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Research article

Immunomodulatory activities of polysaccharides isolated from *Amauroderma rugosum* (Blume and T. Nees) Torrend and their structural characterization

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ABSTRACT

Amauroderma rugosum (Blume and T. Nees) Torrend is a traditionally well-known mushroom that is used for the treatment of cancer. In order to evaluate the pharmacological activities of *A. rugosum* polysaccharides, the mushroom powder was subjected to hot water extraction and pure polysaccharides (ARPs) were isolated by gel-filtration method. Three important APRs called ARP-1, ARP-2 and ARP-5 were identified with average molecular weights of 1494, 450, and 7 kDa respectively. Their antioxidant abilities were estimated by examining free radical scavenging potential against 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid radical ($ABTS^{\bullet+}$), 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$), and hydroxyl radical. Immunomodulatory potentials of these ARPs were determined using murine macrophage cells. These polysaccharides exhibited high antioxidant abilities and stimulated mouse macrophages leading to the generation of tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6). Excellent activities were displayed by ARP-1 and APR-2. Gas chromatography and spectroscopic (FT-IR and NMR) methods were employed in order to carry out their structural characterisation. The two high molecular weight ARPs (ARP-1 and ARP-2) displayed β-(1 → 3)-D-glucan backbone structure with branching of β-(1 → 6)-D-glucopyranosyl. These observations suggest high potential of ARPs for immunotherapeutic applications.

1. Introduction

Consumption of mushrooms serves the dual purpose as food and also provides health benefits [1]. The fungi cell wall is made up of the polymer chains of chitin (or cellulose) and β-glucans which are covalently bonded with hydrogen bridges. Glucans are polysaccharides made up of glucose as the only monomer unit. Increasing the temperature improves their water solubility, but the glucans with protein-conjugation are less soluble [2]. Literature reports indicate that the higher the water solubility the greater will be their bioactivity [2]. Mushroom derived β-glucans that are soluble in water show much greater effect on the human immune system [3] and

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display pharmacological activities that can reduce blood lipids [4], act as antioxidant [5], and have anti-viral and many other activities [6]. For instance, the polysaccharides isolated from *Citrus aurantium* and *Porphyra haitanensis* have displayed high immunomodulatory and antioxidant properties [7]. Recent literature has generated significant attention towards the antioxidant potentials of mushrooms and their polysaccharides. For instance, polysaccharides extracted from *Lepista nuda* [8], *Pleurotus djamor* [9], *Pleurotus ostreatus* [10], and *bachu* mushrooms have exhibited high antioxidant activities against reactive oxygen radicals [11].

Amauroderma rugosum belongs to the basidiomycota division of fungi which also includes oyster, shiitake, split gill and himematsutake mushrooms [12]. The bioactivity of β-1,3-D and β-1,6-D glucans present in this class of mushrooms and other *basidiomycetes*, are the most potent [1,9]. Examples of mushroom β-glucans exhibiting immune enhancing and antitumor activity are *lentinan* (β-1,3; 1, 6-glucan structure) isolated from *Lentinula edodes* (shiitake mushroom) [13], PSP (polysaccharide peptide) and PSK (polysaccharide-Kureha) (protein conjugated β-1,3; 1,6-glucan structure) isolated from *Coriolus versicolour* (Yun Zhi) [13], *schizophyllan* (β-1,3; 1,6-glucan structure) isolated from *Schizophyllum commune* [14], *ganoderma* polysaccharides (β-1,3; 1,6-glucan structure) from *Ganoderma lucidum* (lingzhi) [15], *agaricus* polysaccharides (protein bound β-1,6-glucan structure) isolated from *Agaricus blazei* (Himematsutake mushroom) [16], *pleuran* (β-1,3-glucan containing mannose and galactose in the structure) from *Pleurotus ostreatus* (oyster mushroom) [17], β-1,3; 1,6-glucans isolated from *Grifola frondosa* (maitake mushroom) and *coprinus* polysaccharides (β-1, 3-glucan structure) from *Coprinus comatus* (shaggy ink cap) [17]. Antitumor and immune-stimulating polysaccharides from higher *Basidiomycetes* mushrooms contain the monosaccharides galactose, glucose, mannose, xylose, fucose, arabinose, ribose and glucuronic acid [18]. These polysaccharides may act by preventing oncogenesis, enhancing the immune system or by directly inhibiting tumor growth [19]. The glucans with β-(1 → 3), β-(1 → 4) and β-(1 → 6) structure were proven to be useful for the treatment of several diseases by enhancing the immune system and by antitumor mechanisms [18]. Glucans with β-(1 → 3) backbone structure and β-(1 → 6) branching are favourable for antitumor applications and those with large molecular weight are highly active [18].

A. rugosum is a genus of polypore fungi in the Ganodermataceae family [12]. It is a dietary mushroom traditionally used as an anticancer, anti-diuretic, anti-epilepsy and anti-inflammatory agent [12]. A recent investigation of the *A. rugosum* ethanol extracts exhibited high anti-inflammatory abilities [20]. The water extract of *A. rugosum* exhibited huge potential by decreasing oxidative stress, cytotoxicity, apoptosis and mitochondrial dysfunction of PC12 cells [12].

To date, there are no published reports on *A. rugosum* polysaccharides. Initial investigations in the authors' laboratory demonstrated *A. rugosum* polysaccharides possess immunotherapeutic and antitumor potentials [21]. The present study focuses on the isolation of pure polysaccharides from *A. rugosum* and a study of their antioxidative and immunostimulatory activities. To evaluate the structure-activity relationship of ARPs, a detailed spectroscopic (NMR and FT-IR) characterisation was carried out.

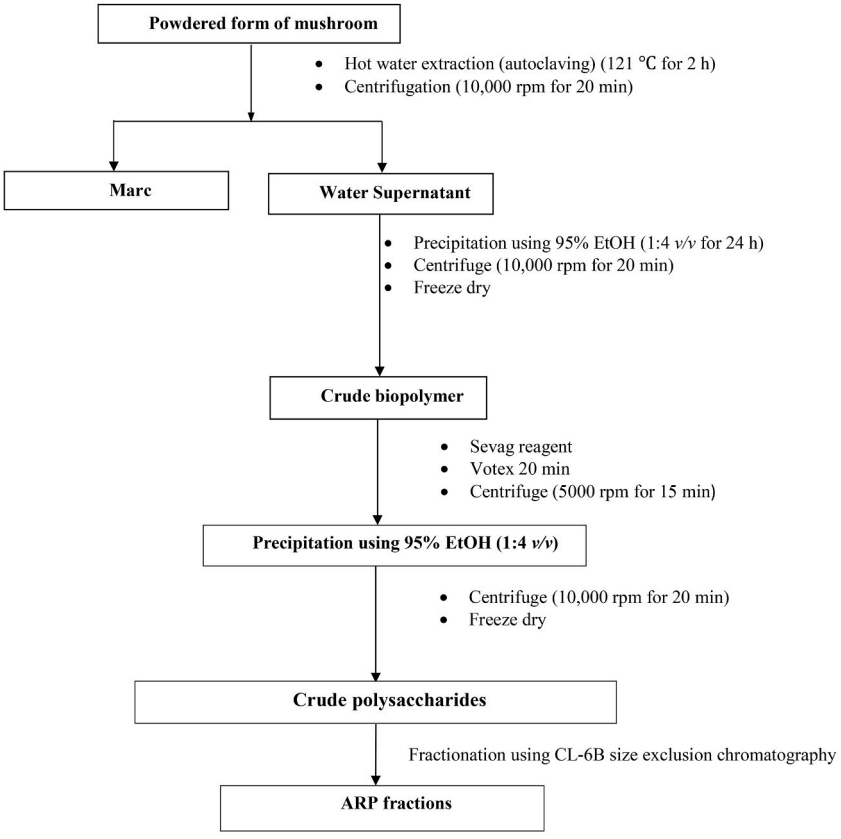

Fig. 1. Flow chart for the extraction of polysaccharides from *A. rugosum*.

2. Materials and methods

2.1. Materials and chemicals

The whole fungus, *Amauroderma rugosum* (called "JiaZhi" in Traditional Chinese Medicine (TCM)), was acquired from Herbal Life Chinese Herbal Medicine shop, Sydney, Australia. A voucher specimen was deposited in the laboratory.

