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Analytical Methods

Extraction optimization, purification and antioxidant activity of procyanidins from hawthorn (*C. pinnatifida* Bge. var. *major*) fruits

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ABSTRACT

The extraction conditions of procyanidins (PC) from the Chinese hawthorn (*Crataegus pinnatifida* Bge. var. *major* N.E.Br.) fruits were optimized by response surface methodology (RSM). Results showed that 93.4 ± 0.21% of the PC could be recovered. The crude extract was then purified by using a LSA-10 resin column, which showed excellent adsorption and desorption properties for PC purification. A fraction with PC content above 83.2% and mainly consisting of EC, a singly-charged dimer and trimer as identified by HPLC/MS, was obtained by isolation on LSA-10 resin. The antioxidant activity of hawthorn PC was tested *in vitro* with different systems. In solution systems, the ·OH and O_2^{-}· scavenging ability of hawthorn PC were higher than vitamin C. In a liposome peroxidation system, hawthorn PC exhibited much higher antioxidant activity than vitamin E. In addition, hawthorn PC (0.02% w/v) was efficient to inhibit lipid peroxidation during enzymatic hydrolysis of porcine meat at 50 °C for 24 h.

1. Introduction

Hawthorn (*Crataegus oxyacantha*) is a traditional medicinal plant and has long been used as a folk medicine and is widely utilized in pharmaceutical preparations mainly because of its beneficial health effects and its low toxicity. The pharmacological effects of *Crataegus* have mainly been attributed to their polyphenolic contents, and oligomeric procyanidins (OPCs) are abundant in hawthorn. The species most often used medicinally are *Crataegus monogyna* and *Crataegus laevigata*, and these two groups are used for standardization and quality control (Sticher & Meier, 1998; Wittig et al., 2002). The active constituents and the antioxidant effects of the extracts of the leaves and flowers of *C. oxyacantha* or *C. monogyna* have been widely studied (Bahorun et al., 2003; Hosseinimehr, Azadbakht, Mousavi, Mahmoudzadeh, & Akhlaghpoor, 2007; Kirakosyan et al., 2003; Sokol-Letowska, Oszmianski, & Wojdylo, 2007; Svedstrom et al., 2002; Tadic et al., 2008).

In China, hawthorn is planted for its edible fruit and its pharmacological history can be dated back to before 300 A.D. The species *Crataegus pinnatifida* Bge. and *Crataegus pinnatifida* Bge. var. *major* N.E.Br. have come to be the predominant species today. Despite its long history as an edible and medicinal fruit, limited data are available on the active constituents and consequently the antioxidant effects of the Chinese hawthorn fruit (Chang, Zhu, Zou, Chow, & Walter, 2001; Kao et al., 2005; Zhang et al., 2001). Recently, the Chinese hawthorn fruit has been shown to have higher PC contents and to be an excellent source of edible PC (Cui et al., 2006), which indicates that it is endowed with compounds that are potentially exploitable as food antioxidants. However, details of the PC and the information on the concentration of OPCs in hawthorn fruits remains unknown. Moreover, despite the many techniques for determining polyphenols, reports on the feasible and economic extraction and purification process of PC from hawthorn fruits in relation to the antioxidant activity of the extract, to our knowledge, are limited in number to date. The significance of antioxidants in preventive medicine is well known. Increasing interest has been devoted to searching for new, effective natural antioxidants because of their beneficial health effects. Therefore, the main objective of this study was to find an optimum extraction method for PC from the Chinese hawthorn fruits (*C. pinnatifida* Bge. var. *major*). A further intent was to develop a purification process based on a LSA-10 macroporous resin that would be suitable for manufacturing of extracts enriched in OPCs. Furthermore, the antioxidant activities of various fractions obtained at every step of purification were tested *in vitro* both in solution and liposome peroxidation systems.

2. Materials and methods

2.1. Materials, chemicals and reagents

Hawthorn fruit (*C. pinnatifida* Bge. var. *major*, Henan province, China); Procyanidins (Tianjin Jianfeng, Natural Product R&D Co. Ltd., Tianjin, China; the purity of procyanidins was 95%); LSA-10 macroporous adsorption resin (Xian Lanxiao, Co. Ltd.); vitamin E

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(V_E, Merck); tris (hydroxymethyl) aminomethane (Tris); thiobarbituric acid (TBA, Shanghai BioTech, Co. Ltd.); Soya bean lecithin (Hei Long Jiang); ferrous sulfate; pyrogallo; 1,10-phenanthroline; vitamin C (V_c); ethylene diamine tetracetate (EDTA); copper acetate. All other chemicals used in the experiments were of analytical grade.

