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Note

Water-soluble polysaccharides from finger citron fruits (*Citrus medica* L. var. *sarcodactylis*)

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ABSTRACT

Four water-soluble polysaccharides, **FCp-1**, **FCp-2**, **FCp-3**, and **FCp-4** were obtained from finger citron fruits (*Citrus medica* L. var. *sarcodactylis*) by hot-water extraction and ethanol precipitation, followed by routine separation procedure. Based on the calibration curve, molecular weights of them were estimated to be 113.9, 32.6, 140.3, and 177.1 kDa respectively. The acid hydrolysis, methylation, IR, GC–MS, and NMR experiments were used for composition analysis. **FCp-1** was a heteropolysaccharide composed of arabinose, galactose, glucose, rhamnose, and xylose, with a molar ratio of 3.0:7.0:4.1:1.0:1.5. **FCp-2** and **FCp-4** were →4)-α-D-Gal*p*A(1→ linking galacturonan differ in molecular weights. **FCp-3** was a →6)-α-D-Glc*p*(1→ linking glucan. According to the results of in vitro assays, **FCp-3** showed significantly and moderately enhancing capacities toward the proliferation of splenocytes and thymocytes respectively. Thus, **FCp-3** or analogs may have further use as immunomodulatory agents.

The finger citron (*Citrus medica* L. var. *sarcodactylis* Hort) (FC) belongs to the family *Rutaceae*, and is widely cultivated in Oriental countries. FC fruit is used in traditional Chinese medicine as adjuvants in the treatment of a variety of chronic diseases, including hypertension and respiratory tract infections,[1,2] and are also consumed as functional foods, being considered beneficial to liver, pancreatic, and stomach function.[3] Previous studies have shown FC fruit contains significant quantities of essential oil, with this material possessing antioxidant activity that could possibly be commercially exploited,[3,4] and which have also demonstrated insulin secretagogue and anti-inflammatory activities.[5] However, a detailed analysis of the structural and biological properties of polysaccharides derived from FC fruit has yet to be reported, except for research on extraction methods of crude polysaccharide.[3] Given the presence and diverse biological activities of polysaccharides throughout nature,[6–11] and their use in many healthcare products, we decided to investigate the polysaccharide profile of FC fruit. In this paper, we report the fractionation (Fig. 1), compositional analysis, structural characterization, and immunological activity of the polysaccharides (Fig. 2) from the FC fruit.

1. Experimental

1.1. General procedures

Ultraviolet–visible (UV–vis) spectra were recorded on a SHIMADZU UV-1800 UV–vis spectrophotometer. IR spectra were recorded from KBr pellets in the 4000–400 cm^{-1} range on a Bruker Alpha-T FT-IR instrument. ^{1}H and ^{13}C NMR spectra of 50 mg compound samples were recorded at 25 °C in D_2O on a Bruker 500 MHz spectrometer. Chemical shifts (δ) are reported in ppm relative to Me_4Si (δ_H 0.0). Gas chromatography–mass spectrometry (GC–MS) analyses were performed on a Thermo Fisher Scientific TRACE DSQ Single Quadrupole GC–MS instrument, using an Agilent HP-5 column (30 m × 0.25 mm i.d). The ionization potential was 70 eV, and the temperature of the ion source was 200 °C.

1.2. Plant material

Fresh FC fruits (*Citrus medica* L. var. *sarcodactylis* Hort) were collected from Jinhua, Zhejiang Province, China, in March 2012, and identified by Dr. Ende Liu (Kunming Institute of Botany, Chinese Academy of Sciences, Kunming, Yunnan Province, China).

1.3. Extraction and isolation of polysaccharides FCp-1, FCp-2, FCp-3, and FCp-4

Whole FC fruit (1.2 kg) was ground to a pulp, and the resulting material was divided into three portions. Each portion was treated with deionized water (2.0 L), and the resulting mixtures were boiled at 100 °C for 3 h and then filtered. The combined filtrates were concentrated to dryness under reduced pressure at 50 °C. The residue (210 g) was suspended in deionized water (800 mL) and washed successively with petroleum ether, EtOAc and finally *n*-BuOH. The remaining aqueous layer was concentrated to a volume of 200 mL under reduced pressure at 50 °C, and the resulting

