of the test sample is measured as a decrease in $DPPH^{\cdot}$ absorbance and was calculated using the following formula:

$$Scavengingrate(\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

2.9.3. $ABTS^{*+}$ radical-scavenging activity

The $ABTS^{*+}$ radical scavenging capacity of LSPP was measured according to the method of Re et al. [61] and Vijaya et al. [77] with minor modification. Briefly, the $ABTS^{*+}$ radical solution (4 mL) was added to 200 μL of the sample solution, mixed thoroughly and incubated for 6 min in the darkness at room temperature. The absorbance of the reaction mixture was measured at 734 nm. Control was prepared as above without any extract. The free radical scavenging capacity of the test sample was measured as the decrease in $ABTS^{*+}$ radical absorbance and was calculated using the following equation:

$$Scavengingrate(\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

2.9.4. NO_2^- scavenging activity

The NO_2^- scavenging capacity of LSPP was measured according to the method of Wang [78] with minor modifications. 2 mL of 5 mL/g solution of $NaNO_2$ was added to the test tube, followed by the addition of 2 mL of the sample solution. After incubation at 25 °C for 30 min, 1 mL of 4 g/L sulfanilic acid solution was added, mixed well and incubated again at 25 °C for 5 min. After incubation, 1 mL of 2 g/L Naphthylenediamine hydrochloride solution was added, followed by 4 mL of distilled water and incubated at 25 °C for 15 min. After this, the absorbance was measured at 538 nm. Control was prepared as above without any extract and distilled water was used instead of the sample solution. NO_2^- scavenging capacity was calculated using the following equation:

$$Scavengingrate(\%) = \left(\frac{A_{control} - A_{sample}}{A_{control}}\right) \times 100$$

2.10. Antiproliferative activity on HepG2

Human hepatoma G2 (HepG2) cells were cultured in 1640 medium (1% streptomycin and penicillin) containing 10% fetal calf serum, and cultured in a humid environment (37 °C) containing 5% CO_2.

Antiproliferative activity was tested by MTT assay [51,82]. HepG2 cells were incubated in 96-well plates for 24 h (5×10^4 cells/well). Then, the cells were cultured for 24 h at different concentrations (25, 50, 100, 150, 200 and 400 μg/mL) of LSPP solution (100 μL). After that, MTT solution with concentration of 1 mg/mL (100 mL) was added and incubated (37 °C, 4 h). Then, DMSO (150 μL) was added and absorbance was measured at 570 nm (multi-functional microplate reader). The antiproliferative capacity of LSPP was calculated according to the following formula:

$$Antiproliferationrate(\%) = \left(\frac{A_c - A_t}{A_c}\right) \times 100$$

where A_t is the absorbance value of the sample group; A_c is the absorbance value of the control group.

2.11. Analysis of polyphenols by HPLC-ESI-MS^n

HPLC analysis was carried out with a reversed-phase column (ZORBAXSB-C18). A 0.1% aqueous acetic acid solution (A) and acetonitrile (B) were used as the mobile phases, respectively. The following gradients were used for elution: 0 ~ 5 min, 5% B; 5–20 min, 5–15% B; 20–35 min, 15–25% B; 35–50 min, 25–35% B; 50–55 min, 35–50% B; 55–60 min, 50–5% B. The sample detection wavelength was 280 nm and the total flow rate of the mobile phase was 1.0 mL/min [82].

ESI-MS analysis used negative ion mode for detection. The gas used for drying and atomization was nitrogen. Detection was recorded across the range m/z 50–2000.

2.12. Statistical analysis

All extractions and measurements were performed in triplicate. The results were expressed as mean values and standard deviation (SD).

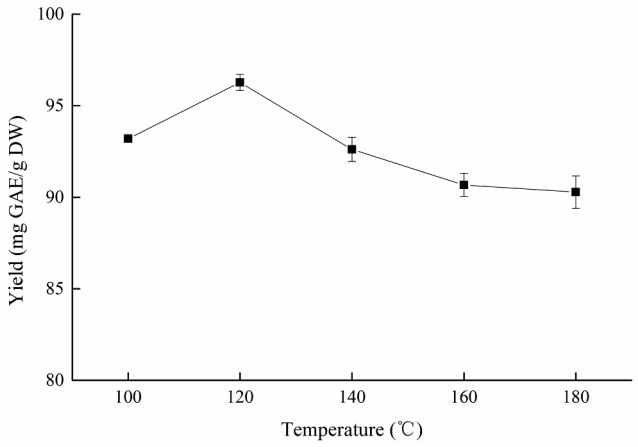

Fig. 1. Effect of extraction temperature on the yield of LSPP.

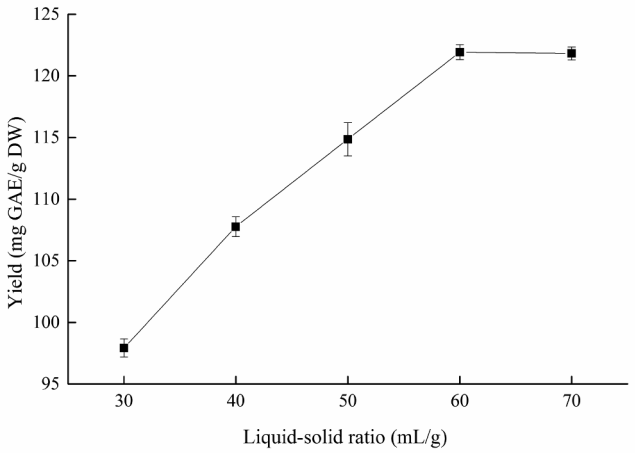

Fig. 3. Effect of extraction liquid-solid ratio on the yield of LSPP.

Origin 9.0 was used to process experimental data. Design-Expert 8.0.6 was used for analysis of variance (ANOVA) of RSM test results.

3. Results and discussion

3.1. Optimization of subcritical water extraction process

3.1.1. Effect of extraction temperature on the yield of LSPP.

Temperature is the key factor of subcritical extraction. As shown in Fig. 1, significant effects ($P < 0.05$) of the extraction temperature on the yield of LSPP could be observed in the temperature range of 100–180 ℃. The yield increased with the increase of extraction temperature, the highest yield was 96.27 mg GAE/g DW, but when the temperature exceeded 120 ℃, the yield began to decease with further temperature increase. This is attributed to the fact that the dielectric constant and polarity of water decrease with the increase of temperature, which is conducive to the dissolution of non-polar compounds such as polyphenols [40,24]. However, after 120 ℃, high temperatures have an adverse effect on polyphenols, leading to degradation of thermally unstable polyphenols [45,67]. Therefore, 120 ℃ was selected as the extraction temperature for RSM experiments.

3.1.2. Effect of extraction time on the yield of LSPP

As shown in Fig. 2, the yield increased at first and then decreased with the extension of extraction time ($P < 0.05$). The highest yield (98.05 mg GAE/g DW) was achieved at 15 min. Within 5–15 min, the yield of polyphenols increased with the extraction time increased. However, when the extraction time was over 15 min, some thermally unstable polyphenols degraded under high temperature conditions [26,45]. Therefore, when the extraction time is longer than 15 min, the yield of polyphenol decreased. Therefore, 15 min was chosen as the optimum extraction time for RSM experiments.

