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Partial Characterization of Glycans from *Daedalea quercina* (L) Fr.

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Heteroglycans were extracted and isolated from the fruiting bodies of wild mushroom *Daedalea quercina* (L) Fr. These were obtained from sequential extraction with hot water, 1% Ammonium oxalate then 5% Sodium hydroxide solution followed by ethanol or acidic precipitation affording crude extracts which were further purified by dialysis membrane (MWCO 12 400). Separation by charge and size were done using DEAE-cellulose column and Sephadex G-100 or Sepharose CL-4B. This resulted to water soluble neutral (W1P-1A) and acidic (W1P-2A) glycan isolates, ammonium oxalate soluble (A1P-1) and two alkali soluble (N1P-1 and N1P-2) glycan isolates. Characterization of the hydrolyzed isolates using HPAEC-PAD and MALDI TOF MS showed glucan-rich heteroglycans (W1P-1A and W1P-2A); glucan and mannan rich heteroglycan, (N1P-1) and galactan-rich heteroglycan (A1P-1). The latter being the first reported galactan-rich glycan found in ammonium oxalate mushroom extract. The hydrolysates had approximately 4-5 degree of polymerization with molecular weights ranging from 689.680 to 851.788 Da. The hydrolysate of isolate N1P-2 is a glucan-rich heteroglycan which also contain N-acetyl glucosamine units. It is composed of polymers with 4-5 hexose units as well as N-acetyl glucosamine containing polymers of 6-8 hexose units with molecular weight range of 689.799 to 851.915 Da and 1055.718 to 1379.584 Da, respectively.

Keywords: *Daedalea quercina; glycans; HPAEC-PAC; MALDI-TOF MS; monosaccharide analysis*

INTRODUCTION

Mushrooms are studied mainly for their health benefits. These were attributed to several bioactive compounds such as polysaccharides, polysaccharide-peptides, nucleosides, triterpenoids and other metabolites which have been identified in a number of species (Wasser, 2002). Among these compounds, polysaccharides are considered the most active compound (Borchers, 1999) as shown by a number of studies on the activity of polysaccharide components of different mushroom species. This includes antitumor mushroom polysaccharides from shiitake (*Lentinus edodes*), reishi (*Ganoderma lucidum*), turkey tail (*Trametes versicolor*), split gill (*Schizophyllum commune*), mulberry yellow polypore (*Phellinus linteus)* and chaga or cinder conk (*Inonotus obliquus*) which are now sold as drugs in countries such as Japan, Korea, Russia and China. Likewise, polysaccharides

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with hypoglycemic effect have also been investigated from mushroom species such as *Agrocybe cylindracea*, *Tremella aurantia* and *Ganoderma lucidum* (Kiho *et al*., 1994, 1995; Xiao *et al*., 2012). It has also been found that hypocholesterolymic activity is associated with polysaccharides from *Auricularia auricular-judae* and *Tramella fuciformis* (Cheung, 1996). Other mushroom polysaccharides also exhibit immunomodulatory and antiviral effects.

The activities of these polysaccharides are closely related to their physico-chemical properties which include molecular size, monosaccharide composition, and configuration/position of glycosidic linkages as well as sequence of the monosaccharides.

Even as many of the mushroom species have already been reported there are still a number of species to be investigated to relate the structure with activity. Hence, we report the isolation and partial characterization of polysaccharides from the wild mushroom *Daedalea quercina* (L) Fr.

Daedaelea quercina (L) Fr. is a wood-rotting fungus of the order Polyporales that grows on decaying oaks and other hardwoods. *D. quercina* have been reported to contain exopolysaccharides of α-linked mannan and α- and β-linked glucan from mycelia culture (Manzoni and Rollini, 2001). It also contains ergosterol (ergosta-7, 22-dien-3β-ol), ergosterol peroxide and 3α-carboxyacetoxy-quercinic acid (Turner *et al*., 1983; Adam *et al*., 1967), 16-O-acetylpolyporenic acid C, 16α-acetoxy-24-methylene-3-oxolanost-8-en-21-oic acid, (+)-24-methylene-3,23-dioxolanost-8-en-26-oic acid, (+)-3b,12b-dihydroxy-24-methyl-23-oxolanost-8-en-26-oic acid and 12b,23-epoxy-3a,23-dihydroxy-24-methyllanost-8-en-26-oic acid (Konig *et al*., 2000), and quercinol, the latter being found to have anti-inflammatory activity (Gebhardt *et al*., 2007).

