Carbohydrate Polymers 88 (2012) 1299–1303

Contents lists available at SciVerse ScienceDirect

Carbohydrate Polymers

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

A water-soluble polysaccharide (EFP-AW1) from the alkaline extract of the roots of a traditional Chinese medicine, *Euphorbia fischeriana*: Fraction and characterization

Jicheng Liu*,[1], Yongxu Sun[1], Lei Liu, Chunlei Yu

Qiqihar Medical University, Qiqihar 161006, China

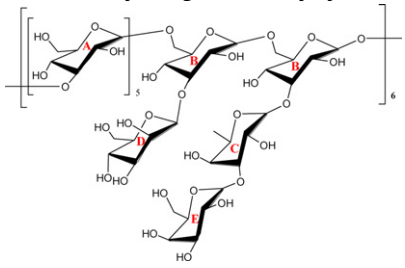
ARTICLE INFO

Article history:
Received 5 July 2011
Received in revised form 12 January 2012
Accepted 5 February 2012
Available online 10 February 2012

Keywords:
Euphorbia fischeriana
Polysaccharide
Structure
Alkaline extracted

ABSTRACT

A water-soluble polysaccharide, designated as EFP-AW1, was isolated from the roots of *Euphorbia fischeriana* and purified to homogeneity by gel-filtration chromatography. Its carbohydrate content was up to 92.34%, which was composed of glucose (Glc), galactose (Gal), mannose (Man) and rhamnose (Rha) in a molar ratio of 14.1:1.9:2.0:1.9. The molecular weight was evaluated to be 10,830 Da as determined by high performance size exclusion chromatography (HPSEC). On the basis of sugar analysis, methylation analysis, periodate oxidation, Smith degradation, and nuclear magnetic resonance spectroscopy (NMR) studies (^{1}H and ^{13}C), the structure of the repeating unit of the polysaccharide was established as:



1. Introduction

Euphorbia fischeriana Steud (Euphorbiaceae) is a traditional Chinese medicine, usually called as "Lang Du", which can treat a group of disease, such as edema, indigestion, as well as liver and lung cancers (Wang et al., 2006). The aboveground and underground parts of *E. fischeriana* were showed in Figs. 1 and 2. Sterols, triterpenes, tannins, and a number of diterpenes compounds had been investigated and in particular diterpenes proved to be more potent antitumor ingredients among all molecules with small molecular weight (Sun, Liu, & Liu, 2011). In an early submitted paper, we had enriched a water-soluble polysaccharide (EFP-W1) from *E. fischeriana* by water-extraction and subsequently column chromatography methods. Later the structural feature of EFP-W1 was elucidated by the combination of chemical and instrumental analysis. Given this, we would report another water-soluble polysaccharide from the roots of *E. fischeriana* extracted by 5% alkali solution. Better constructing the structural characterization would enable us to understand more information about this plant for the therapy of cancer and so on.

2. Materials and methods

2.1. Materials and chemicals

The roots of *E. fischeriana* used in this experiment were collected in Qiqihar, Heilongjiang Province, China. A voucher specimen was deposited in the Herbarium of Qiqihar Medical University, China. DEAE Sepharose Fast Flow, Sephadex G-100, and Sephadex G-25 were purchased from Amersham (Sweden). D-Glucose was form Amresco Inc. T-series dextran was purchased from Sigma Chemical Co. (St. Louis, MO). All other reagents were of analytical grade made in China.

* Corresponding author. Tel.: +86 452 2663371; fax: +86 452 2663371.
E-mail address: jichengliu@yahoo.cn (J. Liu).
[1] These authors contributed equally to this paper.

0144-8617/$ – see front matter © 2012 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carbpol.2012.02.004

Fig. 1. Aboveground parts of *E. fischeriana*.

