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Purification, characterization, and bioactivities of *Physarum polycephalum* microplasmodial intracellular polysaccharides

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Physarum polycephalum is a species of myxomycetes that can produce a significant amount of cell wall-less microplasmodia in liquid media within a short period of time. Our previous study found that the deproteinated IPSs from *P. polycephalum* microplasmodia significantly inhibited cancer cells. In this study, the deproteinated IPSs were further purified by ion exchange chromatography. Based on carbohydrate and molecular weight analysis, three polysaccharide molecules (pp0, pp0.3, and pp0.4) with the molecular weights of 305 kDa, 573 kDa and 1342 kDa, respectively, were obtained from the deproteinated sample. HPLC-UV analysis showed that these polysaccharides had different monomers compositions. Pp0 and pp0.3 had glucose (99.49 % and 75.47 %, respectively) as the major monomer, whereas the main monomer of pp0.4 was galactose (93.09 %). Regarding biological activity, pp0 had a significantly higher antioxidant activity. This polysaccharide also exhibited the highest antiproliferative activity against the tested cancer cells among three fractions. At 1000 µg/mL, pp0 reduced the viability of HeLa and MCF-7 population to 54.54 % and 63.81 %, respectively. Even though these activities were significantly lower than those of the positive control (doxorubicin), pp0 was significantly safer for normal cells. Specifically, only 46.03 % of non-transformed BAE cell population remained viable when treated with doxorubicin, whereas pp0 showed no inhibition toward them.

Keywords: anti-proliferative activity, DPPH scavenging activity, slime molds, DEAE cellulose-52 chromatography, β-glucan.

Polysaccharides are natural carbohydrate polymers (Yang & Zhang 2009). Among the natural sources of polysaccharides, microorganisms have been identified as potential producers of bioactive polysaccharides. Depending on their location in microbial cells, microbial polysaccharides can be classified into three main groups. These are (1) cytosolic polysaccharides, which provide a carbon and energy source for the cells; (2) polysaccharides that make up the cell walls, including peptidoglycans, techoid acids, and lipopolysaccharides; (3) polysaccharides that are excreted into the extracellular environment in the form of capsules or slime, known as exopolysaccharides (Tommonaro et al. 2015). Polysaccharides from various organisms (fungi, bacteria, algae, and plants) have been reported to display a broad spectrum of powerful bioactivities as antioxidants (Wang et al. 2023a) and having antimicrobial (Kungel et al. 2018), antitumor (Mohamed et al. 2018), antiviral (Barcelos et al. 2020), and anti-inflammatory properties (Du et al. 2016).

There is a strong correlation between the physiochemical characteristics of polysaccharides and their bioactivities. For instance, molecular weights of 10^5–10^6, a chemical composition such as having a high sulphated group and glucose content, a molecular structure of consisting of a polymeric backbone such as β-glucan, and a branching degree of 0.2–0.33 were reported to have positive correlation on anticancer activities of polysaccharides (Jin et al. 2003, Cui et al. 2007, Gan et al. 2011, Wang et al. 2023b).

Among the microbial groups, intracellular polysaccharides (IPSs) from fungi have been intensively studied since fungi produce relatively high amounts of biomass compared with bacteria and algae, espe-

