Food Chemistry 136 (2013) 1453–1460

Contents lists available at SciVerse ScienceDirect

Food Chemistry

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

Purification, physicochemical characterisation and anticancer activity of a polysaccharide from *Cyclocarya paliurus* leaves

Jian-Hua Xie [a], Xin Liu [a], Ming-Yue Shen [a,b], Shao-Ping Nie [a], Hui Zhang [a], Chang Li [a], De-Ming Gong [a,c], Ming-Yong Xie [a,*]

[a] State Key Laboratory of Food Science and Technology, Nanchang University, Nanchang 330047, China
[b] Center of Analysis and Testing, Nanchang University, Nanchang 330047, China
[c] School of Biological Sciences, University of Auckland, Private Bag 92019, New Zealand

ARTICLE INFO

Article history:
Received 7 April 2012
Received in revised form 7 August 2012
Accepted 12 September 2012
Available online 29 September 2012

Keywords:
Polysaccharide
Cyclocarya paliurus
Purification
Physicochemical property
Characterisation
Anticancer activity

ABSTRACT

A *Cyclocarya paliurus* (Batal.) Iljinskaja polysaccharide (CPP) was isolated and purified by hot water extraction, ethanol precipitation, deproteinisation and anion-exchange chromatography. Its physicochemical properties were characterised by gel permeation chromatography (GPC), gas chromatography–mass spectrometry (GC–MS), thermal gravimetric analysis (TGA), Fourier transform infrared spectrometry (FTIR), UV–visible spectrophotometry, dynamic light scattering (DLS) and viscometry analysis. The anticancer effect of CPP in human gastric cancer HeLa cells was also evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The results showed that the molecular weight of CPP was 900 kDa, and it contained 64.8% total sugar, 23.5% uronic acid, 9.26% protein, and six kinds of monosaccharides, including glucose, rhamnose, arabinose, xylose, mannose and galactose, with molar percentages of 32.7%, 9.33%, 30.6%, 3.48%, 10.4%, and 13.5%, respectively. Furthermore, the results showed that CPP exhibited a strong inhibition effect on the growth of human gastric cancer HeLa cells.

1. Introduction

Cancer is globally the second most life threatening disease whose mortality follows immediately after that of cardiovascular disease. Research and development of drugs against cancer and its complications have been receiving increasing attention. Surgery and chemotherapy are the mainstream therapeutic methods for cancer in China, but the existing chemotherapeutic drugs have a number of limitations, such as adverse effects, limited efficacy and high rates of secondary failure. Therefore, the research and development of anticancer drugs with high efficiency and low toxicity, particularly the drugs extracted from natural resources, with anticancer activity and no side effects, is of great importance (Efferth et al., 2007).

In recent decades, plant-derived non-starchy polysaccharides have emerged as an important class of bioactive natural products. Increasing attention is being directed towards plant polysaccharides, due to their diverse biological activities, including antihypertensive, antioxidant, anticancer, antimicrobial, antitumor, anti-inflammation, and immunological activities (Chen, Xie, Nie, Li, & Wang, 2008; Ma, Chen, Zhu, & Wang, 2011; Nie & Xie, 2011; Xie et al., 2010a; Zhao et al., 2012). Specifically, most plant polysaccharides are relatively nontoxic and do not cause significant side effects (Xu et al., 2009). Thus, plant polysaccharides are ideal candidates for therapeutics of cancer. Many natural polysaccharides and polysaccharide-protein complexes from Chinese traditional medicine are being extensively explored for their potential treatment and prevention of cancer (Xu et al., 2009).

Cyclocarya paliurus (Batal.) Iljinskaja (*C. paliurus*), commonly known as "sweet tea tree", is a well-known edible and medicinal plant, grown on cloudy and foggy highlands in southern China. Many studies have demonstrated that *C. paliurus* possesses a variety of bioactivities, including antihypertensive activity, hypoglycemic activity, enhancement of mental efficiency, and antioxidant activity (Kurihara et al., 2003; Xie, Li, Nie, Wang, & Lee, 2006; Xie & Xie, 2008; Xie et al., 2010a,b). Chemical studies have shown that this plant contains protein, polysaccharides, triterpenoids, flavonoids, steroids, saponins and phenolic compounds (Xie & Xie, 2008). Among these, *C. paliurus* polysaccharide (CPP) is at the centre of attention as it has unique physical and chemical properties, being recognised, not only for its important functional properties, but also for its potential biological applications. Furthermore, previous studies have shown that some polysaccharides extracted from the leaves of *C. paliurus* exhibit a strong hypoglycemic effect

* Corresponding author. Tel./fax: +86 791 83969009.
E-mail addresses: xmync@163.com, xiejianhua7879@163.com, myxie@ncu.edu.cn (M.-Y. Xie).

0308-8146/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.foodchem.2012.09.078

on diabetic mice (Xie et al., 2006), and high free radical-scavenging (Xie et al., 2010a) and antimicrobial activities (Xie et al., 2012). However, to our knowledge, there is little information on the anticancer activity of this polysaccharide.

Many studies have indicated that the activities of polysaccharide complexes are most closely related to their physicochemical properties, such as polysaccharide content, protein content, chemical composition, molecular weight, viscosity, conformation, types of sugar residues, infrared spectra and degree of branching (Zhang, Cui, Cheung, & Wang, 2007). Therefore, the aim of this study was to evaluate the physicochemical properties and anticancer activity of polysaccharide extracted from the leaves of *C. paliurus*. The physicochemical properties of CPP were determined using different analysis methods, such as gel permeation chromatography (GPC), gas chromatography-mass spectrometry (GC–MS), thermal gravimetric analysis (TGA), Fourier transform infrared spectroscopy (FT-IR), UV–visible spectra, dynamic light scattering (DLS), and viscometry analysis. Moreover, the anticancer effect of the polysaccharide in human gastric cancer HeLa cells was also evaluated by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

2. Materials and methods

2.1. Materials and chemicals

The leaves of *C. paliurus*, cultivated in Xiushui County, Jiangxi Province, China, were provided by Jiangxi Xiushui Miraculous Tea Industry Co. (Jiangxi, China). A voucher specimen was deposited at the State Key Laboratory of Food Science and Technology, Nanchang University, China. The leaves were air-dried and ground into a fine powder in a mill before extraction. The dried leaf powder (5 kg) was first treated with 10 l of 80% ethanol for 24 h to remove the interference components, such as monosaccharides, disaccharides, oligosaccharides, lipids, pigments and polyphenols in the samples. The pretreated materials were then air-dried and used in the subsequent studies.