3.1.3. Effect of extraction liquid-solid ratio on the yield of LSPP

Fig. 3 showed the effect of liquid-solid ratio on the yield ($P < 0.05$). The highest yield was obtained at the liquid-solid ratio of 60 mL/g. Further increase in the liquid-solid ratio resulted in a plateau phenomenon where no further increases in polyphenol yields were observed. The increase in the liquid-solid ratio enhances penetration of solvent and increases contact area between target component and solvent [26,82]. However, this was up to a threshold, beyond which no further extractions were significant. This result has the same trend as the findings of Wang et al. [79]. The liquid-solid ratio of 60 mL/g was selected as the optimal parameter for RSM experiments.

3.1.4. Effect of $NaHSO_3$ addition amount on the yield of LSPP

Overall, the experimental results (Fig. 4) showed that $NaHSO_3$ is a good auxiliary extractant and has a significant effect on yield ($P < 0.05$). The highest yield was obtained for 5‰ $NaHSO_3$ addition amount (W/V), but decreased slightly for 6‰ (W/V). This is attributed to the fact that $NaHSO_3$ can react with dissolved oxygen in water, avoiding the oxidation of polyphenols [75]. Nevertheless, excessive addition of $NaHSO_3$ will have an adverse effect on polyphenols, which is consistent with the results of Zhang et al. [86] and Yan et al. [82]. Therefore, the $NaHSO_3$ addition amount of 5‰ (W/V) was preferred as

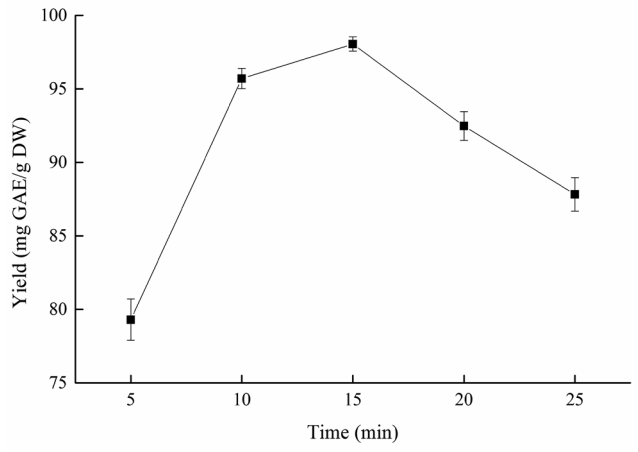

Fig. 2. Effect of extraction time on the yield of LSPP.

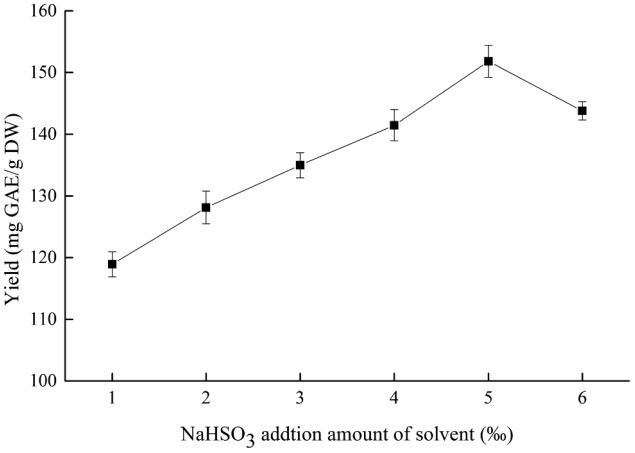

Fig. 4. Effect of extraction $NaHSO_3$ addition amount on the yield of LSPP.

Table 3
Coded values for Box-Behnken design and experimentally observed responses.

Run[a] order	Coded (Independent variables)				Yield (Y) (mg GAE/g DW)
	X_1	X_2	X_3	X_4	
1	−1	0	0	1	152.24
2	0	0	1	1	152.85
3	1	−1	0	0	157.68
4	0	0	−1	−1	133.53
5	0	1	1	0	156.83
6	1	0	1	0	164.82
7	0	−1	−1	0	143.52
8	1	0	−1	0	149.03
9	0	1	−1	0	135.88
10	0	0	0	0	143.76
11	−1	0	−1	0	142.98
12	0	−1	1	0	146.61
13	0	0	0	0	144.19
14	0	1	0	1	148.14
15	0	0	1	−1	153.85
16	−1	1	0	0	148.42
17	0	−1	0	1	151.64
18	−1	0	0	−1	144.31
19	−1	0	1	0	150.32
20	1	0	0	−1	155.02
21	0	0	0	0	142.76
22	1	0	0	1	159.85
23	0	0	0	0	144.31
24	0	0	0	0	145.09
25	0	0	−1	1	148.41
26	−1	−1	0	0	145.69
27	0	1	0	−1	144.85
28	0	−1	0	−1	142.08
29	1	1	0	0	154.83

[a] Randomized.

the appropriate amount for RSM experiments.

3.1.5. Experimental results of RSM optimization

Table 3 shows the LSPP yields (Y) under different SWE conditions. The yield as a function of the independent variables was expressed by the following regression equation: $Y = 144.02 + 4.77X_1 + 0.15X_2 + 5.99X_3 + 3.29X_4 - 1.39X_1X_2 + -2.11X_1X_3 - 0.78X_1X_4 + 4.47X_2X_3 - 1.57X_2X_4 - 3.97X_3X_4 + 6.83{X_1}^2 + 0.70{X_2}^2 + 1.01{X_3}^2 + 2.03{X_4}^2$. Statistical testing of the regression equation was checked by F-test, and ANOVA for the fitted quadratic polynomial model of yield were shown in Table 4. The regression variance model is extremely significant ($P < 0.01$) and three factors (X_1, X_3 and X_4) have significantly ($P < 0.01$) effect on the yield. The results of response surface variance analysis showed that, the lack of fit of the equation is not significant ($P > 0.05$), and the correction coefficients are respectively $R^2 = 0.9955$, $R^2_{Adj} = 0.9911$, suggesting the model is accurate, so it can be used to analyze and predict the process of SWE for LSPP. The combinations of X_1X_2, X_1X_3, X_2X_3, X_2X_4 and X_3X_4 showed extremely significant ($P < 0.01$) interactions, and X_1X_4 also had significant ($P < 0.05$) interactions. This work used Design Expert 8.0.6 software to calculate the regression equation and found that the optimal conditions obtained for extracting polyphenols by subcritical water were: temperature of 139.99 °C, time of 19.97 min, liquid-solid ratio of 70 mL/g and 4‰ (W/V) $NaHSO_3$. The predicted value of yield was 173.67 mg GAE/g DW. Based on the actual conditions, the predicted optimal conditions were adjusted to: temperature of 140 °C, time of 20 min, liquid-solid ratio of 70 mL/g and 4‰ (W/V) $NaHSO_3$. Under these conditions, three parallel experiments were performed to verify the validity of the model equation, the yield of polyphenols was 178.32 mg GAE/g DW, and the error was 4.65 mg GAE/g DW.