Chemicals supplied by Lomb Scientific Pty Ltd. and Sigma-Aldrich (both based in Australia) were utilized in this investigation. These consist of 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($ABTS^{\bullet+}$) (purity ≥98 %, HPLC grade), 2,2-diphenyl-1-picryl-hydrazyl ($DPPH^{\bullet}$) (purity ≥97 %), 1,10-phenanthroline (purity ≥99 %), H_2O_2 (30 % (w/w) in H_2O, contains stabilizer), dimethyl sulfoxide (DMSO) (ACS reagent, purity ≥99.9 %), ferrozine (purity ≥97 %), ascorbic acid, 95 % ethanol, lipopolysaccharide (LPS). Dulbecco's modified Eagle's medium (DMEM) with gluMax and foetal bovine serum (FBS) were bought from BD Bioscience (USA). ELISA kits were acquired from BD Biosciences (USA). Purchase of RAW 264.7 murine macrophages (cell line) was from Sigma-Aldrich.

2.2. Extraction and purification of polysaccharide fractions

The whole dried mushroom (500 g) of *Amauroderma rugosum* was powdered, 2 L water added and then autoclaved for 2 h at 121 °C using an autoclave (Systec DX-200, Systec GmbH, Germany), followed by cooling to 25 °C before collection and precipitation of the supernatant. The Sevag method was employed to deproteinate the crude polysaccharide sample, and gel filtration method was utilized to purify the samples (Gel type: Sepharose CL-6B; Column size: 2.4 × 99 cm, rate of flow: 0.51 mL/min) [19]. The procedure used for extracting and purifying polysaccharides is outlined in Fig. 1. The purified fractions were gathered and dried using freeze-drying method. Samples were preserved at a temperature of −20 °C until further analysis.

2.3. Measurement of average molecular weight

Sepharose CL-6B gel filtration column was first calibrated using standard dextran samples with a range of average molecular weights (2000 kDa–180 Da). A calibration curve was obtained by using these standards [19,21]. The linear equation (1) resulting from data regression, with an R^2 value of 0.9882, was employed to determine the average molecular weights of *A. rugosum* polysaccharides (ARPs).

$$y = -0.2328x + 1.523 \quad (1)$$

2.4. Chemical composition of ARPs

The amount of sugar in the polysaccharide fractions was measured employing the phenol–sulfuric acid method, utilizing glucose as the standard reference [21,22]. The standard data regression resulted in a linear equation (2) with an R^2 of 0.9964, was used to measure the total sugar content of ARPs.

$$y = 0.0018x + 0.0374 \quad (2)$$

The amount of protein was evaluated through the modified Lowry method, with BSA as standard reference [23,24]. BSA data regression produced a linear equation (3) ($R^2 = 0.9923$) which was used to determine the bound protein.

$$y = 0.0017x + 0.0212 \quad (3)$$

The monosaccharide contents of the ARPs were determined by gas chromatography (GC) [19]. Briefly, the polysaccharide samples (2 mg) were taken and 1 mL (2 M) trifluoraccetic acid (TFA) was added and vigorously mixed using a vortex mixer. The samples were hermetically sealed and subjected to a temperature of 121 °C for 1.5 h, then cooled to ambient temperature. The TFA was evaporated at ambient temperature, and 1 mL of methanol was added. Each combination was subjected to N_2 gas bubbling to eliminate methanol, and this process was repeated two times. Then the samples were subjected to hydrolysis followed by acetylation. The mono-sugars were then quantified using gas chromatography (GC) with flame ionisation detection (FID) [19]. The GC instrument used in this research was a Hewlett-Packard 7890B. The resulting chromatograms were compared with those of reference standards for glucose, arabinose, xylose, ribose, rhamnose, galactose, fructose, and fucose.

2.5. FTIR analysis

Three of the purified ARPs were analysed using FTIR spectroscopic technique (TENSOR II FTIR Spectrometer, BRUKER) at 25 °C. The spectra were scanned in the range of 4000 to 450 cm^{-1} with a resolution of 4 cm^{-1} [21].

2.6. NMR spectroscopic study

Proton, carbon and 2D-NMR spectral data were acquired on a Bruker Avance 400 MHz NMR spectrometer [21]. All spectra were

recorded in D_2O as solvent. 1D-NMR data was collected using 64 k data points and then expanded to 128 k points by zero-filling. The data was then Fourier transformed to obtain frequency domain spectra. The 2D-NMR spectra were obtained using a data size of 256 × 2024 points. Each Free Induction Decay (FID) in the F1 dimension was averaged over 48 transients. The FIDs were expanded (by zero-filling) to 1024 points in the F1 dimension and 4096 points in the F2 dimension before subjecting the data to a two-dimensional Fourier transformation. ^{1}H and ^{13}C chemical shifts were referenced using 0.15 % trimethylsilylpropanoic acid (TSP) as an internal reference added to D_2O. All NMR studies were conducted at a temperature of 25 °C. The methodology used for the structural analysis of polysaccharides closely resembles that described in a previous paper [21].

2.7. Bioactivity assays

2.7.1. Antioxidant activity

The $DPPH^{\bullet}$ radical scavenging capacity of ARPs was evaluated by the Blois approach, using ascorbic acid as positive control and pure water as blank. The protocol for this test is consistent with the methods described in earlier publications [21,25]. Absorbance was measured at 492 nm (Multiskan 141 EX, Thermo Electron, United States). Antioxidant activities of ARPs were obtained by using equation (4):

$$DPPH^{\bullet}\ \text{radical scavenging activity}\ (\%) = \frac{OD_{\ neg\_control} - OD_{\ sample}}{OD_{\ neg\_control}} X100\% \quad (4)$$

where, $OD_{neg\_control}$ refers to the absorbance of the DPPH solution without any sample, OD_{sample} represents the absorbance of the test sample (a combination of the DPPH solution and either the test sample or a positive control).

The capacity of polysaccharides to scavenge $ABTS^{\bullet+}$ radical was assessed using the method described in the literature [21]. Ascorbic acid served as the positive control, while a pH 7.4 PBS buffer was employed as the blank. The measurement of absorbance was conducted at a wavelength of 734 nm.

The radical scavenging activities of ARPs were assessed using equation (5):

$$ABTS^{\bullet+}\ \text{scavenging activity}\ (\%) = \frac{OD_{\ neg\_control} - OD_{\ sample}}{OD_{\ neg\_control}} X100\% \quad (5)$$

where, $OD_{neg\_control}$ refers to the absorbance of the $ABTS^{\bullet+}$ radical solution without the sample, OD_{sample} represents the absorbance of the test sample (a combination of the ABTS solution and either the test sample or a positive control).

The $OH^{\bullet}$ radical scavenging capacities were assessed using a modified approach outlined by de Avellar [26]. The specifics of this test are similar to that described in the author's earlier work [21]. The optical density (OD) was measured at a wavelength of 536 nm. Ascorbic acid served as the positive control. The capacity of ARPs to scavenge $OH^{\bullet}$ radicals was assessed using equation (6):

$$OH^{\bullet}\ \text{scavenging activity}\ (\%) = \frac{OD_{\ sample} - OD_{neg\_control}}{OD_{blank} - OD_{neg\_control}} X100\% \quad (6)$$

where, $OD_{neg\_control}$ refers to the absorbance of the reaction mixture without the sample, OD_{blank} represents the absorbance of reaction mixture without the sample and H_2O_2. OD_{sample} represents the absorbance of the test sample (a combination of the reaction mixture with H_2O_2 solution and either the test sample or a positive control).

2.7.2. Measurement of IL-6 production

The protocol for cell culturing and maintaining mouse macrophages (RAW 264.7) adhered to the methodology described in the literature [21]. IL-6 production was assessed using an ELISA kit. All data was collected in triplicate. The calibration curve was constructed using standard IL-6 (mouse), resulting in a linear equation (7) with an R^2 of 0.9935:

$$y = 0.002x + 0.1482 \quad (7)$$

The concentration of IL-6 generated by ARPs was determined using the calibration curve.