Dextrans of different molecular weights were purchased from Pharmacia Biotech (Uppsala, Sweden). Dimethyl sulfoxide (DMSO), MTT, foetal bovine serum (FBS), penicillin and streptomycin were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Pure monosaccharide standards, including mannose, rhamnose, ribose, galactose, xylose, arabinose, fucose, fructose and glucose, were obtained from Merck Co. (Darmstadt, Germany) and Sigma Chemical Co. (St. Louis, MO, USA). Medium RPMI-1640 was purchased from Gibco Invitrogen Co. (Grand Island, NY, USA). HeLa cells, derived from human cervical carcinoma cells, were obtained from Shanghai Institute of Biochemistry and Cell Biology, Chinese Academy of Science (Shanghai, China). Aqueous solutions were prepared with ultra pure water from a Milli-Q water purification system (Millipore, Bedford, MA, USA). All other reagents used in this study were of analytical grade.

2.2. Preparation of C. paliurus polysaccharide

The water-soluble polysaccharide was extracted from the leaves of *C. paliurus* according to Xie, Shen, Nie, Li, and Xie (2011) with some modifications. Briefly, the pretreated samples were extracted three times in 80 °C hot water. The aqueous extract was concentrated to 20% of the original volume under reduced pressure in a rotary evaporator, and proteins were removed by the Sevag method (Navarini et al., 1999). After removal of the Sevag reagent, the solution was decolorised with 30% H_2O_2 and then dialysed for 36 h in tap water and 12 h in ultra pure water (MW cut-off 14 kDa) before concentration in a vacuum evaporator at 55 °C. In order to obtain the crude polysaccharide, the extract was precipitated with three times its volume of 95% ethanol at 4 °C overnight and then centrifuged at 8400*g* for 15 min in a high speed centrifuge (3K3D, Sigma, Germany). The precipitate was then dissolved in distilled water, frozen and freeze-dried.

The crude polysaccharide was further purified through anion-exchange chromatography. An ÄKTA explorer purification system (Amersham Pharmacia Biotech, Uppsala, Sweden), equipped with a P-900 pump, UV-900 monitor, pH/C-900 detector, Frac-950 fraction collector and A-900 auto-sample injector, was employed to purify the crude polysaccharide with an AB-8 macroporous anion-exchange column (3.2 × 70 cm). Briefly, the polysaccharide solution was loaded into the column and then eluted with ultra pure water at a flow rate of 1 ml/min. The separated fractions were collected and enriched according to the results of analysis by a Shimadzu RID-10A Refractive Index Detector. The collected fraction was dialysed in distilled water for 24 h and freeze-dried.

2.3. Physicochemical characterisation of C. paliurus polysaccharide

2.3.1. Measurement of polysaccharide and protein contents

Sugar content was measured using the phenol–sulfuric acid colorimetric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) with D-glucose as a standard at 490 nm. Total uronic acid content was determined colorimetrically by the m-hydroxydiphenyl method (Blumenkrantz & Asboe-Hansen, 1973), using galacturonic acid as a standard. Protein content was analysed by the Coomassie brilliant blue G-250 method, with bovine serum albumin as a standard (Lowry, Rosebrough, Lewsfarr, & Randall, 1951).

2.3.2. Determination of molecular weight by gel permeation chromatography

The molecular weight of the *C. paliurus* polysaccharide was determined by gel permeation chromatography (GPC), using a Sepharose CL-6B column (1.0 × 30 cm). The column was coupled to an ÄKTA Purifier 100 system (Amersham Pharmacia Biosciences). The sample (2.5 mg) was dissolved in ultra-pure water (5 ml) and passed through a 0.45 μm filter. Fractions of 5 ml were collected with a Pharmacia LKB super fraction collector, and the eluent was monitored with a Shimadzu RID-10A Refractive Index Detector. Sample elution was carried out, using ultra pure water as the eluent, at a flow rate of 0.3 ml/min. A standard curve with the elution volume plotted against the logarithm of molecular weight, was constructed using the Dextran T standards (MW: 10,000, 40,000, 70,000, 500,000, 2,000,000 Da) and glucose. According to the elution volume of CPP, its molecular weight was calculated by the calibration curve equation.

2.3.3. Analysis of monosaccharide composition

2.3.3.1. Pre-treatments. The sample (20 mg) was hydrolysed with 2 M H_2SO_4 (5 ml) at 110 °C in a sealed tube for 8 h. After removing the residual acid with $BaCO_3$, the hydrolysates were converted to acetylated aldononitrile derivatives according to conventional protocols. One part of the hydrolysate was analysed by GC–MS, and the others were measured by High-performance anion-exchange chromatography, coupled with pulsed amperometric detection (HPAEC-PAD).

2.3.3.2. GC–MS analysis. Analysis of monosaccharide composition was performed by GC–MS, using an Agilent 6890 N-5973I MSD GC–MS equipped with an HP-5MS elastic quartz capillary column (30 m × 0.25 mm × 0.25 μm). The hydrolysate were reduced by $NaBH_4$, followed by acidification with acetic acid. Approximately 0.5 ml of pyridine and 10 mg of hydroxylamine hydrochloride were added and the tubes were sealed. Samples were incubated in a preheated water bath shaker at 90 °C for 30 min. After incubation, the

tubes were cooled to room temperature and 0.5 ml of acetic anhydride was subsequently added and mixed thoroughly by vortexing. The tubes were then sealed and incubated in a water bath shaker set at 90 °C for 30 min. After cooling, approximately 0.1 ml of clear supernatant was added to autosampler vials with inserts for injection into the GC–MS. The following programme was adopted for GC analysis: injection temperature: 250 °C; detector temperature: 250 °C; column temperature was held at 50 °C for 5 min and increased to 170 °C at 15 °C/min, and then increased to 210 °C at 10 °C/min and finally held for 15 min at 210 °C. Nitrogen was used as the carrier gas and maintained at 40 ml/min. The speed of air and hydrogen gas were 400 and 40 ml/min, respectively. The split ratio was set as 10:1. The temperatures of the ion source and the transfer line were 175 and 280 °C, respectively. Positive ion electron impact mass spectra were recorded at 70 eV ionisation energy, 1 scan/s. Eight monosaccharides were used as the external standards to quantify the monosaccharide content.

2.3.3.3. HPAEC-PAD analysis. High-performance anion-exchange chromatography (HPAEC) was done on a Dionex ICS-2500 system, coupled with pulsed amperometric detection (PAD), equipped with a Carbo PAC™ PA10 (2.0 × 250 mm) column. The hydrolysate (1 mg) was dissolved in pure water (0.02 mg/ml). Twenty-five microlitres of this solution were used for the ionic chromatography analysis by 0HAPEC-PAD of a Dionex ICS-2500 System, eluted with – mixture of water and 250 mM NaOH in the volume ratio of 95:5.

2.3.4. Thermal gravimetric analysis (TGA) and differential thermal analysis (DTA)

Thermal behaviour of the samples was tested using thermal gravimetric analysis (TGA) and differential thermal analysis (DTA) on the Pyris Diamond TG/DTA thermal analyzer (PE, USA). Approximately 5 mg of sample were introduced into the sample pan and heated from 30 to 400 °C with a heating rate of 20 °C/min under nitrogen atmosphere. The gas flow rate was 40 ml/min.