cially the former. However, the challenge with fungal IPSs research is that the fungal cell wall is rather thick and rigid, and cell disruption methods are either time consuming, toxic, or costly. However, these must be performed in order to collect IPS from the fungal biomass (Wang et al. 2022). Previous studies have suggested that fungal IPSs have strong antioxidant and cytotoxicity toward cancer cells. For example, IPS extracted from *Lentinus velutinus* Fr. fruiting bodies (LVP) has exhibited significant antiproliferative activities against HeLa cervical and HepG2 liver cancer cells as well as a strong antioxidant capacity towards DPPH free radicals (Udchumpisai & Bangyeekhun 2020). At 2 mg/mL, the inhibition values of LVP on HeLa and HepG2 cells were 49.97 % and 51.83 %, respectively. Moreover, apoptosis was recorded in both cancer cell lines treated with LVP, characterized by swollen round and adherent losing cells at test concentration higher than 1 mg/mL. This bioactive polysaccharide was composed of only glucose monomers with average molecular weight of 336 kDa (Udchumpisai & Bangyeekhun 2020). A more recent study on an IPS obtained from *Echinodontium tinctorium* (Ellis & Everh.) Ellis & Everh. fruiting bodies was named EtGIPL1a. The EtGIPL1a polysaccharide showed impressive inhibition against U251 glioblastoma cancer cell proliferation, with the half maximal inhibitory concentration (IC50) of 193.2 nM. The polysaccharide was described as having molecular weight of 275 kDa, with a monomer composition of glucose (54.3 %), galactose (19.6 %), mannose (11.1 %), fucose (10.3 %), glucuronic acid (4.0 %), and rhamnose (0.6 %). Moreover, the EtGIPL1a structure consisted of β-1→3-linkage major backbone with β-1→6-linkage branching points. Results of the study also suggested the induction of apoptosis as the mechanism that triggers antiproliferative activity of EtGIPL1a (Zeb et al. 2022).

Myxomycetes (slime molds) are a group of protozoans which have some common characteristics with fungi (e.g., producing fruiting bodies containing reproductive spores) (Stephenson & Stempen 1994). However, myxomycetes have two unique trophic stages (amoebae and plasmodia) in their life cycles (Stephenson & Stempen 1994), which are different from anything found in fungi. In a liquid medium, the plasmodia of some species of myxomycetes (e.g., *Physarum polycephalum* Schwein.) can be fragmented into microplasmodia. Both plasmodia and microplasmodia have no cell wall and they can grow rapidly (Daniel & Rusch 1961, Tran et al. 2015). Our previous research successfully cultured *P. polycephalum* in liquid medium (Truong et al. (2019), which yielded 13.86 g/l biomass after 5-day-cultivation. The fact that this slime mold can be cultured in liquid medium is a significant advantage for IPS research since it is possible to scale up the biomass production. In addition, as previously mentioned, the biomass of *P. polycephalum* microplasmodia are cell wall-less, which makes the extraction of IPSs from *P. polycephalum* microplasmodia much less challenging.

There has been only one previous study of IPSs from *P. polycephalum* microplamodia, and it was carried out by our research group. In our previous study, the crude IPSs and partially purified IPSs (deproteinated by trichloroacetic acid treatment followed by dialysis for small particles removal) from *P. polycephalum* microplasmodia were tested for their antiproliferative activity against MCF-7 and HeLa cancer cells (Do et al. 2021). The partially purified IPSs (1 mg/ml) inhibited about 61 % of Hela cells and 19 % of MCF-7 cells.

It should be noted that crude polysaccharides are collected by ethanol precipitation method. Therefore, the crude polysaccharides would contain different polysaccharide fractions, a small amount of proteins and small particles (e.g, amino acids, peptides, organic acid, and microelements) (Freitas et al. 2011), and the treatments by TCA and dialysis just eliminate the free proteins as well as some small particles from polysaccharide matrix. That being said, the deproteinated IPSs from *P. polycephalum* microplasmodia would contain several fractions of polysaccharides. Thus, to obtain pure polysaccharides from a crude polysaccharide sample, it is crucial to remove proteins from the crude polysaccharide sample, then separate the deproteinated polysaccharides (into different fractions) by chromatography techniques. The polydispersity index (PI) of the deproteinated IPSs in our previous study was found to be 3.160 (Do et al. 2021), which indicated a low homogeneity of the sample, since a PI value equal to 1.0 suggests that the particles are monodispersed (Ge et al. 2023). Therefore, in the present study, the deproteinated IPSs from *P. polycephalum* microplasmodia were further purified by a DEAE cellulose-52 column to obtain separated fractions (purified polysaccharide molecules). The physicochemical characteristics of the obtained purified polysaccharide molecules were then analyzed. In addition, *in vitro* antioxidant activity (against DPPH free radicals) and antiproliferative capacity (towards HeLa and MCF-7 cancer cells) of the polysaccharides were also evaluated. As far as we are aware, this is the first research that attempts to purify myxomycete intrapolysaccharides using

column chromatography technique and characterizes as well as evaluates biological activities of the purified polysaccharides individually.

