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Characterization and antioxidant activities of degraded polysaccharides from *Poria cocos* sclerotium

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ABSTRACT

Poria cocos F.A.Wolf is a Chinese traditional medicine used to treat chronic gastritis, edema, nephrosis, gastric atony, and acute gastroenteric catarrh. Polysaccharides are the main active component of *P. cocos*. We obtained polysaccharides PCP-1, PCP-2, and PCP-3 from the degradation of *P. cocos* polysaccharides (PCP) with different concentrations of H_2O_2 solution. Molecular weights were determined by high performance size exclusion chromatography. HPLC analysis of monosaccharide composition confirmed that PCP-1, PCP-2, and PCP-3 are heteropolysaccharides composed of glucose and arabinose. IR spectra indicated obvious characteristic peaks of polysaccharides. The antioxidant activities of these polysaccharides were evaluated by established *in vitro* systems, including scavenging activity of hydroxyl radicals, ABTS radicals, and ferrous ions. The degradation polysaccharides exhibited obvious and concentration-dependent antioxidant properties. In addition, DNA binding analysis showed that PCP-1 had a stronger capacity than other polysaccharides to interact with DNA. However, each polysaccharide had a certain capacity for DNA damage protection.

1. Introduction

Poria cocos F.A.Wolf is a common traditional East-Asian medicinal fungus that grows around the roots of pine trees in China, Korea, Japan, and North America (Lee & Jeon, 2003). It is widely used as a component in prescriptions of traditional medicines that have invigorating activity and diuretic activity (Tai, Shingu, Kikuchi, Tezuka, & Akahori, 1995).

Polysaccharides are the one of the most abundant natural biopolymers, which exist in almost all organisms. The main functions of polysaccharides in organisms include energy storage, structural support, and antigens. Polysaccharides are important components of the cells and can be derived from plants, animal cell membranes, or microbial cell walls (Zhang, Duan, Zhou, & Yang, 2005). Polysaccharides from natural resources possess immunological properties, such as nonspecific stimulation of host immune systems, resulting in antitumor and antiviral effects, and antioxidant and antimutagenic activities (Sun, Liu, & Kennedy, 2010; Kennedy, 1989; Wang, Chen, Jia, Tang, Ma, & Xu, 2012). After Chihara et al. confirmed the antitumor activity of lentinan for the first time (Chihara, Hamuro, Maeda, Arai, & Fukuoka, 1970), more studies have focused on fungal polysaccharides. Polysaccharides extracted from *Polyporus umbellatus*, *Ganoderma lucidum*, *Auricularia auricula*, and *Polystictus versicolor* had obvious antitumor activity (Zhang, Cui, Cheung, & Wang, 2007).

The main component of *P. cocos* sclerotium is β-(1,3)-D-glucan, but it is water insoluble and shows poor biochemical activity (Ding, Zhang, & Zeng, 1998). To improve water solubility and maximize the development of biochemical activities, research is directed toward modified polysaccharides (Ohno, Kurachi, & Yadomae, 1988; Yang & Du, 2003). The polysaccharide derivatives of *P. cocos* sclerotium exhibited significant water solubility and strong inhibition of S-180 tumor cells after carboxymethylation and phosphorylation (Chen, Xu, Zhang, & Zeng, 2009; Wang & Zhang, 2006). To lower the molecular weight and obtain higher antioxidant activity polysaccharides, ascorbic acid and H_2O_2 were used in combination as degradation reagents (Zhang, Wang, Mo, & Qi, 2012). In general, polysaccharide activity depends on several structural parameters such as sugar type, molecular weight, and degree of functional group substitution (Melo, Feitosa, Freitas, & De Paula, 2002). At present, structural modification is the main method of enhancing polysaccharide activity. While research has been performed on *P. cocos* polysaccharides, H_2O_2 degradation products have not been reported before.

In this study, H_2O_2 was used as a degradation agent and three degraded polysaccharides were obtained. Polysaccharide chemical characterizations and antioxidant activities were investigated using various established *in vitro* systems.

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2. Materials and methods

2.1. Materials

Fresh *P. cocos* sclerotium was excavated from Dali and Yunnan provinces of China. 3-Phenylphenol was purchased from Acros Organics Co. (Geel, Belgium). 2,2′-Azino-bis(3-ethylebenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), ferrozine, and calf thymus DNA (ctDNA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals reagents were of analytical grade from Sinopharm (Shanghai, China).