2.2. General methods

Total sugar and uronic acid contents of polysaccharide were quantified by the phenol–sulphuric acid method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) and m-hydroxydiphenyl analysis (Blumenkrantz & Asboe-Hansen, 1973) using D-glucose and D-glucuronic acid as standard, respectively. The protein content in the polysaccharides was measured by the Bradford's method (Sedmark & Grossberg, 1979), with bovine serum albumin as the standard. The sulfate radical content was determined by barium chloride–gelatin assay (Sun, Liang, Cai, et al., 2009; Sun, Liang, Zhang, Tong, & Liu, 2009). The infrared spectra of polysaccharides were recorded on SPECORD IR spectrometer in a range of 400–4000 cm^{-1}. The samples were analyzed as KBr pellets. UV absorption spectra were recorded with a UV spectrophotometer (Model SP-752, China). Polysaccharide was also analyzed for monosaccharide by GC on a Shimadzu GC-14C instrument (Japan) equipped with a DB-1 capillary column (30 m × 0.25 mm × 0.25 μm) and flame-ionization detector (FID). Gas chromatography–mass spectrometry (GC–MS) was finished on a Shimadzu QP-2010 instrument (Japan) with an HP-5MS quartz capillary column (30 m × 0.25 mm × 0.25 μm). GE Healthcare's ÄKTA Explore 100 purification system was applied to the process of polysaccharide fraction, which was equipped with UV-900 monitor, a P-900 series pump, M-925 mixer, pH/C-900 detector, Frac-950 fraction collector, A-900 auto-sampler and various kinds of columns. Dialysis was carried out using tubing with a Mw cut-off of 500 Da (for globular proteins). All gel chromatography was monitored with phenol–sulfuric acid method.

Fig. 2. Underground parts of *E. fischeriana*.

2.3. The fraction procedure for EFP-AW1

The powdered roots of *E. fischeriana* (0.5 kg) were extracted with 95% ethanol (5000 ml, ×3) at 75 °C for 6 h under reflux to remove lipid. The residue was then extracted with distilled water (5000 ml) at 75 °C for 3 times and 3 h for each time. After centrifugation (1700 × *g* for 15 min), the residue was washed by water until no reaction of phenol–sulfuric acid. The washed sample was extracted in 5% alkali solution for 24 h for three times, and the extraction solution was filtered through line cloth. The suspension was neutralized with hydrochloric acid (0.1 M) and filtered. The supernatant containing water-soluble polysaccharide was dialyzed, concentrated, ethanol precipitated and then dried. The precipitate collected by centrifugation was crude polysaccharides. It was deproteinated by proteinase digestion and the Sevag method (Sun & Liu, 2009), followed by exhaustive dialyzed, concentrated, ethanol precipitated and washed with absolute ethanol, acetone, and ether to yield the crude polysaccharide (CEFP-AW, 21.3 g).

The CEFP-AW was purified on an ÄKTA explore 100 purification system The pretreated sample was loaded to a DEAE Sepharose Fast Flow column (2.6 cm × 40 cm), which was eluted with distilled water and 0.1, 0.3, 0.5 and 1 M NaCl aqueous solutions at a flow rate of 4 ml/min. Total sugar content of each tube was measured at 490 nm by Dubois's method, and protein absorption at 280 nm was recorded for each fraction. Then the water-eluted fraction was purified further on a Sephadex G 100 column (2.6 cm × 100 cm) with 0.15 M NaCl at a flow rate of 1 ml/min, yielding only one fraction of EFP-AW1 (1.8 g), and then was applied to a Sephadex G-25 column (2.6 cm × 40 cm) to remove salts.

2.4. Determination of sugar composition, purity and molecular weight

Polysaccharide was also analyzed for monosaccharide by GC. After hydrolysis with 2 M trifluoroacetic acid (TFA) and conversion of hydrolysate into alditol-acetates as previously described method (Jones & Albersheim, 1972; Oades, 1967), the resulting alditol-acetates were analyzed by GC.

The homogeneity and the molecular weight distribution of EFP-AW1 were determined by HPSEC (Sun & Liu, 2009), which was performed on a SHIMADZU HPLC system fitted with one TSK-G3000 PW_{XL} columns (7.8 mm ID × 30.0 cm) and a SHIMADZU RID-10A detector. The column had been calibrated by molecular mass markers (T-130, 80, 50, 25, 10). The eluent was 0.1 mol/l Na_2SO_4, and the flow rate was 0.5 ml/min at 40 °C, with 1.6 mPa. The molecular weight of EFP-AW1 was estimated by reference to the calibration curve made above.

2.5. Partial hydrolysis with acid

The EFP-AW1 (100 mg) was hydrolyzed with TFA (0.05 M/l) at 95 °C for 3 h, and centrifuged, lyophilized and the precipitate was analyzed by GC. The supernatant was dialyzed against distilled water for 48 h, followed precipitated with ethanol. Precipitation in the sack, supernatant in the sack, and the fraction out of sack were dried and carried out for GC analysis as previously described (Sun et al., 2008).