Materials and methods

Materials

The *Physarum polycephalum* in this study was purchased from Carolina Biological Supply Company (Burlington, North Carolina, USA) in the form of sclerotia (item # 155775).

Two cancer cell lines including cervical carcinorma HeLa and breast carcinorma MCF-7 were purchased from the American Type Culture Collection (Manassas, Rockville, United States). A non-transformed cell line, bovine aortic endothelial cells (BAECs), was purchased from RIKEN (Tsukuba, Japan).

DEAE cellulose–52 ion-exchange was purchased from the Himedia Company (Mumbai, Maharashtra, India).

Microplasmodial culture of *P. polycephalum*

Physarum polycephalum was activated from a sclerotium according to the method described by Tran et al. (2015). The sclerotium was placed on a water agar (WA) plate and then kept in the dark at 27 °C for two days. On the third day, after yellow plasmodia had developed from the sclerotium, sterile oatmeal was added on the surface of the plasmodia and then the latter was incubated for one more day in the dark. A plug of agar (about 2 cm^2) bearing an active plasmodium growing on oatmeal was cut using a sterile blade and placed upside-down onto a new WA. This process was repeated several times until a pure plasmodium was achieved. Then the inoculation preparation was carried out by transferring a block of plasmodium growing WA to new nutrient agar (NA) plate.

The method for developing microplasmodial liquid culture from *P. polycephalum* plasmodia as described by Truong et al. (2019) was followed. A piece of NA with an actively growing plasmodium present was transferred to the wall of a 500 ml flask containing 25 ml nutrient broth (NB) (pH = 4.6). The culture was kept in the dark until the plasmodium spread on the surface of the liquid medium, and then the flask was placed on a rotary shaker (140 rpm, 27 °C), through which the plasmodium was gradually fragmented into microplasmodia. After four days, the *P. polycephalum* microplasmodia had formed. The microplasmodia were then subcultured by transferring 10 % of the growing microplasmodia to a new flask containing sterile NB.

It should be noted that microplasmodia used in this entire research was obtained from the microplasmodial culture generated from one sclerotium mentioned above. After the liquid culture was successfully established (master culture), a set of 100 ml microplasmodial cultures were prepared from the master microplasmodial culture and placed on a rotary shaker (140 rpm, 27 °C) for 4 days. One of the cultures was used as a starter culture for a new batch of microplasmodial culture and microplasmodia were collected from the rest by centrifugation. This process was done until a sufficient amount of microplasmodia were obtained for IPSs extraction and purification. It is possible to stock microplasmodial culture at –20 °C for later use, but from our observation, it will take several attempts to activate the stock culture stored in such condition to have a normal growth rate. Therefore, the best approach is to produce as much of microplasmodia as needed from the master culture once it is established and then terminate the culture. In addition, even though the *P. polycephalum* strain used in our research is axenic, there is no need of adding antibiotics in the liquid culture. However, it is also crucial to check the purity of the culture by microscopic observation to make sure it is free from contamination.

Extraction and purification of intracellular polysaccharides from *P. polycephalum* microplasmodia

Physarum polycephalum microplasmodia were collected from a 5-day-old *P. polycephalum* culture by centrifugation at 6000 rpm, for 15 mins at 25 °C (Truong et al. 2019). The IPS collecting was carried out using a process proposed in our prior study (Do et al. 2021). The obtained biomass was sonicated for cell disruption with a power of 240 W (20 s on, 10 s off) for three minutes, the cell lysate was then centrifugated at 6000 rpm for 15 mins at 25 °C, and pellet was excluded. The precipitation of the achieved supernatant was implemented with chilled ethanol (ratios 1:3, v/v) at 4 °C and left overnight. The precipitated pellet was collected by centrifuging at 8500 rpm, at 25 °C for 20 mins. The pellet, considered as crude IPS, was then dissolved in trichloroacetic acid 5 % (Merck) solution and left overnight at 4 °C for protein removal. The precipitated protein was then discarded by centrifugation at 6000 rpm at 25 °C for 5 mins. The supernatant containing IPS was dialyzed by using a 3.5 kDa molecular weight cut-off membrane (Spectrum Laboratories). The resulting polysaccharide solution was precipitated by chilled ethanol (VN-Chemsol) and

collected by centrifugation following the same procedure as described for crude IPS. The obtained pellet (deproteinated or partially purified IPSs) was then dried at 60 °C (Huynh et al. 2017).