2.3.5. Viscometry analysis

Apparent viscosity measurement was performed at 30 °C in a Brookfield DV-III Ultra Programmable Rheometer (Brookfield Engineering Laboratories, Stoughton, MA, USA), equipped with a CP52 spindle. The effects of shear rate, concentration of CPP, temperature, pH, various salts and sucrose on the viscosity of CPP solution were evaluated.

2.3.6. Particle size and size distribution analysis

The average particle size and distribution of CPP in solutions were determined by the dynamic light scattering (DLS) method. The distribution and time course of polysaccharide size were measured using a Nicomp 380/ZLS Zeta potential/Particle sizer (PSS Nicomp, Santa Barbara, California, USA). The light source was a diode pump solid-state laser (DPSS) with a wavelength of 368 nm, and the scattering angle was 90 degrees. The solutions were diluted to a concentration of 1 mg/ml with ultra pure water, and all measurements were carried out at 25 °C (Liu et al., 2011).

2.3.7. Spectrometric analysis

UV–visible spectra were performed on a ultraviolet–visible spectrophotometer (TU-1900, Pgenenal, Beijing, China) at 25 °C in the range of 220–500 nm, using a quartz cell with 1 cm path length. Each UV spectrum determination was repeated three times.

FT-IR spectra were recorded on a Nicolet 5700 FT-IR spectrometer (Thermo Fisher Scientific Inc., MA, USA) in the frequency range of 500–4000 cm^{-1}. Dried polysaccharide sample (2.0 mg) and KBr powder (spectroscopic grade) were mixed, ground and squashed.

2.4. Anticancer activity

2.4.1. Cell lines and culture

The HeLa cells, were maintained in RPMI-1640 medium supplemented with 10% (v/v) foetal bovine serum (FBS), penicillin (100 U/ml) and streptomycin (100 μg/ml) in an incubator (Thermo Electron Corporation, MA, USA) with a humidified 5% CO_2 at 37 °C.

2.4.2. Growth inhibition assay

The inhibition effect of CPP on the growth of HeLa cells was evaluated *in vitro* by MTT assay. Briefly, the HeLa cells (10^4 cells/well) were incubated in 96-well plates containing 0.1 ml culture medium at 37 °C in humidified atmosphere with 5% CO_2. The cells were permitted to adhere for 12 h. One hundred microlitres of polysaccharide solutions with different concentrations (50, 100, 200, 400 μg/ml), prepared in culture medium, were added to each well. After 24 and 48 h of exposure, the polysaccharide-containing medium was removed. The cells in each well were then incubated in culture medium with 17 μl of MTT solution (5 mg/ml) for 4 h. After the media were removed, 150 μl of DMSO were added to each well. Absorbance at 492 nm was determined by an ELISA plate reader.

The inhibition rate was calculated according to the formula below: Growth inhibition rate (%) = (1 − absorbance of experimental group/absorbance of blank control group) × 100%.

2.4.3. Flow cytometry analysis

The proportion of cells at different phases of cell cycle was monitored by a flow cytometer based on the methods of He, Yang, Jiao, Tiao, & Zhao, 2012 with some modifications. Briefly, HeLa cells were seeded to a culture plate at approximately 5×10^5 and incubated for 24 h, after which 100 μg/ml of CPP were added separately and incubated for a further 48 h. Cells were then washed with PBS, and trypsin/ethylenediaminetetra-acetic acid was added to detach the cells. Next, cells were collected by centrifugation at 2800*g* for 5 min, and the supernatant was removed. Cells were washed with PBS, and PBS containing 75% ethanol was added slowly for incubation at 4 °C for 3 h to fix the cells. Ethanol was removed by centrifugation, and the cells were repeatedly washed with PBS, which was centrifuged three times to remove the supernatants. Then, a mixture of 0.5 ml of RNase A (200 mg/ml), 0.1% Triton X-100 and 0.5 ml of propidium iodide (100 mg/ml) was added and reacted at 4 °C for 45 min in the dark, after which the solution was filtered through a 200 mm membrane filter for cell cycle analysis with a flow cytometer (Coulter Epics XL, Beckman Coulter) at an excitation wavelength of 488 nm.

2.5. Statistical analysis

One-way ANOVA and the student's *t*-test were used to determine the statistical significance of the differences between the values determined for the various experimental and control groups. Data are expressed as means ± SD and the results are taken from at least three independent experiments performed in triplicate. P values of 0.05 or less were considered to be statistically significant.

3. Results and discussion

3.1. Purity and molecular weight of CPP

Crude polysaccharide was isolated from leaves of *C. paliurus* through hot water extraction and ethanol precipitation, and deproteinised by the Sevag method, dialysed against water and dried. The extraction yield of crude polysaccharide was approximately

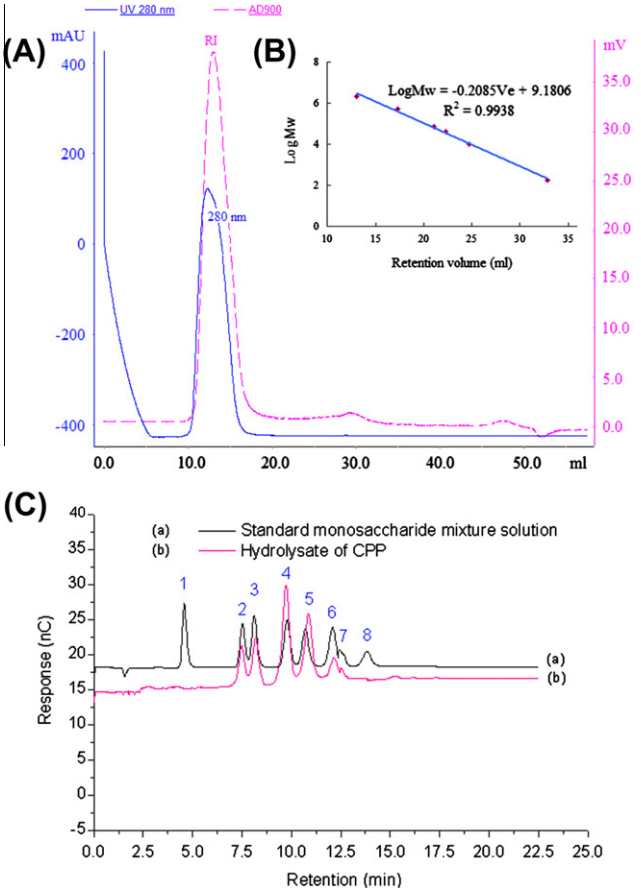


Fig. 1. (A) A gel permeation chromatography of polysaccharide fraction (CPP) on a Sepharose CL-6B packed column (1.0 × 30 cm), using an ÄKTA Purifier 100 system; (B) A calibration curve of various Dextran T-series standards (T-2000, T-500, T-70, T-40, T-10, and glucose); (C) HPAEC-PAD chromatogram profile of standard monosaccharide mixture solution (a) and hydrolysate of CPP (b). Peak identity: 1, fucose; 2, rhamnose; 3, arabinose; 4, galactose; 5, glucose; 6, mannose; 7, xylose; 8, fructose.