2.2. Preparation of the degradation polysaccharides

Original polysaccharides were extracted from *P. cocos* sclerotium in 0.5 M alkaline solution and precipitated by 90% alcohol to give a main fraction, which was named PCP (Huang, Jin, Zhang, Cheung, & Kennedy, 2007). Then, the reaction was moved to a round-bottom flask immersed in a thermostat bath at 70 °C. PCP (10.0 g) was added to the different concentrations of H_2O_2 (1000 mL, 8%, 4%, and 2%, v/v). After stirring for 4 h, the degradation solutions were centrifuged and filtered. The filter liquor was selected and concentrated to a certain volume after using the Sevage method (Sevage, 1934) to remove the protein, and ethanol was added to make the final concentration 80% (v/v). Solutions were kept at 4 °C overnight. Then, precipitates were collected by centrifugation, and dried to get the degradation polysaccharides PCP-1, PCP-2, and PCP-3 in a vacuum oven. The polysaccharide degradation yield (%) was calculated as follows:

$$\text{Yield}\ \ (\%, \text{w/w}) = \frac{\text{dried degradation polysaccharide weight}}{\text{original polysaccharide (PCP) weight}} \times 100$$

2.3. Characterization of degradation polysaccharides

The total sugar content was analyzed with the phenol-sulfuric acid method using glucose as the standard (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Uronic acid was estimated with a modified carbazole method using D-glucuronic acid as the standard (Bitter & Muir, 1962). Biuret reaction was used to determine protein concentration (Gornall, Bardawill, & David, 1949). Free amino acids were analyzed by the ninhydrin reaction (Wu, Hu, Huang, & Jiang, 2013). Iodination was used to measure starch (Li et al., 1994). Phenolic or reducing sugar substance was assayed by the ferric chloride method (Zhou, 1978). Free monosaccharides were found using the benedict reaction (Wu et al., 2013).

Fourier transform infrared (FT-IR) spectra of the degradation polysaccharides were measured by a Thermo Scientific Nicolet iS10 FT-IR spectrometer (Waltham, MA) in KBr disks from 500 to 4000 cm^{-1}.

The molecular size chromatography-refractive index associated with multi-angle laser light scattering techniques (SEC-RI-MALL) measurement was performed on a SEC column (Agilent PL aquagel-OH 40) equipped with a pump (Vavian 210) at 20 °C. A multi-angle laser light photometer (Wyatt-DAWN HELEOS-II, Wyatt Technology Co., USA) and a differential refractive index detector (RI, Wyatt-Optilab T-rex) were simultaneously connected. The concentration of polysaccharide solution was 12.0–20.0 mg/mL in distilled water. After passing through 0.2-μm syringe filters (Whatman, England), 20 μL of solution was injected. The isocratic mobile phase was 0.15 M NaCl aqueous solution, which contained 2 mL acetic acid at a flow rate of 1 mL/min. The specific refractive index increment (dn/dc) value of the polysaccharides in distilled water was 0.147 mL/g at 633 nm in 20 °C. Astra software (version 4.90.07 for Windows, Wyatt Technology Corp.) was used for data acquisition and further analysis.

The molar ratio of monosaccharide composition was analyzed by high performance liquid chromatography-evaporative light scattering detection (SEDEX90 LT-ELSD). Polysaccharides were hydrolyzed with 2 M trifluoroacetic acid (6 h, 100 °C), evaporated to dryness, dissolved in distilled water (4 mL), then subjected to HPLC analysis. Monosaccharides were separated on a lead column (DIKMA Sugar SP0810) at 70 °C. The eluent was HPLC-grade water, with a flow rate of 1.0 mL/min. D-Glucose and D-arabinose were used as references.

2.4. Antioxidant activities

2.4.1. Hydroxyl radical scavenging assay

The scavenging ability of the degraded samples on hydroxyl radicals was investigated as previously described (Smirnoff & Cumbes, 1989), via a well-known Fenton-type reaction with a slight modification. Briefly, 0.1 mL of different sample concentrations were mixed with 0.05 mL $FeSO_4$ (9 mmol/L) and 0.1 mL salicylic acid–ethanol solution (9 mmol/L). Then, 0.1 mL H_2O_2 (8.8 mmol/L) was added before incubation at 25 °C for 60 min. Ascorbic acid was used as positive control, distilled water was used as a blank control. The absorbance of the mixtures was measured at 510 nm. Hydroxyl radical scavenging activity was calculated by the following equation:

$$\text{Scavenging rate}\ \ (\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100,$$

where A_0 is the absorbance of the control group (water instead of test sample solution). A_1 is the absorbance of the test group, and A_2 is the absorbance of the samples only (water instead of H_2O_2 solution).