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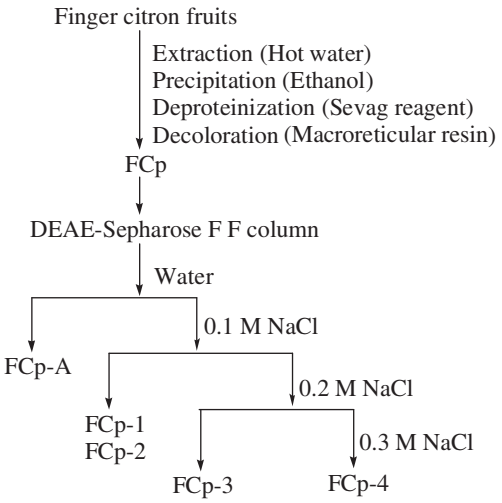

Figure 1. Fractionation procedure of the polysaccharides.

solution was added to 95% aqueous EtOH (3.0 L). This mixture was left to stand for 12 h, and the resulting crude polysaccharide precipitate was collected by filtration. This precipitate was freeze-dried to yield a crude polysaccharide mixture (18.5 g), a portion (10 g) of which was then re-dissolved in deionized water (300 mL). This solution was deproteinated by treatment with Sevag reagent (chloroform/*n*-butanol at a ratio of 4:1, v/v), according to literature methods.[12] The deproteinated solution was decolorized by passing through a macroreticular anion-exchange resin column (D315) and washed with double column volume deionized water, and the resulting solution was concentrated to a volume of 100 mL.[13] This solution was dialyzed in 3500 Da MWCO (molecular weight cut-off) tubing at 37 °C for 48 h, and the solution remaining within the dialysis membrane was freeze-dried to yield polysaccharide (**FCp**; 5.5 g).

A sample of **FCp** (1 g) was dissolved in deionized water (5 mL), and the mixture was centrifuged. The supernatant was separated, and then loaded onto a DEAE-Sepharose Fast Flow (Pharmacia) chromatography column (2.6 × 40 cm), and eluted successively with deionized water, 0.1 M NaCl, 0.2 M NaCl, and 0.3 M NaCl (each 300 mL). The proportion of polysaccharide in each of these eluents was determined by the phenol–sulfuric acid colorimetric method.[14] This analysis revealed that the desired polysaccharide resided in the three NaCl eluents, and dialyzed in 3500 Da MWCO tubing in deionized water at 37 °C for 48 h, respectively. The solution remaining within the dialysis membrane was then concentrated to dryness under reduced pressure, and the residue freeze-dried to give three crude solid. These materials were further purified on a Sephadex G-100 (Pharmacia) gel-filtration chromatography column (2.6 × 40 cm) using 0.01 M NaCl as an eluent. Appropriate fractions were combined, dialyzed, and freeze-dried as before, to yield four homogeneous polysaccharides, denoted **FCp-1** (56 mg), **FCp-2** (84 mg), **FCp-3** (64 mg), and **FCp-4** (220 mg) (Fig. 2).

1.4. Determination of purity and molecular weight of FCp-1, FCp-2, FCp-3, and FCp-4 by high-performance gel-permeation chromatography (HPGPC)

HPGPC was used to determine the purity of **FCp-1**, **FCp-2**, **FCp-3**, and **FCp-4**. Analyses were conducted on a Waters 515 instrument (Milford, PA, USA) equipped with a Waters 2410 refractive index detector and a TSKgel G4000SWxl (Tosoh Biosep, Japan) size-exclusion analytical column (7.8 × 300 mm), using deionized water as the mobile phase and with a sample concentration of 3 mg/mL. T-series dextran standards (Sigma–Aldrich) with defined molecular masses ranging from 10 to 300 kDa were used to calibrate the HPGPC system. All data obtained were collected and analyzed using the Waters Millennium®32 software package.