EXPERIMENTAL

Sampling and Sample Preparation. *Daedalea quercina* fruiting bodies (2 Kg) were collected from *kaingin* (slash and burn farming) sites in Quezon Palawan, Philippines. Sample specimen was lodged in the national museum for taxonomic identification. The air-dried fruiting bodies were cut into thin small pieces prior to sequential extraction.

Extraction. *Water Extraction.* The mushroom samples were soaked in methanol for 24 hours to remove low molecular weight compounds. The mixture was filtered and the methanol extract was set aside. The marc was further extracted with boiling distilled water for 6 hours. The aqueous extracts were combined and concentrated *in vacuo*. The polysaccharides were precipitated from the concentrated extract by addition of excess ethanol (3:1, ethanol:extract) (Carbonero *et al*., 2006) at 4°C for 24 h. The precipitate was washed three times with 70% ethanol and lyophilized.

Ammonium Oxalate Extraction. The marc from water extraction was re-extracted with 1% ammonium oxalate at 100^0C for 6hrs, three times. The extracts were combined and concentrated and precipitated with EtOH (3:1).

Alkali Extraction. The marc from ammonium oxalate extraction was re-extracted with 5% sodium hydroxide three times at 80^0C for 6hrs. The alkali-solubles were chilled to 4^0C and acidified with glacial acetic acid to pH 5 (Buckeridge *et al*., 1999). The precipitate was collected and freeze-dried.

Purification of D. quercina Extracts. *Dialysis.* The lyophilized extracts were re-dissolved with distilled water, and dialyzed through a cellulose membrane (MWCO 12.4 kDa) (Sigma) against distilled water for 3 days. The nondialyzable component was reconcentrated and then fractionally precipitated using ethanol. The resulting precipitate was collected by centrifugation, washed three times with acetone, dried to a constant weight and stored at 4^0C (Xiao, *et al*., 2012).

Ion exchange Chromatography. The supernatant from section 3.1 was applied to a DEAE-cellulose column (3cm x 30 cm) (Sigma) equilibrated with distilled water. Stepwise elution with distilled water afforded neutral polysaccharides. This was followed by a linear

gradient of 0-2 M NaCl to elute acidic polysaccharide. Each fraction was monitored with phenol-sulfuric acid reagent and visualized by UV absorption at 490 nm. The neutral and acidic polysaccharides were pooled separately and concentrated *in vacuo* (Kim *et al*., 2003).

Size Exclusion Chromatography. The concentrated pooled polysaccharides fractions were re-fractionated according to its molecular size on a Sephadex G-100 (Sigma-Aldrich) column for water soluble polysaccharides and Sepharose CL-4B (Sigma-Aldrich) column for water insoluble polysaccharides. Elution was done with 0.1M NaCl (Ke *et al*., 2003) for water soluble polysaccharides and 0.3 M NaOH for alkali soluble polysaccharides. Each fraction was monitored with phenol-sulfuric acid reagent and visualized by UV absorption at 490 nm.

Characterization. Analysis of the monosaccharide composition and masses of hydrolyzed fragments of each sample was done at Academia Sinica, Taiwan using High Performance Anion Exchange Chromatography with Pulsed Amperometric Detection (HPAEC-PAD) and Matrix Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS), respectively.

The samples were hydrolyzed with 4M TFA at 110^0C for 4 h and concentrated *in vacuo*. The concentrated sample was added with 1 mL distilled water, diluted 20 times and analyzed for monosaccharide composition using HPAEC-PAD (Dionex BioLC system).