The purification of the deproteinated IPSs by ion exchange chromatography (IEC) was carried out on a DEAE cellulose-52 column (2.4 × 20 cm). The partially purified IPSs powder (50 mg) was dissolved in 5 ml of 20 mM Tris-HCl (pH = 8.0) (HIMEDIA) and then applied to a DEAE cellulose-52 column (Su et al. 2013, Zhao et al. 2017). The samples were eluted with 20 mM Tris-HCl buffer (pH = 8.0) (Su et al. 2013) and then eluted with 20 mM Tris-HCl buffer (pH = 8.0) containing 0.3, 0.4, 0.6 and 1 M NaCl at a flow rate of 2.0 ml/min. The eluates (5ml/tube) were collected, and the carbohydrate content in each elute was determined using the phenol-sulfuric acid method (Masuko et al. 2005). Subsequently, the fractions were collected, concentrated, dialyzed, and freeze-dried (Zhu et al. 2018, Min et al. 2019). The freeze-dried fraction was stored at 4 °C (no more than two months) in order to have it available for several later tests. Three fractions collected from the IEC of the partially purified IPSs were labeled as pp0, pp0.3 and pp0.4.

Chemical characterization (carbohydrate, protein and sulphated-group contents)

The protein and total carbohydrate contents of the polysaccharide fractions were evaluated by the Bradford method (Bradford 1976) and the phenol-sulfuric acid method (Dubois et al. 1956), respectively. The sulphate group content of the sample was determined by barium chloride-gelatin method (Kawai et al. 1969).

Molecular weight of *Physarum polycephalum* IPS fractions

Number-average molecular weight (Mn), weight-average molecular weight (Mw), and polydispersity index (PI) value of the polysaccharides (pp0, pp0.3, and pp0.4) were determined using gel permeation chromatography (GPC, Agilent 1100 series system, Waters Ultrahydrogel 2000 column, Germany) with a refractive index detector following the protocol in Do et al. (2021). Each sample was prepared at the concentration of 1 mg/ml and then injected into the GPC instrument. The GPC procedure was carried out with a mobile phase consisting of 5 mM Na_2CO_3 (VWR, Belgium) and 10 mM $NaHCO_3$ (Merck), with a flow rate of 1 ml per minute. The column temperature was held at 40 °C.

The GPC system was first calibrated using a 380 kDa pullulan standard, and a random sample was selected for duplicate injection. GPC data were obtained using the add-on Rev.B.01.02 software (Agilent) program with a standard curve built from pullulan standards (Polymer Laboratories) with different molecular weights (23.7, 48, 100 and 380 kDa).

Monosaccharide composition of *Physarum polycephalum* IPS fractions

Monosaccharide composition and content of the three polysaccharides collected by IEC were evaluated by HPLC-UV on an Agilent 1200 series following the protocol proposed by Dai et al. (2010).

The polysaccharide sample was first hydrolyzed by trifluoracetic acid at 100 °C for four hours and the resulted sugar mixture was then derivatized with 1-phenyl-3-methyl-5-pyrazolone (PMP) (Sigma Aldrich). Finally, the PMP-derivatives of hydrolysate for the sample were analyzed by HPLC-UV.

Fourier-transform infrared spectroscopy (FTIR) analysis

FTIR analyses of pp0, pp0.3, and pp0.4 were done with the potassium bromide (KBr) (Merck) pellet method using a Frontier MIR/NIR spectrometer (PerkinElmer Inc., MA, United States) in a range of 400–4000 cm^{-1}. The dried samples and KBr powder were ground (1:40, w/w) and then pressed into 1 mm pellets for FTIR determination (Du et al. 2016).