2.4.2. ABTS radical scavenging assay

The antioxidant activity was also determined by the ABTS radical scavenging activity measured as described by Re, Pellegrini, Proteggente, Pannala, Yang, and Rice-Evans (1999) with some modifications. 2.5 mL of 7 mM ABTS diammonium salt was mixed with 0.5 mL of 15 mM potassium persulfate. After storage in the dark for 24 h, the solution was diluted with distilled water to yield an absorbance of 0.700 at 734 nm. The diluted solution (0.2 mL) was added to 0.05 mL sample solution (2, 4, 6, 8, and 10 mg/mL). The solution was then measured at 734 nm after incubation for 15 min at 20 °C. BHT was used as positive control, distilled water was used as the blank. The scavenging activity of the samples was calculated according to the following equation:

$$\text{Scavenging activity}\ \ (\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100,$$

where A_0 is the absorbance of the control group (water instead of test sample solution). A_1 is the absorbance of the test group, and A_2 is the absorbance of the samples only (water instead of ABTS solution).

2.4.3. Ferrous ion chelating assay

The ferrous ion chelating effect of various polysaccharide fractions was determined according the method reported by Carter (Carter, 1971) with some modifications. A reaction solution, composed of 0.1 mL of different concentrations of the test sample (1.0–10 mg/mL) and 0.01 mL of $FeCl_2$ (2 mM), was shaken well and left to sit for 30 s before it was activated by the addition of 0.02 mL 3-(2-pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine (ferrozine, 5 mM) and 0.27 mL distilled water. After mixing, it was incubated at room temperature for 10 min. Absorbance was measured at 562 nm against a blank. EDTA was

used for comparison. The ferrous ion chelating ability of different concentrations of tested samples was calculated using the following equation:

$$\text{Chelating ability } (\%) = \left(1 - \frac{A_1 - A_2}{A_0}\right) \times 100,$$

where A_0 is the absorbance of the control only, A_1 is the absorbance of tested sample, and A_2 is the absorbance of the tested sample only without $FeCl_2$.

2.5. DNA binding assay

The interaction between polysaccharides and ct-DNA was studied by a ultraviolet (UV) spectrometer described by Huang et al. (Huang, Li, & Yan, 2008; Xiao, Zhang, & Pang, 2009) with slight modifications. Measurements were taken in a quartz cuvette at room temperature using a Lambda 25 Perkinelmer UV Spectrometer. Tris–HCl buffer (50 mM, pH = 8.2) was used to prepare polysaccharide solutions with different concentrations (15, 10, 8, 4, and 2 mg/mL) and ct-DNA solutions (0.1 mg/mL). 0.1 mL polysaccharide solutions with different concentrations were added to the ct-DNA solution (0.2 mL). Reactions took place in the cuvette immersed in a thermostat bath at 37 °C for 15 min. Absorbance curves from 200 to 400 nm were recorded. The ct-DNA solution (0.067 mg/mL) was used as the blank.

2.6. DNA damage protective effect

The DNA damage protective activities of polysaccharides were assayed with Plvx-shrna plasmid DNA, isolated from *Escherichia coli* DH5α by a Fast plasmid mini kit. Plasmid DNA was damaged by H_2O_2 and UV treatment using the method described by Russo et al. (2000) with some modifications. Different structural or conformational forms of plasmid DNA were separated by electrophoresis. The reaction system contained 3 μL of plasmid DNA, 5 μL of polysaccharide (5 mg/mL), and 1 μL of H_2O_2 (15 mmol/L). The mixtures were placed on a sterile bench under a UV lamp (20 W) for 1 min. After irradiation, reaction samples with loading buffer (10×) were separated by electrophoresis on a horizontal 0.8% agarose gel in Tris–borate–EDTA buffer (45 mmol/L Tris–borate, 1 mmol/L EDTA, pH = 8) for 25 min (105 V) (Biso et al., 2010). Rutin (1 mg/mL) was used as a positive control, the damaged and undamaged plasmid DNA was used as a negative and blank control, respectively.