2.7.3. TNF- α production assay

An ELISA kit for TNF-α was used to quantify the production of TNF-α upon treating the cells with ARPs. The protocol for this test closely resembles the methodology described in the author's prior publication [21]. All experiments were conducted in triplicate. A calibration curve was generated using standard TNF-α (mouse), resulting in a linear equation (8) with an R^2 of 0.9921.

$$y = 0.0021x + 0.1634 \quad (8)$$

The concentration of TNF-α produced by ARPs was determined using this calibration curve.

2.7.4. Measurement of toxicity

Cell viability of RAW 264.7 were measured by a MTT assay following the method described previously [21]. The absorbance was determined at 595 nm, and the fraction of live cells was estimated using equation (9):

$$Cell\ viability\ (\%) = \frac{OD_{sample}}{OD_{\ positive\ control}} \text{X } 100\% \quad (9)$$

The positive control was RAW 264.7 cells treated with only the DMEM medium (without the sample and LPS).

2.8. Statistical analysis

All measurements were conducted in triplicate, and the outcome was reported as mean ± standard deviation (SD). Data analysis included the use of Duncan's multiple range and one-way analysis of variance (ANOVA). The statistical computations were conducted using OriginPro 8.5, GraphPad Prism 9.0, and Excel 2016. The statistical difference between the results is considered statistically significant if the p-value is < 0.05.

3. Results and discussion

3.1. Isolation of A. rugosum polysaccharides

A. rugosum polysaccharides (ARPs) were obtained using the hot water extraction method and purified by gel filtration technique (Fig. 1). Five (ARPs) were isolated according to the sugar profile established by the phenol-sulfuric acid assay. These were labelled as ARP-1, ARP-2, ARP-3, ARP-4 and ARP-5 (Fig. 2). Total carbohydrate content of these polysaccharides is given in Table 1. Standard dextran samples were employed to establish the standard calibration curve for the column that was used to obtain the average molecular weights of the isolated polysaccharide. Highest molecular weight of 1455 kDa was displayed by ARP-1 (Fig. 3), followed by ARP-2 (454 kDa) and ARP-3 kDa) (Fig. 3), and ARP-5 had the lowest molecular weight of 3 kDa. It should be noted that the crude polysaccharide samples were subjected to de-proteination using the Sevag method (section 2.2) to remove free protein. Hence, the purified ARPs will only contain bound protein. The bound protein content in ARP-1, ARP-2 and ARP-3 were 21.57 %, 14.95 and 6.95 % respectively (Table 1). Presence of small amounts of protein can also be seen from the NMR spectral data (section 3.3).

Table 1 also gives the chemical composition of the isolated ARPs. It can be seen that ARP-1 consists of only one mono-sugar (100 % glucose). ARP-2 contained glucose (95.05 %) as the major component with small quantities of mannose (2.47 %) and galactose (2.48 %). ARP-5 had glucose (75.35 %), galactose (17.15 %), and small amounts mannose and fucose. ARP-1 and ARP-2 consists mainly of glucose suggesting that they may be glucans. The purification process yielded small amounts ARP-3 and ARP-4 and these were insufficient for further biological and structural studies.

3.2. FT-IR characterisation of active AR polysaccharides

Fig. 4 presents the FT-IR spectra of ARPs. **ARP-1** shows a peak corresponding to β-glycosidic bond (891 cm^{-1}) (Fig. 4a) [19]. Also, there are three intense absorption bands at 995, 1033 and 1061 cm^{-1} (representing C–O stretching vibrations of glycosidic bond) suggesting that ARP-1 contains pyranose sugar. The broad peak at 3316 cm^{-1} represents vibrational stretching of hydroxyl group and the band around 2919 cm^{-1} represents stretching mode of C–H bond [19]. These results suggest that ARP-1 has β-glycosidic bonds and contains pyranose ring structures. Remaining peaks in Fig. 4a represent characteristic vibrational modes of polysaccharide structure. FT-IR results combined with the results from gas chromatography lead to the conclusion that ARP-1 is a β-glucan. **ARP-2** (Fig. 4b) has a band at 891 cm^{-1} consistent with β-glycosidic bonding (Zhang et al., 2014). The three strong absorption bands between 1066 and 1001 cm^{-1} (representing C–O stretching modes of glycosidic bonding) indicate that ARP-2 contains pyranose sugars [19]. The rest of the peaks in Fig. 4b are consistent with a polysaccharide structure. These results suggest that ARP-2 also exhibits β-glycosidic bonding and a pyranose ring structure. FT-IR spectral features combined with the findings of GC (section 3.1) lead to the conclusion that ARP-2 is likely to be a β-glucan.

ARP-5 (Fig. 4c) has a peak at 892.98 cm^{-1} consistent with β-glycosidic bonding [19,24]. FT-IR shows three intense bands in

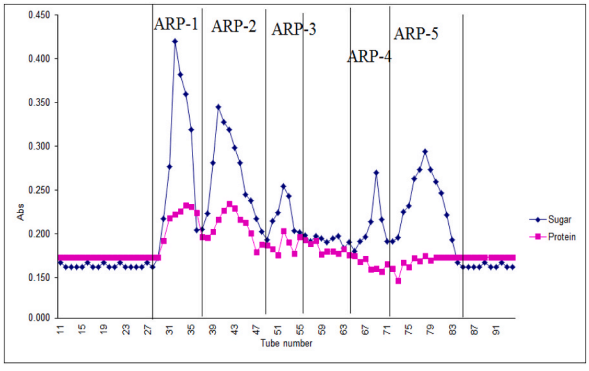

Fig. 2. Gel filtration chromatogram of polysaccharide fractions from *A. rugosum*.

Table 1
Chemical composition (sugar contents) of ARPs.

	ARP-1	ARP-2	ARP-3[a]	ARP-4[a]	ARP-5
Total Protein (%)	21.57	14.95	6.95	1.31	0.32
Total Carbohydrate (%)	78.43	85.05	93.05	98.69	99.68
Monosaccharide (%)					
Rhamnose (%)					
Ribose (%)					
Fucose (%)			3.58	3.21	3.3
Arabinose (%)					
Xylose (%)					
Mannose (%)		2.47	4.65	6.48	4.21
Glucose (%)	100 %	95.05	51.12	50.76	75.34
Galactose (%)		2.48	40.65	39.55	17.15
Unknown (%)					

[a] ARP-3 and ARP-4 were minor fractions and yielded very small quantity. Hence, these fractions were not considered for further study.

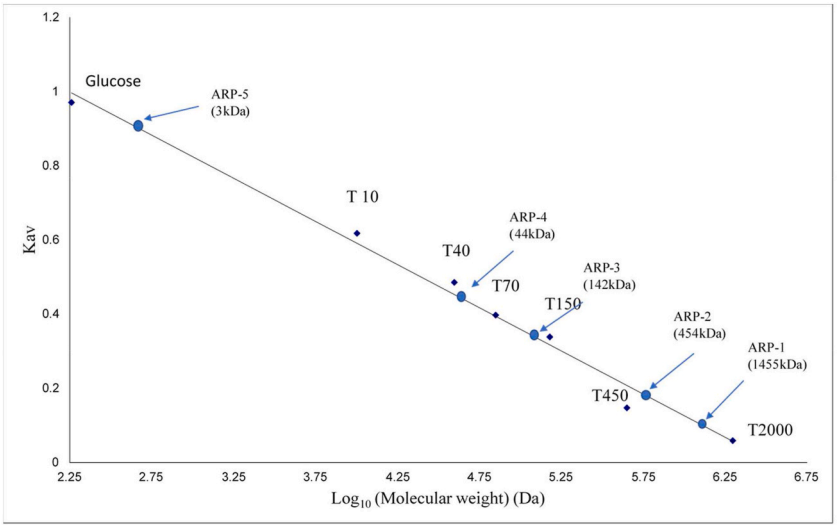

Fig. 3. Calibration curve for the determination of molecular weights of polysaccharides of *A. rugosum* based on the elution volume and the molecular mass of standard dextran series of T2000 (2000 kDa), T450 (450 kDa), T150 (150 kDa), T70 (70 kDa), T40 (40 kDa), T10 (10 kDa) and glucose (180 Da) (**Note**: Kav = $(V_e - V_o)/(V_t - V_o)$, V_o is void volume, V_t is total volume, V_e is elution volume).

