Microwave-assisted Extraction of Polysaccharides from Bamboo (*Phyllostachys acuta*) Leaves and their Antioxidant Activity

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Polysaccharides were isolated from *Phyllostachys acuta* leaves by microwave-assisted extraction under various temperatures and time. The obtained polysaccharides were characterized by acid hydrolysis, the Folin-Ciocalteu method, and Fourier transform infrared spectroscopy (FTIR). The major monosaccharides presented in the extracts were arabinose (258.0 mg/g to 414.5 mg/g), galactose (167.0 mg/g to 289.2 mg/g), and glucose (157.4 mg/g to 246.7 mg/g) along with some mannose, fructose, and xylose. The total phenol yield of the bamboo leaves was 0.31 mg/g to 0.73 mg/g. The FTIR spectra revealed that the polysaccharides mostly consisted of *β*-glycosidic linkages. For the cytotoxicity, the presence of polysaccharides considerably elevated the multiplication of HepG2 cells and showed no growth inhibition for the samples. For the antioxidant activities, the polysaccharides exhibited excellent abilities both in the diphenyl picrylhydrazyl radical potential (DPPH) assay and ferric reducing antioxidant potential (FRAP) assay. The results suggest that bamboo leaf polysaccharides have great potential to be applied in the food, healthcare, and pharmaceutical fields.

Keywords: Polysaccharides; Microwave-assisted extraction; Bamboo leaf; Phyllostachys acuta; Antioxidant

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INTRODUCTION

Bamboo, a perennial green plant belonging to the Gramineae family and Bambusoideae subfamily, is one of the most abundant biomass resources in China. For a long time, bamboo leaves had been considered as residues from traditional applications, and most of them were burned or discarded, which brought in serious environmental pollution and resource waste. It is therefore imperative to find an adequate disposal method for the waste bamboo leaves or means to convert them into useful products.

Many residues, originating from the process of utilization, are important agricultural resources because of their enriched content of bioactive compounds such as proteins, phenolic compounds, phytic acid, and polysaccharides (Zhu and Wu 2009). As a traditional Chinese medicine, bamboo leaves were used as an antipyretic to stop bleeding, and for detoxification and detumescence (Chen *et al*. 1999). Moreover, the antioxidant materials in bamboo have also been considered as a novel natural food antioxidant (Liu *et al*. 2014).

Previous research showed that bamboo leaves could be used as a raw material for obtaining co-products in medical and food industries, such as polysaccharides and flavonoids (Kweon *et al*. 2001) and C-glycosides (Zhang *et al*. 2008). Furthermore,

bamboo leaf extracts have been reported to have great potential for anticancer purposes (Seki and Maeda 2010), protection of immune systems (Seki *et al*. 2010), and inhibiting diseases (Jiao *et al*. 2011).

The most common method used for polysaccharides isolation was through hot water extraction (HWE) (Shi *et al*. 2011). The weaknesses of HWE, including high energy consumption and low yields, had inhibited this method from being widely applied. To optimize the extraction technology, many novel techniques had been employed, such as ultrasonic-assisted extraction (UAE), enzyme-assisted extraction (EAE), supercritical fluid extraction (SFE), and pressurized liquid extraction (PLE) (Kadam *et al*. 2013; Matsunaga *et al*. 2013; Ye *et al*. 2015). These novel extraction technologies had been successfully used in food and pharmaceutical applications for extraction of bioactive compounds (Easmin *et al*. 2015).

Among all the novel extraction technologies, microwave-assisted extraction (MAE) has been considered as one of the predominant methods to extract polysaccharides from plant structures (leaves, seeds, flowers, and roots) because of its simpler operation, shorter extraction time, flexibility, lower energy consumption, and higher efficacy. Moreover, MAE has also been reported to have the potential to minimize the disassembly and possible degradation of the extracted polysaccharides (Marshall *et al*. 2010). Rodriguez-Jasso *et al*. (2011) reported that high yields of fucoidan could be isolated in a very short time with the MAE method.

Vazquez-Delfin *et al*. (2014) elaborated an extraction system based on microwave irradiation, and the results showed lower yields but higher anhydrogalactose content than conventional techniques. Karabegovic *et al*. (2013) optimized the MAE of polysaccharides from cherry laurel leaves with a response surface method (RSM). The previous research suggested that MAE was a promising method for extracting bio-active components, including polysaccharides, flavonoids, and polyphenols.