2.6. Periodate oxidation and Smith degradation

25 mg of EFP-AW1 in 12.5 ml of distilled water was mixed with 12.5 ml of 30 mM $NaIO_4$ and the mixture was kept in darkness

for 48 h at 4 °C. 0.1 ml aliquots were withdrawn from the mixture at 3–6 h intervals and read in a spectrophotometer at 223 nm (Linker, Evans, & Impallomeni, 2001) after dilution 250× with distilled water. Ethylene glycol (2 ml) was added to terminate the periodate oxidation reaction after 3 days. Some of the periodate-oxidized product (2 ml) was used to assess the amount of formic acid by titration with 0.00488 M sodium hydroxide, and the rest was extensively dialyzed against tap water and distilled water for 24 h, respectively. The content inside was concentrated and reduced with $NaBH_4$ (60 mg) for 16 h at 25 °C, neutralized with 50% acetic acid, dialyzed as described above and re-concentrated to 10 ml. One-third of the solution mentioned above was freeze-dried and fully hydrolyzed for GC analysis; others were added to the same volume of 1 M sulfuric acid for 40 h at 25 °C, neutralized to pH 6.0 with $BaSO_4$, and filtered for analysis by Smith degradation. The filtrate was dialyzed (molecular weight cut-off of 3 kDa), and the content out of dialysis sack was desiccated for GC analysis; the content inside the dialysis sack was diluted with ethanol, the supernatant and precipitate were also dried out for GC analysis after centrifugation (Sun, Li, Yang, Liu, & Kennedy, 2010).

2.7. Methylation analysis

EFP-AW1 (20 mg) was methylated three times according to Needs and Selvendran (1993). The methylated products were extracted by chloroform. No absorption peak in the region of 3200–3700 cm^{-1} was detected in the IR spectrum confirmed that the methylated products were completely methylated. The methylated products were hydrolyzed with formic acid and 2 M TFA, and excess acid was evaporated by co-distillation with distilled water. The hydrolyzed product was reduced with $NaBH_4$ for 24 h and acetylated with acetic anhydride–pyridine (1:1) at 100 °C for 2 h. The alditol acetates of the methylated sugars were analyzed by GC–MS.

2.8. NMR analysis

30 mg of EFP-AW1 (deuterium-exchanged) was dissolved in 0.55 ml of deuteroxide (99.99% D). The ^1H and ^{13}C analysis of EFP-AW1 was performed on Bruker AV-600 NMR spectrometer instrument (Sun, Li, et al., 2010).

The above methods applied in this paper are expressed in a conventional way as other papers published by our research group.

3. Results and discussion

3.1. Isolation, purification and characteristic of EFP-AW1

Polysaccharide purification employed on ÄKTA Explore 100 purification system packed with DEAE Sepharose Fast Flow column and Sephadex G 100 column had successfully led to the isolation of one homogeneous and purified polysaccharide from the roots of *E. fischeriana* determined by HPSEC in Fig. 3. Its molecular weight was estimated to be about 10,830 Da.

The total sugar content of EFP-AW1 was determined to be 92.34%. The protein content was 0.1% and showed weak positive response to the Bradford test. No uronic acid and sulfate radical was determined by m-hydroxydiphenyl colorimetric method and barium chloride–gelatin assay, respectively. GC analysis showed that EFP-AW1 was composed of Glc, Gal, Man and Rha in a molar ratio of 14.1:1.9:2.0:1.9, as shown in Fig. 4.

The bands appeared at 1000–1160 cm^{-1}, 1400–1540 cm^{-1}, 2800–2950 cm^{-1}, and 3100–3500 cm^{-1}, which were distinctive absorptions of polysaccharides. The absorption band at 840 and 890 cm^{-1} confirmed the co-existence of α and β-glycosidic bond

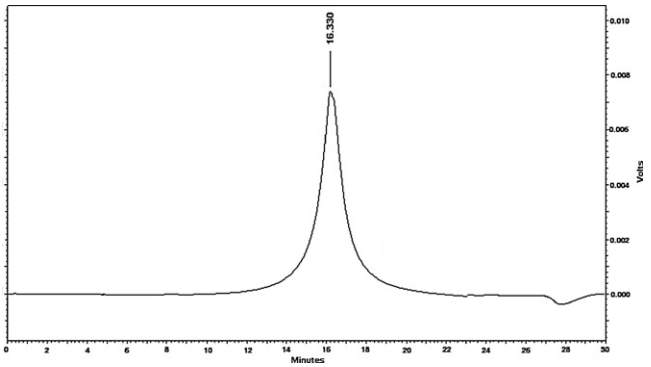


Fig. 3. HPSEC of EFP-AW1.

