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# Structural elucidation of polysaccharide containing 3-*O*-methyl galactose from fruiting bodies of *Pleurotus citrinopileatus*

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## ARTICLE INFO

## ABSTRACT

A water-soluble polysaccharide containing 3-*O*-methyl galactose (PCP60W) was isolated from fruiting bodies of *Pleurotus citrinopileatus* and purified by anion-exchange and gel column chromatography. This polysaccharide has an average molecular weight of $2.74 \times 10^4$ Da and its structure was elucidated using monosaccharide composition and methylation analysis combined with one- and two-dimensional (COSY, TOCSY, NOESY, HMQC and HMBC) NMR spectroscopy. PCP60W was shown to be a linear partially 3-*O*-methylated α-galactopyranan comprised of 6-linked galactose, 6-linked 3-*O*-methyl galactose and 4-linked glucose in a ratio of 3.0:1.0:0.6. This work provides additional evidence for the view that 3-*O*-methyl galactose is common to the genus *Pleurotus*.

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## 1. Introduction

Mushrooms have been valued as edible and medical resources for thousands of years and, more recently, as important sources of bioactive molecules such as antitumor substances. Because of their wide range of biological functions, significant efforts have been applied to obtaining polysaccharides from mushrooms for structure and activity elucidation [1,2]. *Pleurotus citrinopileatus,* also well known as the "golden oyster mushroom", belongs to the genus of *Pleurotus* and is widely consumed in East Asia, in countries including China and Japan, on account of its taste and perceived health values. Recently, increased attention has also been focused on the physiological functions and active constituents of *P. citrinopileatus*. A variety of functions have been attributed to it, including antioxidation [3], antihypertension [4], immunomodulation [5,6], antihyperglycemic activities [7], antitumor activities [6,8], and HIV-1 reverse transcriptase inhibitory activities [8]. A few reports have also partly demonstrated the structural features of polysaccharides isolated from *P. citrinopileatus*, including $(1 \rightarrow 3)$-β-D-glucan and $(1 \rightarrow 6)$-β-D-glucosyl branched $(1 \rightarrow 3)$-β-D-glucan [9], and a branched $(1 \rightarrow 6)$-β-D-glucan (PCP-W1) [10]. However, except for these glucans, there is little available literature on the structural features of other polysaccharides in *P. citrinopileatus*. Moreover, the polysaccharide with a 3-*O*-methyl galactose has not been reported for *P. citrinopileatus*, although it is suggested to be characteristic of the genus *Pleurotus* [11].

In this study, we isolate a water-soluble polysaccharide (PCP60W) from the fruiting bodies of *P. citrinopileatus* that is primarily comprised of galactose and 3-*O*-methyl galactose. The structure features of this polysaccharide, determining its biological functions, are also elucidated by chemical analysis and NMR spectroscopy.

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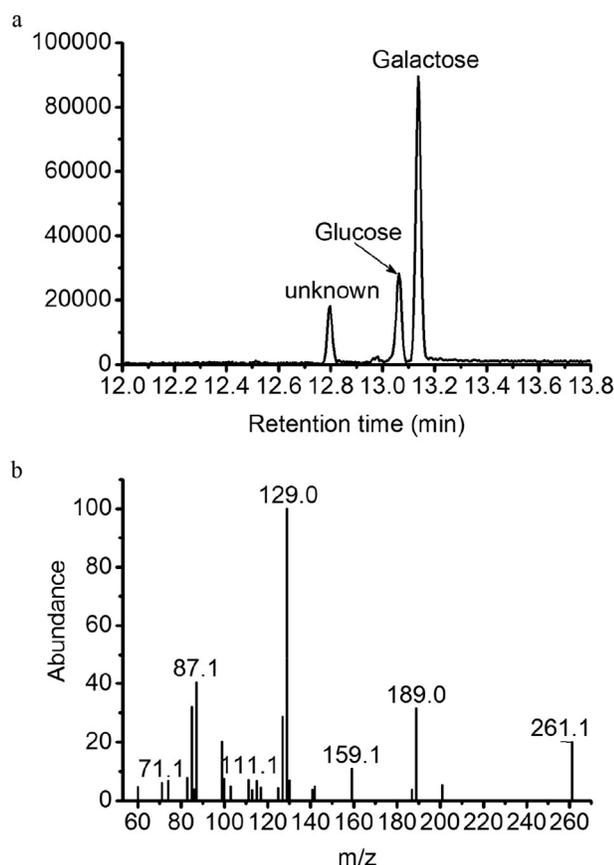
Fig. 1. (a), Total ion chromatogram by GC-MS regarding to monosaccharide blocks in PCP60W from *P. citrinopileatus*; (b), Mass fragments corresponding to the unknown peak at 12.80 min in (a), which were indicatives of 3- or 4-O-methyl-hexose acetate derivative.