2.2. Determination of PC

Standard solutions containing 0, 0.1, 0.2, 0.3, 0.4 and 0.5 mg/ml PC were prepared. Aliquots of 0.5 ml were transferred to five different test tubes, then mixed with 2.5 ml of 30% H_2SO_4 methanol solution, 2.5 ml of 30 g/ml vanillin methanol solution and the mixture was kept at 30 °C for 20 min. The absorbance at 546 nm was checked and then the standard PC absorbance curve was plotted. The sample was then processed in the same way as for the standard PC solutions and the absorbance was determined. The amount of the PC in the sample could be calculated by using the equation deduced from standard solution series.

2.3. Screening of potential OPCs in different plant tissues

Five grams of *Ulmus pumila* Linn skin, *poplar* skin, *hawthorn* fruit, *Glycine max* var. skin, *Horsebean* skin and *adzuki beans* skin was extracted with 70% aqueous ethanol several times until the filtrate contained no PC as detected by $FeCl_3/K_3Fe(CN)_6$. The filtrates were combined and the content of PC was determined. The combined filtrate was then concentrated under reduced pressure, then the solvents were evaporated on a Rotavapor. The residue was extracted with ethyl acetate. PC content in the ethyl acetate phase was assigned as the content of OPCs.

2.4. Design of the experiments to optimize the extraction conditions

Prior to analyses, the seed removed dried hawthorn fruit sample was ground to powder by a coffee grinder. On the basis of the mono-factor test, we selected the hawthorn fruits/solvent ratio (1:20) and extraction times (twice) and designed a series of experiments by response surface methodology (RSM) using Design Expert V7.1 software to optimize the extraction conditions. The main factors selected were (1) the extraction temperature (X_1), (2) the extraction time (X_2) and (3) the ethanol concentration (X_3). Details of the three factors in the experiments are described in Table 1. PC recovery yield was defined as the amount of PC in the crude ethanol extract (g) as a percentage of total amount of PC (g) in the hawthorn fruit.

2.5. Purification of the crude extract

The fractionation of PC from the crude extract was performed on a macroporous adsorbent resins LSA-10 column [1.7 × 42 cm, bed volume (BV) = 70 ml]. LSA-10 has medium polar with pore diameter (0.3 nm−1.25 nm) and high specific surface area (480–520 m^2/g). Sample was dissolved in distilled water at PC content of 14.8 ± 0.08 mg/ml then loaded with a constant current pump at a speed of 2 BV/h. The eluant was step collected (10 ml/tube) and the content of PC was determined. Loading was stopped at the adsorption permeation point (at which the PC content of the eluant was 1/10 of the loading sample). The column was washed with 4 BV water to eliminate the un-adsorbed PC. Eluant was also collected and the PC was determined. The column was then eluted consequently with 4 BV of 30%, 50%, 70% and 90% aqueous ethanol at a speed of 1 BV/h. Fractions were collected separately then lyophilized and the content of PC was determined. Adsorption amount of PC on LSA-10 resin and the recovery yield of the desorption were determined as:

Table 1
Analytical factors and levels for RSM, results of response surface analysis and variance analysis of regression equation.

Results of RSM

Test numbers	X_1	X_2	X_3	Recovery yield (%)
1	1	−1	0	90.2
2	1	0	−1	84.5
3	1	0	1	91.7
4	1	1	0	93.8
5	0	−1	−1	87.1
6	0	−1	1	85.4
7	0	1	−1	94.6
8	0	1	1	90.3
9	−1	−1	0	92.7
10	−1	0	−1	82.1
11	−1	0	1	85.7
12	−1	1	0	84.4
13	0	0	0	91.8
14	0	0	0	92.2
15	0	0	0	92.7

Analytical factors and levels for RSM

		Levels		
Factors	Code	−1	0	1
Temperature (°C)	X_1	40	50	60
Extraction time (h)	X_2	1	1.5	2
Ethanol concentration (%)	X_3	60	70	80

Variance analysis of regression equation

SD	DF	SS	MS	*F* value	Pro > *F*	S
Reg	9	3.2262	1.4452	53.4157	0.0142	*
One	3	2.6529	0.8843	7.5312	0.0266	*
Qua	3	21.6293	7.2098	61.3742	0.0002	**
Int	3	0.0039	0.0013	0.0114	0.6702	
Lack	3	0.0413	0.0138	0.0525	0.9814	
Res	5	0.0881	0.0293			
Error	2	0.5462	0.2731			
Total	14	14.5846				

Note: SD, sources of deviations; DF, degree of freedom; SS, sum of squares; MS, mean square; S, significant.
Reg, regression; One, one-degree term; Qua, quadratic term; Int, interaction term; Lack, lack of fit. Res, residuals; Total, total deviation.
* $p < 0.05$.
** $p < 0.01$.

$$\text{Adsorption amount of procyanidins}(\text{mg/ml}_{\text{resin}}) = (M_T - M_W)/V_B;$$

$$\text{Recovery yield } (\%) = (M_E/M_A) \times 100$$

where M_T was the total amount of PC loaded (mg), M_W was the total amount of PC eluted with 4 BV water (mg), V_B was the bed volume (ml), M_E was the total amount of PC eluted at different ethanol concentration (mg), M_A was the total amount of PC adsorbed on the resin (mg).