2.16% of the leaves. The polysaccharide extract was further purified using anion-exchange chromatography. This purified polysaccharide showed a single and relatively symmetrical peak on GPC, indicating its homogeneity (Fig. 1A). From GPC analysis, a calibration curve was obtained by using various Dextran T-series standards of known molecular weights (Fig. 1B). According to the calibration curve, logMw = 9.21–0.21 Ve (Ve was the retention volume); the average molecular weight of CPP was calculated to be 900 kDa (Fig. 1A).

3.2. Chemical compositions of CPP

Some polysaccharides contain neutral sugar, and are usually conjugated with other components, such as proteins to exhibit various activities (Ma et al., 2011). So it was necessary to analyse the contents of neutral sugar, uronic acid and proteins in the polysaccharide samples. The monosaccharide compositions, and contents of total sugar, uronic acid and proteins of CPP are summarised in Table 1. The total sugar content was determined to be 64.8%, using the phenol–sulfuric acid method. The contents of total uronic acid and proteins in CPP samples were 23.5% and 9.26%, respectively.

Monosaccharide compositions of CPP were determined by H_2SO_4 hydrolysis and GC–MS analysis. The results indicated that glucose and arabinose were the major monosaccharides constructing the backbones of CPP. CPP was composed of glucose, rhamnose, arabinose, xylose, mannose, and galactose with molar percentages

Table 1
Chemical and monosaccharide compositions of *C. paliurus* polysaccharide (CPP)[a].

Chemical composition	CPP (%, w/w)
Total sugar	64.8 ± 1.14
Uronic acid	23.5 ± 1.05
Protein	9.26 ± 0.24
Neutral sugar	Monosaccharide composition in CPP (%, mol)
Glucose	32.7 ± 1.28
Rhamnose	9.33 ± 0.09
Fucose	–[b]
Arabinose	30.8 ± 1.06
Xylose	3.28 ± 0.41
Mannose	10.4 ± 0.17
Ribose	–
Galactose	13.5 ± 0.32

[a] Values are expressed as means ± SD and three replicated independent determinations.
[b] Not detectable.

of 32.7%, 9.33%, 30.6%, 3.48%, 10.4%, and 13.5%, respectively (Table 1). HPAEC-PAD chromatogram profiles of standard monosaccharide mixture solution and hydrolysate of CPP are shown in Fig. 1C. Six monosaccharides (glucose, rhamnose, arabinose, xylose, mannose and galactose) were identified in the hydrolysate of CPP, and their molar percentage were 32.0%, 9.96%, 30.6%, 3.02%, 10.1%, and 14.4%, respectively. The results of HPAEC-PAD analysis were consistent with the characteristics recorded by GC–MS.

In our previous studies (Xie et al., 2010a), a water-soluble polysaccharide from *C. paliurus*, named CPP-1, was composed of xylose, arabinose, glucose, galactose, rhamnose and mannose with molar ratios of 3.23%, 31.2%, 31.3%, 16.0%, 10.6%, and 8.72%, respectively.

3.3. UV spectra and FTIR spectra

UV spectra were recorded on a TU-1900 spectrophotometer. It was found that the weak peak at 280 nm was attributable to the absorption of protein (Fig. 2A). It should be noted that both absorp-

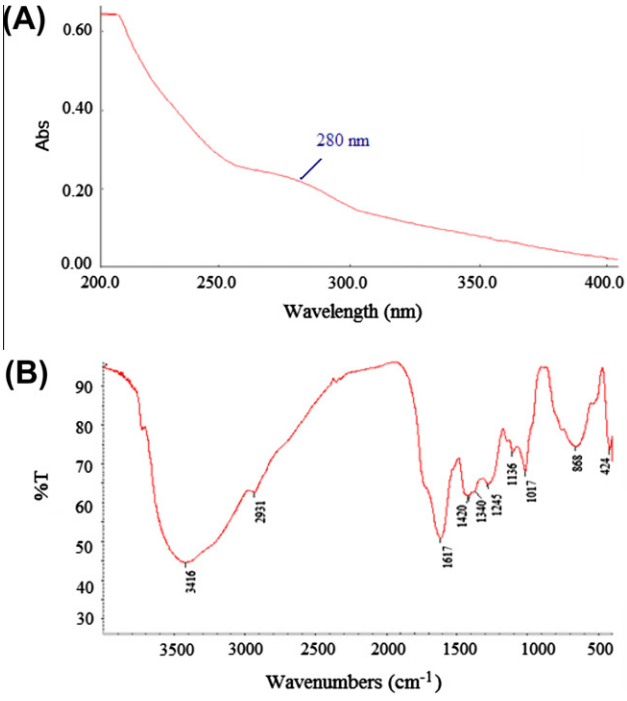


Fig. 2. Spectrum of polysaccharide fraction (CPP) from the leaves of *C. paliurus*. (A) UV spectrum; (B) Infrared spectrum.

tion peaks, detected at RI and UV 280 nm, appeared at approximately the same retention time (Fig. 1A), implying that CPP is a glycoprotein containing polysaccharide chains and protein residues according to our previous study (Xie et al., 2010a).

Fig. 2B illustrates the FTIR spectrum of CPP. The large absorption peak at around 3416 cm^{-1} could be assigned to the stretching vibrations of hydrogen-bonded OH groups. The weak absorption bands at about 3000–2900 cm^{-1} were attributed to C-H stretching vibrations of the free sugar. The strong absorption band at 1617 cm^{-1} was attributed to the C=O asymmetric stretching vibrations of the carboxylate ($-COO^-$) groups. The broad absorption bands with strong intensities, at 1420, 1340 and 1245 cm^{-1}, could be assigned to deforming vibrations of the C–H bond. The wave numbers between 800 and 1200 cm^{-1} represent the finger print region for carbohydrates (Cui, Phillips, Blackwell, & Nikiforuk, 2007). The absorption bands at about 1186, 1136 and 1017 cm^{-1}, were assignable mainly to the C–O–C stretching vibrations and C–O–H bending vibrations. Furthermore, the strong absorption at 1017 cm^{-1} was the stretching vibration of the C–N. These observations of FTIR analysis further confirmed that CPP was a polysaccharide containing proteins and uronic acid.

3.4. Thermal gravimetric analysis

The thermal stability is an important characteristic of materials that may have biological applications, considering the possible need of sterilisation by heating. Thermal stability of the polysaccharide sample was studied using thermal gravimetric analysis, as it can provide a quantitative measurement of mass change in materials associated with dehydration, decomposition and oxidation of a sample with time and temperature.

The thermal gravimetric curve of CPP is presented in Fig. 3. The results showed that heating at a rate of 10 °C/min, from 30 °C to a maximum of 400 °C, resulted in two mass loss events for the polysaccharide samples. The first mass loss, taking place between 30 and 140 °C, may be attributed to the loss of absorbed and structural water of biopolymers or due to desorption of moisture as hydrogen-bound water to the polysaccharide structure (Kittur, Harish Prashanth, Udaya Sankar, & Tharanathan, 2002). The second weight loss event, with an onset of over 250 °C, resulting in a weight loss of about 60%, can be attributed to degradation reactions of thermal decomposition of the polysaccharide and is described by a weight loss onset and oxidation temperature.