## 2. Results and discussion

### 2.1. Isolation and component analysis of PCP60W

Crude polysaccharide was isolated from the fruiting bodies of *P. citrinopileatus* (500 g) by hot water extraction followed by fractional precipitation with alcohol (30 and 60 vol%). These processes produced 0.67 g of 30 vol% fraction and 3.91 g of 60 vol% fraction. The water-soluble polysaccharide PCP60W (0.29 g) was obtained from 3.50 g of 60 vol% fraction by anion exchange and gel chromatography. PCP60W had a total sugar content of 97% and trace of protein of 0.5%. A single symmetrical peak in high-performance liquid chromatography elution profile of PCP60W suggests it is a homogeneous polysaccharide having an average molecular weight of $2.74 \times 10^4$ Da, as determined from a calibration curve of dextran standards.

Sugar analysis of PCP60W as alditol acetates by GC-MS revealed the presence of glucose, galactose and an unknown sugar (Fig. 1a). Based on the major mass fragments of 59.9 87.1, 99.0, 129.0, 189.0, 201.0 and 261.1 for its derivatives (Fig. 1b), this unknown sugar was hypothesized to be 3- or 4-O-methyl-hexose [12], which was further identified as 3-O-methyl-galactose using methylation and NMR analysis (See below).

### 2.2. Structural characterization of PCP60W

Methylation analysis was performed to identify the types of glycosyl linkages present in PCP60W. GC−MS analysis of the resulting partially methylated alditol acetates revealed two peaks in an area ratio of 1:6.7, corresponding to 2,3,6-tri-O-methyl-glucose and 2,3,4-tri-O-methyl-galactose, respectively (Table 1). Given that the monosaccharide compositions demonstrated in Fig. 1a, 2,3,4-tri-O-methyl-galactose is derived from both 6-O-substituted Gal*p* and 6-O-substituted 3-O-methyl-Gal*p*. Taken together, we suggest that PCP60W is comprised of 4-linked Glc*p*, 6-linked Gal*p* and 6-linked 3-O-methyl-Gal*p*.

The $^1$H NMR spectrum of PCP60W (Fig. 2a) presented a distinctive signal at δ 4.99 and a very weak signal at δ 5.34 in the anomeric proton region. The non-anomeric protons were all located between δ 3.40 and δ 4.30. In the $^{13}$C NMR spectrum, anomeric carbon signals were observed at δ 98.48, and nine other carbon signals were observed at δ 79.45, 70.11, 70.00, 69.42, 68.84, 67.84, 67.14, 65.89 and 56.71. The inverted methylene signal in DEPT-135 spectrum revealed the C-6 signals at δ 67.14. Two cross peaks were observed at δ 4.99/3.86 and δ 4.99/3.89 in $^1$H-$^1$H COSY spectrum and the peak at δ 4.99 is believed to correspond to the anomeric proton signals of two residues, which are designated as A and B (Table 2).

The protons of **Residue A** were assigned from H-1/H-2 up to H-4 and H-6/H-5 correlations in COSY and TOCSY spectra. H-5 showed strong NOE correlation to both H-3 and H-4, proving they are within a single sugar ring. The assignments of its corresponding carbon were facilitated by cross peaks in HMQC spectrum (Fig. 2b). Transfer of magnetization of H-1 terminating at H-4 is a signature of *galacto* configuration [13]. Anomeric proton appeared as a singlet ($^3J_{1,2}$<3 Hz) indicates an α-configuration [14]. The downfield shift of C-6 signal suggests the glycosylation of residue A at O-6 [15], confirmed by HMBC correlation H-1/C-6 at δ 4.99/67.14. Thus, residue A was ascribed to →6)-α-D-Gal*p*-(1 → .