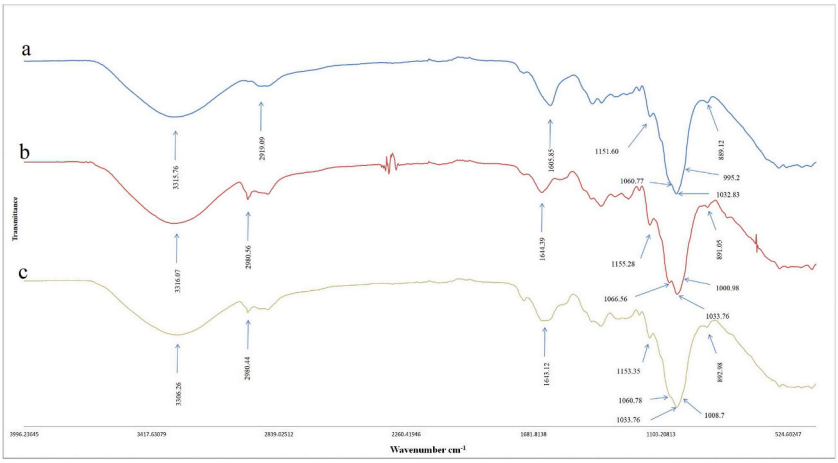

Fig. 4. The FT-IR spectra of the three fractions from *A. rugosum.* (a: ARP-1, b: ARP-2 and c: ARP-5).

1061-1008 cm^{-1} range (representing C–O stretching modes of glycosidic bond) confirming that ARP-5 contains pyranose sugars [19]. The remaining peaks in Fig. 4c conform to a polysaccharide structure.

3.3. NMR characterisation of ARP-1 and ARP-2

ARP-1 and ARP-2 are found to be the most active *A. rugosum* polysaccharides (section 3.4 and 3.5). A detailed NMR spectroscopic characterization of these two ARPs was therefore carried out in order to understand structure-activity relationship. One-dimensional (proton and carbon) NMR spectra of ARP-1 and ARP-2 are given in Figs. 5 and 6 respectively. It can be seen from the results presented in Fig. 5 that the spectral features between 3 and 4.6 ppm are very similar for ARP-1 and ARP-2 (except that the former gave sharper proton resonances). Fig. 5 also indicates that these ARPs are protein conjugates (weak signals in the region between 0.8 and 2.5 ppm). These results are consistent with those reported in the literature for bioactive mushroom polysaccharides [19]. Similar conclusions can be drawn from ^{13}C NMR spectra presented in Fig. 6 that displays weak protein peaks within the carbon spectral region between 10 and 50 ppm.

13-carbon spectra of ARP-1 and ARP-2 exhibited very similar resonance positions with minor changes indicating that these two ARPs are structurally similar (Fig. 6A and B). The detailed resonance assignments of ARP-2 were done by means of 2D-NMR spectra. Fig. 7a and b presents 2D-g-COSY and 2D-HSQC spectra of ARP-2. These 2D-NMR correlations were used to assign the ^{1}H and ^{13}C resonances of ARP-2 (Table 2). Following are the prominent features used in this assignment process (refer to Fig. 9 for the proposed structure of ARP-2):

1. C-3 carbon chemical shifts are relatively large in the rings A, B and C compared to that of ring D, indicating that C-3 of the rings A, B and C are involved in glycosidic linkages but not ring D. Hence, the ring D is likely to be part of a branched side chain. This is also evident from the HSQC correlations (Fig. 7b).
2. C-1 carbon chemical shifts are relatively large in all the rings (A, B, C and D) and hence this carbon in all the rings must be involved in glycosidic linkages (this is also evident from HSQC correlations). The features highlighted in dot-points 1 and 2 indicate that the rings A, B and C are part of the main chain and are involved in (1 → 3)-glycosidic linkages.
3. C-6 carbon chemical shift of the ring B stands out and this value is relatively large compared to those of the other rings. This feature indicates that the C-6 carbon of ring B is the branching point. The features highlighted in dot-points 1, 2 and 3 indicate that the C-1 of ring D and the C-6 of ring B are involved in (1 → 6)-glycosidic linkage with the ring D as branched side chain.

These assignments are consistent with the NMR spectral correlations presented in Fig. 7 and are also in agreement with literature findings for β-glucans isolated from medicinal mushrooms [27]. In addition, the Distortionless Enhancement by Polarization Transfer (DEPT) spectrum of ARP-2 (Fig. 8) provides additional confirmation of C-6 carbon resonance assignments (negative peaks for CH_2-carbons). Three –CH_2 resonances were identified at 62.98 ppm (C-6 of D-ring), 63.48 ppm (C-6 of A and C rings), and 71.6 ppm (C-6 of B-ring where branching occurs) from 2D-NMR and DEPT spectra (Table 2, Figs. 7 and 8). NMR results along with those of GC and FT-IR demonstrate that ARP-2 possesses β-(1 → 3)-D-glucan structure in the backbone and β-(1 → 6)-D-glucopyranosyl branching as represented in Fig. 9. It should be noted that the NMR structure and the spectral correlations of ARP-2 obtained in this study are very similar to those of β-D-glucans derived from other closely related family of mushroom *Ganoderma lucidum* [19,27,28]. It is therefore conclusive that the NMR structure of ARP-2 (Fig. 9) derived in this study is unambiguous.

Very similar proton and carbon NMR spectra of ARP-1 and ARP-2 (Figs. 5 and 6) suggest that ARP-1 exhibits β-(1 → 3)-D-glucan structure similar to that presented in Fig. 9.

Below is a summary of findings of spectroscopic and GC characterisation methods:

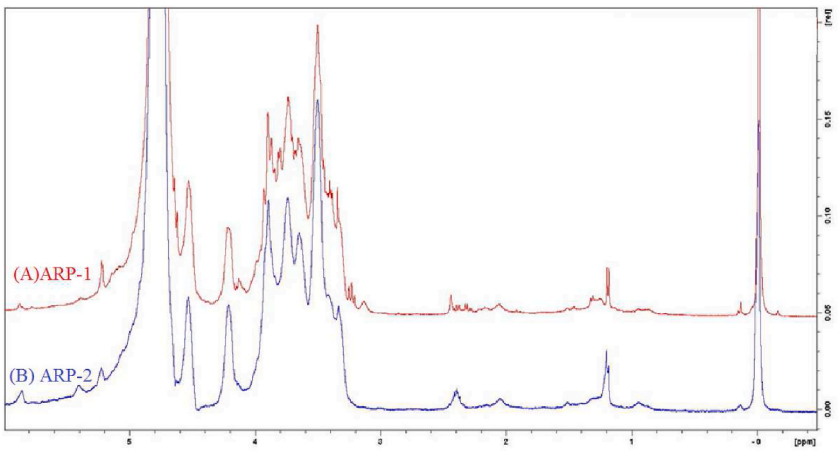

Fig. 5. ^{1}H NMR spectra of *A. rugosum* polysaccharides: (A) ARP-1 and (B) ARP-2. **Parameters used**: number of scans = 128; relaxation delay ≈4 s; data points = 64k (zero filled to 128 k before Fourier transform).

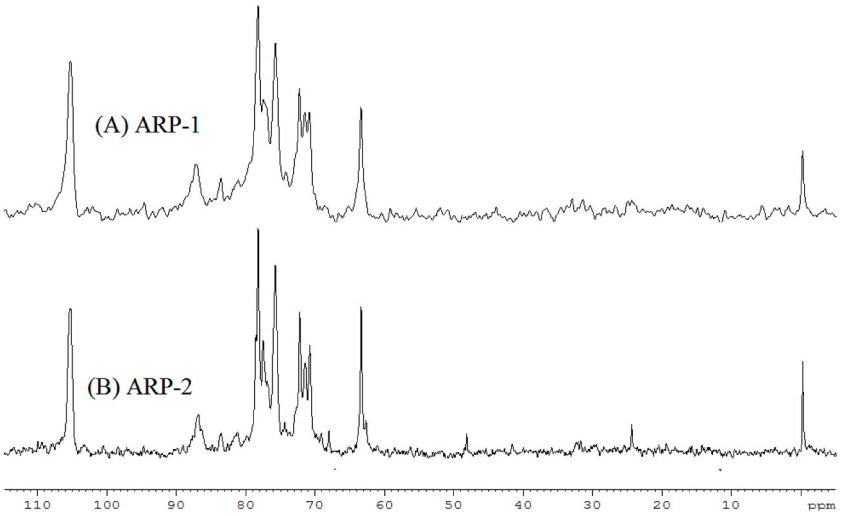

Fig. 6. ^{13}C NMR spectra of *A. rugosum* polysaccharide: (A) ARP-1 and (B) ARP-2.**Parameters used**: number of scans = 2048; relaxation delay ≈3.36 s; data points = 64k (zero filled to 128 k before Fourier transform).