Temperature is the key factor of subcritical water extraction. Our research group has extracted polyphenols from sorghum bran using subcritical water and the optimized temperature for SWE was 144.5 °C [45]. Cvetanović et al. [14] used subcritical water to extract polyphenols from chamomile. The results showed that the dominant phenolic compound apigenin achieved the maximum yield at 115 °C, which was lower than this research. Polyphenols of sage (*Salvia officinalis* L.) herbal dust were extracted with subcritical water and the optimum extraction temperature for total polyphenols yield was 201.5 °C, which was higher than this research [58]. Aliakbarian et al. [1] used subcritical water to extract polyphenols from grape pomace. The optimum extraction temperature was 140 °C, which was similar with this research. These may be due to that different plant material matrix and extraction system results in the difference in optimal subcritical water extraction temperature for polyphenols.

3.1.6. The yield of LSPP by conventional extraction (HWE)

The yield of LSPP extracted by HWE is 146.15 mg GAE/g DW, which is significantly lower than SWE. This is attributed to the fact that subcritical water has a higher temperature and a lower polarity, which facilitates the dissolution of target compounds such as polyphenols [37,42,54]. Besides, high temperatures reduce the viscosity and surface tension of water, which contributes to an increase in molecular diffusion rate [60,72,82].

3.2. Antioxidant activity of LSPP

In order to evaluate the antioxidant activity of LSPP more comprehensively, four methods were used for evaluation. Previous researches have shown that lotus seedpod polyphenols have good antioxidant activity [36,81]. According to Table 5, LSPP showed good antioxidant ability ($P < 0.05$). Compared with HWE, LSPP extracted by SWE had better reducing power and ability to scavenge $DPPH^{\cdot}$, $ABTS^{*+}$ and ${NO_2}^-$.

The reducing power indirectly reflects the antioxidant capacity of polyphenols which is measured by their ability to convert the Fe^{3+}/ferricyanide complex to the ferrous form [43]. As shown in Table 5, as the polyphenol concentration increased, the reducing power of the polyphenol also increased. These results showed that LSPP possessed significant reducing power ($P < 0.05$). When an antioxidant (proton donating substances) was encountered, $DPPH^{\cdot}$ accepts electron or hydrogen radical to become stable diamagnetic molecule [70]. Polyphenols scavenge $DPPH^{\cdot}$ radicals in a concentration-dependent manner. When the polyphenol concentration was 7.5 μg/mL, the $DPPH^{\cdot}$ scavenging rates were 49.9% (SWE) and 43.4% (HWE), respectively. In addition, we also found that the $ABTS^{*+}$ radical scavenging rates of LSPP increased with increasing concentration, when the LSPP concentration was 50 μg/mL, the $ABTS^{*+}$ scavenging rates reached 62.4% (SWE) and 57.2% (HWE), respectively. Besides, LSPP scavenges ${NO_2}^-$ in a concentration-dependent manner with superior scavenging effects, when the polyphenol concentration was 100 μg/mL, the ${NO_2}^-$ scavenging rates reached 52.8% (SWE) and 48.3% (HWE), respectively, (Table 5). In summary, the lotus seedpod polyphenols obtained by SWE have better antioxidant activity than by HWE, and the antioxidant capacity is positively correlated with the concentration.

Xiao et al. [81] used boiling distilled water to extract lotus seedpod proanthocyanidins and evaluated their antioxidant capacity. When the polyphenol concentration was 9.10 ± 0.11 μg/mL, $DPPH^{\cdot}$ scavenging rate was 50%, and the scavenging ability was lower than SWE. Kim and Shin [36] obtained lotus seedpod extracts by 80% ethanol. $DPPH^{\cdot}$ and $ABTS^{*+}$ radical scavenging capacities of the water fraction of lotus seedpod extracts were 94.5% and 95.2% at 0.8 mg/mL, and the scavenging ability was also lower than SWE. This indicates that subcritical water extraction technology is very promising in the field of polyphenol extraction. Wang et al. [79] extracted anthocyanins from *Lycium ruthenicum Murr* using subcritical water. When the anthocyanin concentration was 100 μg/mL, the scavenging rates of $DPPH^{\cdot}$ and $ABTS^{*+}$ were 85.97% and 69.81%, respectively, and the scavenging ability was lower than LSPP. Cvetanović et al. [13] used subcritical

Table 4
Analysis of variance (ANOVA) for second-order polynomial model for all investigated responses.

Source	Sum of squares	df[a]	Mean square	F-value	p-Value	Significant level
Model	1325.77	14	94.70	223.30	< 0.0001	**
X_1	273.26	1	273.26	644.37	< 0.0001	**
X_2	0.25	1	0.25	0.60	0.4526	
X_3	431.17	1	431.17	1016.74	< 0.0001	**
X_4	129.99	1	129.99	306.52	< 0.0001	**
X_1X_2	7.78	1	7.78	18.34	0.0008	**
X_1X_3	17.84	1	17.84	42.06	< 0.0001	**
X_1X_4	2.41	1	2.41	5.69	0.0318	*
X_2X_3	79.78	1	79.78	188.13	< 0.0001	**
X_2X_4	9.85	1	9.85	23.22	0.0003	**
X_3X_4	63.10	1	63.10	148.78	< 0.0001	**
X_1^2	302.61	1	302.61	713.57	< 0.0001	**
X_2^2	3.20	1	3.20	7.55	0.0157	*
X_3^2	6.64	1	6.64	15.66	0.0014	**
X_4^2	26.70	1	26.70	62.97	< 0.0001	**
Residual	5.94	14	0.42			
Lack of Fit	3.02	10	0.30	0.41	0.8824	Not significant
Pure Error	2.92	4	0.73			
Cor Total	1331.71	28				
$R^2 = 0.9955$;	$R^2_{Adj} = 0.9911$					

* $P < 0.05$ is significant.
** $P < 0.01$ is extremely significant.

Table 5
Antioxidant activity of polyphenols in lotus seedpod.

Antioxidant capacity assays	Concentration (μg/mL)	Extraction method	
		SWE	HWE
Reducing power (absorbance at 700 nm)	12.5	0.158 ± 0.005	0.147 ± 0.007
	25	0.243 ± 0.008	0.209 ± 0.009
	50	0.419 ± 0.013	0.346 ± 0.008
	75	0.564 ± 0.01	0.482 ± 0.005
	100	0.71 ± 0.013	0.595 ± 0.023
DPPH· Scavenging rate (%)	2.5	19.7 ± 0.56	17.1 ± 0.31
	5	34.4 ± 0.55	31.4 ± 1.49
	7.5	49.9 ± 0.28	43.4 ± 1.20
	10	60.3 ± 0.71	52.5 ± 0.19
	15	76.3 ± 0.39	70.6 ± 1.11
ABTS*+ Scavenging rate (%)	12.5	23.5 ± 0.59	22.9 ± 0.54
	25	40.5 ± 0.23	36.9 ± 1.35
	50	62.4 ± 2.23	57.2 ± 0.65
	75	82.1 ± 0.74	75.1 ± 0.69
	100	94.4 ± 0.53	88.9 ± 0.45
NO_2^- Scavenging rate (%)	25	31.9 ± 0.82	26.7 ± 1.37
	50	36.9 ± 1.88	30.5 ± 2.08
	75	46.5 ± 1.96	42.8 ± 0.97
	100	52.8 ± 0.21	48.3 ± 0.63
	200	63.8 ± 0.80	57.1 ± 1.07
	400	67.4 ± 1.21	63.2 ± 1.03

water to extract polyphenolic compounds from *Aronia melanocarpa* leaves. The IC_{50} values of DPPH· and ABTS*+ radicals scavenging rates were 30.45 ± 0.94 μg/mL and 32.94 ± 0.54 μg/mL, respectively. DPPH· scavenging ability is lower than LSPP, but ABTS*+ scavenging ability is better than LSPP. All these findings indicate that lotus seedpod is a good source of antioxidants.

