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ORIGINAL ARTICLE

Antioxidant and antimicrobial properties of water soluble polysaccharide from *Arachis hypogaea* seeds

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Abstract The water soluble crude polysaccharide (AHP) was obtained from the aqueous extracts of the *Arachis hypogaea* seeds through hot water extraction followed by ethanol precipitation. Antioxidant activities and inhibitory activities against the bacteria of AHP were investigated. AHP at 2 mg/mL was found to inhibit the formation of superoxide anion (55.33 %) and hydroxyl radicals (30.85 %), to scavenge the DPPH radical (57.43 %) and to chelate iron ion (27.83 %) in in vitro systems. AHP also exhibited the antibacterial activities. AHP at 12.5 mg/mL could inhibit the growth of the Gram-positive bacteria, implying that the Gram-positive bacteria were more sensitive to AHP than the Gram-negative bacteria. Polysaccharide with antioxidant and antibacterial activities in the "Chang Sheng Guo" further increased the nutritive values of peanuts as well as the natural health product potential.

Keywords Antimicrobial · Antioxidant · Peanut · Polysaccharide · Seeds

Introduction

Peanut (*Arachis hypogaea*) belongs to the *Fabaceae* family and *Arachis* genus. It has been shown to present several valued dietary constituents (Zhao et al. 2011), including dietary fibers, proteins, micronutrients, monounsaturated fats, and polysaccharides. Peanut has been consumed as a perfect food for their good nutritional value for a long time and is called "Chang Sheng Guo" (meaning in English "long-life nuts") in China.

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"Chang Sheng Guo" means literally that eating peanut constantly could prolong human life. Maybe it sounds a bit exaggerated, but proofs from modern scientific researches prove that many active components in peanut can elicit several biological effects, including cardio-protective (Thompkinson et al. 2012), anti-inflammatory, anticancer and others. Djoko et al. (2007) has found that peanut stilbenoids had the immunological activities. The stilbenoids, arachidin-1, arachidin-3 and resveratrol, have been characterized as antioxidant and anti-inflammatory agents (Abbott et al. 2010). Resveratrol induces programmed cell death and possesses anticancer activity (Huang et al. 2010). Peanut phytoalexins possess antioxidant, antimicrobial, anti-diabetic and anticancer effects (Holland and O'Keefe 2010). Among the bioactive components in peanut, polysaccharides are the important types.

Polysaccharides, which are widely distributed in animals, plants and microorganisms, exhibit varied biological activities and offer health benefits, such as lowering blood cholesterol and protecting bodies against infections, inflammation and tumor (Yuan et al. 2010). Polysaccharides can control free radicals generated in the body and protect the body tissue, thereby help in preventing various diseases caused by tissue damage (Wang et al. 2012). Hence they are considered to possess an important role as radical scavenger for the prevention of oxidative damage (Annegowda et al. 2011).

Recently, more and more published data indicate that polysaccharides from edible and medicinal plants, for example, *Pteridium aquilinum* (Xu et al. 2009), *Ficus carica* L. (Yang et al. 2009), *Lycium barbarum* L. (Lin et al. 2009) and *Portulaca oleracea* L. (Chen et al. 2009), are demonstrated to have strong antioxidant activities, such as scavenging activities against DPPH radicals, superoxide radicals and hydroxyl radicals. But little information is available about the antioxidant and antimicrobial effects of peanut polysaccharides. Therefore, the objective of this work was to evaluate the scavenging ability of DPPH, superoxide

anion and hydroxyl radicals, the chelating ability of iron ion and reducing power of peanut polysaccharides in vitro. Moreover, the effect of the polysaccharides on some bacteria was also evaluated.

Materials and methods

Plant materials Peanut (*A. hypogaea*) seeds were kept at −80 °C. After sterilizing by 70 % (v/v) ethanol for 40–60 s and followed with treatment of 5 % (v/v) sodium hypochlorite (NaOCl) for 20 min, the seeds were washed with sterile water to remove residual NaOCl.