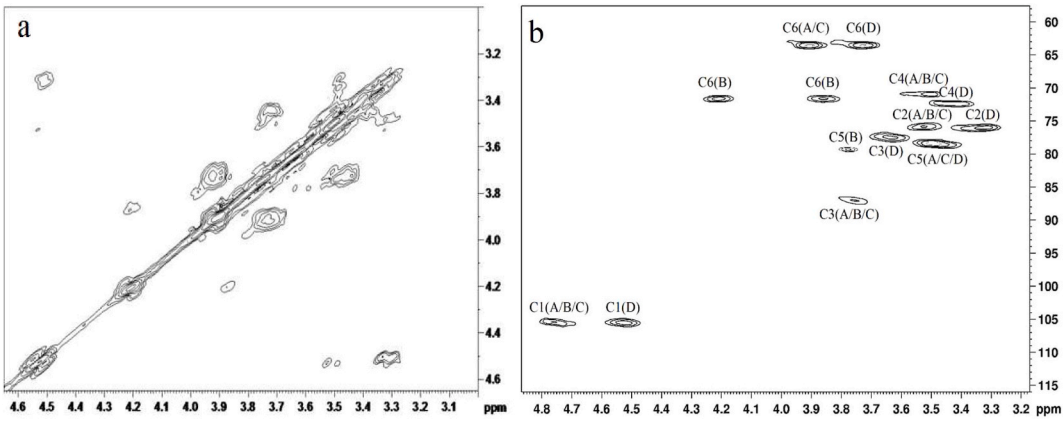

Fig. 7. (a) g-COSY and (b) HSQC spectra of ARP-2.**Parameter used for g-COSY**: Number of scans = 48; relaxation delay ≈ 1.68s; data points: 2048 in t_2 dimension and 256 in t_1 dimension (zero filled to 4 k and 1 k before Fourier transform in t_2 and t_1 dimensions respectively).**Parameter used for HSQC**: Number of scans = 48; relaxation delay ≈ 1.6s; data points: 2024 in t_2 dimension and 256 in t_1 dimension (zero filled to 4 k and 1 k before Fourier transform in t_2 and t_1 dimensions respectively).PS: sugar rings A, B, C and D are represented in Fig. 9.

Table 2
Proton and carbon NMR chemical shifts (ppm) of ARP-2.

	H1/C1 (ppm)		H2/C2 (ppm)		H3/C3 (ppm)		H4/C4 (ppm)		H5/C5 (ppm)		H6/C6 (ppm)	
A[a]	4.75	105.47	3.52	75.89	3.75	87.09	3.50	70.91	3.49	78.33	3.89	63.49
B[a]	4.75	105.47	3.52	75.89	3.75	87.09	3.50	70.91	3.76	78.65	4.13	71.61
C[a]	4.75	105.47	3.52	75.89	3.75	87.09	3.50	70.91	3.49	78.33	3.89	63.49
D[a]	4.53	105.33	3.32	75.89	3.64	77.64	3.40	72.37	3.49	78.33	3.74	62.99

[a] Sugar rings A, B, C and D are represented in Fig. 9.

- GC results (section 3.1) demonstrated that ARP-1 and ARP-2 contain glucose as the major mono-sugar.
- FT-IR spectral features (section 3.2) suggest that ARP-1 and ARP-2 exhibit pyranose ring structures with β-glycosidic bonding.
- NMR spectral assignments demonstrate that ARP-1 and ARP-2 have β-D-(1 → 3)-glucopyranosyl structure in the main chain and β-(1 → 6)-glucopyranosyl units in the side chain (Fig. 9).

The structural features of ARP-1 and ARP-2 presented in this paper closely resemble those of schizophyllan extracted from *Schizophyllum commune* [27].

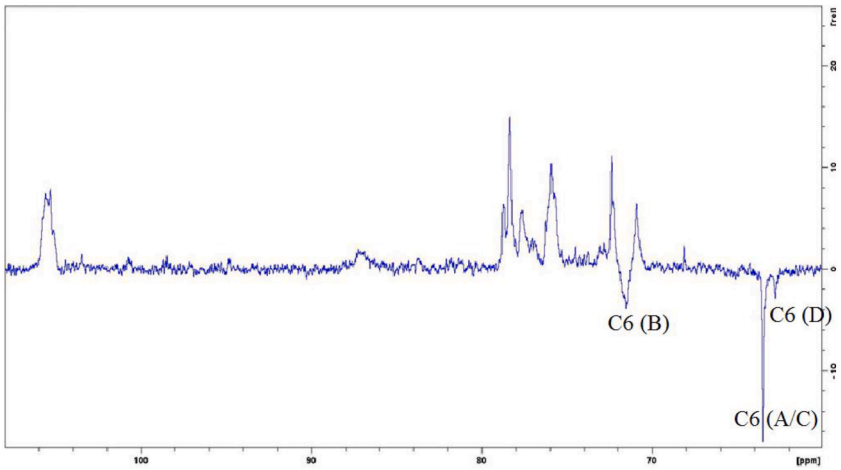

Fig. 8. DEPT spectra of ARP-2.Parameters used: Number of scans = 3000; relaxation delay ≈3.36 s; data points = 64k (zero filled to 128k before Fourier transform); DEPT pulse angel = 135°.PS: sugar rings A, B, C and D are represented in Fig. 9.

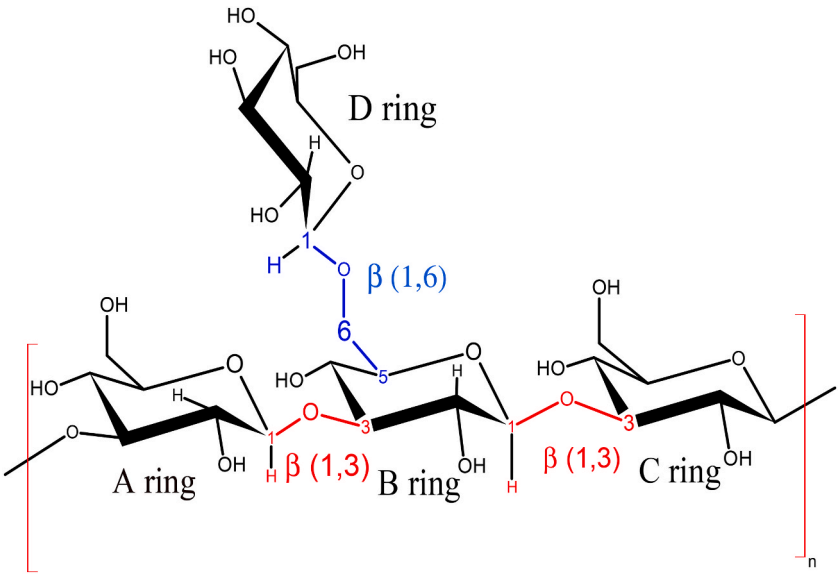

Fig. 9. Structure of ARP-2.