3.3. Antiproliferation activity of LSPP extracted by SWE on HepG2

Our research group has demonstrated that lotus seedpod procyanidins have good antiproliferative activity on mouse B16 melanoma cells [16]and human hepatoma G2 cells [18]. In the present study, we also found that, LSPP extracted by SWE has a good antiproliferative effect on HepG2 cells (Fig. 5, $P < 0.05$). When the polyphenols concentration was 200 μg/mL, the antiproliferation rate reached 57.21%. As the

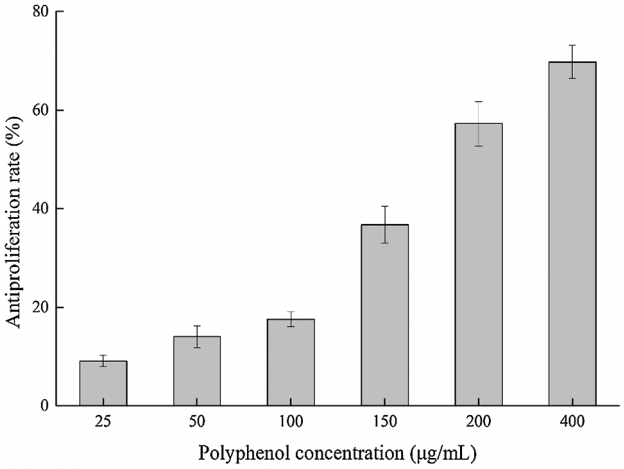

Fig 5. Antiproliferative ability of LSPP on HepG2.

concentration of polyphenols increased, the antiproliferative ability of LSPP was better as HepG2 cells proliferation was inhibited in a concentration-dependent manner, which are consistent with previous studies [45,46]. This indicates that lotus seedpod is a promising source of antiproliferative components. Many researches have also demonstrated that each compound in LSPP has significant antiproliferative activity on HepG2 cells [3,8,27,28,32,49,56,62,82,84]. Jain et al. [32] found that catechin and its liposomes could induce apoptosis in HepG2 cells. Pi et al. [59] demonstrated that quercetin could induce severe apoptosis in HepG2 cells through arrest of cell cycle and disruption of mitochondria membrane potential. Azab et al. [3] discovered that quercetin and kaempferol derivatives exhibited significant cytotoxicity against HepG2 cells. Pace et al. [56] found that cyanidin-3-*O*-glucoside inhibited HepG2 cell proliferation in a time- and dose-dependent manner, and its thermal degradation products and primary metabolites in vivo also produced significant antiproliferative effects on HepG2 cells. You et al. [84] found that isoquercetin has a significant growth inhibitory effect on HepG2 cells. Guo et al. [27] demonstrated by experiments that the antiproliferative activity of *Hippophae rhamnoides L.* extracts on HepG2 cells was related to its flavonoid aglycones (isorhamnetin, quercetin and kaempferol). These findings may help explain our results and

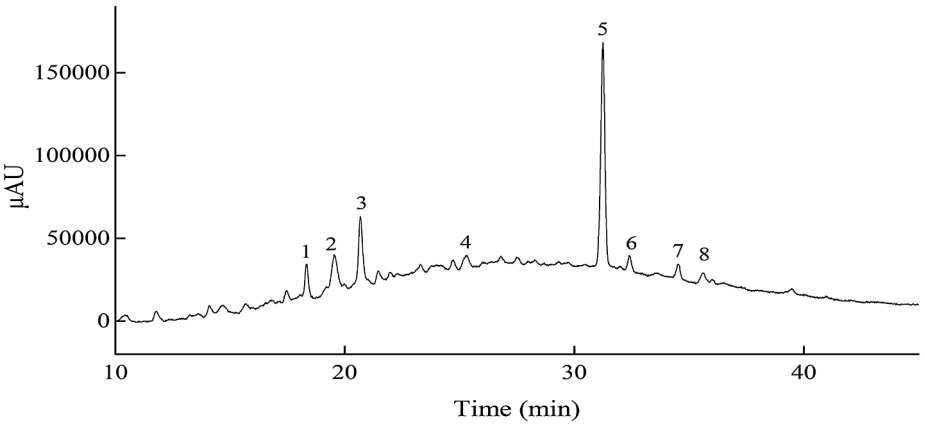

Fig. 6. HPLC chromatogram of LSPP (280 nm).

require further research. In addition, previous researches have also demonstrated that the bioactive polyphenolic components obtained from other plant materials using subcritical water also have good antiproliferative capacity on tumour cells [13,14,45,82].

3.4. Identification of LSPP extracted by SWE via HPLC-ESI-MS^n

Fig. 6 shows the chromatograms of LSPP marked peaks (1–8) following an elution order. Detailed HPLC-ESI-MS^n analyses with literature was carried out and contrasted with authentic standards (catechin, quercetin-3-*O*-glucuronoside and isoquercetin) for the identification of 8 polyphenol compounds in lotus seedpod. MS^n information and molecular structure of 8 polyphenol compounds are also shown in Table 6.

For compound 1 (t_R = 18.35 min), MS gave four fragments at *m/z* 577.09, *m/z* 288.96, *m/z* 425.04 and *m/z* 407.14, respectively (Table 6). The presence of ($[M-H]^-$) fragment ion at *m/z* 577.09 suggested that the molecular mass of compound 1 was 578. For compound 2 (t_R = 19.55 min), MS gave four fragments at *m/z* 577.01, *m/z* 288.98, *m/z* 425.00 and *m/z* 407.14, respectively (Table 6). The presence of ($[M-H]^-$) fragment ion at *m/z* 577.01 suggested that the molecular mass of compound 2 was 578. Therefore, compound 1 and 2 were isomers. By comparison with the literature [65], compound 1 and 2 were identified as proanthocyanidin dimer 1 and 2, respectively.

According to Table 6, compound 3 (t_R = 20.68 min) exhibited a molecular ion ($[M-H]^-$) at *m/z* of 288.97, and three MS^2 fragments (244.89, 204.92 and 178.93 *m/z*). Based on mass spectrometry information and compared to a real standard, compound 3 can be identified as catechin [35,82].

Compound 4 (t_R = 25.27 min) produced the molecular ion ($[M-H]^-$) at *m/z* of 449.12. MS^2 gave three fragments at *m/z* of 287.18, 259.21 and 269.27 (Table 6). The fragment of 287.18 *m/z* was a cyanidin moiety [41] resulted from the loss of hexose (162 units, glucose or galactose) [7,41,82]. Compound 4 was identified as cyanidin-3-*O*-glucoside. The identification of compound 4 was consistent with that of Zhang et al. [88].

Compound 5 with t_R = 31.25 min, molecular ion ($[M-H]^-$) at *m/z* 476.98 and one MS^2 fragment (301 *m/z*). Compound 6 with t_R = 32.39 min, molecular ion ($[M-H]^-$) at *m/z* 462.95 and one MS^2 fragment (301 *m/z*). MS^2 = 301 *m/z* was identified as quercetin, which had been described by Burri et al. [6]. Therefore, compounds 5 and 6 were derivatives of quercetin (loss of 176 and 162 units, respectively). Thus, they were identified as quercetin-3-*O*-glucuronoside [38] and isoquercetin [31]respectively by contrasting with authentic standard.