2.6. HPLC/MS analysis of hawthorn PC

HPLC/MS analyses were performed using an HP 1100 Series HPLC/MSD (Agilent Technologies) equipped with an API–ES ionization chamber. Briefly, separations of PC were performed on a Pinnacle II C18 column (5 μm, 4.6 × 250 mm) at 30 °C using a 20 μl injection volume. The ternary mobile phase consisted of (A) water–2% formic acid, and (B) 100% methanol. Gradient elution separation programs at a flow rate of 1 ml/min was as follows: 0–5 min, 95%A + 5%B; 5–25 min, 95%A + 5%B → 100%B; 25–30 min, 100%B. Data were collected using UV detector at 280 nm. Conditions for mass spectral analysis in the negative ion mode

include a capillary voltage of 4.2 kV, the fragmentor at 50 V and the drying gas temperature at 350 °C. Data were collected on an HP Chem-Station using scan mode over a mass range of m/z 100–1500.

2.7. NMR spectroscopy

^{13}C NMR spectra was measured at 30 °C with a using 3 mm tubes with MeOH-d_4 as the solvent. ^{13}C chemical shifts was given in ppm relative to tetramethylsilane (TMS) as an internal standard.

2.8. Antioxidant activity of hawthorn preparations

2.8.1. Hydroxyl radical-scavenging activity

The hydroxyl radical-scavenging assay was carried out using the method described by De Avellar et al. (2004) with some modifications. 1,10-Phenanthroline (0.75 mmol/l) and Fe^{2+}–EDTA (0.75 mmol/l) were dissolved in phosphate buffer (pH 7.4) and mixed thoroughly. H_2O_2 (0.1 ml/l) and the hawthorn PC fractions were added. The mixture was incubated at 37 °C for 60 min, then the absorbance was measured at 536 nm. Hydroxyl radical-scavenging activity (I_1) was determined using the equation:

$$I_1\ (\%) = [(A_s - A_1)/(A_0 - A_1)] \times 100$$

where A_s is the absorbance of the sample, A_1 is the absorbance of the control solution containing 1,10-phenanthroline, $FeSO_4$ and H_2O_2, and A_0 is the absorbance of the blank solution containing 1,10-phenanthroline and $FeSO_4$.

2.8.2. Superoxide radical-scavenging activity

Superoxide radical-scavenging activity was estimated at 25 °C by spectrophotometric monitoring of the inhibition of pyrogallol auto-oxidation as described by Marklund and Marklund (1974) with some modifications. Pyrogallol solution (0.4 ml; 2.5 mmol/l) was added to a tube containing 0.1 ml hawthorn PC fraction previously dissolved in 4.5 ml Tris/HCl/EDTA buffer (0.1 mol/l, pH 8), mixed thoroughly then incubated at 25 °C for 4 min. Reaction was stopped by addition of 2 drops of HCl (8 mol/l). The absorbance was measured in triplicate at 325 nm. Superoxide radical-scavenging activity (I_2) was determined as:

$$I_2\ (\%) = [(A_b - A_s)/A_b] \times 100$$

where A_b is the absorbance of the blank (0.1 ml deionized water instead of sample), A_s is the absorbance of the sample.

2.8.3. Antioxidant activity of hawthorn procyanidins in liposome system

Lecithin liposomes are prepared according the method described by Huang and Frankel (1997). Lecithin (2.4 g) was suspended in deionized water at a concentration of 8 mg/ml then by stirring with a glass rod and sonicating for 15 min. In the Erlenmeyer flasks containing 50 ml of liposome samples, 1 ml of antioxidant ethanol solutions was added, the mixture was sonicated for 5 min, then 20 μl of cupric acetate (30 mM) was added and mixed thoroughly. The open mouth oxidation test in darkness was performed in a shaker water bath at 37 °C. The oxidative stability of these samples was determined by measuring the content of propionaldehyde (MDA) formed in the system. TBA method (Hiroshi, Ohishi, & Yagi, 1979) was used to determine the MDA concentration. The antioxidant activity of hawthorn PC was expressed by the inhibition rate of MDA formation which was calculated as follows:

$$\text{Inhibition}\ \% = (1 - C_s/C_0) \times 100$$

where C_s and C_0 were the concentration (μM) of MDA with and without antioxidant, respectively.