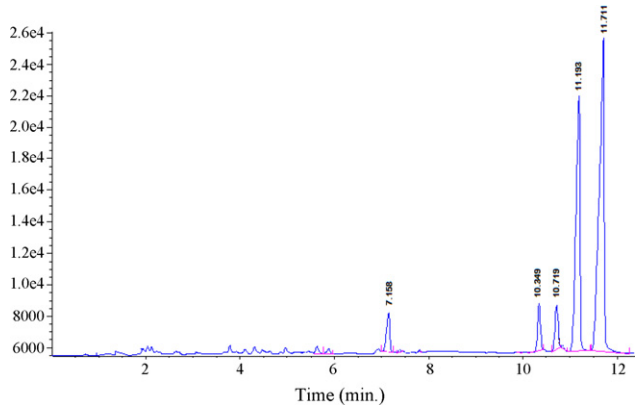


Fig. 4. GC of EFP-AW1.

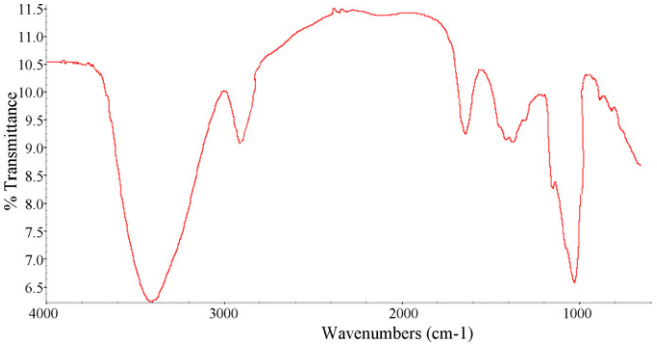


Fig. 5. The FTIR spectra of EFP-AW1.

(Fig. 5), which was in good agreement with the following results of NMR analysis for EPF-AW1.

3.2. Structural characterization of EFP-AW1

The fully methylated EFP-AW1 was hydrolyzed with acid, converted into alditol acetates, and analyzed by GC/MS (Table 1). The results showed the presence of five fractions, including 2,4,

Table 1
The results of methylation analysis of EFP-AW1.

Peak no.	Methylated sugar	Molar ratio	Linkage type
Residue A	2,4,6-Me_3-Glc*p*	5	1,3-Linked-α-D-Glc
Residue B	2,4-Me_2-Glc*p*	2	1,3,6-Linked-β-D-Glc
Residue C	2,4,-Me_2-Rha*p*	1	1,3-Linked-α-L-Rha
Residue D	2,3,4,6-Me_4-Man*p*	1	1-Linked-β-D-Man
Residue E	2,3,4,6-Me_3-Gal*p*	1	1-Linked-α-D-Gal

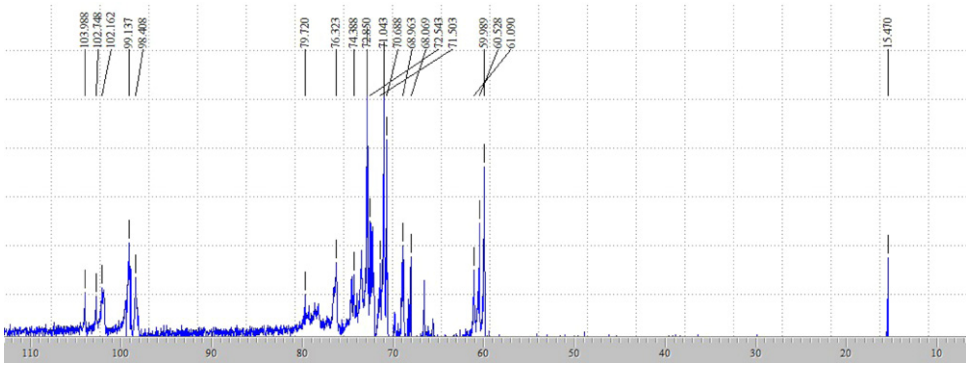


Fig. 6. ^{13}C NMR (150 M) spectra of EFP-AW1.