Phyllostachys acuta leaf is a forestry residue with a variety of bio-active components. Feedstocks used to isolate bio-components were generally confined to medicinal plants. To our best knowledge, there has been a lack of reports about ordinary bamboo leaf polysaccharide extraction. Thus, the *Phyllostachys acuta* leaf was used as feedstock to isolate crude polysaccharides for bio-antioxidants. The results revealed that the crude polysaccharides had potent antioxidant activities, no cytotoxicity, and great potential to be used in food and pharmaceutical areas.

EXPERIMENTAL

Raw Materials

Bamboo leaves were harvested from Yunnan province of China, in 2013, from three-year-old bamboo (*Phyllostachys acuta*). The raw material was first dried at room temperature for 15 days and then ground into a fine powder to obtain a fraction under 20-mesh. The powder was stored in a desiccator at room temperature.

Chemicals and Reagents

2, 2’-Diphenyl-1-picryl-hydrazyl (DPPH), 1, 3, 5’-tri (2’-pyridyl) -2, 4, 6’-triazine (TPTZ), and ascorbic acid were purchased from Sinopharm Chemical Company (Beijing, China). All chemicals were of analytical grade and were used without any further purification.

Extraction Process

The polysaccharide extraction was based on a previously reported method with some modifications (Liu *et al*. 2015). The extraction process was carried out in a 500-mL beaker, followed by heating in a microwave oven (MicroSYNTH, Milestone, USA). Then, 20 g (oven-dried weight) of bamboo leaf flour was added to 300 mL of ultra-pure water in the beaker. After that, the beaker was subjected to microwave heating for predetermined temperatures (60, 70, 80, and 90 °C) and time intervals (20, 40, and 60 min).

Once the reaction was complete, the supernatant and sediments were separated by vacuum filtration. The residues were washed with ultra-pure water three times and then discarded. The solutions were condensed to approximately 100 mL using a rotary evaporator at 60 ºC under vacuum. Anhydrous ethanol (300 mL) was slowly added to the resulting solution, which was then precipitated overnight at 4 °C for 24 h. Subsequently, the precipitated polysaccharide fractions were centrifuged and freeze-dried to obtain crude polysaccharides.

Except for polysaccharides, a small amount of monosaccharide, protein, as well as flavones could also be precipitated. The main components were polysaccharides, so the extracted solid was considered as crude polysaccharides. The extraction yield was utilized to optimize the extraction conditions.

FTIR Analysis

The functional groups of the extracted crude polysaccharides were identified on a FTIR spectrophotometer (NicoletiN10, Thermo, USA). The crude polysaccharides were ground into powder and pressed into 1 mm disks for FTIR measurement in the frequency range of 4,000 to 400 cm^{-1}.

Monosaccharide Component Analysis

The total neutral monosaccharide and uronic acids of the samples were determined by high-performance anion exchange chromatography (HPAEC). Each sample (0.03 g) was first hydrolyzed with 0.03 mL of 72% sulfuric acid at 30 °C for 1 h. During this procedure, a stirring rod was used to stir the sample every 5 to 10 min. Subsequently, the hydrolysates were diluted with 8.4 mL of ultra-pure water, and then the tubes were sealed and incubated in a bake oven at 105 °C for 1 h. After that, the sample solutions were diluted with ultra-pure water and filtered *via* a 0.22-μm filter membrane before injecting into the HPAEC system (Dionex ISC 300, USA) with an amperometric detector, a CarbopacTMP-20 column (4 mm × 250 mm, Dionex), and a guard PA-20 column (3 mm × 30 mm, Dionex). The peaks were identified by comparing the retention time to the standards.

Total Phenol Analysis

Total phenol analysis was conducted according to the method reported by Jerez *et al.*(2007). Briefly, 0.5 mL of the extracted crude polysaccharide solution (0.1 g/L) was added to 2.5 mL of Folin-Ciocalteu reagent (2.0 M). The mixture was shaken continuously and incubated for 30 min. Then, 2.5 mL of Na_2CO_3 solution (7.5 g/mL) was added to the previous mixture. After maintaining for 1.5 h at 37 °C, the mixture was measured at 765 nm against a blank (ultrapure water), and the total phenol content was calculated according to the standard curve of gallic acid solutions (0 to 0.06 g/L).