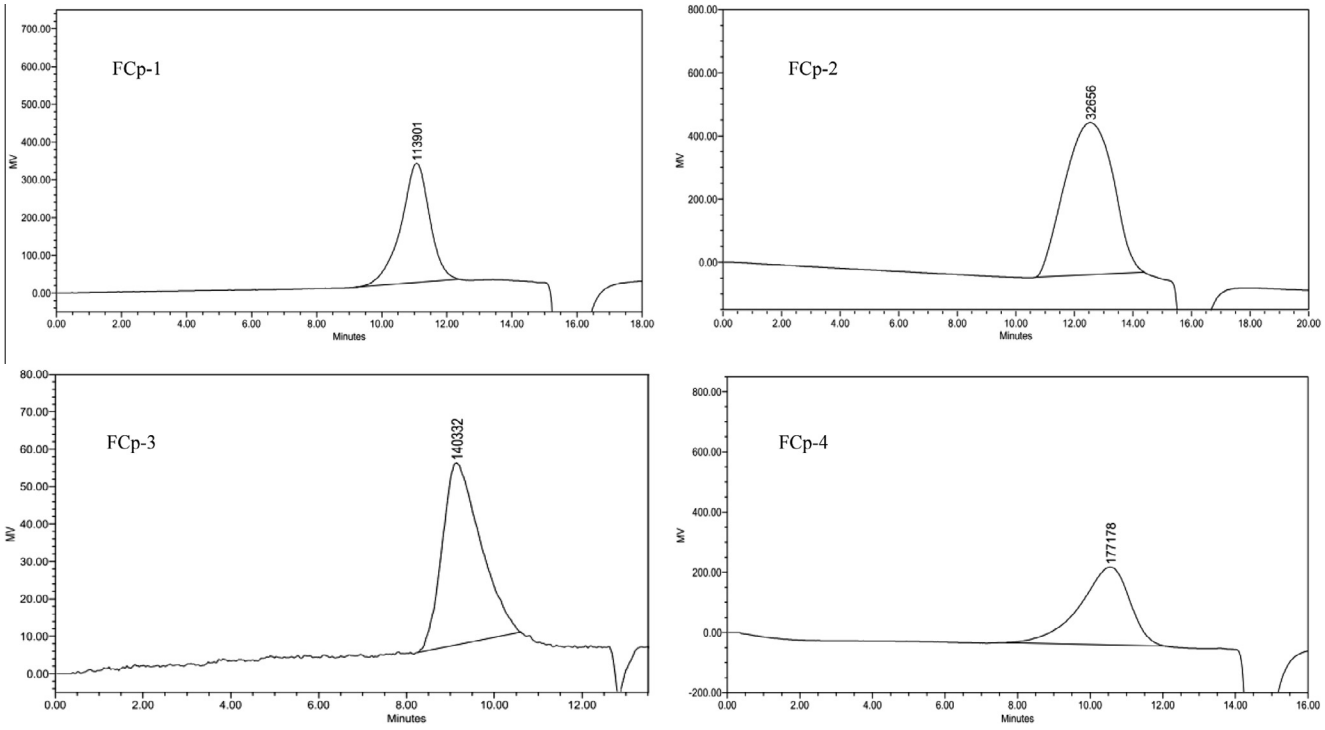

Figure 2. HPGPC profiles for obtained polysaccharides **FCp-1** (PD = 1.16), **FCp-2** (PD = 1.06), **FCp-3** (PD = 1.22), and **FCp-4** (PD = 1.41). (PD = Mw/Mn; PD, polydispersity; Mw, weight-average molecular mass; Mn, number-average molecular mass).

1.5. Component analysis of polysaccharides FCp-1, FCp-2, FCp-3, and FCp-4

Samples of polysaccharides **FCp-1** to **FCp-4** (each 5 mg) were hydrolyzed in 2 M trifluoroacetic acid (TFA; 5 mL) at 120 °C for 6 h, and then evaporated to dryness under reduced pressure. The residue was divided into two portions. One portion was used for paper chromatography (PC), which was performed on Whatman 3 MM filter paper, eluting with either (a) EtOAc/HOAc/H_2O (3:3:1) or (b) *n*-BuOH/pyridine/H_2O (6:4:3) solvent systems. Papers were visualized by aniline–diphenylamine–phosphoric acid solution (a) or *p*-anisidine solution (b),[15] and authentic standards (L-arabinose, D-glucose, D-mannose, D-galactose, D-ribose, D-xylose, L-rhamnose, D-glucuronic acid and D-galactonic acid) were used as reference compounds. The other portion of crude residue was treated with $NaBH_4$ (15 mg), and the resulting mixture stirred at room temperature for 12 h. The reaction mixture was then treated with a mixture of Ac_2O and pyridine (1.0 mL of each) at 120 °C for 6 h,[16] and then concentrated to dryness under reduced pressure. The resulting alditol acetates were re-dissolved in CH_2Cl_2 (1 mL) for GC–MS analysis, from which their structures were identified by their retention times and electron-impact profiles.