2.8.4. Inhibition of lipid peroxidation during enzymatic hydrolysis of porcine meat

Forty grams of minced fillet porcine meat were suspended in 20 ml of distilled water at ambient temperature. The mixture was placed in a water bath maintained at 50 °C. Enzymatic hydrolysis was started by the addition of enzymes at an enzyme/protein (E/S) ratio of 2% (Alcalase:Flavourzyme = 1:1, w/w). At the end of the hydrolysis, the mixture was heated in a 100 °C water bath for 5 min to inactivate enzymes and centrifuged at 3000 rpm for 10 min to separate lipid from the hydrolysate. Twenty millilitres of light petroleum was added to the lipids fraction then stirred thoroughly to extract lipids, then 5 ml 95% aqueous ethanol was added, stirred thoroughly followed by centrifugation at 3000g for 5 min at ambient temperature. The light petroleum phase was pipetted into a culture dish to remove the solvents in a draught cupboard for 5 h. Determination of the peroxide value, POV (based on AOCS Official Method Cd 8-53) was then carried out on the isolated lipids.

2.9. Statistical analysis

The results were processed using Origin v7.5. The data were subjected to analysis of variance (ANOVA). P values < 0.05 were regarded as significant. All experiments were carried out in triplicate.

3. Results and discussion

3.1. General

In a preliminary study, a series of plant tissues was screened for potential sources of OPCs. The PC contents of *U. pumila* L. skin, *poplar* skin, *hawthorn* fruit, *Glycine max* var. skin, *Horsebean* skin and *adzuki beans* skin were 3.28 ± 0.12%, 1.32 ± 0.08%, 2.96 ± 0.14%, 0.33 ± 0.03%, 3.87 ± 0.53%, and 0.84 ± 0.74%, respectively, in which OPCs/PC ratio was 18.6%, 10.4%, 61.6%, 0.0%, 5.7% and 13.7%. The highest OPCs/PC ratio was observed for hawthorn fruit, indicating that Chinese hawthorn fruit was a good source of OPCs.

3.2. Optimization of extraction of PC from hawthorn fruit

Extraction conditions of PC from hawthorn fruits was optimized by RSM. By taking the PC recovery yield as the response value (Y), 15 experiments were designed, in which 1–12 were factorial experiments, 13–15 were zero-point tests. Zero-point tests were performed in triplicate to estimate the errors. Results and the variance analysis of regression equation are shown in Table 1. The one-degree term was significant, the quadratic term was extremely significant, the interaction term was not significant, indicating that the relationship between response value and the factors was not simply a linear one. Dependent variables and all independent variables were significant. Error of lack of fit was not significant. The regression equation was fitted by RSM with the experiment results as follows:

$$\begin{aligned} Y = {} & 2.3451 + 0.1356X_1 - 0.2045X_2 + 0.0570X_3 + 1.4955X_1^2 \\ & + 0.9580X_2^2 - 0.2805X_3^2 + 0.0340X_1X_2 - 0.7985X_1X_3 \\ & - 0.2685X_2X_3 \end{aligned}$$

By first order partial derivation of the equation, the extraction conditions were optimized as follows: X_1 = 54.7 °C, X_2 = 1.6 h, X_3 = 67.5%. This method resulted in 94.6% recovery of total PC from raw hawthorn fruit. To test the accuracy of the regressive model, experiments under the optimum conditions were repeated, the PC recovery yield was 93.4 ± 0.21%. Based on one-way ANOVA analysis, no significant difference was observed between the RSM

model and the verification model ($p > 0.05$), which meant that the conditions optimized by RSM could be applied to the extraction of PC from hawthorn fruits.

3.3. Purification of PC on the LSA-10 column

Prior to being applied on the LSA-10 column, the crude alcohol extracts were adjusted to 80% aqueous ethanol then centrifuged at 3000 rpm for 10 min to remove proteins and sugars. The solvent of the supernatant was evaporated off on a Rotavapor under reduced pressure, then partitioned with light petroleum (1:2, v/v) to remove lipids and to obtain the aqueous fraction (F1). The PC content of the F1 was 11.4 ± 0.12% (dry basis). The aqueous fraction was then further partitioned with ethyl acetate to remove residual sugars and red colorants, and the crude extract (F2) was obtained by removal of ethyl acetate then lyophilized. F2, with determined PC content up to 48.1 ± 0.25% (dry basis), was further purified on the LSA-10 resin column described in Section 2.5.