3.5. Viscometry analysis

Besides chemical properties, applicability of polysaccharide is largely dependent on its thermal and rheological behaviour

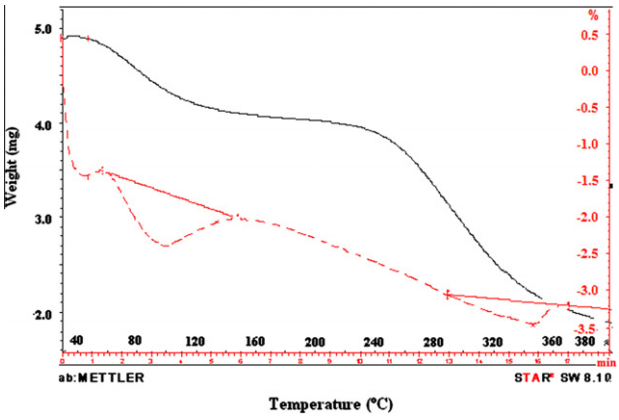


Fig. 3. TGA/DTA curves of polysaccharide fraction (CPP) from the leaves of *C. paliurus*.

(Marinho-Soriano & Bourret, 2005). Viscosity is an important physical property of CPP. It is important, from an application point of view, to study the dependency of apparent viscosity on the concentration of CPP. The effect of shear rate on viscosity of the polysaccharide solution (0.125–1 mg/ml) at 25 °C is presented in Fig. 4a. The apparent viscosity of CPP solution was slightly concentration-dependent. As the concentration of CPP solution increased, the viscosity increased. For the concentration of 0.125 mg/ml, the viscosity was 1.6 mPa s. At a concentration of 1 mg/ml, the viscosity was approximately 3.1 mPa·s. The apparent viscosity of the solution decreased sharply as the rate of shear increased. Typical shear-thinning flow behaviour was observed for all CPP solutions at all concentrations tested (Fig. 4a). This is in agreement with reports of other polysaccharide solutions, such as *Acacia tortuosa* gum (Munoz et al., 2007) and peach gum (Qian, Cui, Wang, Wang, & Zhou, 2011). This effect might be due to the disruption of ordered structure of polymer solution caused by shear force, which would lead to decreased viscosity with increased shear rate.

The effect of temperature on the apparent viscosity of CPP solution, at the concentration of 1 mg/ml, was examined in the range 10–45 °C. The viscosity was 3.1 mPa·s at the minimum temperature of 10 °C, and 1.2 mPa·s at the maximum temperature of 45 °C (Fig. 4b). A reduction in the apparent viscosity of CPP solution was observed with increase in temperature. This may be due to the fact that the interactions of the molecules in solution became weaker at high temperatures.

Fig. 4c represents the effect of pH on the viscosity of CPP at a shear rate of 40 s^{-1}. Viscosity was highest at the neutral state, pH 7.0. The viscosity of the CPP at the concentration of 1 mg/ml decreased gradually at pH values below or above 7.0. Those data were fully consistent with a report by Amin, Ahmad, Yin, Yahya, and Ibrahim (2007) that viscosity of *Durio zibethinus* gum solution decreased under more acidic and more alkaline conditions. The application of more acidic and alkaline conditions to the polymer solution degraded galactomannan, which would lead to decreased viscosity at high and low pH values.

Fig. 4d shows the effects of NaCl, $CaCl_2$ and sucrose concentrations on the viscosity of the CPP solution. The viscosity of CPP solutions changed only slightly with addition of each salt or not at all over a wide range of salt concentrations. The viscosity of CPP solution at the concentration of 1 mg/ml increased with increasing $CaCl_2$ and sucrose concentrations. In more concentrated solution, the presence of $CaCl_2$ may promote interaction between chains and an increase in viscosity. This increase of viscosity is possibly due to complete opening of polypeptide chain to random chain and intermolecular hydrodynamic interaction. This kind of property was also observed for other polysaccharide gels, such as polysaccharides from the red seaweed *Gracilaria dura* (Marinho-Soriano & Bourret, 2005).

3.6. Particle size and size distribution analysis

Fig. 5 presents the average size distribution of CPP in aqueous solution. The size distribution was in the range 150–300 nm. The size distribution of the CPP was narrow and uniform with minor particle size, which further confirmed that the CPP was a relatively homogeneous polysaccharide.

3.7. Anticancer activity

It has been reported that polysaccharides play a certain role in anti-tumor activity (Nie & Xie, 2011). Polysaccharide extracted from *C. paliurus* was reported to possess a plethora of biological activities, including antioxidant, antimicrobial, and hypoglycemic activities (Xie et al., 2006, 2010a, 2012). However, there is little information regarding the antimicrobial potentials of polysaccha-