Separate samples were hydrolyzed to low molecular weight oligosaccharides (2M TFA 50 °C, 2h) and then purified by gel-filtration chromatography on a Sephadex G-15 (Sigma-Aldrich) column using double distilled water as eluent. The oligosaccharides were mixed with 10 mM of 2, 5-DHB (1:1) then ran on MALDI-TOF MS (Ultraflex™ TOF/TOF mass spectrometer) (Hung., *et al*., 2008).

RESULTS

Polysaccharide Extraction. The sequential extraction of *D. quercina* fruiting bodies and subsequent precipitation afforded W1 (H_2O extract, 32.25 g), A1 ($(NH_4)_2C_2O_4$ extract, 11.61 g), N1 (NaOH extract, 43.09 g) and of N2 (alkali insoluble extract, 311. 8 g) which represent the different types of glycan components of *D. quercina* cell walls based on extraction technique.

The fungi cell wall is generally comprised of the water soluble outer layer (proteins, glycoproteins and polysaccharides), alkali-soluble amorphous layer (glucan) and the alkali-insoluble inner layer (glucan-chitin complex) (Latgé, 2007, Klis, 1994; Ruiz-Herrera, 1992). Aside from water, the use of chelating agents such as ammonium oxalate also extract pectic polysaccharides (Selvendran and Rayden, 1990).

Some of these extracts were tested for hypoglycemic and anticancer activities and will be discussed in a separate paper.

Purification. *Dialysis of W1, A1 and N1*. Precipitates obtained from dialyzed extracts, W1P (3.2982 g), A1P (2.8183 g) and N1P (10.6410 g) were obtained. This corresponds to 10.22 %, 24.28 % and 24.69% extraction yields. This suggests that majority of the crude extracts are polar low molecular weight metabolites.

Purification Of Precipitates (W1P). Fractionation of the sample (W1P) by ion exchange chromatography (IEC) on a DEAE-cellulose column separated the acidic and neutral polysaccharides. Three distinct peaks were observed from the elution curve representing three pooled fractions W1P-1, W1P-2 and W1P-3 (Figure. 1). Fraction W1P-1 which was eluted with water contains neutral glycans. The other two fractions W1P-2 and W1P-3 were eluted using increasing concentration of NaCl to elute acidic glycans that remained bound to the resin. The high absorbance in the elution curve of fraction W1P-2 suggests that it contains the highest concentration of glycans among the three fractions.

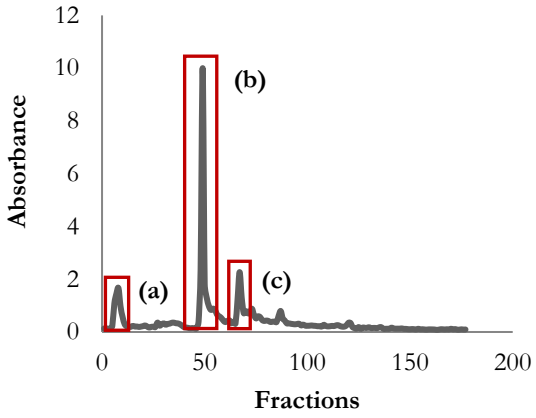

Figure 1. Elution Curve of W1P on DEAE-Cellulose Column; (a) W1P-1; (b) W1P-2; (c) W1P-3.

Only one pure fraction (W1P-1A, 0.0063 g) was obtained from the size exclusion chromatography (SEC) of the neutral glycan fraction W1P-1 (Figure 2).

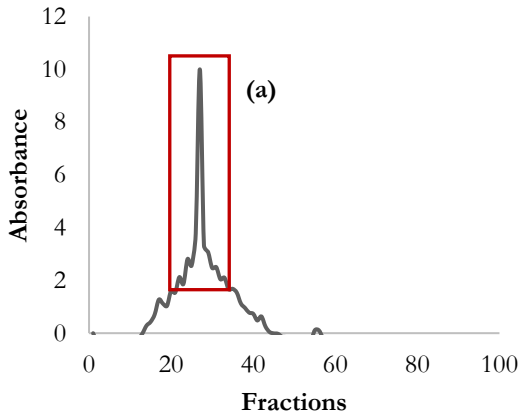

Figure 2. Elution Curve of W1P-1 on Sephadex G-100 Column (W1P-1A (a)).