Antioxidant activities IPS fractions *in vitro*

The *in vitro* antioxidant activity of the purified polysaccharides (pp0, pp0.3, and pp0.4) were evaluated using a DPPH free radical scavenging assay. The procedure followed Hassan et al. (2016). The polysaccharides were dissolved in distilled water to reach various concentrations (0, 2.5, 5, 7.5, and 10 mg/ml). A 0.2 mM DPPH (Sigma Aldrich) solution was prepared in absolute ethanol. A volume of 100 µl of DPPH solution was added to a 100 µl polysaccharide solution with a known concentration in a 96-well-plate. The reaction mixture was then shaken for 15 s on a microtiter plate shaker and kept at room temperature for 15 min in the dark. The absorbance at 517 nm of the solution was measured against the blank which consisted of the corresponding sample and ethanol. The negative control solution was prepared by mixing all reagents except the test compound. Ascorbic acid (VWR Prolabo) was used as the positive control.

In vitro antioxidant was measured by the following equation:

Scavenging activity (%) = $(A_{control}–A_{sample})/A_{control} \times 100$

whereas $A_{control}$: absorbance of the negative control (without test compound)

A_{sample}: absorbance of the test solution (with test compound)

In vitro antiproliferative activities of IPS fractions

The antiproliferative activities against two cancer cell lines (HeLa and MCF-7) and a non-transformed cell line (BAEC) of the purified IPSs was evaluated by the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay described by Liu et al. (2011). The cancer cells were seeded in 96-well microplate with a volume of 180 µl containing 5000 cells each well then incubated under the standard conditions at 37 °C and 5 % CO2 for 24 hours. The serially diluted solution of the IPS fractions in phosphate buffered saline (PBS pH7.4, 0.01 M) was added to each well with a volume of 20 µl then incubated for another 24 hours. Each well was then washed with 100 µl of PBS. After that, 50 µl of Dulbecco's Modified Eagle Medium (DMEM) (Cytiva) containing 2 mg/ml MTT (Sigma Aldrich) was injected into each well, the wells were then incubated for four hours. Finally, 200 µl of dimethyl sulfoxide (DMSO) (VWR Prolabo) was added into each well to dissolve the MTT formazan and then the light absorbance of the mixture at 540 nm was recorded. PBS and doxorubicin 50 µg/ml (LC Laboratories) were used as the negative and positive controls, respectively.

Cell viability ratio (%) was measured using the following equation:

$$\text{Viability (\%)} = [OD_{sample}/OD_{control}] \times 100 \ \%$$

OD_{sample} and $OD_{control}$ represent the light absorbtion at 540 nm of the test sample and the negative control, respectively.

Statistical analysis

The data were represented by the means ± standard deviations of three replicates. Statistical analysis was performed using one-way ANOVA using SPSS software version 25. Differences were considered significant if $p < 0.05$.

Results

The deproteinated IPSs solution from *P. polycephalum* microplasmodia was fractionated by ion exchange chromatography on a DEAE-cellulose 52 column. After loading onto the column, the initial IPSs was eluted with a gradient of NaCl concentration including 0, 0.3, 0.4, 0.6, and 1 M. The carbohydrate content of the obtained elutes was analyzed by the phenol-sulfuric acid method (Dubois et al. 1956). Carbohydrate was not detected with the fractions eluted with NaCl concentrations of 0.6 M and 1 M (data not shown). The polysaccharides, hence, were collected only from the elution of the buffer with 0 M, 0.3 M, and 0.4 M of NaCl (Fig. 1). There were three fractions obtained and they were sequentially labeled as pp0, pp0.3, and pp0.4. The yields of the three fractions were 20.71±1.56 %, 15.35±3.25 % and 38.38±4.21 % of the total IPSs