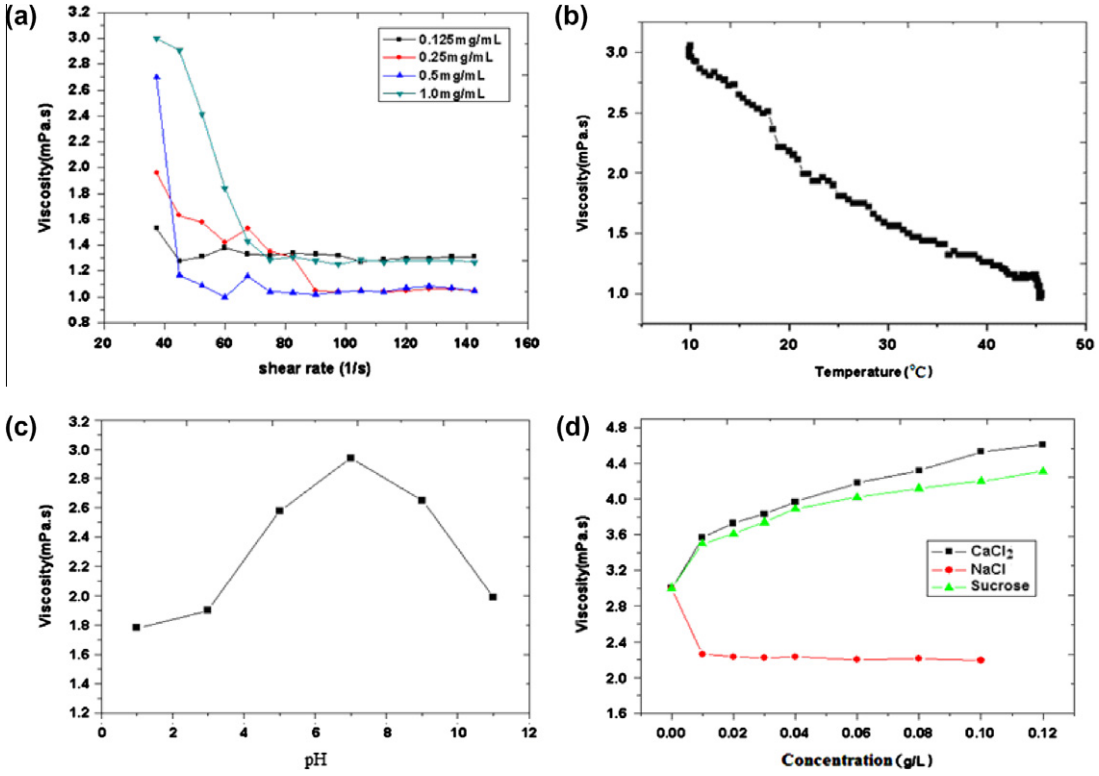


Fig. 4. Viscoelastic properties of CPP solution. (a) Effect of shear rate on the viscosity of CPP solution at different concentrations at 25 °C; (b) effect of temperature on the viscosity of a 1 mg/ml CPP solution at a shear rate of 40 s^{-1} and a heating rate of 20 °C/min; (c) effect of pH on the viscosity of a 1 mg/ml CPP solution at a shear rate of 40 s^{-1} and 25 °C; (d) effect of salts and sucrose at different concentrations on the viscosity of a 1 mg/ml CPP solution at a shear rate of 40 s^{-1} and 25 °C.

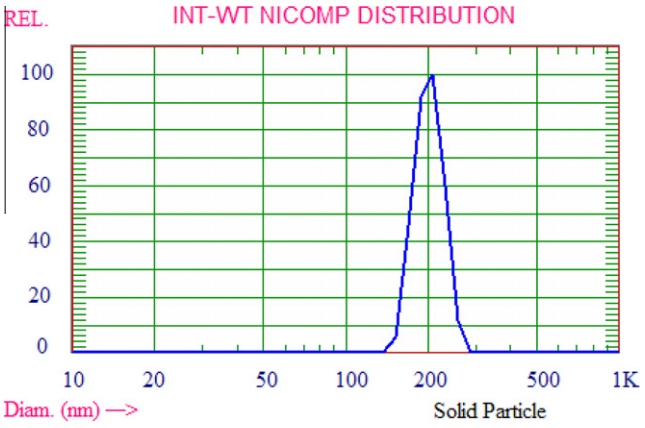
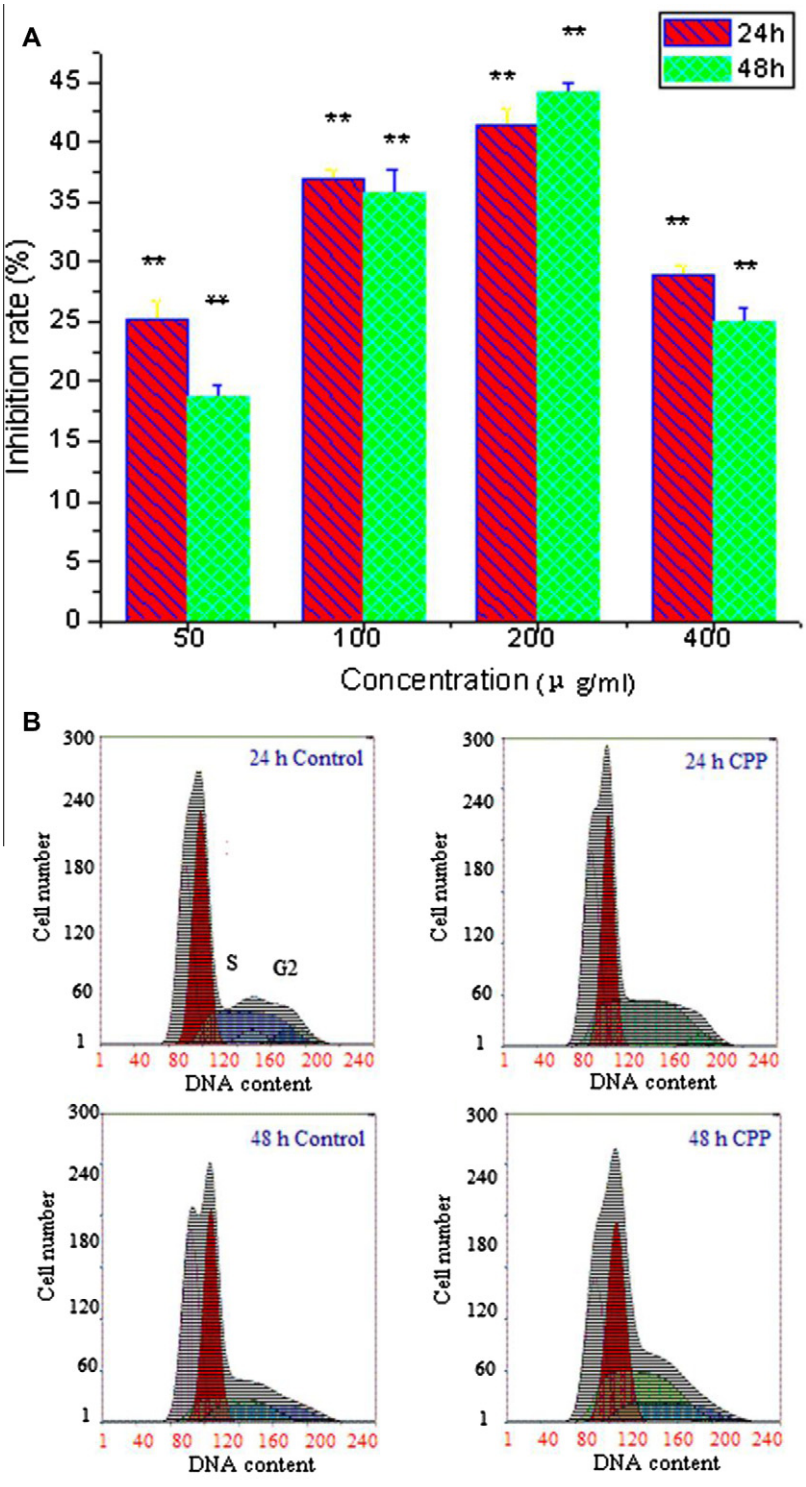


Fig. 5. Size distribution profiles of polysaccharides (10 μg/ml) in 0.15 M NaCl solution at 25 °C.

rides from *C. paliurus*. In the present study, therefore, the anticancer activity of CPP, *in vitro*, was evaluated. The viability of HeLa cancer cells treated with CPP polysaccharides for 24 h was determined using a colorimetric MTT-based assay (Fig. 6A). The polysaccharides exhibited a dose-dependent activity within the concentration range 50–200 μg/ml, and the inhibitory effects of CPP on HeLa cells increased significantly ($P < 0.05$) with the increase of sample concentration. The inhibition rates of CPP increased from 16.2% to 35.7% when the concentrations varied from 50 to 100 μg/ml. The highest inhibition rate on HeLa cancer cells was 43.2%, at the concentration of 200 μg/ml. These results are in agreement with the report of wolfberry fruit polysaccharides (He et al., 2012). However, when the concentration of CPP was 400 μg/ml, the inhibition rate on HeLa cancer cells decreased. We speculate that this effect might be due to the fact that the cells could induce resistance at high drug concentration. To better understand the bioactivity of CPP, our further study on the mechanism of its anticancer activity against HeLa cancer cells is currently underway. The results indicated that CPP could directly inhibit the proliferation of HeLa cancer cells. This was in agreement with the reports on other polysaccharides, such as polysaccharides from *Phellinus linteus* (Li et al., 2004) and *Asparagus officinalis* (Zhao et al., 2012).