For **Residue B**, the majorities of its protons and carbons were assigned following the same procedures as used for residue A. Despite the lack of a H-3/C-3 correlation peak in HMQC spectrum, C-3 was assigned to δ 79.45 from the long-range correlation resonances H-1/C-3 (δ 4.99/79.45) and H-4/C-3 (δ 4.29/79.45) in HMBC spectrum. The $δ_H$ 3.46 and $δ_C$ 56.71 signals were an indication of the presence of O-CH$_3$, while the peak at δ 3.46/79.45 in HMBC spectrum demonstrated the presence of O-CH$_3$ group on residue B at C-3, which also contributed to the downfield shift of the C-3 signal. Thus, residue B was identified as →6)-α-3-O-Me-D-Gal*p*-(1→,

**Table 1**
GC-MS results of partial methylated alditol acetates derivatives of PCP60W.

| RT (min) | PMAAs[a] | Major mass fragments (m/z) | Area ratio[b] | Substituted sugar unit | Molar ratio[d] |
|---|---|---|---|---|---|
| 10.791 | 2,3,6-Me$_3$-Glc*p* | 59,71,87,101,117,129,143,161,173,189,233 | 1.0 | 4-O-substituted Glc*p* | 0.6 |
| 11.308 | 2,3,4-Me$_3$-Gal*p* [c] | 43,87,99,101,117,129,161,173,189,233 | 6.7 | 6-O-substituted Gal*p* | 3.0 |
| | | | | 6-O-substituted 3-O-Me-Gal*p* | 1.0 |

[a] 2,3,6-Me$_3$-Glc*p* corresponds to 2,3,6-tri-O-methyl-1,4,5-tri-O- acetylglucitol.
[b] Calculated from the integration areas of peaks in total ion chromatogram.
[c] Corresponded to two residues, 6-O-substituted Gal*p* and 6-O-substituted 3-O-Me-Gal*p*, in this work.
[d] Estimated from their integration areas ratios of protons in $^1$H NMR spectrum (as shown in Fig. 2a).

which was also confirmed by the reported chemical shift data for this unit [16]. The NMR assignment of residue B, 3-*O*-methyl-galactose, confirmed the results of monosaccharide composition and methylation analysis.

In addition to the signal at δ 4.99 for residue A and B, the other anomeric proton signal at δ 5.34 might be attributable to →4)-α-D-Glc*p*-(1→ based on the results of methylation analysis. However, this assignment could not be corroborated owing to absence of

relevant correlation resonances in two-dimensional spectra.