The adsorption permeation curve of PC on LSA-10 resin was as shown in Fig. 1. At the adsorption permeation point, the loading volume was about 3.7 BV (260 ml). Total amount of PC loaded (M_T) and eluted with 4 BV water (M_W) were, respectively, 3833.3 mg and 181.0 mg. Therefore, 94.4% of the loaded PC was adsorbed on the resin and the adsorption amount of PC was 52.37 mg/ml$_{resin}$. Step-gradient elution with increasing concentrations of aqueous ethanol showed that the total PC recovery yield was up to 94.8% (Table 2), in which 50% aqueous ethanol was proved to be the most efficient to elute PC adsorbed on the resin, with 64% recovery yield and the PC content (dry basis) up to 83.2%, indicating that fractions with relatively higher content of OPCs could be obtained by eluting with a low ethanol concentration. Based on the above findings, it can be concluded that LSA-10 resin has excellent adsorption/desorption properties in PC purification and could be further applied to large-scale preparation.

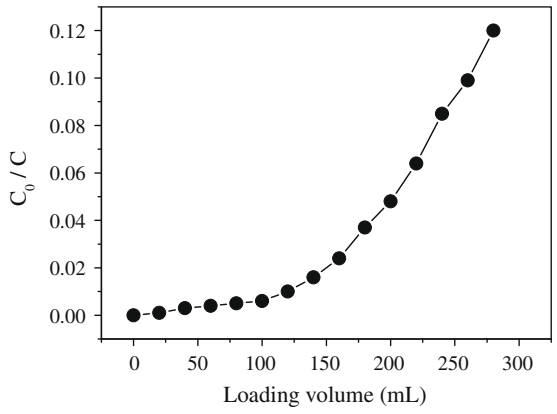

Fig. 1. Adsorption permeation curve of procyanidins on LSA-10 resin. C_0, PC concentration of the eluant (mg/ml); C, PC concentration of the loading sample (mg/ml).

3.4. HPLC/MS analyses of F4

F4 fraction was dissolved in methanol then filtrated through a 0.45 μm membrane. The filtrate was used for RP-HPLC/ESI-MS analysis. The profile of RP-HPLC showed three sharp peaks (P1, P2 and P3) (Fig. 2A). The peak of P3 (41.89%, retention time from 19.28 to 20.33 min) was substantially higher than P1 (28.38%, retention time from 16.80 to 18.05 min) and P2 (17.57%, retention time from 18.05 to 19.28 min). This meant P3 fraction was the major PC of F4. It should be noted that P2 was consisted of 3 peaks at retention times 18.24, 18.50, 18.90 min accounting, respectively, for 1.35%, 10.81% and 5.41%.

Mass spectral data showed that a wide mass range was recorded of peak 1 (Fig. 2B). The dominant species observed come from OPCs (*m/z* 577 and 865) and were compounded by the trace appearance of ions from other species (*m/z* 385.2, 499.3, 645.2 and 787.2). Without authentic standards the OPCs were assigned as a singly-charged dimer (*m/z* 577) and a trimer species (*m/z* 865), or doubly charged tetramer (*m/z* 576) and hexamer species (*m/z* 864), or the triply charged nonamers (*m/z* 864). It was reported that the mDP (mean degree of polymerization) of the PC in the Chinese hawthorn fruit is around 2 (Cui et al., 2006). This value is lower than that for European hawthorn fruit and far lower than that for the leaves and flowers of hawthorn (Svedstrom et al., 2002). This might be related to the high level of organic acids in the Chinese hawthorn fruit (Gao, Feng, & Qin, 1995) and the decomposition of PC into smaller molecular fragments in acidic conditions. The acidic conditions inhibit ionization so that singly-charged ions dominate. It was also reported that EC and PC-B2 were the two most abundant components, with 348 and 374 mg/100 g in the fruits (Chang, Zuo, Chow, & Ho, 2006). Based on the forgoing, the major PC of peak 1 was mostly proved to be a singly-charged dimer (*m/z* 577) and a trimer species (*m/z* 865). No OPCs was observed of peak 2 (Fig. 2C). Mass spectral data showed 4 molecular ions at *m/z* 154.6, 284.9, 363.2 and 379.1. Upon review of the mass spectral data in literature, the molecular ion at *m/z* = 284.9 was possibly kaempferol, a dominant flavonol existing in the Chinese hawthorn fruits. As for peak 3, mass spectral data indicated the presence of predominantly one monomer, which was assigned as (+)-catechin or (−)-epicatechin (*m/z* 289.0). Further analysis by ^{13}C NMR spectrum showed the following data: 28.9 (C-4), 66.9 (C-3), 79.4 (C-2), 95.5 (C-8), 96.2 (C-6), 99.7 (C-4a), 115. 3 (C-2′), 115.6 (C-5′), 119.3 (C-6′), 132.1 (C-1′), 145.3 (C-3′), 145.5 (C-4′),157.0 (C-5), 157.5 (C-7), 157.6 (C-8a). It was proved to be epicatechin, which was in agreement with the reported results that PC occured in *Crataegus* was primarily composed of (−)-epicatechin (Chang et al., 2006; Svedstrom et al., 2002; Zhang et al., 2001).