Apoptosis is a physiological and crucial process that is regarded as the preferred way to eliminate cancer cells. Based on the MTT assay, the cell cycle analysis of CPP after 24 and 48 h of treatment is shown in Fig. 6B. Treatment with100 μg/ml of CPP for 24 h resulted in a decreased percentage of cells in the G2 phase, and the distribution of G2/M phase cells decreased from 10.7 ± 1.56% to 4.20 ± 0.28%. In contrast, a reversed tendency was observed for the S phase ratio, with a ratio of 45.1 ± 1.55%, at 100 μg/ml for 48 h, which was substantially higher than the control treatment (29.7 ± 1.41%). These results suggested that CPP could inhibit the growth of HeLa cancer cells through cell-cycle arrest in the S phase, which may induce apoptosis.

Polysaccharides have been found to play a crucial biological role in many biological processes. Polysaccharides extracted from plants may prove to be one of the promising candidates from search of non-toxic and low-cost natural products with significant anticancer activity. It has been reported that the anticancer activity of polysaccharides is probably a consequence of the stimulation of the cell-mediated immune response (Ooi & Liu, 2000). For instance, anticancer activities were found in the polysaccharides from *Panax ginseng* and *Ganoderma lucidum*, suggesting that the immunostimulatory effect might be the main mechanism for the anti-tumor activities of polysaccharides (Cao & Lin, 2004; Shin et al., 2002). In addition, the anticancer activity of polysaccharide is usually



Cycle distribution / Time (h)	Control		CPP	
	S (%)	G2 (%)	S (%)	G2 (%)
24	25.50±1.13	10.70±1.55	35.65±1.76*	4.20±0.28
48	29.70±1.41	3.20±1.13	45.10±1.55*	1.15±0.21

Fig. 6. (A) The inhibition rate of HeLa cancer cells by CPP samples at concentrations from 50 to 400 μg/ml. The values are presented as means ± S.D. from three independent experiments, in which each treatment was repeated three times. Significant differences from control were evaluated using the *t*-test: $^{**}P < 0.01$; (B) Flow cytometric analysis of HeLa cancer cells treated with CPP at the concentration of 100 μg/ml for 24 and 48 h. Significant differences from control were evaluated using the *t*-test: $^{*}P < 0.05$.

not influenced by one single factor but is combined with other factors. It has been reported that the level of anti-oxidation and reactive oxygen species is correlated well with the generation and malign transformation of cancer cells. Leng, Liu, and Chen (2005) reported that compounds that can enhance the level of anti-oxidation and clear the reactive oxygen species in cancer cells may inhibit the growth of cells. In our previous report, we demonstrated a fraction from *C. paliurus* polysaccharides with strong scavenging activity *in vitro* (Xie et al., 2010a,2012). Therefore, the higher anticancer activities of CPP might be related to their relatively stronger antioxidant activities. In this study, the polysaccharide fraction (CPP) isolated from *C. paliurus* effectively inhibited the proliferation of HeLa cancer cells *in vitro*. Our data suggested that CPP might be a potential, natural apoptosis-inducing anticancer agent.

4. Conclusions

On the basis of the above results, it could be concluded that the water extract of the leaves of *C. paliurus* contained one predominant polysaccharide (CPP). Chemical and instrumental analytical methods were applied to evaluate the physicochemical properties of the purified CPP by anion-exchange chromatography. Gel permeation chromatography (GPC) showed that the molecular weight (Mw) of CPP was 900 kDa. Monosaccharide analysis revealed that the CPP is a heteropolysaccharide, which was composed of glucose, rhamnose, arabinose, xylose, mannose, and galactose with molar percentages of 32.7%, 9.33%, 30.6%, 3.48%, 10.4%, and 13.5%, respectively. In addition, CPP (at the concentration of 200 μg/ml) exhibited a strong inhibition effect on the growth of HeLa cancer cells. These studies have demonstrated that CPP has an inhibitory action on the growth of HeLa cells *in vitro*, which is related to the induction of S phase arrest and the promotion of apoptosis. Work on the possible anticancer mechanism of CPP is worthy of future studies.

Acknowledgements

The authors gratefully acknowledge the financial supports by the Program of the National Natural Science Foundation of China (No. 31130041), the National Key Technology R&D Program of China (No. 2012BAD33B06), the Research Foundation for Young Scientists and Objective-Oriented Project of State Key Laboratory Food Science and Technology, Nanchang University, China (Nos. SKLF-QN-201104 and SKLF-MB-201001), and the Natural Science Foundation for Young Scientists of Jiangxi Province, China.

References

Amin, A. M., Ahmad, A. S., Yin, Y. Y., Yahya, N., & Ibrahim, N. (2007). Extraction, purification and characterization of durian (*Durio zibethinus*) seed gum. *Food Hydrocolloids, 21*(2), 273–279.

Blumenkrantz, N., & Asboe-Hansen, G. (1973). New method for quantitative determination of uronic acid. *Analytical Biochemistry, 54*(2), 484–489.

Cao, Q. Z., & Lin, Z. B. (2004). Antitumor and anti-angiogenic activity of *Ganoderma lucidum* polysaccharides peptide. *Acta Pharmacologica Sinica, 25*(6), 833–838.

Chen, Y., Xie, M. Y., Nie, S. P., Li, C., & Wang, Y. X. (2008). Purification, composition analysis and antioxidant activity of a polysaccharide from the fruiting bodies of *Ganoderma atrum*. *Food Chemistry, 107*(1), 231–241.

Cui, S. W., Phillips, G. O., Blackwell, B. A., & Nikiforuk, J. (2007). Characterisation and properties of *Acacia senegal* L. Willd. Var. senegal with enhanced properties (Acacia (sen) SUPERGUM™): Part 4. Spectroscopic characterisation of *Acacia senegal* var. senegal and Acacia (sen) SUPERGUM™ arabic. *Food Hydrocolloids, 21*(3), 347–352.

Dubois, M., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, F. (1956). Colorimetric method for determination of sugars and related substances. *Analytical Chemistry, 28*(3), 350–356.

Efferth, T., Fu, Y. J., Zu, Y. G., Schwarz, G., Konkimalla, V. S. B., & Wink, M. (2007). Molecular target-guided tumor therapy with natural products derived from traditional Chinese medicine. *Current Medicinal Chemistry, 14*(19), 2024–2032.