3.4. Antioxidant capacities of ARPs

The free radical scavenging abilities of the three *A. rugosum* polysaccharides (ARP-1, ARP-2 and ARP-5) together with that of the standard (ascorbic acid) are presented in Fig. 10. The results presented in Fig. 10 were calculated relative to the activity of ascorbic acid taken as 100 %. The three ARPs exhibited excellent $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities (Fig. 10A and B). ARP-1 displayed highest activity against $DPPH^{\bullet}$ (72 %) and the least active fraction is ARP-5 (62 %). The activities against $ABTS^{\bullet+}$ were very similar with ARP-1 exhibiting 74 % activity and ARP-5 displayed 65 % activity. The IC_{50} value for ARP-1 against DPPH radical was 222 μg/mL, ARP-2 was 322 μg/mL and ARP-5 at 500 μg/mL. The IC_{50} for ARP-1 against $ABTS^{\bullet+}$ was about 139 μg/mL. The hydroxyl ($OH^{\bullet}$) scavenging activities ARPs are given in Fig. 10C. ARP-1 exhibited significantly high $OH^{\bullet}$ scavenging ability (>80 %) and ARP-5 was the least active. The IC_{50} value for ARP-1 against the hydroxyl radical was about 165 μg/mL. Comparison of these radical scavenging activities with other fungal polysaccharides reveal that the polysaccharides from *A. rugosum* showed higher DPPH scavenging activity than the polysaccharides from *Ganoderma lucidum* (PSP) that has an IC_{50} value of 0.28 mg/mL [29]. A water soluble galactoglucan extracted from food mushroom *Pleurotus djamor* had an IC_{50} value of 3.83 mg/mL [9]; and a glucan-rich heteropolysaccharide isolated from *Inonotus obliquus* exhibited an IC_{50} value of 1.3 mg/mL [30]. In addition, ARP-1 showed significantly higher ABTS and hydroxyl radical scavenging abilities than other mushroom polysaccharides, such as, polysaccharides isolated from the wild mushrooms *Paxillus involutus* and *Lepista nuda* [8,31].

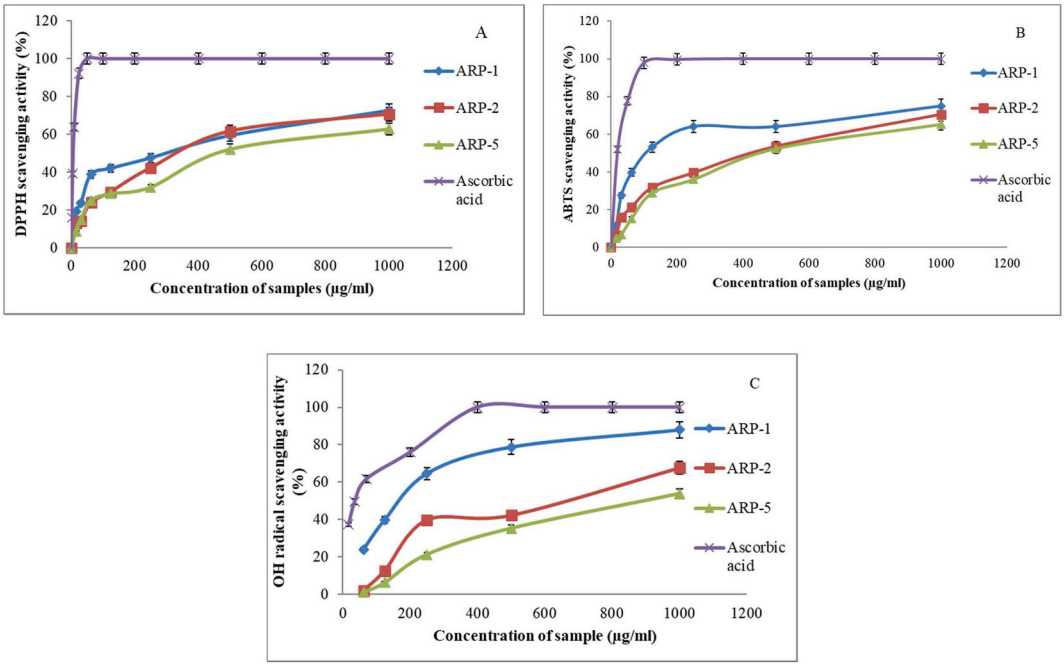

Fig. 10. Antioxidant activities of polysaccharide fractions of *A. rugosum*. (A) DPPH free radical scavenging activity of the ARPs. (B) ABTS and free radical scavenging activity of the ARPs. (C) Hydroxyl radical scavenging activity of the ARPs. Results are the mean ± SD of three separate experiments, each in triplicate, all of the results were compared with standard (ascorbic acid) in line chart.

Literature reports indicate that certain structural features are responsible for the high antioxidant capacity of botanical polysaccharides [24]. These features include: (i) higher molecular weight (>90 kDa) is preferable [31], (ii) presence of β-glycosidic linkages [31,32] improves activity, and (iii) protein conjugation increases radical scavenging ability [32]. For instance, PSP and PSK that have protein conjugation displayed high superoxide free radical and hydroxyl free radical scavenging potentials [33].

The observed structural features and antioxidant activities of ARP-1 are consistent with the literature cited earlier. ARP-1, which displayed the highest antioxidant activity in this study has (i) largest molecular mass (1494 kDa) (Figs. 3 and 10), (ii) comprises of β-glycosidic bonds (Fig. 4a), (iii) is bound to protein (20 % protein content), and (iv) solely contains glucose as mono-sugar (Table 1).

3.5. Immunomodulatory activities of ARPs

The immunomodulatory activities of *A. rugosum* polysaccharides are presented in Fig. 11. They displayed significant immunomodulatory activities by increasing TNF-α and IL-6 production in a concentration dependant manner (Fig. 11A to B). Results shown in Fig. 11A and B suggest that ARP-1 and ARP-2 display excellent immunomodulatory activities that are greater than LPS (at 1 μg/mL) (known positive control). It is clear from the results (Fig. 11C and D) that the immunomodulatory potentials of ARP-1, ARP-2 and ARP-5 rise dramatically at the concentrations larger than 10 μg/mL. These results are illustrated by the observation that ARP-1 displayed: (i) more than 16-fold increase in the production of TNF-α (at 100 μg/mL) (Fig. 11C), and (ii) over 20-fold rise in the production of IL-6 in comparison with the negative control (macrophages without treatment with sample) (Fig. 11D). Similarly, excellent immunomodulatory properties of ARP-2 at 100 μg/mL are observed, where, (i) more than 13-fold rise in the production of TNF-α (Fig. 11C), and (ii) about 15-fold rise in the production of IL-6 were observed in comparison with the negative control (macrophages without treatment with sample) (Fig. 11D). ARP-5 showed relatively lower immunostimulatory potential amongst all the ARPs. However, the immunostimulatory abilities of ARP-5 are superior when compared with the activities of polysaccharides from many traditional herbs. These results are extremely significant and confirm that the ARPs are important agents to promote the immune system.

Literature demonstrates that β-glucans display immunomodulatory property by interacting with immune cell receptors [34]. This mode of interaction activates both innate and adaptive immune system responses that triggers the formation IL-1, IL-6 and TNF-α [34]. Such an action of β-D-(1 → 3)-glucans accounts for indirect antitumor activity that occurs via the stimulation of immune defence.

β-glucans have the ability to bind and interact with two kinds of immune system receptors: (i) the dectin-1 (known as β-glucan receptor) that recognises and binds with β-(1 → 3)-glucans, and activates macrophages and dendritic cells (DCs) [34], and (ii) toll like receptors (TLR), particularly TLR-2 and TLR-4 that can interact with β-(1 → 3)-glucans and stimulate macrophages leading to the production and secretion of cytokines [35]. The published literature [35] shows that the size (molecular weights) of β-glucans is one of the crucial factors in this mode of action and this is in agreement with the findings of this research. For instance, largest immunostimulatory activity was displayed by ARP-1 that also has the highest molecular mass amongst all the ARPs. In this research, the structures of ARP-1 and ARP-2 (sections 3.2 and 3.3) were identified as β-(1 → 3)-glucans with β-(1 → 6) linked branching, and this