6-tri-O-methyl-gluctiol (Residue A: 1,3-linked Glc), 2,4-di-O-methyl-gluctiol (Residue B: 1,3,6-linked Glc), 2,4-di-O-methyl-rhamnitol (Residue C: 1,3-linked Rha), 2,3,4,6-tetra-O-methyl-mannitol (Residue D: 1-linked Man) and 2,3,4,6-tetra-O-methyl-galactitol (Residue E: 1-linked Gal) in a relative molar ratio of 5:2:1:1:1. This showed a good correlation between terminal and branched residues, and these molar ratios agreed with the overall monosaccharide composition described above. The results from analysis of GC–MS, which were consistent with the results from partial acid hydrolysis, periodate oxidation and Smith degradation, indicated that 1,3-linked Glc and 1,3,6-linked Glc residues were major components of the backbone structure, 1,3-linked Rha residue were distributed in branches, and residues of branches terminated with 1-linked Man or 1-linked Gal residues.

The ^{13}C NMR spectra (Fig. 6) showed five strong signals at 98.408–103.988 ppm. In the anomeric carbon region of EFP-AW1, signals at 99.137 ppm could be attributed to C-1 of Residue A (α-glycosidic bond); 102.748 ppm to C-1 of Residue B (β-glycosidic bond); 103.988 ppm to C-1 of Residue C (α-glycosidic bond); 102.162 ppm to C-1 of Residue D (β-glycosidic bond); 98.408 ppm to C-1 of Residue E (α-glycosidic bond). The signals for unsubstituted C-6 of Residue A, D and E range from 59.989 to 61.090 ppm and for substituted C-6 of Residue B in the lower field from 68.069 to 68.963 ppm. A typical peak at 15.470 was attributed to C-6 of Residue C exclusively. The signal of substituted C-3 of Residues A, B and C had moved downfield to 79.720, 76.323 and 74.388 ppm, respectively. Accordingly there five signals appeared in the 600-MHz ^{1}H NMR spectrum (data not shown), namely 4.971, 4.454, 4.943, 4.839 and 5.074 for Residues A, B, C, D and E, respectively. The special anomeric carbons and their protons chemical shift confirmed that sugar residues were linked by α and β-glycosidic bond, which agreed with co-presence of an IR band 840 and 890 cm^{-1} (Barker, Bourne, Stacey, & Whiffen, 1954). The other proton signals (H2–H5) of EFP-AW1 were not assigned due to overlapping peaks. All the NMR chemical shifts were compared with the literature values (Carilllo et al., 2009; Das et al., 2008; Dey et al., 2010; Maity et al., 2011; Pieretti et al., 2009; Sun, Liu, Yang, & Kennedy, 2010b; Sun et al., 2008).

4. Conclusion

From the above analysis, we elucidated that the structural feature of EFP-AW1 from the roots of *E. fischeriana* had the following structure: the backbone consisted of 6 repeating units of [→3)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→3,6)-α-D-Glc*p*-(1→3,6)-α-D-Glc*p*-(1→]; the side chain [→1)-α-L-Rha*p*-(3→1)-α-D-Gal*p*] was attached to the backbone through O-3 of Glc residues; the terminal residue of [→1)-β-D-Man*p*] was attached to O-3 of Glc residues. The further detailed structure elucidation would continue in our later research.

Acknowledgement

This study was supported by the National Natural Science Foundation of China (Grant No. 30973902).

References

Barker, S. A., Bourne, E. J., Stacey, M., & Whiffen, D. H. (1954). Infra-red spectra of carbohydrates. Part I: Some derivatives of D-glucopyranose. *Journal of the Chemical Society*, 171–176.

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

Carilllo, S., Silipo, A., Perino, V., Lanzetta, R., Parrilli, M., & Molinaro, A. (2009). The structure of the O-specific polysaccharide from the lipopolysaccharide of *Burkholderia anthina*. *Carbohydrate Research*, *344*, 1697–1700.

Das, D., Maiti, D., Chandra, K., Mondal, S., Ojha, A. K., Roy, S. K., et al. (2008). NMR and MALDI-TOFMS analysis of a heteroglycan isolated from hot water extract of edible mushroom, *Volvariella bombycina*. *Carbohydrate Research*, *343*, 2258–2265.