1.6. Methylation analysis of polysaccharides FCp-1 and FCp-3

Per-O-methylation of polysaccharide samples **FCp-1** and **FCp-3** was performed in a dry solution of DMSO and CH_3I, using NaOH as a catalyst.[17] To a solution of polysaccharide (20 mg) in dry DMSO (5 mL) and under a positive pressure of dry N_2 was added dry NaOH powder (200 mg), and the resulting mixture was stirred overnight at room temperature. Subsequently, CH_3I (2 mL) was slowly added to the reaction mixture, and the resulting mixture stirred for a further 4 h at room temperature. At this time, deionized water (1 mL) was added to stop the reaction, and the mixture was subjected to dialysis and freeze-drying conditions (as previously described). This whole process was repeated three times, until the resulting residue displayed no –OH absorption band in the IR spectrum, at which point the mixture was hydrolyzed by treatment with 2 M TFA (5 mL) at 120 °C for 8 h. The residue was then reduced with $NaBH_4$ (15 mg) at room temperature and followed by adding Ac_2O and pyridine (1.0 mL of each) to acetylate at 120 °C for 8 h, then concentrated to dryness under reduced pressure. The resulting partially methylated alditol acetates were re-dissolved in CH_2Cl_2 (1 mL) for further GC–MS analysis.

1.7. Assay of effect of polysaccharides FCp-1, FCp-2, FCp-3, and FCp-4 on splenocyte and thymocyte proliferation

Cells of the spleen and thymus were prepared from immunized mice under aseptic conditions by homogenization in Hank's balanced salt solution (HBSS),[18] followed by addition of Tris–NH_4Cl solution to lyse the erythrocytes. The resulting solution was centrifuged, and the pellet was re-suspended in complete RPMI 1640 (Sigma–Aldrich) medium, and the cell concentration was adjusted with medium to 1×10^6 cells/mL. The viability of cells was estimated as being >90% by the Trypan Blue dye exclusion method.

Next, a sample of cells (180 μL) was seeded in each well of a 96-well microtiter plate, and incubated with 20 μL of various concentrations (12.5, 25, 50, 100 and 200 μg/mL) of samples **FCp-1**, **FCp-2**, **FCp-3**, and **FCp-4** at 37 °C in a 5% CO_2 incubator. Lipopolysaccharide (LPS, L6511 of *Salmonella enterica* serotype Typhimurium, Sigma–Aldrich, 4 μg/mL) and Concanavalin A (Con A, L7647 of *Canavalia ensiformis*, Sigma–Aldrich, 10 μg/mL) were used as positive controls for splenocyte and thymocyte stimulation assays, respectively. After 48 h, MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide] (20 μL, 5 mg/mL) was added to each well and the plates were incubated for a further 4 h. The supernatant was then removed by aspiration, and the resulting formazan crystals were dissolved in DMSO (150 μL). The absorbance of these DMSO solutions was measured at 570 nm with a plate reader.[19,20] Proliferation of splenocytes (% Splenocyte Proliferation Index or % SPI) and thymocytes (% Thymocyte Proliferation Index or % TPI) was expressed as mean ± standard deviations. All experiments were conducted in quadruplicate, and repeated three times.

2. Results and discussion

Four water-soluble polysaccharides, **FCp-1**, **FCp-2**, **FCp-3**, and **FCp-4** were obtained from FC by hot-water extraction and ethanol precipitation, followed by routine separation procedure. Based on the calibration curve, molecular weights of them were estimated to be 113.9, 32.6, 140.3, and 177.1 kDa respectively (Fig. 2). Samples were all white powders, and did not absorb at 280 and 260 nm in the UV–visible spectrum, suggesting the absence of protein and nucleic acid. Samples also did not cause darkening of iodine solution, suggesting the absence of starch.