Cytotoxicity Analysis

Cytotoxicity was evaluated using the MTS (3-(4,5-dimethylthiazol-2-yl)-5(3-carboxymethoxyphenyl)-2-(4-sulfopheny)-2H-tetrazolium, inner salt) assay. In detail, HepG2 cells were cultivated in trypsin-EDTA medium and 5% CO_2 atmosphere at 37 ºC in advance. Then, a density of 1×10^5 HepG2 cells were seeded into 96-well plates and treated with polysaccharide (extracted at 70 °C and 40 min) solutions (0, 0.01, 0.05, 0.1, 0.25, 0.5, and 1 mg/mL) for 0, 4, or 12 h. At predetermined time intervals,1.0% MTS solution was added to the medium, and then the mixtures were subjected to incubation for 4 h at 37 ºC. After that, the absorbance of the resulting solutions at 490 nm was measured by a spectra-photometric plate reader (Multiscan MK3, Thermo Fisher Scientific, USA).

DPPH Radical Scavenging Activity

The DPPH radical scavenging activity of the extracted polysaccharides was carried out according to previous methods with minor modifications (Yin *et al*. 2010). In detail, the DPPH powder was dissolved in methanol to obtain 25 mg/L solutions. Then, 3 mL of DPPH fluid was added to 0.5 mL of polysaccharide liquor (1, 2, 4, 6, 8, and 10 mg/mL, respectively). The mixtures were shaken vigorously and maintained in the darkness for 30 min. After that, the absorbance was measured at 517 nm using ascorbic acid as a positive control. All tests were carried out in parallel. The scavenging activity on DPPH radicals was calculated by the following equation,

$$\text{DPPH radical scavenging activity (\%)} = \frac{A_{DPPH} - A_{sample}}{A_{DPPH}} \times 100 \quad (1)$$

where A_{DPPH} is the absorbance of the DPPH radical solution with methanol and A_{sample} is the absorbance of the DPPH radical solution with polysaccharide fluid.

Ferric Reducing Activity

The FRAP assay was carried out according to a previously described method with some modifications (Tian *et al*. 2009). The FRAP solution consisted of 250 mL of acetate buffer (300 mM, pH 3.6), 25 mL of TPTZ solution (10 mM in 40 mM HCl), and 25 mL of $FeCl_3 \cdot 6H_2O$ solution (20 mM). After preheating to 37 ºC, 3 mL of FRAP reagent was mixed with 0.12 mL sample solutions (0.5, 1, 2, 3, 4, and 5 mg/mL, respectively) and incubated for 30 min. Subsequently, the absorbance was measured at 593 nm, and the final result was calculated as the concentration of $FeSO_4 \cdot 7H_2O$.

RESULTS AND DISCUSSION

Extraction of Polysaccharides from Bamboo Leaves

The effects of time and temperature on crude polysaccharides yield were investigated with a heating power of 800 W, and the mass ratio of raw material to water was fixed at 1:15. As shown in Figure 1, the yield of crude polysaccharides was positively correlated with increasing temperature (from 60 to 90 °C) and time (from 20 to 60 min). At 70 °C and 40 min, the yield reached 1993 mg/L. After that, increasing temperature and time only slightly increased the yield. Harsher extraction conditions, in terms of time and temperature, may have led to an efficient opening of the cell wall network and hence to a higher yield. In detail, when the temperature was set to be 60 °C, increasing reaction time from 20 to 60 min brought in only 126 mg/L polysaccharides of augmented yield. On the

other hand, from 70 to 90 °C, the solid product yield could be significantly enhanced by increasing reaction time from 20 to 40 min. With further prolonging of the reaction time to 60 min, the solid yield was almost the same as that of 40 min. The results suggested that both reaction time and temperature played an essential role in the extraction processes. Furthermore, 60 °C was not strong enough for sufficient polysaccharides dissolution. At higher reaction temperatures, small particles of polysaccharides could be isolated from the cell wall efficiently within 20 min; increasing reaction time contributed to polysaccharides dissolution, as well as degradation. From the beginning to 40 min, the dissolution reaction was dominant, and after that, the degradation rate was enhanced and comparable with dissolution rate, resulting in the slight increase of production yield at longer reaction time. Compared with the hot water extraction reported by Cui *et al.* (2014), the extraction time in this study was effectively reduced to less than 60 min.