He, M. W., Yang, X. B., Jiao, Y. B., Tiao, L. M., & Zhao, Y. (2012). Characterisation of antioxidant and antiproliferative acidic polysaccharides from Chinese wolfberry fruits. *Food Chemistry, 133*(3), 978–989.

Kittur, F. S., Harish Prashanth, K. V., Udaya Sankar, K., & Tharanathan, R. N. (2002). Characterization of chitin, chitosan and their carboxymethyl derivatives by differential scanning calorimetry. *Carbohydrate Polymers, 49*(2), 185–193.

Kurihara, H., Fukami, H., Kusumoto, A., Toyoda, Y., Shibata, H., Matsui, Y., Asami, S., & Tanaka, T. (2003). Hypoglycemic action of *Cyclocarya paliurus* (Batal.) Iljinskaja in normal and diabetic mice. *Bioscience, Biotechnology, and Biochemistry, 67*(4), 877–880.

Leng, B., Liu, X. D., & Chen, Q. X. (2005). Inhibitory effects of anticancer peptide from Mercenaria on the BGC-823 cells and several enzymes. *FEBS Letters, 579*(5), 1187–1190.

Li, G., Kim, D. H., Kim, T. D., Park, B. J., Park, H. D., Park, J. I., Na, M. K., Kim, H. C., Hong, N. D., Lim, K., Hwang, B. D., & Yoon, W. H. (2004). Protein-bound polysaccharide from *Phellinus linteus* induces G2/M phase arrest and apoptosis in SW480 human colon cancer cells. *Cancer Letters, 216*(2), 175–181.

Liu, C. M., Zhong, J. Z., Liu, W., Tu, Z. C., Wan, J., Cai, X. F., & Song, X. Y. (2011). Relationship between functional properties and aggregation changes of whey protein induced by high pressure microfluidization. *Journal of Food Science, 76*(4), E341–E347.

Lowry, O. H., Rosebrough, N. J., Lewsfarr, A., & Randall, R. J. (1951). Protein measurement with the folin reagent. *Journal of Biological Chemistry, 193*, 265–275.

Ma, L. S., Chen, H. X., Zhu, W. C., & Wang, Z. S. (2011). Effect of different drying methods on physicochemical properties and antioxidant activities of polysaccharides extracted from mushroom *Inonotus obliquus*. *Food Research International*. http://dx.doi.org/10.1016/j.foodres.2011.05.005.

Marinho-Soriano, E., & Bourret, E. (2005). Polysaccharides from the red seaweed *Gracilaria dura* (Gracilariales, Rhodophyta). *Bioresource Technology, 96*(3), 379–382.

Munoz, J., Rincon, F., Carmen Alfaro, M. C., Zapata, I., de la Fuente, J., Beltran, O., & Leon de Pinto, G. (2007). Rheological properties and surface tension of *Acacia tortuosa* gum exudates aqueous dispersions. *Carbohydrate Polymers, 70*(2), 198–205.

Navarini, L., Gilli, R., Gombac, V., Abatangelo, A., Bosco, M., & Toffanin, R. (1999). Polysaccharides from hot water extracts of roasted *Coffea arabica* beans: Isolation and characterization. *Carbohydrate Polymers, 40*(1), 71–81.

Nie, S. P., & Xie, M. Y. (2011). A review on the isolation and structure of tea polysaccharides and their bioactivities. *Food Hydrocolloids, 25*(2), 144–149.

Ooi, V. E., & Liu, F. (2000). Immunomodulation and anti-cancer activity of polysaccharide–protein complexes. *Current Medicinal Chemistry, 7*(7), 715–729.

Qian, H. F., Cui, S. W., Wang, Q., Wang, C., & Zhou, H. M. (2011). Fractionation and physicochemical characterization of peach gum polysaccharides. *Food Hydrocolloids, 25*(5), 1285–1290.

Shin, J. Y., Song, J. Y., Yun, Y. S., Yang, H. O., Rhee, D. K., & Pyo, S. (2002). Immunostimulating effects of acidic polysaccharides extract of *Panax ginseng* on macrophage function. *Immunopharmacology and Immunotoxicology, 24*(3), 469–482.

Xie, J. H., Shen, M. Y., Nie, S. P., Li, C., & Xie, M. Y. (2011). Decolorization of polysaccharides solution from *Cyclocarya paliurus* (Batal.) Iljinskaja using ultrasound/H_2O_2 process. *Carbohydrate Polymers, 84*(1), 255–261.

Xie, J. H., Shen, M. Y., Xie, M. Y., Nie, S. P., Chen, Y., Li, C., Huang, D. F., & Wang, Y. X. (2012). Ultrasonic-assisted extraction, antimicrobial and antioxidant activity of *Cyclocarya paliurus* (Batal.) Iljinskaja polysaccharides. *Carbohydrate Polymers, 89*(1), 177–184.

Xie, J. H., Xie, M. Y., Nie, S. P., Shen, M. Y., Wang, Y. X., & Li, C. (2010a). Isolation, chemical composition and antioxidant activities of a water-soluble polysaccharide from *Cyclocarya paliurus* (Batal.) Iljinskaja. *Food Chemistry, 119*(4), 1626–1632.

Xie, J. H., Xie, M. Y., Shen, M. Y., Nie, S. P., Wang, Y. X., & Li, C. (2010b). Optimisation of microwave-assisted extraction of polysaccharides from *Cyclocarya paliurus* (Batal.) Iljinskaja using response surface methodology. *Journal of the Science of Food and Agriculture, 90*(8), 1353–1360.

Xie, M. Y., Li, L., Nie, S. P., Wang, X. R., & Lee, F. S. C. (2006). Determination of speciation of elements related to blood sugar in bioactive extracts from *Cyclocarya paliurus* leaves by FIA-ICP-MS. *European Food Research and Technology, 223*(2), 202–209.

Xie, M. Y., & Xie, J. H. (2008). Review about the research on *Cyclocarya paliurus* (Batal.) Iljinskaja. *Journal of Food Science and Biotechnology, 27*(1), 113–121.

Xu, H. S., Wu, Y. W., Xu, S. F., Sun, H. X., Chen, F. Y., & Yao, L. (2009). Antitumor and immunomodulatory activity of polysaccharides from the roots of *Actinidia eriantha*. *Journal of Ethnopharmacology, 125*(2), 310–317.

Zhang, M., Cui, S. W., Cheung, P. C. K., & Wang, Q. (2007). Antitumor polysaccharides from mushrooms: A review on their isolation process, structural characteristics and antitumor activity. *Trends in Food Science & Technology, 18*(1), 4–19.

Zhao, Q. S., Xie, B. X., Yan, J., Zhao, F. C., Xiao, J., Yao, L. Y., Zhao, B., & Huang, Y. X. (2012). In vitro antioxidant and antitumor activities of polysaccharides extracted from *Asparagus officinalis*. *Carbohydrate Polymers, 87*(1), 392–396.