From these results, it can be concluded that the degree of polymerization is 1–3 and there was a notable absence of higher oligomers. Smaller PC, monomer to trimer, have been reported to be absorbed in the human intestine directly *in vitro* (Deprez, Mila, Huneau, Tome, & Scalbert, 2001; Holt et al., 2002; Sano et al., 2003). As for the significance of these results, the fruits of Chinese hawthorn may become a preferred resource of edible OPCs.

Table 2
Effect of step-gradient elution on procyanidins absorbed on LSA-10 resin column.

Fractions (eluting solvent)	PC (mg) (±SD)	PC recovery (%)	PC content (%, dry basis)	Degree of enrichment
F3 (30% aqueous ethanol)	201 ± 3	5.5	15.2	5.14
F4 (50% aqueous ethanol)	2347 ± 7	64.0	83.2	28.11
F5 (70% aqueous ethanol)	436 ± 2	11.9	45.1	15.24
F6 (90% aqueous ethanol)	682 ± 5	18.6	6.7	2.26
Total	3476 ± 6	94.8		

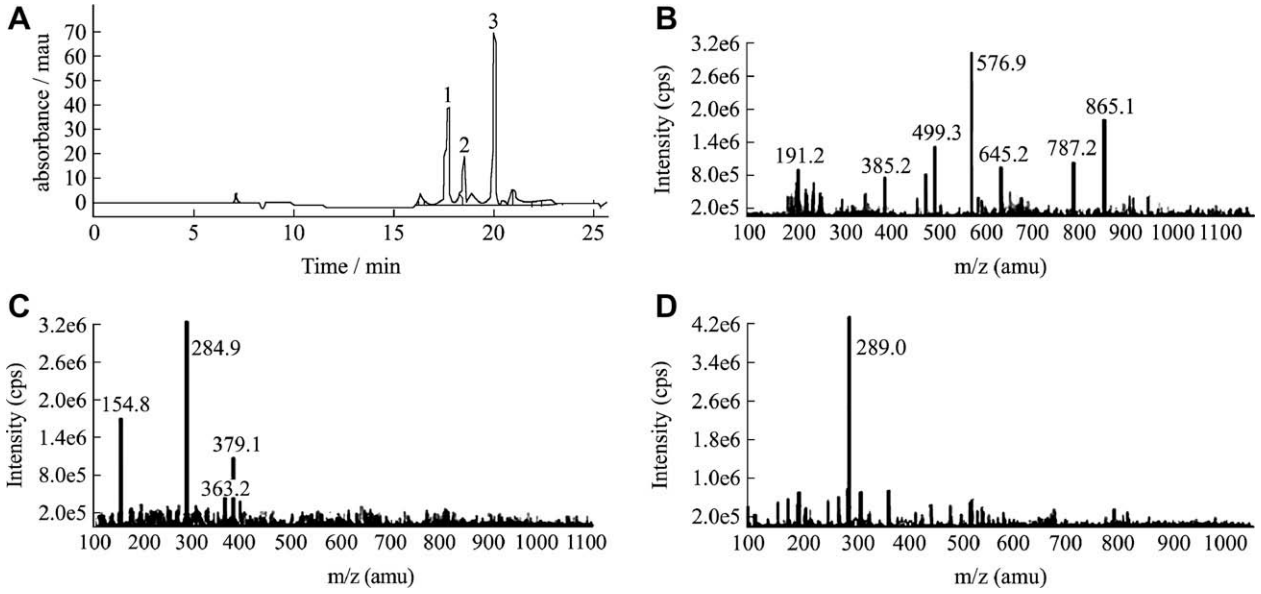

Fig. 2. RP-HPLC and ESI-MS of F4. (A) Procyanidins profile using RP-HPLC; (B) mass spectrum of peak 1 of RP-HPLC; (C) mass spectrum of peak 2 of RP-HPLC; (D) mass spectrum of peak 3 of RP-HPLC.