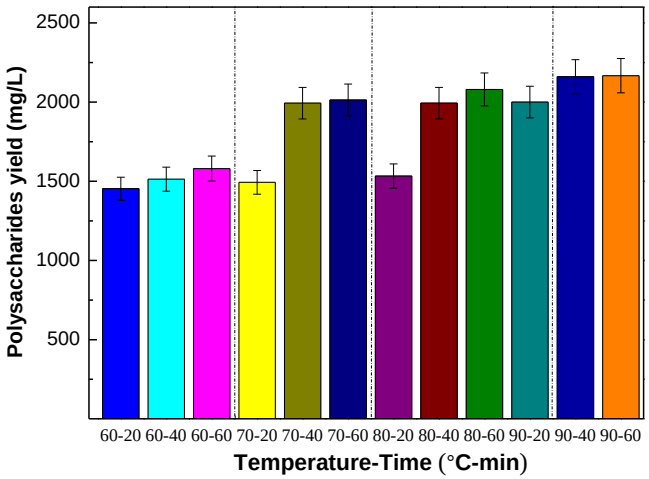

Fig. 1. Yield of crude polysaccharides extracted under various conditions

FTIR Analysis

FTIR spectra demonstrated that no considerable differences were observed between the fractions under both mild and severe conditions, suggesting that the tested range of temperature (60 to 90 °C) and time (20 to 60 min) had no enormous effects on the structure of the extracted crude polysaccharides. The samples gave signals for -OH stretching vibration at 3280 cm^{-1} and for C-H stretching at approximately 2930 cm^{-1} (asymmetrical stretching)and 2862 cm^{-1} (symmetrical stretching) (Buranov and Mazza 2012). The peaks at 400 to 2000 cm^{-1} are the characteristic absorptions of polysaccharides (Luo *et al*. 2012; Peng *et al*. 2013).The strong absorption signals at 1613 cm^{-1} can be assigned to the stretching vibration of the CHO and C=O bonds (Mao *et al*. 2013). The bands at approximately 1413 cm^{-1} can be attributed to C-H bending vibration of -CH_2-. The absorption peaks at 1328 cm^{-1} are related to C-O stretching vibration in the C-OH groups or C-C stretching vibration in the carbohydrate structure (Sandula *et al*. 1999). There are intense peaks at around 1100 cm^{-1}, which can be assigned to the uronic acids that had not been broken by the extraction process (Ying *et al*. 2011). As shown in Figure 2, S6 showed a lower absorption peak than other extracted solids at this region. It was assumed that the uronic acids were instable at the extraction conditions. When prolonging the reaction time from 40 min to 60 min, the uronic acids degraded much faster than its formation, resulting in lower uronic acids content and weak absorption signals at around 1100 cm^{-1}. In addition,

the absorption signals at approximately 900 cm^{-1} can be attributed to the β-glycosidic linkages between sugar units (Sun *et al*. 2003). In conclusion, the FTIR spectra revealed the general characteristic absorption peaks of polysaccharides, and no novel absorbance signals were found.

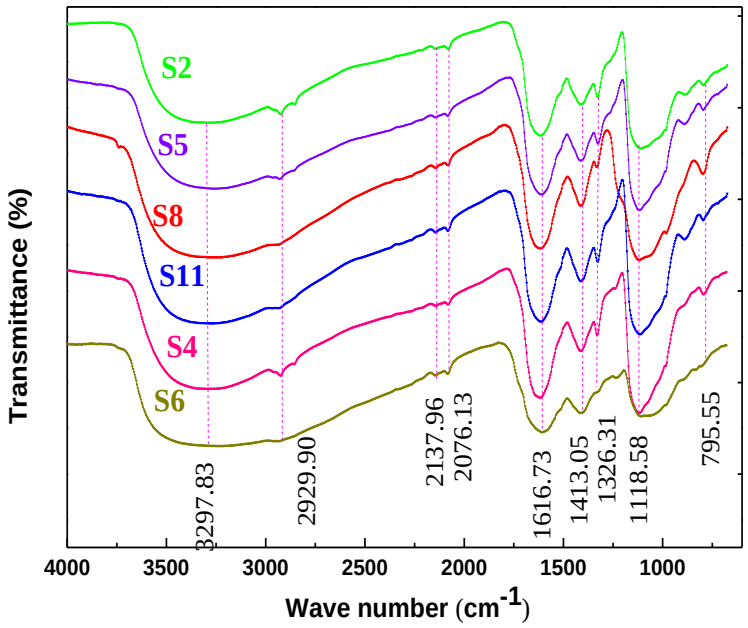

Fig. 2. FTIR spectra of crude polysaccharides extracted under various conditions (S2, S4, S5, S6, S8, and S11 represent samples extracted at 60 °C and 40 min, 70 °C and 20 min, 70 °C and 40 min, 70 °C and 60 min, 80 °C and 40 min, and 90 °C and 40 min, respectively)