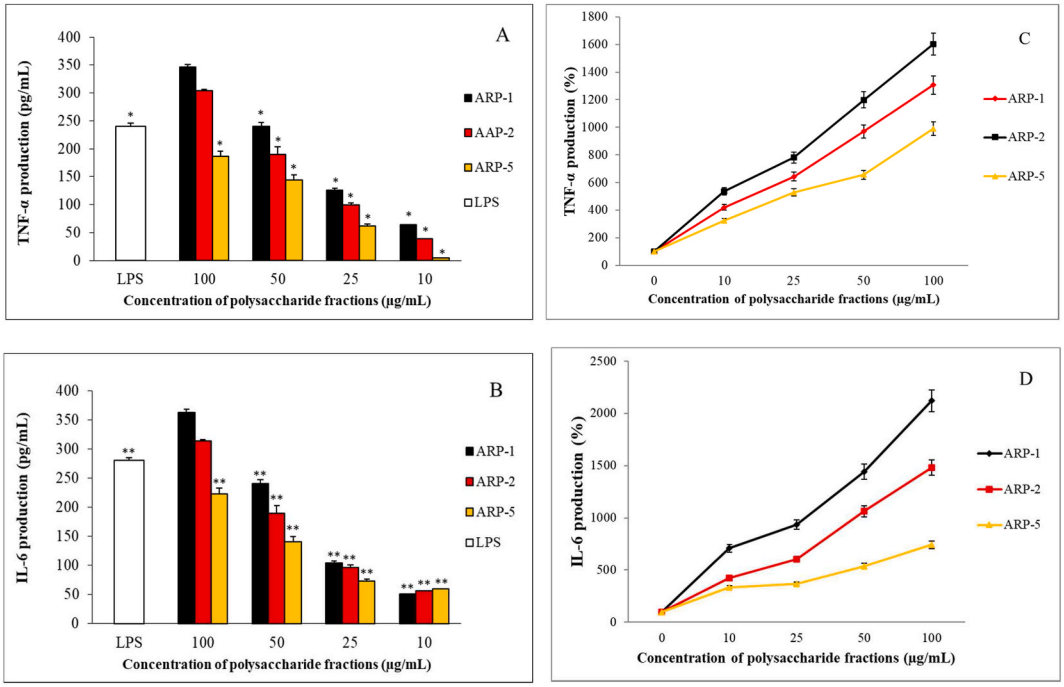

Fig. 11. Effects of *A. rugosum* polysaccharides on murine RAW 264.7 macrophages. (A and C): represent tumour necrosis factor-α (TNF-α) production, and (B and D): represent interleukin 6 (IL-6) production. * Statistical difference for the positive control (LPS treated group) and the samples was significant, n = 3, $p < 0.05$ ** Statistical difference for the positive control (LPS treated group) and the samples was significant, n = 3, $p < 0.04$.

structure is beneficial for immunostimulatory activities [35]. It has been demonstrated by the literature findings that several β-glucans isolated from various mushrooms display significant immune-enhancing and anticancer properties [1]. A comparison of the immune-enhancing potential of β-glucans from *A. rugosum* with the published information on β-glucans reveal a great potential of ARPs for developing immunotherapeutic and antitumor formulations [1].

3.6. Cell viability

Cell viability studies on the three ARPs are presented in Fig. 12. These observations lead to the conclusion that *A. rugosum* polysaccharides are non-toxic even at 100 μg/mL. These findings agree with the reports that botanical/mushroom polysaccharides display low toxicity [21].

4. Conclusion

Three major polysaccharides with significant biological importance were isolated from *A. rugosum* (ARP-1, ARP-2 and ARP-5). The structures of two new and highly potential immunomodulatory polysaccharides (ARP-1 and ARP-2) were determined. They possess a β-D-(1 → 3)-glucan structure in the main chain and β-D-(1 → 6) structure in the side chain. These novel ARPs also exhibited excellent

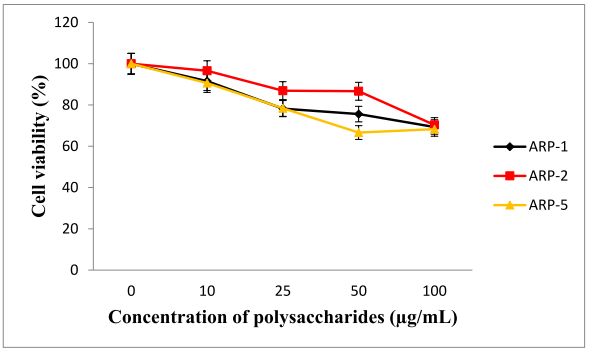

Fig. 12. Cell viabilities of isolated polysaccharide fractions from *A. rugosum* at different concentrations.

antioxidative capacities and low toxicity. The findings of this research reveal that the ARPs present huge potential to be used as immune-enhancing agents. Clearly, *A. rugosum* polysaccharides have great prospect for developing immune-enhancing agents that have relevance in cancer treatment. These polysaccharides have simultaneously displayed antioxidant as well as immunomodulatory properties indicating that they present great possibility for cancer immunotherapy. These findings corroborate well with the fact that *A. rugosum* is widely used by traditional practitioners to formulate antitumor agents.

An exciting future study with ARPs is to initiate a detailed investigation of their anticancer potentials. Studies involving animal and human subjects to further evaluate their immune-enhancing abilities will be beneficial to develop effective therapeutics. Such studies will provide cues for developing potent immunotherapeutic and anticancer formulations by appropriately combining the most potent mushroom polysaccharides.

Additional information

No additional information is available for this paper.

Data availability statement

Data will be made available on request. Data is stored at the following repository at the Western Sydney University and is only available on request.

OneDrive - Western Sydney University\AAAA-NReddy-LZhang_AR results\A rugosum unprocessed results.

CRediT authorship contribution statement

Lin Zhang: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Cheang Soo Khoo:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Sundar Rao Koyyalamudi:** Writing – review & editing, Supervision, Methodology, Conceptualization. **Narsimha Reddy:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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References

[1] P. Maity, I.K. Sen, I. Chakraborty, S. Mondal, H. Bar, S.K. Bhanja, S. Mandal, G.N. Maity, Biologically active polysaccharide from edible mushrooms: a review, Int. J. Biol. Macromol. 172 (2021) 408–417, https://doi.org/10.1016/j.ijbiomac.2021.01.081.

[2] O. Rop, J. Mlcek, T. Jurikova, Beta-glucans in higher fungi and their health effects, Nutr. Rev. 67 (2009) 624–631, https://doi.org/10.1111/j.1753-4887.2009.00230.x.

[3] K.F. Cheng, P.C. Leung, General review of polysaccharopeptides (PSP) from C. versicolor: pharmacological and clinical studies, Cancer Ther. 6 (2008) 117–130.

[4] Y. Wang, J. Jia, X. Ren, B. Li, Q.J. Zhang, Extraction, preliminary characterization and in vitro antioxidant activity of polysaccharides from Oudemansiella radicata mushroom, Int. J. Biol. Macromol. 120 (2018) 1760–1769, https://doi.org/10.1016/j.ijbiomac.2018.09.209.

[5] A. Kiss, P. Grünvald, M. Ladányi, V. Papp, I. Papp, E. Némedi, I.J.F. Mirmazloum, Heat treatment of reishi medicinal mushroom (ganoderma lingzhi) basidiocarp enhanced its β-glucan solubility, antioxidant capacity and lactogenic properties, Foods 10 (9) (2021) 2015, https://doi.org/10.3390/foods10092015.

[6] L. Gomes, C. Borges, M.L.M. Serralheiro, M. Minhalma, R. Pacheco, Cork processing wastewaters components fractioned by ultrafiltration membranes–studies of antioxidant and antitumoral activity, J. Chem. Technol. Biotechnol. 93 (3) (2018) 861–870, https://doi.org/10.1002/jctb.5441.

[7] Q.M. Liu, S.S. Xu, L. Li, T.M. Pan, C.L. Shi, H. Liu, M.J. Cao, W.J. Su, G.M. Liu, In vitro and in vivo immunomodulatory activity of sulfated polysaccharide from Porphyra haitanensis, Carbohydr. Polym. 165 (2017) 189–196, https://doi.org/10.1016/j.carbpol.2017.02.032.

[8] X. Shu, Y. Zhang, J. Jia, X. Ren, Y. Wang, Extraction, purification and properties of water-soluble polysaccharides from mushroom Lepista nuda, Int. J. Biol. Macromol. 128 (2019) 858–869, https://doi.org/10.1016/j.ijbiomac.2019.01.214.

[9] G.N. Maity, P. Maity, S. Khatua, K. Acharya, S. Dalai, S. Mondal, Structural features and antioxidant activity of a new galactoglucan from edible mushroom Pleurotus djamor, Int. J. Biol. Macromol. 168 (2021) 743–749, https://doi.org/10.1016/j.ijbiomac.2020.11.131.