Fractionation of the acidic fraction W1P-2 by SEC afforded one pure fraction (W1P-2A, 0.0566 g) (Figure 3). The glycans eluted in the void volume must have a molecular weight exceeding the molecular weight range of sephadex G-100 (1KD-100 KD). The purity of this fraction will be determined from its LC/MS/MS data.

No pure fraction was obtained from the fractionation of the acidic fraction W1P-3 by SEC.

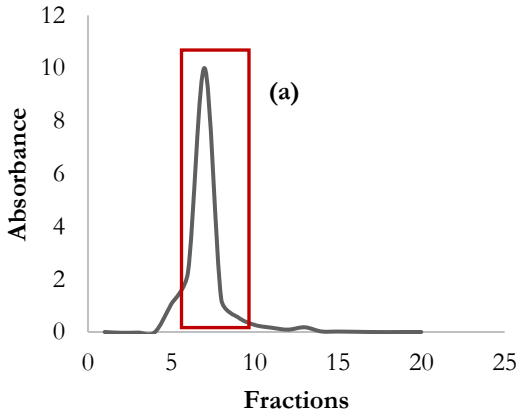

Figure 3. Elution Curve of W1P-2 of Sephadex G-100 Column (W1P-2A (a)).

Purification of $(NH_4)_2C_2O_4$ and NaOH Dialyzed and Precipitated Extracts. The semi-purified $(NH_4)_2C_2O_4$ extract A1P was rechromatographed by size exclusion chromatography on sepharose CL-4B using 0.3 M NaOH as eluent. This resulted in the isolation of a relatively pure fraction A1P-1 (0.7348 g) Figure 4.

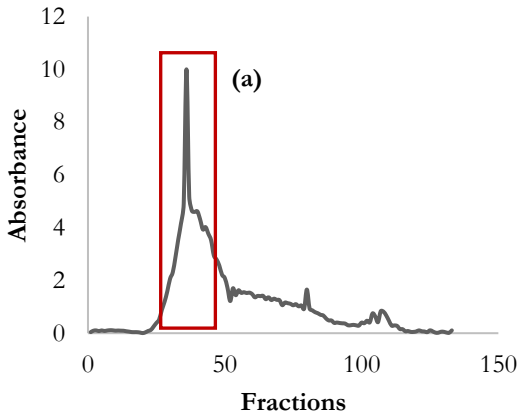

Figure 4. Elution Curve of A1P on Sepharose CL-4B Column (A1P-1 (a)).

N1P-1 (0.1161 g) and N1P-2 (0.032 g) were obtained from N1P by SEC (Figure. 5). N1P-1 was eluted in the void volume suggesting that its molecular weight exceeded the molecular weight range of Sepharose CL-4B (30 KD-5000 KD). The purity of this fraction will be determined from its LC/MS/MS data. In contrast, N1P-2 which was eluted in the included volume must be pure.

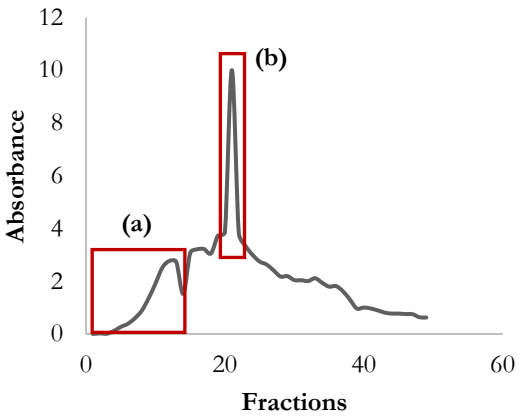

Figure 5. Elution Curve of N1P on Sepharose CL-4B Column (N1P-1 (a); N1P-2 (b)).