2.7. Statistical analysis

All the experimental data were analyzed using Origin (version 7.5 for Windows, OriginLab, CO, USA) and recorded as mean ± standard deviation (SD) for at least three replicates. Differences were considered significant at $P < 0.05$.

3. Result and discussion

3.1. Preparation of the degradation polysaccharide from PCP

White and water-soluble powders were obtained after drying in a vacuum oven. The H_2O_2 concentration, reaction temperature, and reaction time have a great impact on the degradation of polysaccharides (Hou, Wang, Jin, Zhang, & Zhang, 2012). The degradation yields were 10.53 ± 0.83%, 6.89 ± 0.92%, and 2.27 ± 0.85% from PCP-1 to PCP-3, respectively. We found that the degradation ratio of PCP increased as the concentration of the H_2O_2 increased.

3.2. Polysaccharide degradation assay

The chemical properties of the three degradation polysaccharides are listed in Table 1. The ninhydrin, biuret, iodination, ferric chloride, benedict, and carbazole tests were negative for the three polysaccharides, revealing that there are no free amino acids, proteins, starch, free monosaccharides, free reducing sugars, or polyphenols. However, there was uronic acid present. The total sugar content was 84.15 ± 6.18%, 79.94 ± 8.50%, and 72.02 ± 7.10%, respectively. The molar ratio of monosaccharides revealed that the samples were mostly glucose, which agrees with the conclusion that the main polysaccharide component of *P. cocos* sclerotium is a linear β-(1,3)-D-glucan (Wang et al., 2004). SEC-RI-MALLS was used to determine molecular weights, which were 2.33×10^3 Da, 3.20×10^3 Da, and 2.85×10^3 Da, respectively.

3.3. Infrared spectrum

As shown in Fig. 1, the main peaks are similar among the three polysaccharides. All samples showed a broad and intense band from 3440 to 3400 cm^{-1}, which was assigned to the hydrogen-bonded hydroxyl groups (Wang, Zhang, Di, Liu, & Wu, 2011). A weak band at around 2930 cm^{-1} was attributed to C—H group antisymmetrical stretching vibration (Zhang, Ye, & Wang, 2010). The band at around 1615 cm^{-1} was assigned to the stretching vibrations of the CHO and C=O bonds (Tian et al., 2012). Some weak bands from 1400 to 1200 cm^{-1} were also FT-IR characteristic absorptions of a polysaccharide (Coimbra, Barros, Barros, Rutledge, & Delgadillo, 1998). In addition, the spectra showed several weak and specific bands in the 1200–1000 cm^{-1} region, which were attributed to ring vibrations overlapped with stretching vibrations of (C—OH) side groups, and C—O—C glycosidic bond vibrations (Coimbra et al., 1998). These results indicate that PCP-1, PCP-2, and PCP-3 possessed typical absorption peaks of polysaccharides.

3.4. Antioxidant activity

Samples showed obvious, concentration-dependent antioxidant properties. Furthermore, the samples showed more significant scavenging activity to hydroxyl radicals than to ferrous ion or ABTS radicals. Therefore, we concluded that this was relevant to the combination mechanism between the polysaccharides and the free radicals, which needs to be further researched.

3.4.1. Hydroxyl radical scavenging assay

Hydroxyl radicals are considered the most harmful free radicals among the reactive oxygen species, and they can impact biomacromolecules in living cells (Gülcin, 2006). As shown in Fig. 2, the scavenging activity increased with increasing sample concentration. The EC_{50} values were 1.457 mg/mL, 2.109 mg/mL, and 1.754 mg/mL, respectively. The scavenging activity of PCP-1 was slightly higher than the others. At a concentration of 5 mg/mL, the polysaccharides were over 90% effective, the scavenging effects was up to 99% when the concentration of ascorbic acid was at 1 mg/mL. These results indicate that degradation polysaccharides should be explored as potential antioxidants.

3.4.2. ABTS radical scavenging assay

The ABTS assay is often used to determine the total antioxidant power of single compounds or complex mixtures of various plants (Katalinic, Milos, Kulisic, & Jukic, 2006). Concentration-dependent radical scavenging activity is also shown in Fig. 3. Scavenging activity decreased in the order: PCP-3 > PCP-2 > PCP-1, and the EC_{50} values were 5.392 mg/mL, 8.507 mg/mL, and 9.521 mg/mL respectively. The scavenging effect of BHT ranged from 45.3% to 85.8% at the concentration from 0.01 mg/mL to 0.1 mg/mL. This indicates

Table 1
Comparison of the chemical properties of the three polysaccharides.