Further chemical analysis of them was conducted by treating samples with 2 M TFA at 120 °C for 8 h. PC of the resulting solutions showed that only galacturonic acid was present in **FCp-2** and **FCp-4**, which was confirmed by examination of its NMR spectral data. Glucose was found to be present in **FCp-3**, which was further confirmed by GC–MS and NMR analysis (see Supplementary information). **FCp-1** was found to be composed of arabinose, galactose, glucose, rhamnose, and xylose, in a molar ratio of 3.0:7.0:4.1:1.0:1.5, This corresponded to the results of GC–MS (Table 1). The absolute configuration of the polysaccharides was determined by GLC of their acetylated glycosides, using (+)-2-butanol, as per the literature method.[21] The results showed that both galacturonic acid in **FCp-2** and **FCp-4** and glucose in **FCp-3** have the D-configuration. And the galactose, glucose, and xylose in FCp-1 have the D-configuration, while the arabinose and rhamnose have the L-configuration.

The IR spectrum of **FCp-2** and **FCp-4** had strong absorption bands at 3425, 2938, 1612, 1416, 1331, 1100, 1088, 1016, 953, 835, and 595 cm^{-1}. The strong band at 1612 cm^{-1} was due to the carbonyl group, while the band at 2938 cm^{-1} was ascribed to a C–H stretching vibration. The strong band at 3425 cm^{-1} was

Table 1
Profile of partially O-methylated alditol acetates obtained from methylation analysis of **FCp-1** and **FCp-3**

Compound	*O*-Me-alditol acetate	mol (%)
FCp-1	2,3,5-Me_3-Ara	1
	2,3,4-Me_3-Xyl	2
	2,3,4-Me_3-Ara	1
	2,3,4-Me_3-Rha	1
	2,4-Me_2-Ara	11
	2,3,4,6-Me_4-Glc	1
	2,3-Me_2-Ara	5
	2,3,4,6-Me_4-Gal	2
	2,3-Me_2-Xyl	7
	2,3-Me_2-Rha	4
	2-Me_1-Rha	1
	2,3,6-Me_3-Gal	3
	2,3,6-Me_3-Glc	7
	2,3,4-Me_3-Gal	12
	2,3,4-Me_3-Glc	14
	3,6-Me_2-Gal	1
	2,3-Me_2-Glc	3
	2,3-Me_2-Gal	8
	2,4-Me_2-Gal	16
FCp-3	2,3,4,6-Me_4-Glc	Trace
	2,3,4-Me_3-Glc	100

attributed to a hydroxyl stretching vibration, and the characteristic absorption signal at 835 cm^{-1} indicated the presence of α-pyranoid-type glycosidic linkage.[22–24] In the IR spectrum of **FCp-3**, a hydroxyl stretching vibration band was seen at 3422 cm^{-1}, and a C–H stretching vibration band at 2935 cm^{-1}. The presence of three absorption bands at 1098, 1084, and 1021 cm^{-1} indicated the presence of a pyranoid ring, while the characteristic absorption band at 838 cm^{-1} suggested the presence of α glycosidic linkage of the polysaccharide.[22–24]

GC–MS of per-O-methylated samples of **FCp-3** showed one main peak, which corresponded to 2,3,4-tri-*O*-methyl-Glc, while a series of peaks were present in the GC–MS profile of **FCp-1** (Table 1).

In the ^{1}H NMR spectra (see Supplementary information), **FCp-2** and **FCp-4** displayed five strong proton signals at δ_H 5.09, 4.82, 4.44, 4.02, and 3.79. Six strong carbon signals at δ_C 175.2 (C_6), 98.9 (C_1), 77.7 (C_4), 71.1 (C_5), 68.6 (C_3) , and 67.9 (C_2) could be observed in the corresponding ^{13}C NMR spectrum, with the most downfield of these obviously representing the C_6-carbonyl group of the alduronic acid unit, which was corresponding to PC results. And heteronuclear correlations observed in the HMQC spectrum (see Supplementary information) further confirmed **FCp-2** and **FCp-4** as being →4)-α-D-Gal*p*A(1→ linked galacturonans.