Because of the good separation of H-4 of residue A and B from other signals in ¹H NMR spectrum (Fig. 2a), the integration area ratio between these was useful for determining the relative ratio of residue A and B. From areas of signals at δ_H 5.34, 4.29 and 4.04, →4)-α-D-Glc*p*-(1→ and residue A and B were determined to be in a ratio of 0.6:1.0:3.0 (Table 1). The ratio of →4)-α-D-Glc*p*-(1→ to the sum of residue A and residue B is 0.6:4.0, which is in good
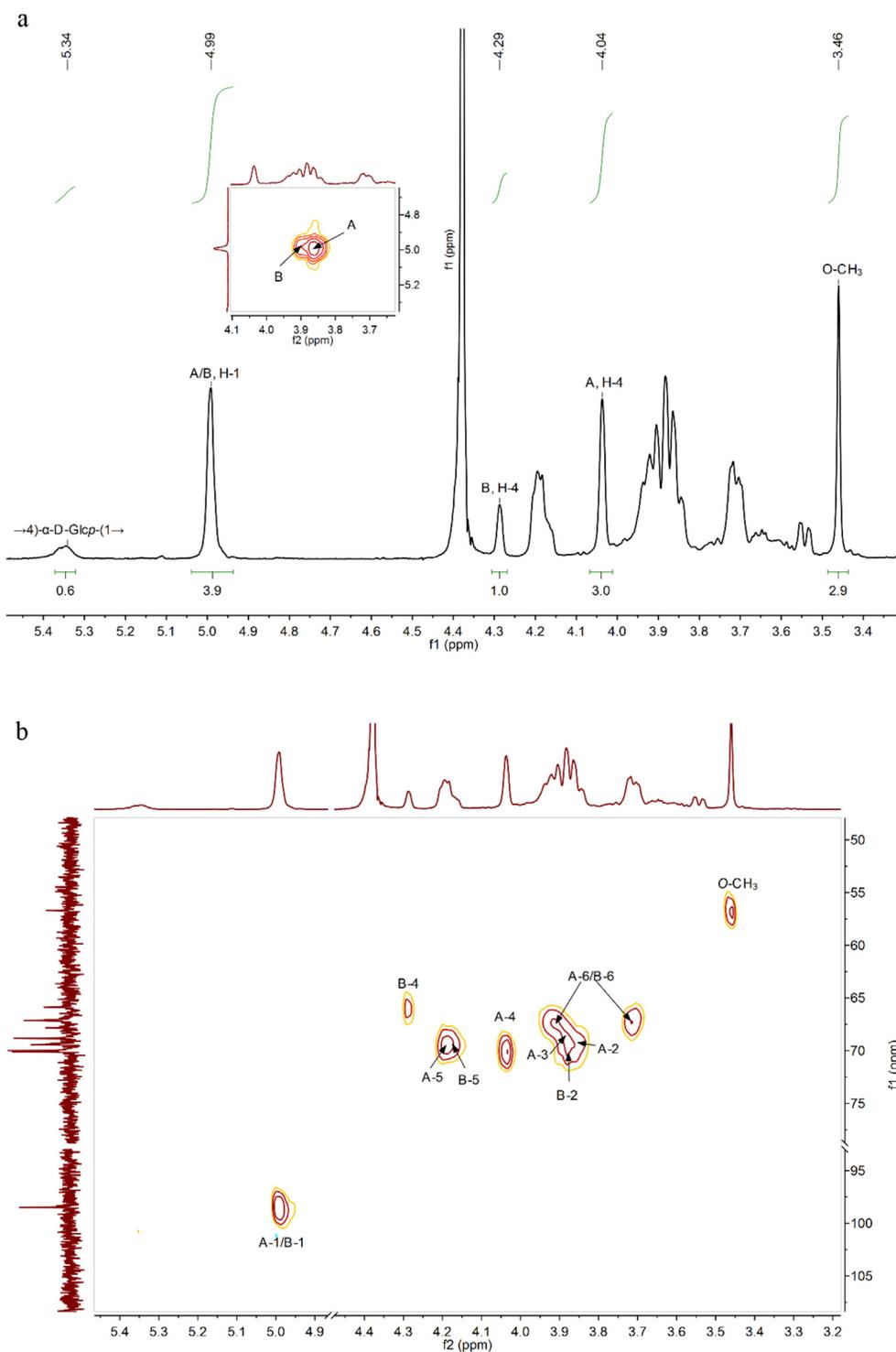


**Fig. 2.** NMR spectra of PCP60W from *P. citrinopileatus* in D₂O at 60 °C. (a), ¹H NMR (500 MHz) spectrum, insertion is a partial ¹H–¹H COSY spectrum; (b), ¹H–¹³C HMQC spectrum. Arabic numerals refer to the carbons in the sugar residues denoted by capital letters as shown in Table 2. Integration area ratios are given below the ¹H NMR spectrum.

**Table 2**
$^1$H and $^{13}$C NMR chemical shifts [expressed as δ (ppm)] of PCP60W.

| Residues | | Protons or carbons | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 3 | 4 | 5 | 6 | O-Me |
| →6)-α-D-Gal*p* (**A**) | H | 4.99 | 3.86 | 3.88 | 4.04 | 4.19 | 3.71[a], 3.92[b] | |
| | C | 98.48 | 68.84 | 70.00 | 70.11 | 69.42 | 67.14 | |
| →6)-α-3-O-Me-D-Gal*p* (**B**) | H | 4.99 | 3.89 | 3.54 | 4.29 | 4.17 | 3.72[a], 3.93[b] | 3.46 |
| | C | 98.48 | 67.84 | 79.45 | 65.89 | 69.42 | 67.14 | 56.71 |

[a] H-6a.
[b] H-6b.

agreement with the result of methylation analysis (1:6.7).