Samples	Total sugar (%)	Uronic acids (%)	Mw (kDa)	Neutral sugar (mole ratio, glucose as 1)[a]	
				Ara	Glu
PCP-1	84.15 ± 6.18	8.75 ± 1.47	2.33 ± 3.63%	0.02	1
PCP-2	79.94 ± 8.50	8.07 ± 1.12	3.20 ± 3.09%	0.01	1
PCP-3	72.02 ± 7.10	7.19 ± 0.85	2.85 ± 2.71%	0.03	1

[a] Ara: arabinose; Glu: glucose.

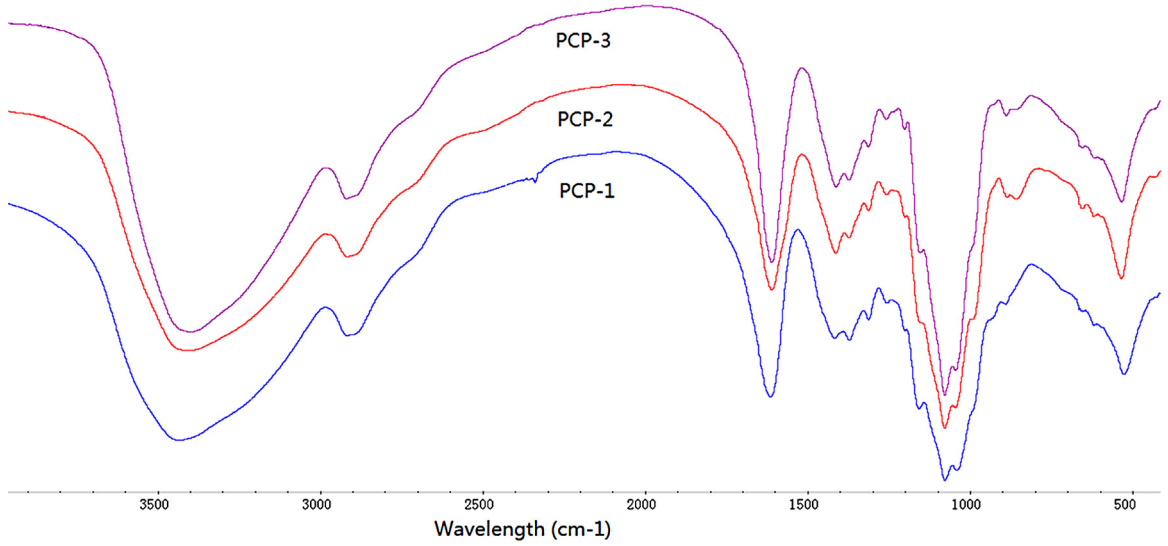

Fig. 1. IR of the PCP-1, PCP-2 and PCP-3.

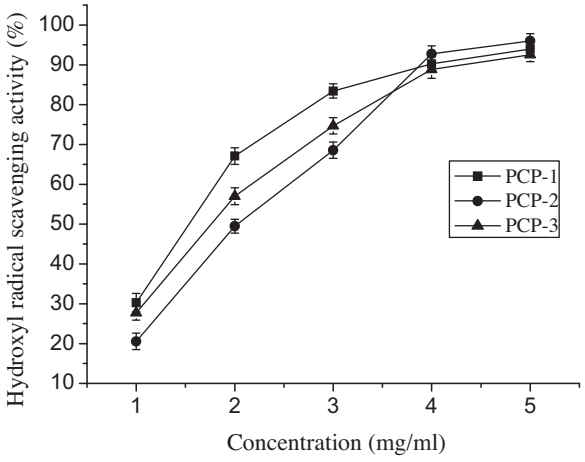

Fig. 2. Scavenging effects of polysaccharides on hydroxyl radical.