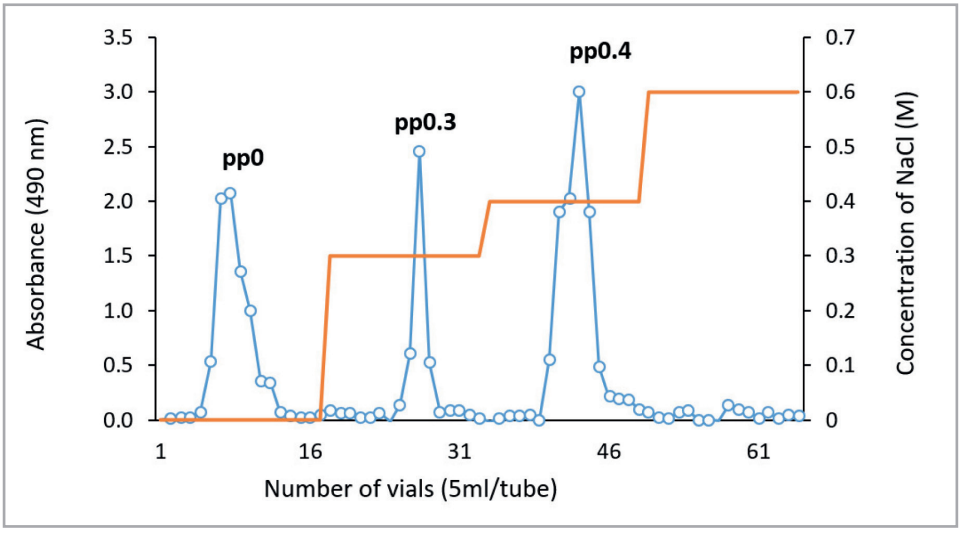

Fig. 1. Elution curve of polysaccharide fractions obtained from the deproteinated IPSs of *P. polycephalum* microplasmodia on a DEAE cellulose-52 column.

Tab. 1. Polysaccharide fractions obtained from the purification of *P. polycephalum* deproteinated IPSs by ion exchange chromatography, their mass recovery (relative to the weight of the IPSs prior fractionation), and percentages of their major chemical components.

Fraction	Mass recovery (%)	Major chemical components			Total percentages (%)
		Carbohydrate content (%)	Protein content (%)	Sulphated group (%)	
pp0	20.71±1.56	92.54±1.14[b]	0.39±0.04[a]	ND	92.93
pp0.3	15.35±3.25	72.83±4.36[a]	0.57±0.03[b]	0.066±0.014[a]	73.47
pp0.4	38.38±4.21	74.68±0.25[a]	0.31±0.02[a]	ND	74.99

ND = not detected. The different letters (a, b) imply significant differences *($p < 0.05$)*. Total percentages were calculated by adding together the contents of carbohydrate, protein, and sulfate (if any) detected in each sample.

prior to the column chromatography treatment, respectively (Tab. 1).

Chemical composition and molecular weight

The chemical compositions of purified polysaccharides are presented in Tab. 1. The carbohydrate contents of purified polysaccharide fractions are determined through the phenol-sulphuric acid method (Dubois et al. 1956). The pp0 sample yielded the highest carbohydrate content (92.54±1.14 % w/w) *($p < 0.05$)*, while pp0.3 and pp0.4 contained the similar carbohydrate contents of 72.83±4.36 % and 74.68±0.25 %, respectively (Tab. 1) *($p > 0.05$)*.

The protein contents of all polysaccharide samples were measured by the Bradford assay (Bradford 1976). For the three fractions, the protein percentage values for pp0.3 were the highest (0.57 %), significantly higher *($p < 0.05$)* than those of pp0 and pp0.4 (0.31 % and 0.39 % *($p > 0.05$)*, respectively).

Sulfated group content was also analyzed using the barium chloride-gelatin method (Kawai et al. 1969). Only the pp0.3 sample possessed a sulfated group in its composition (0.066±0.014 %).

The molecular weights (MWs) of the purified fractions of *P. polycephalum* IPS were analyzed by gel permeation chromatography and the results are shown in Tab. 2, the distributions of MWs in the three samples are presented in Fig. 2. In general, all the purified polysaccharides possessed relatively high MWs. Specifically, the three polysaccharides pp0, pp0.3, and pp0.4 had MW of 305, 573, and 1342 kDa, respectively. Moreover, the polydispersity index (PI) values of pp0, pp0.3, and pp0.4 were 1.283, 1.976, and 1.644 (Tab. 2), correspondingly.