3.5. Antioxidant activity of hawthorn PC

3.5.1. Scavenging effect of hawthorn PC on $O_2^{-\cdot}$ and ·OH

The superoxide radical is known to be very harmful to cellular components as a precursor of more active oxidative species such as singlet oxygen and hydroxyl radicals (Kanatt, Chander, & Sharma, 2007). Furthermore, it is considered to play an important role in the peroxidation of lipids. Therefore studying the superoxide radical-scavenging effect of the fractionated hawthorn PC is most necessary. The dose-dependent superoxide radical-scavenging abilities of the hawthorn PC (F1, F2 and F4) and V_c were as presented in Fig. 3A. Hawthorn PC showed higher hydroxyl radicals-scavenging activity. Addition of F4 led to 92.2% hydroxyl radical suppression at 0.1 mg/ml, while addition of V_c at the same concentration resulted in only 21.1% hydroxyl radical suppression, which was much lower than that of hawthorn PC at 0.02 mg/ml (31.3%). The IC_{50} value, calculated from the regression equation was 0.023, 0.033, 0.053 and 0.11 mg/ml, respectively, for F1, F2, F4 and V_c.

Among the oxygen radicals the hydroxyl radical is the most reactive free-radical and severely damages adjacent bio-molecules such as proteins, DNA, polyunsaturated fatty acids, nucleic acids and almost any biological molecule it touches (Aruoma, 1998). Therefore removal of the hydroxyl radical is probably one of the most effective defenses of a living body against various diseases. The hydroxyl radical-scavenging abilities of the hawthorn PC and V_c were presented in Fig. 3A. The hydroxyl radical-scavenging abilities increased in a dose-dependent way. For $O_2^{-\cdot}$, the scavenging ability of hawthorn PC was higher than V_c too, with IC_{50} being 0.014, 0.028, 0.05 and 0.17 mg/ml for F1, F2, F4 and V_c, respectively.

3.5.2. Antioxidant activities of hawthorn PC in liposome system

During oxidation at 37 °C, in the presence of copper ions, hawthorn PC inhibited MDA formation (Fig. 3B). This inhibition rate increased in a dose-dependent way from 10 to 40 mg/ml. Inhibition of hawthorn PC (F4) at 10, 25 and 40 μg/ml after 48 h was 16.3%, 44.7% and 85.9%, respectively. In contrast to the great difference in the $O_2^{-\cdot}$ and ·OH scavenging ability among F1, F2 and F4 in solution system, only slight difference in the inhibition of MDA formation was observed. At the same PC content (40 mg/ml), the amount of MDA formed was lowest in the presence of F1 followed by F2 and F4. At 25 mg/ml, V_E had no antioxidant activity in inhibiting MDA formation after 24 h of oxidation ($p > 0.05$). At 150 μg/ml, V_E showed some antioxidant activity ($p < 0.05$) with the inhibition rate of 31.2%. These data indicated that, in the oxidation of liposome with added copper, hawthorn PC showed higher antioxidant activity than that of V_E. Lecithin liposome was charged polar substrates and provides more suitable models to study the activities of hydrophilic antioxidants (Huang & Frankel, 1997). The greater affinity of hydrophilic antioxidants such as PC for the polar lecithin liposome bilayers is expected to increase their antioxidant activity. Since the inhibition rate of MDA formation was also dose-dependent, the mechanism might be related to the increased concentration of hawthorn PC at the lecithin liposome bilayers, thus affording better protection. The better antioxidant activity of PC in this system can also be ascribed to the additive effect of their metal chelation ability. In part, the higher hydrogen-donating and free-radical-scavenging abilities of PC may thus explain its better antioxidant activity relative to that of V_E.

3.5.3. Inhibition of lipid peroxidation during the hydrolysis of porcine meat

Fig. 3C presented the antioxidant activities of hawthorn PC on the POV of porcine meat after 24 h enzymatic hydrolysis at 50 °C. The initial POV of porcine meat was 0.89 meq/kg and it increased to 27.92 meq/kg in the absence of antioxidant. However, in the presence of 0.02% hawthorn PC, POV was much lower. POV were 6.87, 7.12 and 7.52 meq/kg, respectively, for F1, F2 and F4. These values were slightly higher than that of TBHQ (5.73 meq/kg) at the same concentration. Similar to TBHQ, hawthorn PC was a phenolic antioxidant, it can react with the free lipid peroxide radicals by donating electrons to convert them to more stable products and terminating the free-radical chain reaction. Enzymatic hydrolysis is an efficient way to recycle food proteins from the waste. To make full use of the enzymes and to obtain higher hydrolysis degree, enzymatic hydrolysis was mostly carried out at 40–60 °C for a long time, during which the oxidation/deterioration of fat would result in the unfavorable smell of the hydrolysate, especially for protein sources of animal origin which contain higher lipid. The present