Compound 7 (t_R = 34.51 min) showed the molecular ion ($[M-H]^-$) at *m/z* of 460.94 and one fragment at *m/z* of 284.98 (loss of 176 units) (Table 6), which suggested that the molecular mass of compound 7 was 462. Therefore, compound 7 was identified as kaempferol-3-*O*-glucuronide, in agreement with the findings of Viacava et al. [76].

Compound 8 (t_R = 35.60 min) showed the molecular ion ($[M-H]^-$) at *m/z* of 315.2 and one fragment at *m/z* of 300.22 (Table 6), which indicated that the molecular mass of compound 8 was 316. MS^2 = *m/z* 300.22 was obtained by lose of a moiety (methyl group, 15 units). Compound 8 was identified as isorhamnetin, which was consistent with the results of Simirgiotis et al. [69].

3.5. Total polyphenol and flavonoid content of LSPP extracted by SWE and HWE

The total polyphenol and total flavonoid content of LSPP obtained by SWE and HWE were shown in Table 7. The TPC and TFC of SWE are significantly higher than HWE. This is advantageous in explaining macroscopically the reason why the antioxidant activity of LSPP obtained by SWE is higher than that of HWE.

4. Conclusion

In this study, single factor experiments were used as basis for RSM. Response surface method was applied successfully for the optimization of SWE conditions to obtain the polyphenols from lotus seedpod. Under

Table 6
Chromatographic properties and main MS peaks of polyphenols from lotus seedpod.

Peak	t_R (min)	MW	$[M-H]^-$ (*m/z*)	Typical MS^2 ions (*m/z*)	Identification
1	18.35	578	577.09	288.96, 425.04, 407.14	Proanthocyanidin dimer 1 [65]
2	19.55	578	577.01	288.98, 425.00, 407.14	Proanthocyanidin dimer 2 [65]
3	20.68	290	288.97	244.89, 204.92, 178.93	Catechin [35]
4	25.27	449	449.12	287.18, 259.21, 269.27	Cyanidin-3-*O*-glucoside [88]
5	31.25	478	476.98	301	Quercetin-3-*O*-glucuronoside [38]
6	32.39	464	462.95	301	Isoquercetin [31]
7	34.51	462	460.94	284.98	Kaempferol-3-*O*-glucuronide [76]
8	35.60	316	315.2	300.22	Isorhamnetin [69]

Table 7
Total polyphenol and flavonoid content of LSPP (SWE and HWE).

Extraction technique	Total polyphenol content (TPC) (mg GAE/g DW)	Total flavonoid content (TFC) (mg RE/g DW)
SWE	815.4 ± 1.12	1012.05 ± 5.88
HWE	785.6 ± 5.14	932.56 ± 2.22

optimal conditions, the yield of polyphenols was 178.32 mg GAE/g DW, significantly higher than that of hot water extraction (HWE). Furthermore, Lotus seedpod polyphenols extracted by SWE has better antioxidant activity than by HWE. Lotus seedpod polyphenols obtained by subcritical water also exhibited excellent and significant anti-proliferative ability on HepG2 cells. In addition, eight polyphenols were identified by HPLC-ESI-MS^n. This work can be a reference for the development of lotus seedpod as bioactive materials, and the utilization of high-efficiency and eco-friendly subcritical water in the field of phenolic compound extraction. Moreover, we may further purify the LSPP in future studies to elucidate the composition and structure-activity relationships of LSPP.

Declaration of Competing Interest

The authors declare no competing financial interests or any others regarding this study.

Acknowledgment

The authors are grateful for National Natural Science Foundation of China (31201456, 31371734), Key Research and Development Plan of Jiangsu Province (BE2016350) and the Priority Academic Program Development of Jiangsu Higher Education Institutions (PAPD).

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.seppur.2019.116217.