Preparation of crude polysaccharides from A. hypogaea seeds The seeds were thoroughly dried to constant weight at 50 °C with thermostatic blower dryer (YY881-1, Xinyiyang Corporation, China), finely powdered with a mixer (XA-1, Huachenghuanxuan Corporation, China) for 10 min and filtered through 60 mesh sieve. The powder was subjected to successive extraction with n-hexane (powder: n-hexane=1:3) to remove lipids at −20 °C. Then hot 80 % ethanol was used to remove the monosaccharide and oligosaccharide (5 times, 2 h each). The dried powder was then subjected to water extraction. The extraction conditions which had been optimized were described as follows: 100 g defatted powder and 1,500 mL water were added into a flask and extracted at 90 °C for 3 h with stirring. The supernatant was collected by centrifugation (3,000 rpm, 20 min), then condensed by rotary evaporation (RE-85Z, Beilun Corporation, China) and subjected to Sevag method (1938) to remove free proteins. The volume ratio of sample to Sevag agent (chloroform: n-butyl alcohol=4:1) was 2:1. Then the mixture was centrifuged at 5,000 rpm for 15 min to remove free protein. The anhydrous alcohol was slowly added into the deproteinized solution to the final concentration of 67 % and kept for 12 h at 4 °C to precipitate the crude polysaccharides. After centrifugation, the precipitate was washed with anhydrous ethanol and freeze dried to get the crude polysaccharides, which was kept in dark at 4 °C before use. The content of polysaccharides was determined by the phenol-sulphuric acid method (Dubois et al. 1956). In brief, different volumes of 0.1 mg/mL standard glucose solution and the polysaccharides sample were mixed with 1 mL of 5 % phenol solution and 5 mL of 96 % sulphuric acid at 25–30 °C for 20 min. The absorbance was measured at 490 nm. The amount of polysaccharides was calculated by using the standard graph. The crude polysaccharides obtained from the seeds of *Arachis hypogaea* (AHP) were dissolved in water, yielding a series of sample solution with different concentrations (0.4, 0.8, 1.2, 1.6, 2 mg/mL).

Reducing power The reducing power was determined by the method of Mathew and Abraham (2006). Reaction mixture containing 2.5 mL of different concentrations of samples in PBS (0.2 M, pH 6.6) was incubated with 2.5 mL $K_3Fe(CN)_6$ (1 %, w/v) at 50 °C for 20 min. The reaction was terminated by adding 2.5 mL trichloroacetic acid (10 %). The mixture was centrifuged at 2,000 rpm for 10 min. The upper layer of solution (2.5 mL) was mixed with 0.5 mL $FeCl_3$ (0.1 %, w/v) and 2.5 mL distilled water. The absorbance was measured at 700 nm against a blank. A higher absorbance indicated a higher reducing power. Ascorbic acid was used for comparison.

DPPH (1-diphenyl-2-picrylhydrazyl) radical scavenging activity The radical scavenging activity of the samples was measured according to the procedure described by Negro et al. (2003). The 0.2 mM solution of DPPH in methanol was prepared freshly before the measurements. The AHP solutions (2 mL) with different concentrations were thoroughly mixed with 2 mL DPPH solution, and allowed to stand for 30 min in the dark. The control solution contained equivalent distilled water instead of the sample solution. The absorbance was measured at 517 nm against a blank control without the sample. The capability to scavenge the DPPH radical was expressed as percentage of DPPH scavenging relative to blank. Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The experiments were carried out in triplicate and the data were averaged. BHT (butylated hydroxytoluene) was used as positive control. The scavenging rate was calculated according to the equation: $\text{scavenging rate}(\%) = \left(A_{\text{control}} - A_{\text{sample}}\right) \times 100\%/A_{\text{control}}$.

Superoxide anion radical scavenging activity The superoxide radical scavenging activity of the samples was assessed by Chen's method (2011). The AHP samples were dissolved in 50 mM Tris–HCl buffer (pH 8.2) to form varying concentrations (0.4, 0.8, 1.2, 1.6, 2 mg/mL). The reaction systems contained 5 mL AHP solution and 10 μl of 50 mM pyrogallol solution. The absorbance variation was detected within 2 min at 325 nm. For the blank control, the sample was replaced by the Tris–HCl buffer. The scavenging rate was calculated as: $\text{scavenging rate}(\%) = \left(\Delta A_{\text{control}} - \Delta A_{\text{sample}}\right) \times 100\%/\Delta A_{\text{control}}$. $^{\triangle}A_{\text{sample}}$ and $^{\triangle}A_{\text{control}}$ were the absorbance variations of the AHP solutions and the control within 2 min, individually.