Chemical Components

The HPAEC analysis (Table 1) showed that the component sugars of the extracted crude polysaccharides were mannose, fructose, xylose, glucose, galactose, and arabinose. Arabinose, galactose, and glucose were the predominant monosaccharides (relative mass 258.0 mg/g extracted solids to 414.5 mg/g extracted solid, 167.0 mg/g extracted solids to 289.2 mg/g extracted solids, and 157.4 mg/g extracted solids to 246.7 mg/g extracted solids, respectively), along with low levels of mannose (relative mass 6.9 mg/g extracted solids to 14.6 mg/g extracted solids), fucose (relative mass 7.8 mg/g extracted solids to 13.0 mg/g extracted solids), and xylose (relative mass 40.9 mg/g extracted solids to 109.0 mg/g extracted solids). The differences in monosaccharide contents were associated with extraction conditions, which was seen varied at different temperatures and time. In addition to monosaccharides, the extraction conditions also had an apparent effect on the release of total phenol in the final products. At 40 min, there was a higher total phenol content than that with other conditions, except for that at 90 °C (the highest phenol yielded at 20 min). The results could be assumed that the dissolved phenol particles were unstable in the reaction conditions. When the reaction temperature was sever (at 90 °C, for example), it would degraded sharply, resulting in low yield. As relatively severe reaction conditions were favorable for the phenol dissolution, the highest final phenol release reached as high as 0.73 mg/g from bamboo leaves at 70 °C, 40 min.

Table 1. The Monosaccharide Compositions and Phenol Contents of the Crude Polysaccharides Extracted at Different Time and Temperatures*

T (°C)	*t* (min)	Man (mg/g)	Fuc (mg/g)	Xyl (mg/g)	Glu (mg/g)	Gal (mg/g)	Ara (mg/g)	Phenol** (mg/g)	Others*** (mg/g)
60	20	12.8	9.0	55.3	182.8	282.6	293.3	0.31	1.74
	40	9.7	9.8	42.5	182.8	175.0	412.9	0.34	2.17
	60	12.3	8.0	69.8	199.8	273.8	294.3	0.35	2.12
70	20	10.2	10.0	58.4	173.8	289.2	318.4	0.48	2.28
	40	9.9	10.2	43.4	182.0	176.5	414.6	0.73	3.54
	60	12.5	11.7	76.2	223.7	259.8	277.8	0.42	3.66
80	20	11.8	13.0	62.9	246.7	243.7	266.3	0.31	1.89
	40	8.0	9.1	40.9	157.4	203.3	416.5	0.58	3.96
	60	14.6	8.9	109.0	237.4	231.0	258.0	0.44	4.64
90	20	11.8	7.8	65.2	244.4	238.4	260.6	0.50	3.97
	40	6.9	9.1	56.3	167.0	167.0	413.8	0.39	5.03
	60	10.7	8.5	73.9	171.9	244.4	275.3	0.43	5.69

*_T_: temperature; _t_: time; Man: mannose; Fuc: fucose; Xyl: xylose; Glu: glucose; Gal: galactose; Ara: arabinose. All the monosaccharide content was calculated as mass fraction of extracted solid materials.
** The phenol content was calculated as mass fraction of raw bamboo leaves.
*** Others were represented as other particles in the crude polysaccharides like protein, fat, and so on. Its content was calculated as mass fraction of raw bamboo leaves.