[10] J.R. Barbosa, M.M.S. Freitas, L.C. Oliveira, L.H.S. Martins, A.O. Almada-Vilhena, R.M. Oliveira, J.C. Pieczarka, B. Davi do Socorro, R.N. Junior, Obtaining extracts rich in antioxidant polysaccharides from the edible mushroom Pleurotus ostreatus using binary system with hot water and supercritical CO2, Food Chem. 330 (2020) 127173, https://doi.org/10.1016/j.foodchem.2020.127173.

[11] D. Zeng, S. Zhu, Purification, characterization, antioxidant and anticancer activities of novel polysaccharides extracted from Bachu mushroom, Int. J. Biol. Macromol. 107 (2018) 1086–1092, https://doi.org/10.1016/j.ijbiomac.2017.09.088.

[12] J. Li, R. Li, X. Wu, R.L.C. Hoo, S.M.Y. Lee, T.M.Y. Cheung, B.S.Y. Ho, G.P.H. Leung, Amauroderma rugosum protects PC12 cells against 6-OHDA-induced neurotoxicity through antioxidant and antiapoptotic effects, Oxid. Med. Cell. Longev. 2021 (2021) e6683270, https://doi.org/10.1155/2021.

[13] X. Zhang, Z. Cai, H. Mao, P. Hu, X. Li, Isolation and structure elucidation of polysaccharides from fruiting bodies of mushroom Coriolus versicolor and evaluation of their immunomodulatory effects, Int. J. Biol. Macromol. 166 (2021) 1387–1395, https://doi.org/10.1016/j.ijbiomac.2020.11.018.

[14] Y. Sugiyama, Polysaccharides. *Immunotherapy of Cancer: an Innovative Treatment Comes of Age*, Springer, Tokyo, 2016, pp. 37–50.
[15] Z.A. Bhat, A.H. Wani, J.M. War, M.Y. Bhat, Major bioactive properties of Ganoderma polysaccharides: a review, Asian J. Pharmaceut. Clin. Res. 14 (3) (2021) 11–24, https://doi.org/10.22159/ajpcr.2021v14i3.40390.
[16] M.H. Lin, K.M. Lee, C.Y. Hsu, S.Y. Peng, C.N. Lin, C.C. Chen, C.K. Fan, P.C. Cheng, Immunopathological effects of Agaricus blazei Murill polysaccharides against Schistosoma mansoni infection by Th1 and NK1 cells differentiation, Int. Immunopharm. 73 (2019) 502–514, https://doi.org/10.1016/j.intimp.2019.05.045.
[17] I. Mirończuk-Chodakowska, K. Kujawowicz, A.M. Witkowska, Beta-glucans from fungi: biological and health-promoting potential in the COVID-19 pandemic era, Nutrients 13 (11) (2021) 3960, https://doi.org/10.3390/nu13113960.
[18] X. Meng, H. Liang, L. Luo, Antitumor polysaccharides from mushrooms: a review on the structural characteristics, antitumor mechanisms and immunomodulating activities, Carbohydr. Res. 424 (2016) 30–41, https://doi.org/10.1016/j.carres.2016.02.008.
[19] L. Zhang, N. Reddy, S.R. Koyyalamudi, Isolation, characterization, and biological activities of polysaccharides from medicinal plants and mushrooms, in: F.R. S. Atta-ur-Rahman (Ed.), Studies in Natural Products Chemistry, 2014. UK.
[20] P.M. Chan, G. Kanagasabapathy, Y.S. Tan, V. Sabaratnam, U.R. Kuppusamy, Amauroderma rugosum (Blume & T. Nees) Torrend: nutritional composition and antioxidant and potential anti-inflammatory properties. Evid.-based Complement, Alternative Med. 2013 (2013) e304713, https://doi.org/10.1155/2013.
[21] L. Zhang, N. Reddy, C. Khoo, S.R. Koyyalamudi, C.E. Jones, Antioxidant and immunomodulatory activities and structural characterization of polysaccharides isolated from Lobelia chinensis Lour, Pharmacologia 9 (4) (2018) 157–168, https://doi.org/10.5567/pharmacologia.2018.157.168.
[22] M. Dubois, K.A. Gilles, J.K. Hamilton, P.T. Rebers, F. Smith, Colorimetric method for determination of sugars and related substances, Anal. Chem. 28 (3) (1956) 350–356, https://doi.org/10.1021/ac60111a017.
[23] O.H. Lowry, N.J. Rosebrough, A.L. Farr, R.J. Randall, Protein measurement with the Folin phenol reagent, J. Biol. Inorg. Chem. 193 (1951) 265–275.
[24] S.R. Thambiraj, M. Phillips, S.R. Koyyalamudi, N. Reddy, Antioxidant activities and characterisation of polysaccharides isolated from the seeds of Lupinus angustifolius, Ind. Crops Prod. 74 (2015) 950–956, https://doi.org/10.1016/j.indcrop.2015.06.028.
[25] M.S. Blois, Antioxidant determinations by the use of a stable free radical, Nature 181 (4617) (1958) 1199–1200, https://doi.org/10.1038/1811199a0.
[26] I.G. de Avellar, M.M. Magalhães, A.B. Silva, L.L. Souza, A.C. Leitão, M. Hermes-Lima, Reevaluating the role of 1, 10-phenanthroline in oxidative reactions involving ferrous ions and DNA damage, Biochim. Biophys. Acta Gen. Subj. 1675 (1–3) (2004) 46–53, https://doi.org/10.1016/j.bbagen.2004.08.006.
[27] H. Kono, N. Kondo, K. Hirabayashi, M. Ogata, K. Totani, S. Ikematsu, M. Osada, Two-dimensional NMR data of a water-soluble β-(1→ 3, 1→ 6)-glucan from Aureobasidium pullulans and schizophyllan from Schizophyllum commune, Data Brief 15 (2017) 382–388, https://doi.org/10.1016/j.dib.2017.09.067.
[28] W.A. Bubb, NMR spectroscopy in the study of carbohydrates: characterizing the structural complexity, Concepts Magn. Reson. Part A: An Educational Journal 19 (1) (2003) 1–19, https://doi.org/10.1002/cmr.a.10080.
[29] I.C. Ferreira, S.A. Heleno, F.S. Reis, D. Stojkovic, M.J.R. Queiroz, M.H. Vasconcelos, M. Sokovic, Chemical features of Ganoderma polysaccharides with antioxidant, antitumor and antimicrobial activities, Phytochemistry 114 (2015) 38–55, https://doi.org/10.1016/j.phytochem.2014.10.011.
[30] Y. Hu, Y. Sheng, M. Yu, K. Li, G. Ren, X. Xu, J. Qu, Antioxidant activity of Inonotus obliquus polysaccharide and its amelioration for chronic pancreatitis in mice, Int. J. Biol. Macromol. 87 (2016) 348–356, https://doi.org/10.1016/j.ijbiomac.2016.03.006.
[31] Y. Liu, Y. Zhou, M. Liu, Q. Wang, Y. Li, Extraction optimization, characterization, antioxidant and immunomodulatory activities of a novel polysaccharide from the wild mushroom Paxillus involutus, Int. J. Biol. Macromol. 112 (2018) 326–332, https://doi.org/10.1016/j.ijbiomac.2018.01.132.
[32] H. Wang, Y.M. Liu, Z.M. Qi, S.Y. Wang, S.X. Liu, X. Li, H.J. Wang, X.C. Xia, An overview on natural polysaccharides with antioxidant properties, Curr. Med. Chem. 20 (23) (2013) 2899–2913.
[33] J.M.F. Wan, Polysaccharide Krestin (PSK) and Polysaccharopeptide (PSP), Handbook of Biologically Active Peptides: Fungal Peptides, Elsevier Inc., USA, 2013, pp. 180–184.
[34] J.J. Volman, J.D. Ramakers, J. Plat, Dietary modulation of immune function by β-glucans, Physiol. Behav. 94 (2) (2008) 276–284, https://doi.org/10.1016/j.physbeh.2007.11.045.
[35] H.A. El Enshasy, R. Hatti-Kaul, Mushroom immunomodulators: unique molecules with unlimited applications, Trends Biotechnol. 31 (12) (2013) 668–677, https://doi.org/10.1016/j.tibtech.2013.09.003.