Hydroxyl radical scavenging activity The scavenging activity of polysaccharides against the hydroxyl radical was investigated by using Fenton's reaction ($Fe^{2+} + H_2O_2 \rightarrow$

Fe^{3+} + OH^- + ·OH). The hydroxyl radical scavenging activity of AHP was measured with the salicylic acid method with some modifications (Zhong et al. 2010). The reaction mixture was prepared in order: 1 mL $FeSO_4$ (9.1 mM), 1 mL salicylic acid (9.1 mM) and 1 mL AHP sample with different concentration. One mL H_2O_2 (8.8 mM) was added to the mixture to start the reaction. The reaction mixture was incubated at 37 °C for 60 min and then centrifuged at 14,000 g for 6 min. The absorbance of the solution was measured at 510 nm. A similar procedure was used to prepare the control solution and sample solutions. The control solution contained all reagents except water in place of the samples. The experiments were carried out in triplicate and the data were averaged. BHT was used as positive control. The scavenging rate was calculated according to the equation: $\text{scavenging rate}(\%) = \left[A_{control} - \left(A_{sample} - A_0\right)\right] \times 100\%/A_{control}$

A_0 was the absorbance for background (instead of H_2O_2).

Metal chelating assay The ferrous ion chelating ability of AHP was investigated with slightly modified method of Li et al. (2007). Samples (1 mL) with different concentration were mixed with $FeCl_2$ (0.1 mL, 2 mM) and ferrozine (0.4 mL, 5 mM). The mixture was shaken well and allowed to stand for 10 min at room temperature. Then the absorbance of the mixture was determined at 562 nm. In the control, the sample was substituted with water. EDTA was used as positive control. The ferrous ion chelating activity was given by the following equation: $\text{chelating ability}(\%) = \left(A_{control} - A_{sample}\right) \times 100\%/A_{control}$

Assay for antimicrobial activities *Staphylococcus aureus* (ATCC6538), *Bacillus subtilis* (ATCC9372) and *Escherichia coli* (ATCC8099) were inoculated into separate nutrient broths and cultured at 37 °C for 12 h. Broths of the test microorganism (0.4 mL) were evenly spread on the individual nutrient agar plate under sterile conditions. Then AHP solutions with different concentration (6.25, 12.5, 25, 50, 75, 100 mg/mL) were absorbed onto sterile filter paper discs (diameter: 7 mm) individually. Each disc with the sample at different concentration was placed at the intersected space of the same agar plate. Filter paper discs having sterile H_2O and ampicillin were used as controls. One agar plate had six sample discs and two control discs totally. The plates were incubated at 37 °C. After 24 h, the diameter of any clear inhibition zone around the discs in the bacteria plates was measured in three different directions. All experiments were repeated for six times.

Statistical analysis The antioxidant and antibacterial data were analyzed by using analysis of variance (ANOVA) in SPSS statistics software (Version 18, IBM Corporation, New York, USA) for significance.

Results and discussion

A. hypogaea is not only known as a good source of oil and protein, but also it possesses great diversity of bioactive components, which have potential therapeutic and other biological functions (Lopes et al. 2011). AHP, obtained from the *Arachis hypogaea* seeds by hot water extraction followed by ethanol precipitation, was white to pale, odorless and soluble powder. An average yield of the AHP was 8.28 % (W/W, dry weight). The antioxidant and antimicrobial activities of AHP were studied.

Reducing power A compound with reducing power may serve as a potential antioxidant (Arabshahi-Delouee and Urooj 2007). By donating electrons, antioxidant substances are able to block radical chain reaction by converting reactive oxygen species to more stable products. In this reducing power assay, AHP could reduce the Fe^{3+}/ferricyanide complex to the ferrous form, which was monitored by measuring the formation of Perl's Prussian blue at 700 nm. The result showed that the absorbance, reflecting the reducing power, increased with the increasing concentration of polysaccharides (Fig. 1a). The reducing power of AHP was much lower than that of ascorbic acid.

DPPH radical scavenging activity The DPPH method is widely used to evaluate the free radical scavenging ability of natural compounds (Kedare and Singh 2011). DPPH radical-scavenging activity is conceived to be due to their hydrogen-donating ability. In the DPPH test, the antioxidant is able to reduce the stable DPPH radical to the yellow-colored diphenylpricrylhydrazine (Chen et al. 2008). DPPH radical scavenging activity of AHP was determined by using different concentrations of the extract. As shown in Fig. 1b, radical scavenging activity increased significantly with AHP concentrations ($p<0.05$). The scavenging activity of AHP at 2 mg/mL was 57.43 %, whereas the scavenging activity of BHT at 0.2 mg/mL was over 67.07 %. The results indicated that AHP could scavenge DPPH free radicals, especially at high dosages. However, the scavenging activity of AHP was lower than that of BHT in this study.

Superoxide anion radical scavenging activity Excessive production of superoxide anion radical has been regarded as the beginning of reactive oxygen species accumulation in cells, resulting in redox imbalance and related harmful physiological consequences (Li et al. 2010). As shown in Fig. 1c, the concentration of AHP was positively correlated with its scavenging activity on superoxide anion radical. The scavenging activity of AHP at 2 mg/mL was 55.33 %.