Cytotoxicity Analysis

In order to be used in food and pharmaceutical industry applications, the extracted crude polysaccharides must exhibit minimal toxicity. To evaluate the effects of crude polysaccharides on cell proliferation, the cytotoxicity was evaluated against HepG2 cells, taking the samples extracted at 70 °C and 40 min as arepresentative. Cells were exposed to increasing doses of extracted crude polysaccharides solutions with concentrations of 0.01 mg/mL to 1 mg/mL for 0, 12, or 24 h, and the cell viability was determined by the MTS assay. As shown in Figure3, cell viabilities of HepG2 were markedly increased after exposure to extracted crude bamboo leaf polysaccharides for 12 h in a dose-dependent manner. The proliferation of HepG2 cells was increased in correspondence to the increasing extracted crude polysaccharides concentrations (from 0.01 to 0.5 mg/mL). When further increasing the dose to 1 mg/mL, the cell proliferation rate was decreased and resulted in a comparable OD 490 value of the blank sample. The inhibition ratio of HepG2 cells was considerably decreased after exposure to the crude polysaccharides in a dose- and time- dependent manner. The inhibition ratio was -26.77% after 24 h of exposure to 0.5 mg/mL crude polysaccharides, while a -3.02% inhibition ratio was observed after 24 h of exposure to 1.0 mg/mL crude polysaccharides. The results revealed that the extracted crude polysaccharides had no obvious cytotoxicity against HepG2 cells. In addition, in the presence of the bamboo leaf extracts, the cell multiplication rate was markedly accelerated.

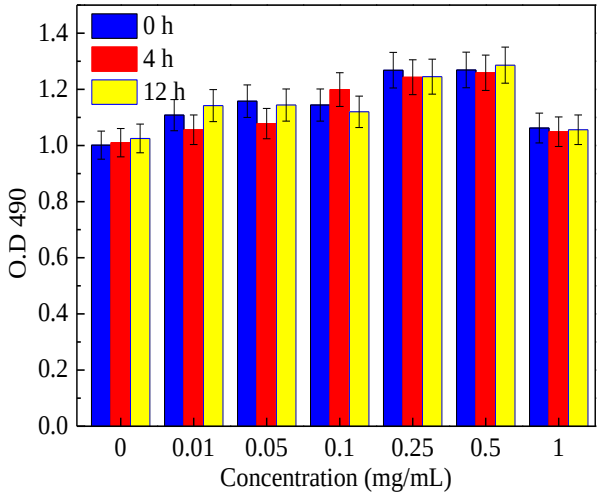

Fig. 3. Cytotoxicity profile of crude polysaccharides extracted at 70 ºC and 40 min against HepG2 cells

Scavenging Activity of DPPH Radical Analysis

DPPH is a stable free radical that has a maximum absorption at 517 nm in methanol. When encountering a proton-donating substance (antioxidants), DPPH radicals are scavenged and the absorbance at 517 nm is reduced. Based on this principle, the antioxidant activity of a substance can be measured. Figure 4 shows that the scavenging activities of all the extracted crude polysaccharides were clearly observed under all tested concentrations and correlated well with increased concentrations up to 10 mg/mL. At 10 mg/mL, the antioxidant effects of all the samples were higher than 85% with less than 3.5 mg/L DPPH residue. The resulting solid material from the extracts at 40 min and 70 °C had the highest antioxidant activity, with an IC_{50} of 1.15 mg/mL. The IC_{50} of ascorbic acid was 0.30 mg/mL, indicating that the DPPH scavenging activity of ascorbic acid was much higher than that of extracted bamboo leaf crude polysaccharides. However, the antioxidant activity was remarkably higher than that of other polysaccharides, such as CMCP from *Cucurbita moschata* (IC_{50} = 7.8 mg/mL, tested with 20 mg/mL DPPH solution) (Wu *et al.* 2014). These results indicated that at appropriate extraction conditions, the extracted crude polysaccharides could have noticeable scavenging effects on free radicals. The mechanism of the extracted crude polysaccharides radical scavenging can be explained as follows: hemiacetal hydroxyl in reducing sugars, phenolic hydroxyl groups, and other components acted as hydrogen donators to react with DPPH radicals to convert them to more stable products.

Ferric Reducing Activity Power

The FRAP assay is commonly used for the routine analysis of single antioxidant and total antioxidant activity of plant extracts (Pulido *et al.* 2000; Schlesier *et al.* 2002). When colorless Fe (III)-TPTZ form reacts with electron-donating antioxidants, a colored Fe (II)-TPTZ form is generated. Antioxidants with higher efficiency generate more Fe (II)-TPTZ, and a deeper color is observed. Figure 5 shows that the reducing power of all samples was notable at all tested concentrations and positively correlated with increased concentrations up to 5.0 mg/mL.