Dey, B., Bhunia, S. K., Maity, K. K., Patra, S., Mandal, S., Maiti, S., et al. (2010). Chemical analysis of an immunoenhancing water-soluble polysaccharide of an edible mushroom, *Pleurotus florida* blue variant. *Carbohydrate Research*, *345*, 2736–2741.

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*, 350–356.

Jones, T. M., & Albersheim, P. (1972). A gas chromatographic method for the determination of aldose and uronic acid constituents of plant cell wall polysaccharides. *Plant Physiology*, *49*, 926–936.

Linker, A., Evans, L. R., & Impallomeni, G. (2001). The structure of a polysaccharide from infectious strains of *Burkholderia cepacia*. *Carbohydrate Research*, *335*, 45–54.

Maity, K. K., Patra, S., Dey, B., Bhunia, S. K., Mandal, S., Das, D., et al. (2011). A heteropolysaccharide from aqueous extract of an edible mushroom, *Pleurotus ostreatus* cultivar: Structural and biological studies. *Carbohydrate Research*, *346*, 366–372.

Needs, P. W., & Selvendran, R. R. (1993). Avoiding oxidative degradation during sodium hydroxide/methyl iodide-mediated carbohydrate methylation in dimethyl sulfoxide. *Carbohydrate Research*, *245*, 1–10.

Oades, J. M. (1967). Gas–liquid chromatography of alditol acetates and its application to the analysis of sugars in complex hydrolysates. *Journal of Chromatography*, *28*, 246–252.

Pieretti, G., Nicolaus, B., Poli, A., Corsaro, M. M., Lanzetta, R., & Parrilli, M. (2009). Structural determination of the O-chain polysaccharide from the haloalkaliphilic *Halomonas alkaliantarctica* bacterium strain CRSS. *Carbohydrate Research*, *344*, 2051–2055.

Sedmark, J. J., & Grossberg, S. E. (1979). A rapid, sensitive, and versatile assay for protein using Coomassie brilliant blue G250. *Analytical Biochemistry*, *79*, 544–552.

Sun, Y. X., Li, X., Yang, J. F., Liu, J. C., & Kennedy, J. F. (2010). Water-soluble polysaccharide from the fruiting bodies of *Chroogomphis rutilus* (Schaeff.: Fr.) O. K. Miller: Isolation, structural features and its scavenging effect on hydroxyl radical. *Carbohydrate Polymers*, *80*, 720–724.

Sun, Y. X., Liang, H. T., Cai, G. Z., Guan, S. W., Tong, H. B., Yang, X. D., et al. (2009). Sulfated modification of the water-soluble polysaccharides from *Polyporus albicans* mycelia and its potential biological activities. *International Journal of Biological Macromolecules*, *44*, 14–17.

Sun, Y. X., Liang, H. T., Zhang, X. T., Tong, H. B., & Liu, J. C. (2009). Structural elucidation and immunological activity of a polysaccharide from the fruiting body of *Armillaria mellea*. *Bioresource Technology*, *100*, 1860–1863.

Sun, Y. X., & Liu, J. C. (2009). Structural characterization of a water-soluble polysaccharide from the roots of *Codonopsis pilosula* and its immunity activity. *International Journal of Biological Macromolecules*, *43*, 279–282.

Sun, Y. X., Liu, J. C., & Liu, D. Y. (2011). Chemical constituents and biological activities of *Euphorbia fischeriana* Steud. *Chemistry and Biodiversity*, *8*, 1205–1214.

Sun, Y. X., Liu, J. C., Yang, X. D., & Kennedy, J. F. (2010). Purification, structural analysis and hydroxyl radical-scavenging capacity of a polysaccharide from the fruiting bodies of *Russula virescens*. *Process Biochemistry*, *45*, 874–879.

Sun, Y. X., Wang, S. S., Li, T. B., Li, X., Jiao, L. L., & Zhang, L. P. (2008). Purification, structure and immunobiological activity of a new water-soluble polysaccharide from the mycelium of *Polyporus albicans*(Imaz) Teng. *Bioresource Technology*, *99*, 900–904.

Wang, Y. B., Huang, R., Wang, H. B., Jin, H. Z., Lou, L. G., & Qin, G. W. (2006). Diterpenoids from the roots of *Euphorbia fischeriana*. *Journal of Natural Product*, *69*, 967–970.