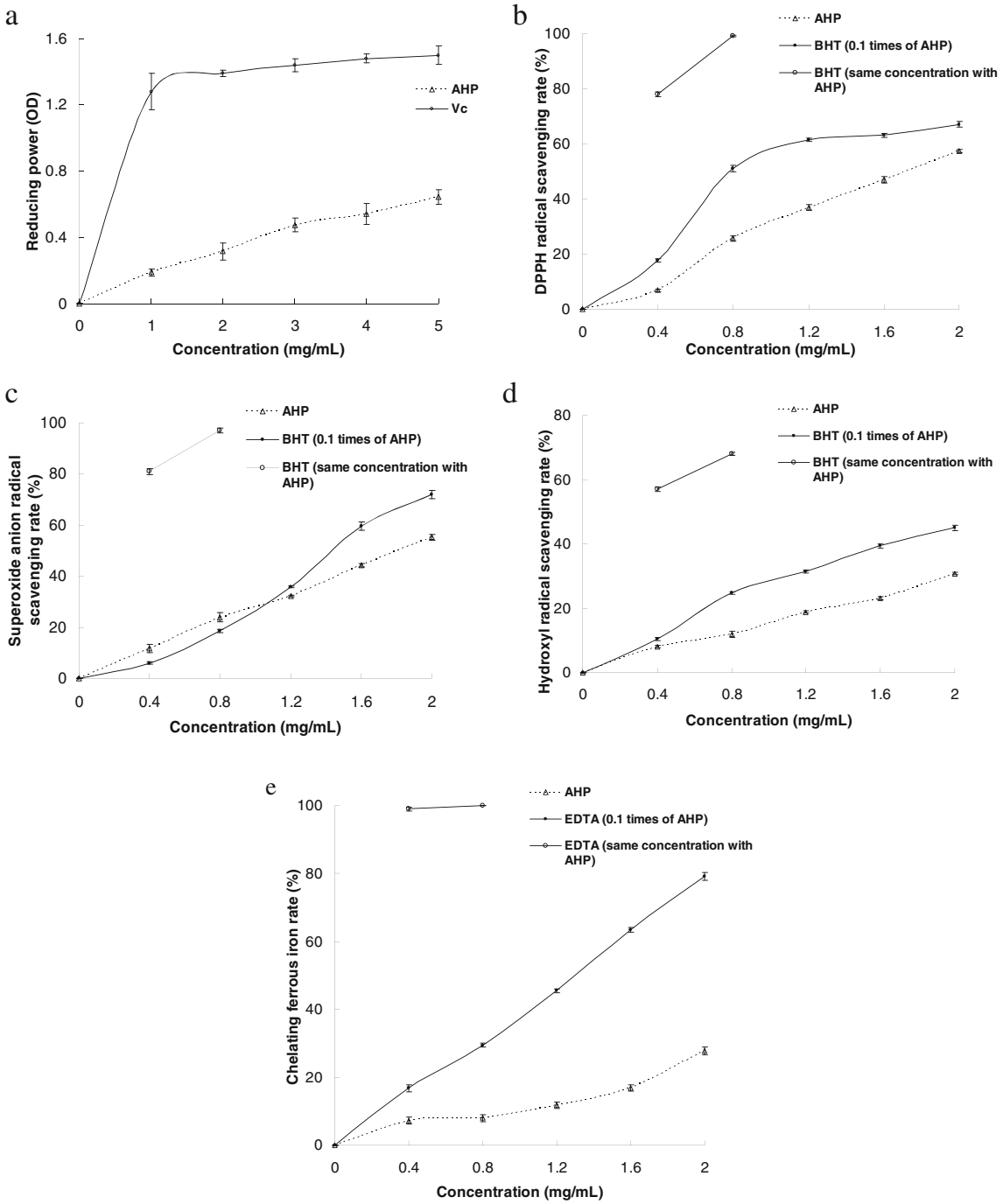

Fig. 1 Reducing power (compared to ascorbic acid); DPPH scavenging activity (compared to BHT); superoxide anion radical scavenging activity (compared to BHT); hydroxyl radical scavenging activity (compared to BHT) and Chelating activity (compared to EDTA) of water soluble crude peanut polysaccharide (AHP) as a function of concentration (n=3). The data are mean ± SD. The *bars* are SD values

Hydroxyl radical scavenging activity Hydroxyl radical can be generated in biological cells through the Fenton reaction and is thought to initiate cell damage in vivo (Zhong et al. 2010). The hydroxyl radical scavenging activities of AHP and BHT were shown (Fig. 1d). Both exhibited significant hydroxyl radical scavenging activities which increased with the concentration ($p<0.05$). The scavenging activity of AHP was 30.85 % at 2 mg/mL.