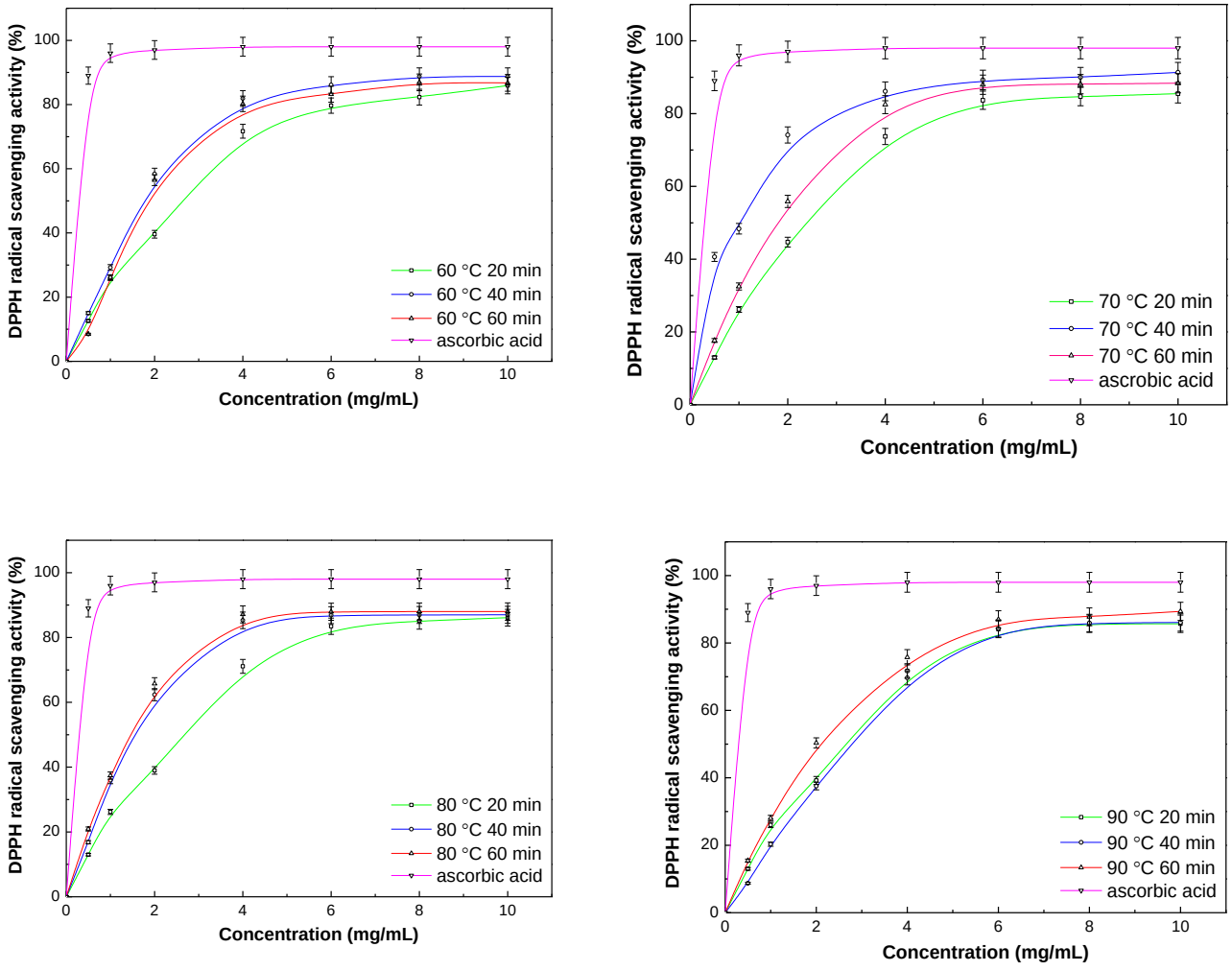

Fig. 4. DPPH scavenging activities of samples extracted under various conditions

The FRAP values of the extracted crude polysaccharides were from 0.77 to 1.80 mM at 5 mg/mL which was remarkably higher than that of the polysaccharide PD3 extracted from soybean sprout (0.80 mM FRAP value at 24 mg/mL polysaccharide concentration) (Yuan *et al.* 2015). The resulting solid material extracted at 40 min and 70 °C exhibited outstanding ferric reducing activity power. The results were perfectly aligned with the DPPH assay.

To explain the reason for the efficient antioxidant activity of crude polysaccharides extracted at 70 ºC and 40 min, one should take the chemical components into account. Compared to other extracted crude polysaccharides, the products extracted at 70 ºC and 40 min have more total phenol content. Therefore, it is assumed that both polysaccharides and polyphenols could contribute to the radical scavenging process, and polyphenols may play a more essential role.