The glycosidic linkages between residues were identified by NOESY and HMBC spectra. An α-(1 → 6)-linkage was identified from the strong correlations δ 4.99/3.72 (H-1/H-6a) and δ 4.99/3.93 (H-1/H-6b), which was also confirmed by the cross peaks at δ 4.99/67.14 (H-1/C-6) and δ 3.72/98.48 (H-6/C-1) in HMBC spectrum. Taken together, we conclude that PCP60W is a linear partially 3-O-methylated (1 → 6)-linked α-galactopyranans in which O-methyl substitution occurs in about a quarter of the galactosyl units and that a small amount of →4)-α-D-Glc*p*-(1 → is occasionally inserted. Polysaccharides of very similar structure to PCP60W, but containing no 4-linked α-D-Glc*p*, have been previously isolated from *Pleurotus eryngii* and *Pleurotus ostreatoroseus* [17]. To the best of our knowledge, this is the first time that a galactopyranan, but not a glucan, has been isolated from *P. citrinopileatus*. Moreover, PCP60W is also the first polysaccharide containing 3-O-methyl-galactose reported from *P. citrinopileatus*, even though such polysaccharides have been suggested to be characteristic of the genus *Pleurotus* [11]. Our findings provide additional support for the opinion that some common characteristics are shared in the genus *Pleurotus*.

## 3. Experimental

### 3.1. Materials and chemicals

DEAE Sepharose Fast Flow and High-Resolution Sephacryl S-300 were purchased from GE Healthcare Co., Ltd. trifluoroacetic acid, dextrans, monosaccharide standards (D-glucose, D-galactose, D-mannose, D-xylose, L-rhamnose, L-fucose), NaBH$_4$, CH$_3$I and DMSO were from Sigma Chemicals Co., Ltd. Other reagents were of A.R. grade and made in China.

### 3.2. General analysis

Total sugar content was determined by phenol-sulfuric acid method using glucose as standard [18]. The protein content was determined using an Enhanced BCA Protein Assay Kit (Beyotime, China).

### 3.3. Isolation and purification

After pretreatment by immersion in 80% alcohol over night for three times, the dried fruiting bodies of *P. citrinopileatus* (500 g) were extracted three-times (2 h each time) with boiling water. All aqueous extracts were combined and concentrated into one-tenth of the original volume under reduced pressure, followed by fractional precipitation with slow addition of 95% alcohol to a final alcohol concentration of 30 and 60 vol%. The 60 vol% fraction was subjected to purification by binding to a diethylaminoethyl sepharose fast flow (XK 26 mm × 100 cm) with step-wise salt elution. The fraction eluting with water was harvested and further purified by chromatography on Sephacryl S-300 gel (XK 16 mm × 100 cm). The main fraction eluting with water was

collected, concentrated, lyophilized and termed as PCP60W.

### 3.4. Homogeneity and molecular weight

The homogeneity and molecular weight of PCP60W was determined by high performance size-exclusion chromatography (HPSEC). HPSEC was performed with a TSK gel G4000PWxL column on a Waters 2695 system equipped with a Waters 2414 refractive index detector, eluted with distilled water at 1.0 mL/min. A set of T-series dextran standards (Mw: 5 kDa, 12 kDa, 80 kDa, 150 kDa, 270 kDa and 670 kDa) was used to prepare the external calibration curves for estimating of molecular weight of PCP60W.

### 3.5. Monosaccharide compositions analysis

Monosaccharides of PCP60W were determined using gas chromatography-mass spectroscopy (GC–MS) after acetylation. First, PCP60W was hydrolyzed with trifluoroacetic acid (2 M, 4 mL) at 110 °C for 2 h. The resulting products were reduced using NaBH$_4$ (10 mg/mL, 3 mL) for 3 h after removing excess trifluoroacetic acid. Finally, the reduced mixtures were acetylated with acetic anhydride to give the alditol acetylates which was analyzed by GC–MS [19]. GC–MS was performed using an HP–5 capillary column (30 m × 0.32 mm × 0.25 μm). The oven temperature was programed from 120 °C (hold for 1 min) to 240 °C (hold for 6.5 min) at 10 °C/min. Both temperature of inlet and detector were 250 °C. Helium was used as carrier gas. The mass scan range was 40.0–500.0 amu.

### 3.6. Methylation analysis

Methylation analysis was performed according to the method of Needs and Selvendran [20]. PCP60W was completely dissolved in DMSO (0.6 mL) and then methylated after addition of NaOH/DMSO slurry (0.6 mL) and CH$_3$I (0.6 mL). This reaction was terminated with water and the products was extracted with CHCl$_3$ and evaporated to dryness. Complete methylation was confirmed by the disappearance of the OH band (3200–3700 cm$^{-1}$) in the IR spectrum. Following hydrolysis of the permethylated polysaccharide in sequence with formic acid (88%) at 100 °C for 3 h and TFA (2 M) at 100 °C for 6 h, the partially methylated alditol acetylates was obtained by reduction and acetylation and analyzed by GC–MS as described in section 3.5.