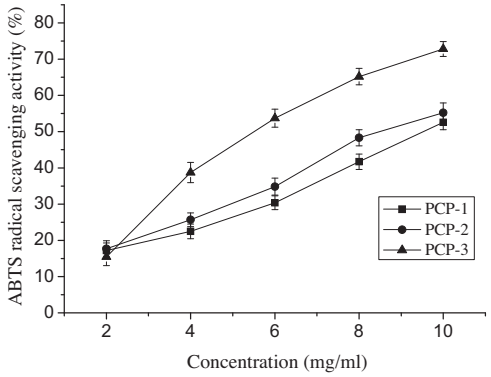

Fig. 3. Scavenging effects of polysaccharides on ABTS radical.

that PCP-3 had a better effect than others. PCP-3 had a radical scavenging rate of 72.6% at a concentration of 10 mg/mL.

3.4.3. *Ferrous ion chelating assay*

Metal chelating activity is generally acknowledged as an antioxidant mechanism because it reduces the concentration of the catalyzing transition metal in lipid peroxidation (Chen, Ju, Li, & Yu, 2012). Ferrozine is a sensitive reagent, which can mix with ferrous ions and form colored species (iron (II)–ferrozine complex). After antioxidants are introduced to the system, they compete with ferrozine for ferrous ions, decreasing the absorbance of the solution. As shown in Fig. 4, the metal chelating rate increased from 79.0% to nearly 100.0%, when the concentration of PCP-1 was increased from 1.0 to 10.0 mg/mL. However, EDTA could Exhibit 91.7% scavenging effect at a low concentration of 0.08 mg/mL. This indicates that PCP-1 has strong and stable scavenging ability. Chelating ability of the samples decreased in the order: PCP-1 > PCP-2 > PCP-3.

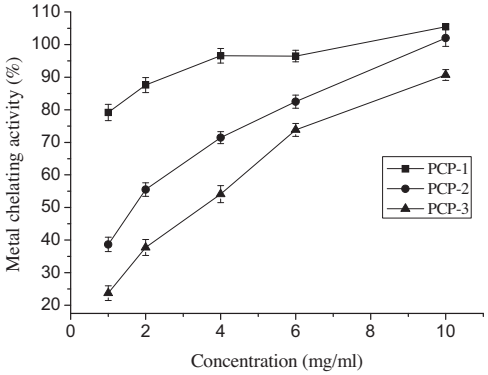

Fig. 4. Scavenging effects of polysaccharides on ferrous ion.

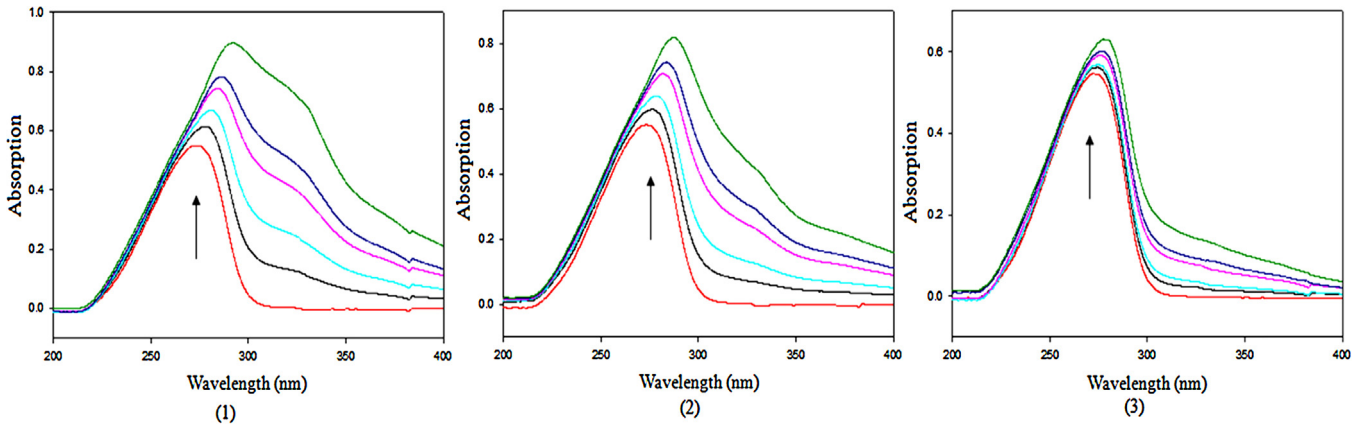

Fig. 5. Absorption spectra for PCP-1, PCP-2, and PCP-3 with different concentrations. Absorption spectra for PCP-1 (1), PCP-2 (2), and PCP-3 (3) in quartz cuvettes at different concentrations were recorded with a UV–visible spectrophotometer at room temperature. Arrows show the absorbance changes upon increasing sample concentration. The bottom curve is the standard control of ct-DNA.