Characterization. *Monosaccharide Composition by HPAEC-PAD.* Hydrolysates from water and NaOH extracts (W1P-1A, W1P-2A, N1P-1 and N1P-2) are glucan-rich heteroglycans. Each fraction has glucose as their major component, with W1P-1A having the highest glucose percentage (59.97%). Fraction N1P-1 has the highest mannose content (37.14) compared to all the other fractions. In contrast, the fraction from $(NH_4)_2C_2O_4$ extract, A1P-1, differ from the other fractions; glucose is a minor component (16.89%) whereas galactose is the major component (55.40%).

To summarize, all fractions contain mannose, xylose, arabinose, rhamnose and fucose as minor constituents in varied percentages. No uronic acid-type or sulfated monosaccharides characteristic of acidic fractions were identified. (Figure 6).

These glycan fractions represent glycan fractions from outer to inner cell wall composition. The glycan fractions from water extract (W1P-1A and W1P-2A) derived from the outer layer is consistent with previous reports where glucose is the major component. The acidic fraction W1P-2A has no arabinose and rhamnose as compared to the neutral fraction W1P-1A.

A1P-1 is a mixed heteroglycan with galactose as the major component. Few mushrooms have been reported as galactan producers. While *Pleurotus eryngii* (Carbonero *et al.*, 2008), *Pleurotus ostreatoroseus* (Rosado *et al.* 2002, 2003) and *Inonotus levis* (Vinogradov and Wasser, 2005) have galactose as the major sugar component, their galactan-rich metabolites were derived from water. This is the first report of a galactan-rich fraction A1P-1 derived from ammonium oxalate mushroom extract.

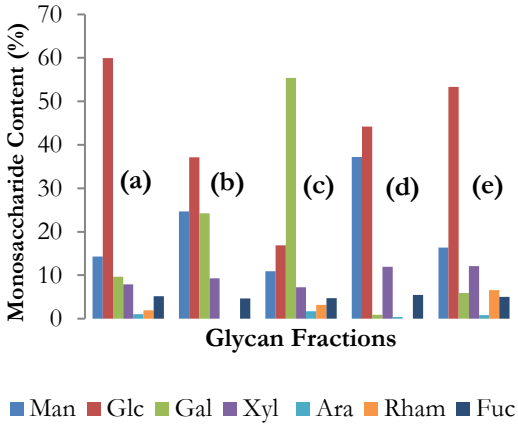

Figure 6. Monosaccharide Composition of Daedalea quercina Fractions: W1P-1A (a), W1P-2A (b); A1P-1 (c), N1P-1 (d), N1P-2 (e).

The inner cell wall glycan fractions from NaOH extract, N1P-1 and N1P-2, contain glucose as the major component. Fraction N1P-2 has higher glucose content (53.30%) than N1P-1 (44.18%). N1P-1 shows higher percentage of mannose but lower amount of glucose and absence of rhamnose as compared to N1P-2. This means that the alkali fractions are mostly composed of glucans and mannans. Alkali-soluble mushroom glycans in general contain different amounts and combination of glucose, galactose, arabinose, and glucuronic acid with glucose or glucuronic acid as the most abundant monosaccharide component (Wu *et al.*, 2013; Amaral *et al.*, 2008; Chen *et al.*, 2014).

MALDI-TOF MS of the Hydrolysis Products of the Isolates from D. quercina. The Mass spectra (MS) of the acid hydrolyzed isolates from *D. quercina* (W1P-1A, W1P-2A, A1P-1, N1P-1 and N1P-2) were obtained using Matrix Assisted Laser Desorption Ionization - Time of flight analyzer (MALDI-TOF) MS. Each isolate was observed as sodiated ions.

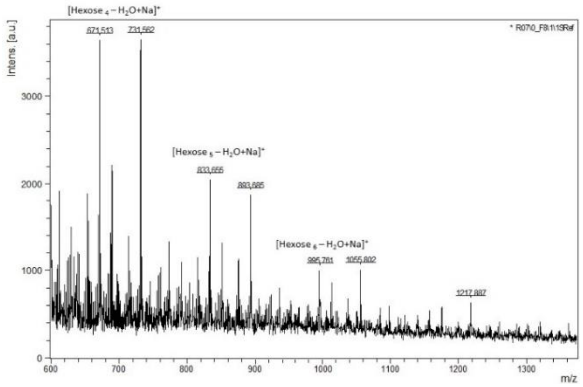

Figure 7. MALDI TOF MS of W1P-2A on 2, 5-DHB as Matrix.