### 3.7. NMR analysis

After drying in a vacuum over P$_2$O$_5$ for 72 h, 30 mg of PCP60W was deuterium exchanged three times with D$_2$O and finally dissolved in 0.5 mL of D$_2$O for NMR analysis. NMR spectra were recorded on a Varian INOVA 500 NMR spectrometer (Bruker, Switzerland). $^1$H NMR were carried out at 25 °C and 60 °C, while $^{13}$C NMR was conducted only at 25 °C. 2D NMR spectra were also recorded, including $^1$H-$^1$H COSY, TOCSY, HMQC, NOESY and HMBC.

HOD ($\delta$ 4.78) was used for calibration of ^{1}H chemical shifts and 4,4-dimethyl-4-silapentane-1-sulfonic acid ($\delta$ 0.00) for ^{13}C chemical shifts.

## References

[1] S.S. Ferreira, C.P. Passos, P. Madureira, M. Vilanova, M.A. Coimbra, Carbohydr. Polym. 132 (2015) 378–396.
[2] X. Meng, H. Liang, L. Luo, Carbohydr. Res. 424 (2016) 30–41.
[3] Y.-L. Lee, G.-W. Huang, Z.-C. Liang, J.-L. Mau, LWT-Food Sci. Technol. 40 (2007) 823–833.
[4] S.H. Hu, Z.C. Liang, Y.C. Chia, J.L. Lien, K.S. Chen, M.Y. Lee, J.C. Wang, J. Agric. Food Chem. 54 (2006) 2103–2110.
[5] F. Sheu, P.-J. Chien, H.-K. Wang, H.-H. Chang, Y.-T. Shyu, J. Sci. Food Agric. 87 (2007) 1550–1558.
[6] J.-N. Chen, C.-Y. Ma, P.-F. Tsai, Y.-T. Wang, J.S.-B. Wu, J. Agric. Food Chem. 58 (2010) 12117–12122.
[7] S.-H. Hu, J.-C. Wang, J.-L. Lien, E.-T. Liaw, M.-Y. Lee, Appl. Microbiol. Biotechnol. 70 (2005) 107–113.
[8] Y.R. Li, Q.H. Liu, H.X. Wang, T.B. Ng, BBA Gen. Subj. 1780 (2008) 51–57.
[9] J. Zhang, G. Wang, H. Li, C. Zhuang, T. Mizuno, H. Ito, C. Suzuki, H. Okamoto, J. Li, Biosci. Biotechnol. Biochem. 58 (1994) 1195–1201.
[10] J. Liu, Y. Sun, H. Yu, C. Zhang, L. Yue, X. Yang, L. Wang, J. Liu, Carbohydr. Polym. 87 (2012) 348–352.
[11] F.R. Smiderle, L.M. Olsen, E.R. Carbonero, R. Marcon, C.H. Baggio, C.S. Freitas, A.R.S. Santos, G. Torri, P.A.J. Gorin, M. Iacomini, Phytochemistry 69 (2008) 2731–2736.
[12] H. Stepan, C. Bleckmann, H. Geyer, R. Geyer, E. Staudacher, Carbohydr. Res. 345 (2010) 1504–1507.
[13] K. Gheysen, C. Mihai, K. Conrath, J.C. Martins, Chem. Eur. J. 14 (2008) 8869–8878.
[14] D. Rout, S. Mondal, I. Chakraborty, M. Pramanik, S.S. Islam, Carbohydr. Res. 340 (2005) 2533–2539.
[15] P.K. Agrawal, Phytochemistry 31 (1992) 3307–3330.
[16] A.Q. Zhang, M. Xu, L. Fu, P.L. Sun, Carbohydr. Polym. 92 (2013) 236–240.
[17] E.R. Carbonero, A.H.P. Gracher, M.C.C. Rosa, G. Torri, G.L. Sassaki, P.A.J. Gorin, M. Iacomini, Phytochemistry 69 (2008) 252–257.
[18] M. Dubois, K.A. Gilles, J.K. Hamilton, P. Rebers, F. Smith, Anal. Chem. 28 (1956) 350–356.
[19] P. Albersheim, D.J. Nevins, P.D. English, A. Karr, Carbohydr. Res. 5 (1967) 340–345.
[20] P. Needs, R. Selvendran, Carbohydr. Res. 254 (1994) 229–244.