References

[1] B. Aliakbarian, A. Fathi, P. Perego, F. Dehghani, Extraction of antioxidants from winery wastes using subcritical water, J. Supercrit. Fluids 65 (2012) 18–24.
[2] A. Antoniolli, A.R. Fontana, P. Piccoli, R. Bottini, Characterization of polyphenols and evaluation of antioxidant capacity in grape pomace of the cv, Malbec. Food Chem. 178 (2015) 172–178.
[3] S.S. Azab, M. Abdel-Daim, O.A. Eldahshan, Phytochemical, cytotoxic, hepatoprotective and antioxidant properties of Delonix regia leaves extract, Med. Chem. Res. 22 (9) (2013) 4269–4277.
[4] M.S. Blois, Antioxidants determination by the use of a stable free radical, Nature 4617 (1958) 1199–1200.
[5] M.E. Brglez, H.M. Knez, M. Škerget, Z. Knez, U. Bren, Polyphenols: extraction methods, antioxidative action, bioavailability and anticarcinogenic effects, Molecules 21 (7) (2016) 901.
[6] S.C.M. Burri, A. Ekholm, A. Hakansson, E. Tornberg, K. Rumpunen, Antioxidant capacity and major phenol compounds of horticultural plant materials not usually used, J Funct Foods. 38 (2017) 119–127.
[7] C. Carazzone, D. Mascherpa, G. Gazzani, A. Papetti, Identification of phenolic constituents in red chicory salads (*Cichorium intybus*) by high-performance liquid chromatography with diode array detection and electrospray ionisation tandem mass spectrometry, Food Chem. 138 (2–3) (2013) 1062–1071.
[8] M. Chen, H. Meng, Y. Zhao, F. Chen, S. Yu, Antioxidant and in vitro anticancer activities of phenolics isolated from sugar beet molasses, BMC Complement. Altern. Med. 15 (2015) 313.
[9] A. Cvetanović, J. Švarc-Gajić, P. Mašković, S. Savić, L. Nikolić, Antioxidant and biological activity of chamomile extracts obtained by different techniques: perspective of using superheated water for isolation of biologically active compounds, Ind. Crop. Prod. 65 (2015) 582–591.
[10] A. Cvetanović, J. Švarc-Gajić, Z. Zeković, P. Mašković, S. Đurović, G. Zengin, C. Delerue-Matos, J. Lozano-Sánchez, A. Jakšić, Chemical and biological insights on aronia stems extracts obtained by different extraction techniques: From wastes to functional products, J. Supercrit. Fluids. 128 (2017) 173–181.
[11] A. Cvetanović, J. Švarc-Gajić, U. Gašić, Ž. Tešić, G. Zengin, Z. Zeković, S. Đurović, Isolation of apigenin from subcritical water extracts: Optimization of the process, J. Supercrit. Fluids 120 (2017) 32–42.
[12] A. Cvetanović, J. Švarc-Gajić, Z. Zeković, U. Gašić, Ž. Tešić, G. Zengin, P. Mašković, M.F. Mahomoodally, S. Đurović, Subcritical water extraction as a cutting edge technology for the extraction of bioactive compounds from chamomile: Influence of pressure on chemical composition and bioactivity of extracts, Food Chem. 266 (2018) 389–396.
[13] A. Cvetanović, G. Zengin, Z. Zeković, J. Švarc-Gajić, S. Ražić, A. Damjanović, P. Mašković, M. Mitić, Comparative in vitro studies of the biological potential and chemical composition of stems, leaves and berries Aronia melanocarpa's extracts obtained by subcritical water extraction, Food Chem. Toxicol. 121 (2018) 458–466.
[14] A. Cvetanović, J. Švarc-Gajić, Z. Zeković, J. Jerković, G. Zengin, U. Gašić, Ž. Tešić, P. Mašković, C. Soares, M. Fatima Barroso, C. Delerue-Matos, S. Đurović, The influence of the extraction temperature on polyphenolic profiles and bioactivity of chamomile (*Matricaria chamomilla L.*) subcritical water extracts, Food Chem. 271 (2019) 328–337.
[15] F. Dahmoune, B. Nayak, K. Moussi, H. Remini, K. Madani, Optimization of microwave- assisted extraction of polyphenols from Myrtus communis L. leaves, Food Chem. 166 (2015) 585–595.
[16] Y. Duan, H. Zhang, F. Xu, B. Xie, X. Yang, Y. Wang, Y. Yan, Inhibition effect of procyanidins from lotus seedpod on mouse B16 melanoma in vivo and in vitro, Food Chem. 122 (1) (2010) 84–91.
[17] Y. Duan, H. Zhang, B. Xie, Y. Yan, J. Li, F. Xu, Y. Qin, Whole body radioprotective activity of an acetone–water extract from the seedpod of *Nelumbo nucifera Gaertn*, Seedpod. Food Chem. Toxicol. 48 (2010) 3374–3384.
[18] Y. Duan, H. Xu, X. Luo, H. Zhang, Y. He, G. Sun, X. Sun, Procyanidins from Nelumbo nucifera Gaertn. Seedpod induce autophagy mediated by reactive oxygen species generation in human hepatoma G2 cells, Biomed. Pharmacother. 79 (2016) 135–152.
[19] K.S. Duba, A.A. Casazza, H.B. Mohamed, P. Perego, L. Fiori, Extraction of polyphenols from grape skins anddefatted grape seeds using subcritical water: experiments and modeling, Food Bioprod. Process. 94 (2015) 29–38.
[20] S. Erşan, Ö.G. Üstündağ, R. Carle, R.M. Schweiggert, Subcritical water extraction of phenolic and antioxidant constituents from pistachio (*Pistacia vera L.*) hulls, Food Chem. 253 (2018) 46–54.
[21] F.A. Espinosa-Pardo, V.M. Nakajima, G.A. Macedo, J.A. Macedo, J. Martínez, Extraction of phenolic compounds from dry and fermented orange pomace using supercritical CO_2 and cosolvents, Food Bioprod. Process. 101 (2017) 1–10.
[22] J.P. Fan, J. Cao, X.H. Zhang, J.Z. Huang, T. Kong, S. Tong, Z.Y. Tian, Y.L. Xie, R. Xu, J.H. Zhu, Optimization of ionic liquid based ultrasonic assisted extraction of puerarin from Radix Puerariae Lobatae by response surface methodology, Food Chem. 135 (4) (2012) 2299–2306.
[23] M. García-Marino, J.C. Rivas-Gonzalo, E. Ibáñez, C. García-Moreno, Recovery of catechins and proanthocyanidins from winery by-products using subcritical water extraction, Anal. Chim. Acta 563 (1–2) (2016) 44–50.
[24] P. Putnik, F.J. Barba, I. Španić, Z. Zorić, V. Dragović-Uzelac, D. Bursać Kovačević, Green extraction approach for the recovery of polyphenols from Croatian olive leaves (Olea europea), Food Bioprod. Process. 106 (2017) 19–28.
[25] Y. Gong, L. Liu, B. Xie, Y. Liao, E. Yang, Z. Sun, Ameliorative effects of lotus seedpod proanthocyanidins on cognitive deficits and oxidative damage in senescence-accelerated mice, Behav. Brain Res. 194 (2008) 100–107.
[26] Y. Gong, X.F. Zhang, L. He, Q.L. Yan, F. Yuan, Optimization of subcritical water extraction parameters of antioxidant polyphenols from sea buckthorn (*Hippophaë rhamnoides L.*) seed residue, J. Food Sci. Technol. 52 (3) (2015) 1534–1542.
[27] R. Guo, X. Guo, T. Li, X. Fu, R.H. Liu, Comparative assessment of phytochemical profiles, antioxidant and antiproliferative activities of Sea buckthorn *(Hippophae rhamnoides L.)* berries, Food Chem. 221 (2017) 997–1003.
[28] H. Guo, W. Lin, X. Zhang, X. Zhang, Z. Hu, L. Li, Z. Duan, J. Zhang, R. Feng, Kaempferol induces hepatocellular carcinoma cell death via endoplasmic reticulum stress-CHOP-autophagy signaling pathway, Oncotarget 8 (47) (2017) 82207–82216.
[29] C. Hafsi, H. Falleh, M. Saada, M. Rabhi, K. Mkadmini, R. Ksouri, C. Abdelly, A. Smaoui, Effects of potassium supply on growth, gas exchange, phenolic composition, and related antioxidant properties in the forage legume Sulla carnosa, Flora 223 (2016) 38–45.
[30] K. Hartonen, K. Inkala, M. Kangas, M.L. Riekkola, Extraction of polychlorinated biphenyls with water under subcritical conditions, J. Chromatogr. A 785 (1997) 219–226.
[31] G.H. Jang, Heon W. Kim, M.K. Lee, S.Y. Jeong, A.R. Bak, D.J. Lee, J.B. Kim, Characterization and quantification of flavonoid glycosides in the Prunus genus by UPLC- DAD-QTOF/MS, Saudi J. Biol. Sci. 25 (8) (2018) 1622–1631.
[32] P. Jain, N. Kumar, V.R. Josyula, H.V. Jagani, N. Udupa, C. Mallikarjuna Rao, P. Vasanth Raj, A study on the role of (+)-catechin in suppression of HepG2 proliferation via caspase dependent pathway and enhancement of its in vitro and in vivo cytotoxic potential through liposomal formulation, Eur. J. Pharm. Sci. 50 (3–4) (2013) 353–365.
[33] Z. Jia, M. Tang, J. Wu, The determination of flavonoid contents in mulberry and their scavenging effects on superoxide radicals, Food Chem. 64 (1999) 555–559.
[34] G.H. Jiang, S.H. Nam, S.H. Yim, Y.M. Kim, H.G. Gwak, J.B. Eun, Changes in total phenolic and flavonoid content and antioxidative activities during production of juice concentrate from asian pears *(Pyrus pyrifolia Nak*ai), Food Sci. Biotechnol. 25 (1) (2016) 47–51.
[35] Y. Jiao, D. Chen, M. Fan, S. Young Quek, UPLC-QqQ-MS/MS-based phenolic quantification and antioxidant activity assessment for thinned young kiwifruits, Food Chem. 281 (2019) 97–105.
[36] M.J. Kim, H.S. Shin, Antioxidative effect of lotus seed and seedpod extracts, Food Sci. Biotechnol. 21 (6) (2012) 1761–1766.