Metal chelating assay Ferrum is an important lipid oxidation prooxidant due to its high reactivity. The ferrous state of ferrum can accelerate lipid oxidation by breaking down

hydrogen and lipid peroxidase to reactive free radicals. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of other chelating agents, the complex formation is disrupted. Measurement of specific OD value therefore is used to estimate the metal chelating activity (Wang et al. 2009). The metal chelating activities of AHP and EDTA were shown in Fig. 1e. A maximum chelating effect of 27.83 % was achieved for AHP at the concentration of 2 mg/mL, which was lower than that attained by EDTA (29.48 %) at 0.08 mg/mL.

Under both normal and pathological conditions, it is of great significance to discover some safe compounds with radicals scavenging activity for the oxidative stress in human body (Zhou et al. 2010). BHT has been widely used as a syntherical food additive in the food technology. AHP had the advantage and the extensive future over BHT in the sensitive food-safety issue because AHP was the safe, inartificial and nutritious component of peanut.

Assay for antimicrobial activities The antibacterial activity of AHP was tested against *S. aureus*, *B. subtilis* and *E. coli*. AHP showed different activities in vitro against the three tested bacteria at the concentrations which ranged from 6.25 mg/mL to 100 mg/mL. To determine the antibacterial sensitivity of AHP, *S. aureus* was compared with ampicillin (10 mg/mL), and *B. subtilis* and *E. coli* were compared with 50 mg/mL ampicillin under the same experimental conditions. As shown in Table 1, the antibacterial activity of AHP against *S. aureus* was highest at 12.5 mg/mL and the inhibitory zone diameter was 24.3±0.62 mm. Not all the concentrations of AHP could inhibit the bacteria. Further analyses showed that antibacterial sensitivity of AHP varied with the bacteria species. *S. aureus* and *B. subtilis* were sensitive to AHP with low concentration (12.5 and 25 mg/mL), while *E. coli* could be inhibited by higher concentrations of AHP solution (50, 75 and 100 mg/mL). It indicated that the Gram-positive bacteria were found to be more sensitive to AHP than the Gram-negative bacteria. The first possible reason involved the different cellular structures of the Gram-positive and Gram-negative bacteria. The cellular structures might influence the sensitivities to AHP of the bacteria. The second aspect related to polysaccharides property. As a kind of carbohydrate, polysaccharides might be partially used by the bacteria. So the highest concentration of AHP tested didn't have the significant inhibitory effect against all the test bacteria. That was to say, the inhibitory effect was not dose-dependent.

Polysaccharides with antioxidant and antibacterial activities in peanut further add to the peanut nutritive value of peanuts as well as the natural health products potentials. Including peanuts in daily diet allows one to easily meet a reasonable amount of the hygienical requirements. The current work will provide new reference data for the uses of peanut as the potential nourishment and health treasure in food and pharmaceutical industries.

Conclusion

In conclusion, AHP had the reducing power. It had the moderate free radical scavenging abilities on DPPH and superoxide anion radicals, and indicated a little weak activity on scavenging hydroxyl radical and on chelating ferrous iron ion. All the antioxidant activities increased with the polysaccharides concentration. Gram-positive bacteria were more sensitive to AHP than the Gram-negative bacteria. And the inhibitory effect was not dose-dependent. The current work will provide new reference data for the uses of peanut as the potential nourishment and health treasure in food and pharmaceutical industries.

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Table 1 Diameters of the inhibitory zones against the bacteria of peanut polysaccharides

Bacteria	Concentration of peanut polysaccharides (mg/mL)						Control	
	6.25	12.5	25	50	75	100	H_2O	Ampicillin
S. aureus	-	24.3 ± 0.62^{B}	12.6 ± 0.81^{C}	-	-	-	-	$49.7\pm1.52^{\triangle A}$
B. subtilis	-	8.2 ± 0.46^{B}	7.2 ± 0.52^{B}	-	-	-	-	$11.6\pm1.05^{*A}$
E. coli	-	-	-	7.1 ± 0.51^{C}	8.2 ± 0.54^{C}	9.7 ± 0.63^{B}	-	$23.3\pm1.14^{*A}$

Ampicillin: 50 mg/mL (*), 10 mg/mL ($^{\triangle}$). The symbol (-) means that there is no effective inhibitory zone. The diameter is mean ± SD (n=6) and the unit is millimeter. Values with different superscripts differ significantly ($p<0.01$) (n=6)

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