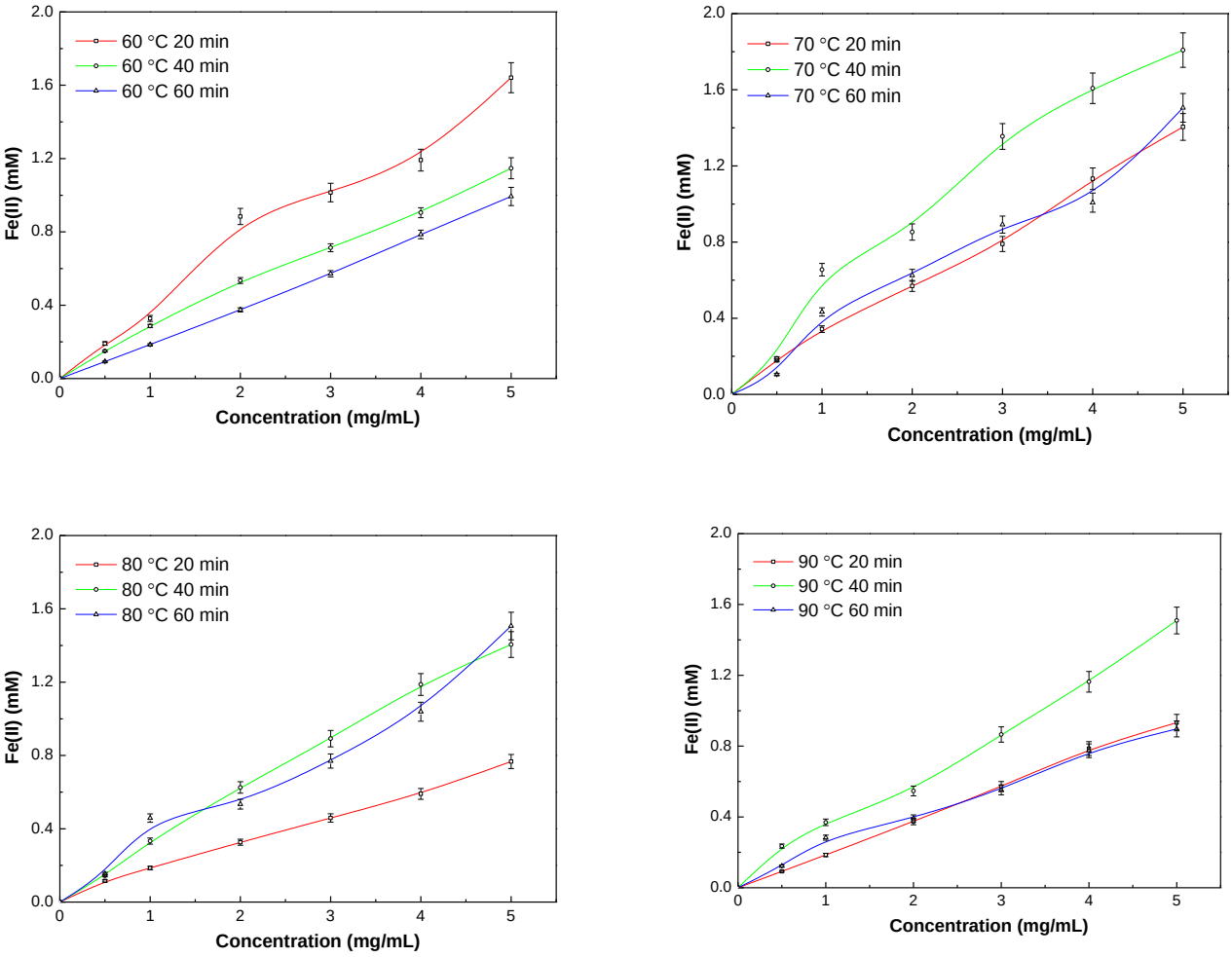

Fig. 5. Ferric reducing power of polysaccharides extracted under various conditions

CONCLUSIONS

1. Over 2000 mg/L of bamboo leaf crude polysaccharides were isolated with the MAE method, and all the extracted solids exhibited excellent antioxidant activities in both DPPH and FRAP assays.
2. Cytotoxicity experiments against HepG2 cells showed that the extracted crude polysaccharides had no inhibitory effects on cell proliferation.
3. There is great potential for the crude polysaccharides extracted from bamboo leaves to be applied as functional food ingredients and pharmaceuticals.

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