[37] W.J. Kim, B. Veriansyah, Y.W. Lee, J. Kim, J.D. Kim, Extraction of mangiferin from Mahkota Dewa (*Phaleria macrocarpa*) using subcritical water, J. Ind. Eng. Chem. 16 (3) (2010) 425–430.
[38] S. Kumar, A. Singh, B. Kumar, Identification and characterization of phenolics and terpenoids from ethanolic extracts of Phyllanthus species by HPLC-ESI-QTOF-MS/MS, J. Pharm. Anal. 7 (4) (2017) 214–222.
[39] M.J. Ko, H.L. Kwon, M.S. Chung, Pilot-scale subcritical water extraction of flavonoids from satsuma mandarin (*Citrus unshiu Markovich*) peel, Innovative Food Sci. Emerg. Technol. 38 (2016) 175–181.
[40] D. Lachos-Perez, A.M. Baseggio, P.C. Mayanga-Torres, M.R. Maróstica, M.A. Rostagno, J. Martínez, T. Forster-Carneiro, Subcritical water extraction of flavanones from defatted orange peel, J. Supercrit. Fluids 138 (2018) 7–16.
[41] J.H. Lee, M.G. Choung, Identification and characterisation of anthocyanins in the antioxidant activity-containing fraction of Liriope platyphylla fruits, Food Chem. 127 (4) (2011) 1686–1693.
[42] B. Li, Y. Yang, Y. Gan, C.D. Eaton, P. He, A.D. Jones, On-line coupling of subcritical water extraction with high-performance liquid chromatography via solid-phase trapping, J. Chromatogr. A 873 (2000) 175–184.
[43] Y. Liu, S.S. Ma, S.A. Ibrahim, E.H. Li, H. Yang, W. Huang, Identification and antioxidant properties of polyphenols in lotus seed epicarp at different ripening stages, Food Chem. 185 (2015) 159–164.
[44] X.P. Luo, Y.Q. Duan, W.Y. Yang, H.H. Zhang, C.Z. Li, J.X. Zhang, Structural elucidation and immunostimulatory activity of polysaccharide isolated by subcritical water extraction from Cordyceps militaris, Carbohydr. Polym. 157 (2017) 794–802.
[45] X.P. Luo, J.M. Cui, H.H. Zhang, Y.Q. Duan, Subcritical water extraction of polyphenolic compounds from sorghum (*Sorghum bicolor L.*) bran and their biological activities, Food Chem. 262 (2018) 14–20.
[46] X.P. Luo, J.M. Cui, H.H. Zhang, Y.Q. Duan, D. Zhang, M.H. Cai, G.Y. Chen, Ultrasound assisted extraction of polyphenolic compounds from red sorghum (*Sorghum bicolor L.*) bran and their biological activities and polyphenolic compositions, Ind. Crop. Prod. 112 (2018) 296–304.
[47] S. Martin, E. Gonzalez-Burgos, M.E. Carretero, M.P. Gomez-Serranillos, Neuroprotective properties of Spanish red wine and its isolated polyphenols on astrocytes, Food Chem. 128 (1) (2011) 40–48.
[48] P. Mašković, V. Veličković, M. Mitić, S. Đurović, Z. Zeković, M. Radojković, A. Cvetanović, J. Švarc-Gajić, J. Vujić, Summer savory extracts prepared by novel extraction methods resulted in enhanced biological activity, Ind. Crop. Prod. 109 (2017) 875–881.
[49] L. Menghini, L. Leporini, N. Scanu, G. Pintore, R. La Rovere, E.S. Di Filippo, T. Pietrangelo, S. Fulle, Effect of phytochemical concentrations on biological activities of cranberry extracts, J. Biol. Regul. Homeost. Agents 25 (1) (2011) 27–35.
[50] J.K. Monrad, K. Srinivas, L.R. Howard, J.W. King, Design and optimization of a semicontinuous hot-cold extraction of polyphenols from grape pomace, J. Agric. Food. Chem. 60 (22) (2012) 5571–5582.
[51] T. Mosmann, Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays, J. Immunol. Methods 65 (1983) 55–63.
[52] M.T. Munir, H. Kheirkhah, S. Baroutian, S.Y. Quek, B.R. Young, Subcritical water extraction of bioactive compounds from waste onion skin, J. Cleaner Prod. 183 (2018) 487–494.
[53] A. Naffati, J. Vladić, B. Pavlić, R. Radosavljević, A. Gavarić, S. Vidović, Recycling of filter tea industry by-products: Application of subcritical water extraction for recovery of bioactive compounds from *A. uva-ursi* herbal dust, J. Supercrit. Fluids 121 (2017) 1–9.
[54] E.S. Ong, J.S.H. Cheong, D. Goh, Pressurized hot water extraction of bioactive or marker compounds in botanicals and medicinal plant materials, J. Chromatogr. A 1112 (1–2) (2006) 92–102.
[55] M. Oyaizu, Studies on products of browning reactions: antioxidative activities of product of browning reaction prepared from glucosamine, Jpn. J. Nutrit. 44 (1986) 307–315.
[56] E. Pace, Y. Jiang, A. Clemens, T. Crossman, H.P.V. Rupasinghe, Impact of thermal degradation of cyanidin-3-*O*-glucoside of haskap berry on cytotoxicity of hepatocellular carcinoma HepG2 and breast cancer MDA-MB-231 cells, Antioxidants (Basel) 7 (2018) 2.
[57] C. Pasten, N.C. Olave, L. Zhou, E.M. Tabengwa, P.E. Wolkowicz, H.E. Grenett, Polyphenols downregulate PAI-1 gene expression in cultured human coronary artery endothelial cells: molecular contributor to cardiovascular protection, Thromb Res. 121 (1) (2007) 59–65.
[58] B. Pavlić, S. Vidović, J. Vladić, R. Radosavljević, M. Cindrić, Z. Zeković, Subcritical water extraction of sage (*Salvia officinalis L.*) by-products—Process optimization by response surface methodology, J. Supercrit. Fluids 116 (2016) 36–45.
[59] J. Pi, B. Li, L. Tu, H. Zhu, H. Jin, F. Yang, H. Bai, H. Cai, J. Cai, Investigation of quercetin-induced HepG2 cell apoptosis-associated cellular biophysical alterations by atomic force microscopy, Scanning 38 (2) (2016) 100–112.
[60] L. Ramos, E.M. Kristenson, U.A.T. Brinkman, Current use of pressurised liquid extraction and subcritical water extraction in environmental analysis, J. Chromatogr. A 975 (2002) 3–29.
[61] R. Re, N. Pellegrini, A. Proteggente, A. Pannala, M. Yang, C. Rice-Evans, Antioxidant activity applying an improved ABTS radical cation decolorization assay, Free Radical Biol. Med. 26 (1999) 1231–1237.
[62] A. Ribas-Latre, J.M. Del Bas, L. Baselga-Escudero, E. Casanova, A. Arola-Arnal, M.J. Salvadó, C. Bladé, L. Arola, Dietary proanthocyanidins modulate the rhythm of BMAL1 expression and induce RORα transactivation in HepG2 cells, J. Funct. Food. 13 (2015) 336–344.
[63] I. Rodríguez-Meizoso, F.R. Marin, M. Herrero, F.J. Señorans, G. Reglero, A. Cifuentes, E. Ibáñez, Subcritical water extraction of nutraceuticals with antioxidant activity from oregano. Chemical and functional characterization, J. Pharm. Biomed. Anal. 41 (5) (2006) 1560–1565.