FCp-3 had seven groups of signals at δ_H 5.00, 4.00, 3.92, 3.77, 3.73, 3.59, and 3.54 in its ^{1}H NMR spectrum. In its ^{13}C NMR spectrum, six strong carbon signals at δ_C 97.6 (C_1), 71.3 (C_2), 73.3 (C_3), 69.4 (C_4), 70.1 (C_5), and 65.5 (C_6) could be observed, with the most downfield of these being the anomeric carbon, which correlates with the anomeric proton at δ_H 5.00 in the HMQC spectrum. The two methylene protons (δ_H 4.00 and 3.77) of C_6 (δ_C 65.5) were coupled to the anomeric carbon at δ_C 97.6, demonstrating the location of the connecting sugar unit, which could also be clearly observed in the HMBC spectrum. The correlations of other protons in the sugar unit with their neighboring carbon atoms were also observed in the HMBC spectrum, indicating that the glucose units were linked in 1 → 6 position in a →6)-α-D-Glc*p*(1→ linking mode. This corresponded to the results of GC–MS.

In **FCp-1**, the anomeric protons signals were present at δ_H 5.0–5.5 in the ^{1}H NMR spectrum, and the other protons (except for methyl protons of rhamnose units) were at δ_H 3.2–4.8. In the ^{13}C NMR spectrum, anomeric carbons were present at δ_C 98.0–110, and other carbons (except for methyl carbon of rhamnose units at δ_C 16.37) were distributed in δ_C 60.0–85.0 (see Supplementary information).

The immunological activity of **FCp-1**, **FCp-2**, **FCp-3**, and **FC-p4** was evaluated by splenocyte and thymocyte proliferation experiments. The results are represented as mean ± standard deviation, and are compared against the activity of a phosphate-buffered saline (PBS) control, according to a literature method.[19] **FCp-2** and **FCp-4** exhibited no effect on splenocyte or thymocyte proliferation, while **FCp-1** displayed slight activity at doses >100 μg/mL. However, **FCp-3** significantly enhanced splenocyte proliferation at doses ⩾25 μg/mL, and demonstrated a moderate effect on thymocyte proliferation (Fig. 3). These data suggest that **FCp-3** could be used as an immunomodulatory agent.

In conclusion, this is the first report describing the identity of the four main water-soluble polysaccharides present in finger citron (*Citrus medica* L. var. *sarcodactylis* Hort) fruit. In future work, we will describe the synthesis and biological properties of **FCp-3** analogs.

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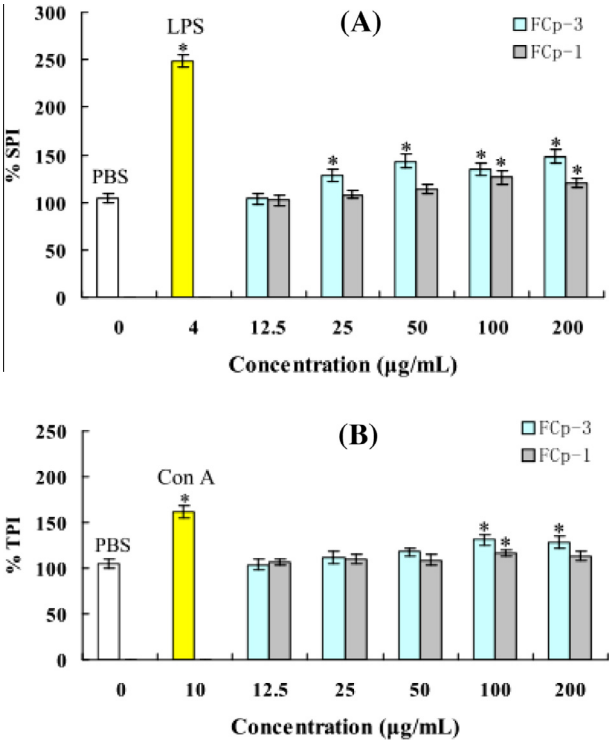

Figure 3. Effects of different concentrations of **FCp-1** and **FCp-3** on splenocytes (A) and thymocytes (B) proliferation; LPS (lipopolysaccharide) and Con A (concanavalin A) were as positive control (*Significantly different from PBS control, $p < 0.05$).

Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.carres.2013.12.020.