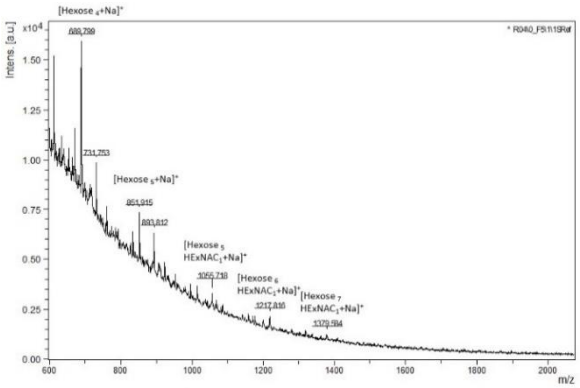

Figure 8. MALDI TOF MS of N1P-2 on 2, 5-DHB Matrix.

The MALDI-TOF mass spectrum of the acidic glycan, W1P-2A (Figure 7) shows sodium adducts of repeating units of hexose. Loss of m/z 162 were noted. This is consistent with glycans with repeating units of β-(1→3) linkage specifically of glucan (Hung 2008; Yang 2012; Chan and Tang 2003). The high mass resolution of some peaks were calculated as (162.1424) n + 22.998977 Da or (162.1424) n + 22.998977 + 18.01524 (mass of reducing end residue) Da where n is the number of hexose units. The molar mass of the hydrolyzed products ranged from 689.6 to 1013.9 Da. It can be concluded that W1P-2A hydrolysis products consisted of polymers of 4-6 hexose units. The actual molar mass of the intact polysaccharide is undetermined. This is about the same information that was derived from all the other fractions except fraction N1P-2 derived from the NaOH extract.

Fraction N1P-2 is an exception to the types of sugar composition expected from an alkali extract. The mass spectrum of the second alkali soluble fraction N1P-2 (Figure 8) shows the presence of hexose polymers of 4-5 units as well as N-acetyl glucosamine containing fragments with 6-8 hexose units. For glucosamine-containing fragments the calculation of the mass is 162.1424n + 203.1950 (HexNAC) + 22.998977 + 18.01524 (mass of reducing end residue) Da. The molar mass of the oligosaccharides range from 689.799 to 851.915 Da for the hexose polymer and 1055.718 to 1379.584 Da for the N-acetyl glucosamine containing hexose polymer. The presence of N-acetyl derivative (NAC) suggests the presence of glycan-chitin complex. The glycan-chitin complex is expected from acidic extraction with some exceptions (Zhang *et al*., 2003, Kim *et al*., 2006).

No MALDI-TOF MS data was obtained for $(NH_4)_2C_2O_4$ isolate A1P-1.

CONCLUSIONS

Five relatively pure fractions were obtained in this study: two from H_2O extract (W1P-1A, W1P-2A), one from $(NH_4)_2C_2O_4$ extract (A1P-1), and two from NaOH extract (N1P-1, N1P-2). Monosaccharide and MALDI-TOF MS analysis of the hydrolysates of each fraction showed that neutral (W1P-1A) and acidic (W1P-2A) water soluble fractions are glucan-rich heteroglycans (671.5-995.8 Da). Galactan-rich heteroglycan was identified from the ammonium oxalate solubles (A1P-1). Glucan and mannan-rich heteroglycans from alkali solubles N1P-1 (689.680 to 851.788 Da). Another glucan-rich heteroglycans (N1P-2) (689.799- 851.915 Da) from the alkali extract also showed the presence of N-acetyl glucosamine (1055.718 to 1379.584 Da).

The MS data of the five fractions which showed a series of m/z 162.1 Da differences between line spectrum peaks is indicative of the presence of a β-(1 →3) monosaccharide linkages among monosaccharide units (Hung 2008; Yang 2012; Chan and Tang 2003).

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