[64] P. Rodsamran, R. Sothornvit, Extraction of phenolic compounds from lime peel waste using ultrasonic-assisted and microwave-assisted extractions, Food Biosci. 28 (2019) 66–73.
[65] P.J. Sarnoski, J.V. Johnson, K.A. Reed, J.M. Tanko, S.F. O'Keefe, Separation and characterisation of proanthocyanidins in Virginia type peanut skins by LC–MS^n, Food Chem. 131 (3) (2012) 927–939.
[66] I. Sereewatthanawut, S. Prapintip, K. Watchiraruji, M. Goto, M. Sasaki, A. Shotipruk, Extraction of protein and amino acids from deoiled rice bran by subcritical water hydrolysis, Bioresour. Technol. 99 (2008) 555–561.
[67] A. Tomšik, B. Pavlić, J. Vladić, M. Cindrić, P. Jovanov, M. Sakač, A. Mandić, S. Vidović, Subcritical water extraction of wild garlic (Allium ursinum L.) and process optimization by response surface methodology, The Journal of Supercritical Fluids 128 (2017) 79–88.
[68] C.Y. Shen, J.G. Jiang, C.L. Huang, W. Zhu, C.Y. Zheng, Polyphenols from blossoms of *citrus aurantium L. var. amara Engl.* show significant anti-complement and anti-inflammatory effects, J. Agric. Food Chem. 65 (41) (2017) 9061–9068.
[69] M.J. Simirgiotis, J. Bórquez, G. Schmeda-Hirschmann, Antioxidant capacity, polyphenolic content and tandem HPLC–DAD–ESI/MS profiling of phenolic compounds from the South American berries Luma apiculata and L. chequén, Food Chem. 139 (2013) 289–299.
[70] J.P. Singh, A. Kaur, N. Singh, L. Nim, K. Shevkani, H. Kaur, D.S. Arora, In vitro antioxidant and antimicrobial properties of jambolan (*Syzygium cumini*) fruit polyphenols, LWT – Food Sci. Technol. 65 (2016) 1025–1030.
[71] V.L. Singleton, J.A. Rossi, Colorimetry of total phenolics with phosphomo-lybdic-phosphotungstic acid reagents, Am. J. Enol. Vitic. 16 (3) (1965) 144–158.
[72] R.M. Smith, Extractions with superheated water, J. Chromatogr. A 975 (2002) 31–46.
[73] J. Švarc-Gajić, A. Cvetanović, A. Segura-Carretero, I.B. Linares, P. Mašković, Characterisation of ginger extracts obtained by subcritical water, J. Supercrit. Fluids 123 (2017) 92–100.
[74] C.C. Teo, S.N. Tan, J.W. Yong, C.S. Hew, E.S. Ong, Pressurized hot water extraction (PHWE), J. Chromatogr. A 1217 (16) (2009) 2484–2494.
[75] H.K. Trivedi, M.C. Patel, A stability indicating method for the determination of the antioxidant sodium bisulfite in pharmaceutical formulation by RP-HPLC technique, Sci. Pharm. 55 (2011) 4395–4406.
[76] G.E. Viacava, S.I. Roura, D.M. Lopez-Marquez, L.A. Berrueta, B. Gallo, R.M. Alonso-Salces, Polyphenolic profile of butterhead lettuce cultivar by ultrahigh performance liquid chromatography coupled online to UV–visible spectrophotometry and quadrupole time-of-flight mass spectrometry, Food Chem. 260 (2018) 239–273.
[77] K.R.C. Vijaya, D. Sreeramulu, M. Raghunath, Antioxidant activity of fresh and dry fruits commonly consumed in India, Food Res. Int. 43 (2010) 285–288.
[78] S.C. Wang, Food hygiene Inspection Technology, Chemical Industry Press, Beijing, 1998.
[79] Y. Wang, G. Luan, W. Zhou, J. Meng, H. Wang, N. Hu, Y. Suo, Subcritical water extraction, UPLC-Triple-TOF/MS analysis and antioxidant activity of anthocyanins from *Lycium ruthenicum Murr*, Food Chem. 249 (2018) 119–126.
[80] J. Xi, D. Shen, S. Zhao, B. Lu, Y. Li, R. Zhang, Characterization of polyphenols from green tea leaves using a high hydrostatic pressure extraction, Int. J. Pharm. 382 (1–2) (2009) 139–143.
[81] J. Xiao, S. Li, Y. Sui, X. Li, Q. Wu, R. Zhang, M. Zhang, B. Xie, Z. Sun, In vitro antioxidant activities of proanthocyanidins extracted from the lotus seedpod and ameliorative effects on learning and memory impairment in scopolamine-induced amnesia mice, Food Sci. Biotechnol. 24 (4) (2015) 1487–1494.
[82] Z. Yan, X. Luo, J. Cong, H. Zhang, H. Ma, Y. Duan, Subcritical water extraction, identification and antiproliferation ability on HepG2 of polyphenols from lotus seed epicarp, Ind. Crops Prod. 129 (2019) 472–479.
[83] J. Yi, H. Qu, Y. Wu, Z. Wang, L. Wang, Study on antitumor, antioxidant and immunoregulatory activities of the purified polyphenols from pinecone of Pinus koraiensis on tumor-bearing S180 mice in vivo, Int. J. Biol Macromol. 94 (2017) 735–744.
[84] H.J. You, H.J. Ahn, G.E. Ji, Transformation of rutin to antiproliferative quercetin-3-glucoside by Aspergillus niger, J. Agric. Food Chem. 58 (20) (2010) 10886–10892.
[85] Z. Zeković, M. Kaplan, B. Pavlić, E.O. Olgun, J. Vladić, O. Canlı, S. Vidović, Chemical characteri- zation of polyphenols and volatile fraction of coriander *(Coriandrum Sativum L)* extracts obtained by subcritical water extraction, Ind. Crops Prod. 87 (2016) 54–63.
[86] H.H. Zhang, J.F. Li, Y.Q. Duan, H.L. Ma, F.F. Xu, Y. Qin, Extraction and stability of procyanidins from chestnut shells, Food Sci. 32 (8) (2011) 5–9.
[87] G. Zhang, M. Hu, L. He, P. Fu, L. Wang, J. Zhou, Optimization of microwave-assisted enzymatic extraction of polyphenols from waste peanut shells and evaluation of its antioxidant and antibacterial activities in vitro, Food Bioprod. Process. 91 (2) (2013) 158–168.
[88] H. Zhang, M. Jordheim, D.H. Lewis, S. Arathoon, Ø.M. Andersen, K.M. Davies, Anthocyanins and their differential accumulation in the floral and vegetative tissues of a shrub species (*Rhabdothamnus solandri A. Cunn*), Sci. Hortic. 165 (2014) 29–35.
[89] H.H. Zhang, Y. Cheng, X.P. Luo, Y.Q. Duan, Protective effect of procyanidins extracted from the lotus seedpod on immune function injury induced by extremely low frequency electromagnetic field, Biomed. Pharmacother. 82 (2016) 364–372.
[90] Y.J. Zhang, Y. Zhang, A.A. Taha, Y. Ying, X.P. Li, X.Y. Chen, C. Ma, Subcritical water extraction of bioactive components from ginseng roots (*Panax ginseng C.A. Mey*), Ind. Crops Prod. 117 (2018) 118–127.