References

1. Pharmacopoeia Commission of People's Republic of China *Chin. Pharmacopoeia* **2010**, *I*, 167.
2. Chen, Y. S.; Gong, Z. L. *Food Sci. Technol.* **2003**, *3*, 92–94 (In Chinese).
3. Wu, Z.; Li, H.; Tu, D. W.; Yang, Y.; Zhan, Y. *Ind. Crops Prod.* **2013**, *44*, 145–151.
4. Wu, Z.; Li, H.; Yang, Y.; Zhan, Y.; Tu, D. W. *Ind. Crops Prod.* **2013**, *46*, 311–316.
5. Peng, C. H.; Ker, Y. B.; Weng, C. F.; Peng, C. C.; Huang, C. N.; Lin, L. Y.; Peng, R. Y. *J. Agric. Food Chem.* **2009**, *56*, 8812–8819.
6. Wiater, A.; Paduch, R.; Choma, A.; Pleszczyńska, M.; Siwulski, M.; Dominik, J.; Janusz, G.; Tomczyk, M.; Szczodrak, J. *Int. J. Biol. Macromol.* **2012**, *15*, 1014–1023.
7. Pires, N. R.; Cunha, P. L.; Maciel, J. S.; Angelim, A. L.; Melo, V. M.; de Paula, R. C.; Feitosa, J. P. *Carbohydr. Polym.* **2013**, *91*, 92–99.
8. Costa, L. S.; Fidelis, G. P.; Cordeiro, S. L.; Oliveira, R. M.; Sabry, D. A.; Câmara, R. B.; Nobre, L. T.; Costa, M. S.; Almeida-Lima, J.; Farias, E. H.; Leite, E. L.; Rocha, H. A. *Biomed. Pharmacother.* **2010**, *64*, 21–28.
9. Tabarsa, M.; Lee, S.-J.; You, S. G. *Carbohydr. Res.* **2012**, *361*, 141–147.
10. Dourado, F.; Madureira, P.; Carvalho, V.; Coelho, R.; Coimbra, M. A.; Vilanova, M.; Mota, M.; Gama, F. M. *Carbohydr. Res.* **2004**, *339*, 2555–2666.
11. Yang, B.; Wang, J. S.; Zhao, M. M.; Liu, Y.; Wang, W.; Jiang, Y. M. *Carbohydr. Res.* **2006**, *341*, 634–638.
12. Sevag, M. G.; Lackman, D. B.; Smolens, J. *J. Biol. Chem.* **1938**, *124*, 425–436.
13. Hu, C. F.; Kong, Q. J.; Yang, D. Y.; Pan, Y. J. *Carbohydr. Polym.* **2011**, *84*, 402–406.
14. Dubois, M.; Gilles, K. A.; Hamilton, J. K.; Rebers, P. A. *Anal. Chem.* **1956**, *28*, 350–356.
15. Bailey, R. W.; Bourne, E. J. *J. Chromatogr. A* **1960**, *4*, 4206–4213.
16. Wolfrom, M. L.; Thrompson, A. *Methods in Carbohydrate Chemistry*; Academic Press: New York, 1963; pp 65–68.
17. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
18. Maiti, S.; Bhutia, S.; Mallick, S. K.; Kumar, A.; Khadgi, N.; Maiti, T. K. *Environ. Toxicol. Pharmacol.* **2008**, *26*, 187–191.
19. Mosmann, T. *J. Immunol. Methods* **1983**, *65*, 55–63.
20. Sarangi, I.; Ghosh, D.; Bhutia, S. K.; Mallick, S. K.; Maiti, T. K. *Int. Immunopharmacol.* **2006**, *6*, 1287–1297.

21. Gerwig, G. J.; Kamerling, J. P.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1978**, *66*, 349–357.
22. Ahrazem, O.; Gómez-Miranda, B.; Prieto, A.; Barasoaín, I.; Bernabé, M.; leal, J. A. *Mycol. Res.* **2000**, *104*, 603–610.
23. Wu, Y. L.; Hu, N.; Pan, Y. J.; Zhou, L. J.; Zhou, X. X. *Carbohydr. Res.* **2007**, *342*, 870–875.
24. Barker, S. A.; Bourne, E. J.; Stacey, M.; Whiffen, D. H. *J. Chem. Soc.* **1954**, 171–176.