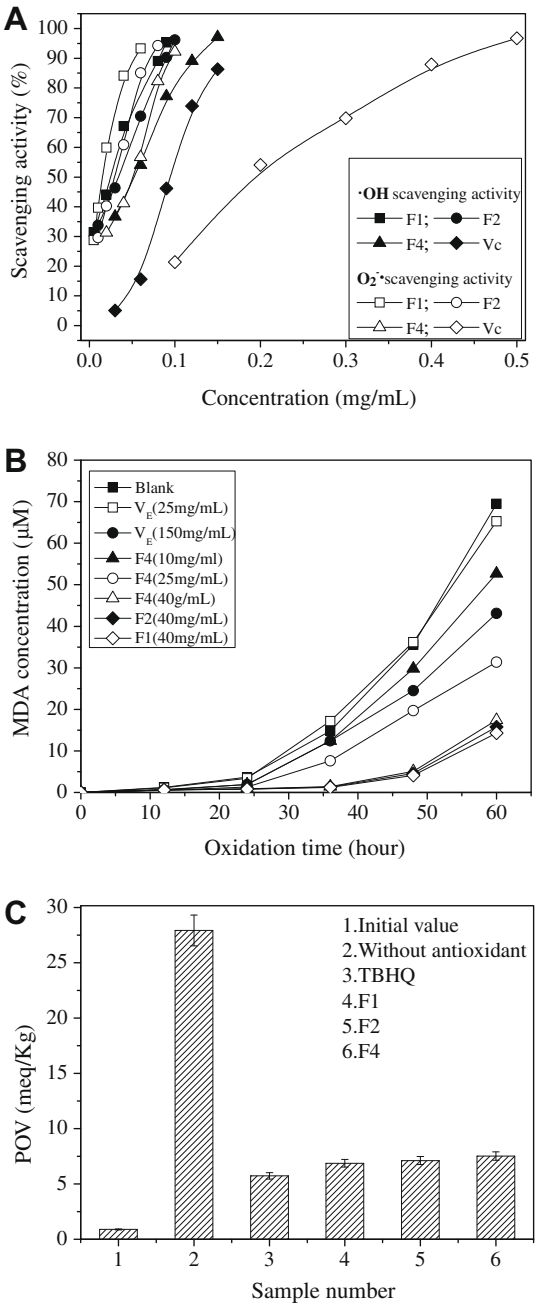

Fig. 3. Antioxidant activity of hawthorn PC. (A) $O_2^{-\cdot}$ and ·OH scavenging activity; (B) antioxidant activities of hawthorn PC in liposome system; (C) inhibition of lipid peroxidation during the enzymatic hydrolysis of porcine meat.

results show that the hawthorn PC can prevent the lipid oxidation during enzymatic hydrolysis and improve the flavor of hydrolysate, but without any effect on the hydrolysis degree (data not shown).

As shown in this study, the antioxidant activity found in the crude extracts (F1 and F2) is more active than that of purified one. In a further study, the hawthorn PC preparation had higher antioxidant activity than that of (−)-epicatechin and procyanidin B2 which were isolated from that preparation at the same concentration (Sokol-Letowska et al., 2007). Generally, monomers have lower antioxidant activity than dimers and others (Ariga, Koshiyama, & Fukushima, 1988; Jayaprakasha, Singh, & Sakariah, 2001). The crude extracts had a more complex series of procyanidins comprising not only the same classes of PC in the purified one, but also other PC of higher mDP, such as tetramers, pentamers and hexamers which express higher antioxidant activity (Sokol-Letowska et al., 2007). In addition, other polyphenols, phenolic acid and triterpenoids, such as hyperoside, isoquercitrin, chlorogenic acid, oleanolic acid and ursolic acid, which have been detected (Cui et al., 2006) in the matured fruits of Chinese hawthorn, were possibly presented in the crude extracts. Therefore, the better antioxidant activity of the crude extracts can be mostly ascribed to the synergism of the complex phenolic compounds acting on various radicals.

4. Conclusions

This work presented an efficient extraction method of PC from hawthorn fruits and a purification process based on LSA-10 macroporous adsorption resin that would be further applied to large-scale preparation of extracts enriched in OPCs. Results showed that the Chinese hawthorn fruits which are traditionally used as food and herbs are a preferred resource for edible OPCs and endowed with potentially exploitable antioxidant activities. Despite that, it still remains unknown why the crude extracts were stronger than the purified one in their antioxidant activity, especially in the solution system. Future investigations will concern in-depth analyses (both in terms of chemistry and antioxidant activity) to better understand the synergism of the main active components involved in the oxidative power. Investigations are also needed on the possible use of hawthorn extracts as the antioxidant substances in traditional Chinese food, such as sausages and moon cake.

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