The EC_{50} of PCP-2 and PCP-3 were 1.69 mg/mL and 3.39 mg/mL. Various polysaccharide fractions exhibited an outstanding capacity for Fe^{2+} chelation, further demonstrating their strong antioxidant capability.

3.5. DNA binding assay

The absorption curves of PCP-1 (1), PCP-2 (2), and PCP-3 (3) are recorded in Fig. 5. The bottom curves are ct-DNA absorption curves (blank control). After the addition of polysaccharides (the polysaccharide solutions have no absorption at the wavelength of maximum absorption), significant hyperchromism and red shift effects were observed. Furthermore, the absorbance and maximum absorption wavelength increased with increasing concentrations of samples, suggesting that the polysaccharides had a strong interaction with the ct-DNA double helix (Xiao, Lin, Wang, & Yang, 2001). The hyperchromism and red-shift effects were the most apparent with PCP-1, indicating that PCP-1 has the strongest interaction with the ct-DNA double helix.

3.6. DNA damage protective effect

The DNA migration assay is a sensitive biomarker of DNA damage (Tepe, Degerli, Arslan, Malatyali, & Sarikurkcu, 2011). As seen in Fig. 6, DNA derived from Plvx-shrna plasmids showed bands on agarose gel (lane 6). UV irradiation and H_2O_2 (lane 5) resulted in the cleavage of supercoiled circular DNA (Sc DNA) to an open circular form (Oc DNA) (Kumar & Chattopadhyay, 2007). After the degradation polysaccharides were added to the reaction mixture, a clear Sc DNA band was observed on the electrophoretic pattern (lanes 1–3). This revealed that the polysaccharides can protect the native Sc DNA against UV irradiation and H_2O_2. The positive control (lane 4, rutin, 1 mg/mL) showed a clearer Sc DNA band than lanes 1–3. Results obtained from the protective effect on DNA damage indicated a correlation with the antioxidant potential activity of polysaccharides. Therefore, further research should be undertaken to investigate their relationships.

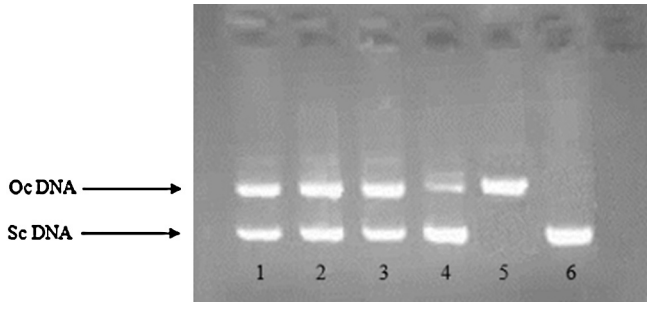

Fig. 6. Agarose gel electrophoretic pattern showing forms of plasmid DNA. Lanes 1–3: UV + H_2O_2 treated with PCP-3, PCP-2, and PCP-1, respectively. Lane 4: UV + H_2O_2 treated with rutin (positive control). Lane 5: H_2O_2 + UV treated without polysaccharides. Lane 6: untreated DNA (control). Supercoiled circular DNA (Sc DNA); open circular DNA (Oc DNA).

4. Conclusions

We obtained water-soluble and antioxidant polysaccharides (PCP-1, PCP-2, and PCP-3) from the *P. cocos* polysaccharide fraction via H_2O_2 degradation. Typical absorption peaks from the infrared spectrum indicate that degradation is a feasible method to acquiring low-molecular-weight polysaccharides. *In vitro* antioxidant activity studies showed that PCP-1, PCP-2, and PCP-3 exhibit a strong ability to scavenge free radicals and protect against DNA damage. The strong interaction between the ct-DNA double helix and polysaccharides is interesting and some work is being carried out to discover whether this phenomenon is related to the antioxidant activity or DNA repair activity of the polysaccharides. Various factors influenced the antioxidant activity of the polysaccharides. Therefore, further research is required to explore their complete structure and mechanism of antioxidant activity to develop the polysaccharides as functional foods and potential therapeutic agents.

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