Monosaccharide compositions and FTIR spectra

The results of monosaccharide analysis presented as HPLC-UV spectra are shown in Fig. 3. The

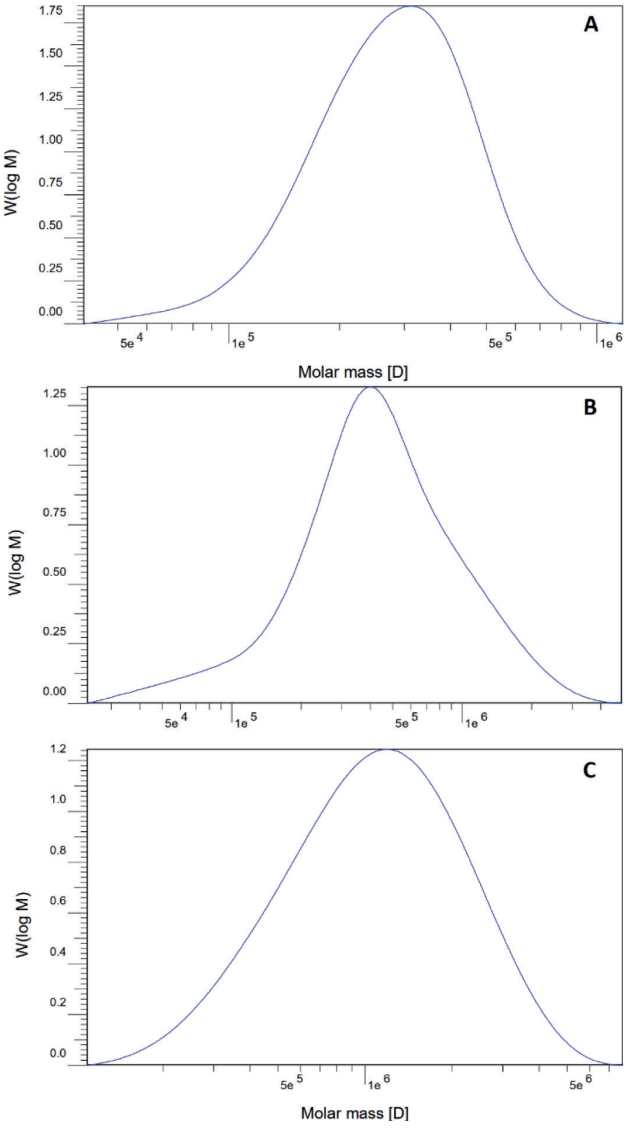

Fig. 2. Molecular weight distributions of polysaccharide molecules in pp0 (**A**), pp0.3 (**B**), and pp0.4 (**C**).

Tab. 2. Number-average molecular weight (Mn), weight-average molecular weight (Mw), and polydispersity index (PI) of purified polysaccharide molecules obtained from the deproteinated IPSs of *P. polycephalum* microplasmodia.

Sample	Number-weighted molecular weight (Mn)	Mass-weighted molecular weight (Mw)	Polydispersity (Mw/Mn)
Pp0	238 kDa	306 kDa	1.283
Pp0.3	290 kDa	573 kDa	1.976
Pp0.4	816 kDa	1342 kDa	1.644

monosaccharide content analysis pointed out that each polysaccharide fraction was composed of four or five monomer types (Tab. 3). There are three monosaccharides that exist in all samples, including mannose, glucose, and galactose. Glucose had the highest percentage in the composition of both pp0 and pp0.3, with the percentages of 99.49 % and 75.47 %, respectively. On the other hand, pp0.4 had galactose as the main monomer, with a content of 93.09 %. Fucose was found only in pp0.3 and pp0.4, with pp0.3 having a higher content (1.28 %) of this monomer, while pp0.4 contained 0.35 %. Ribose was presented only in the pp0.4 polysaccharide with a relatively low content, only 0.10 %. Similarly, arabinose existed only in the pp0 sample with a content of 0.09 %.

FTIR spectra of three polysaccharides are shown in Fig. 4. Typical bands of polysaccharide functional groups and structures are observed in their spectra.

Antioxidant activities

The *in vitro* antioxidant activities of the samples are presented in Fig. 5. In general, all samples exhibit the DPPH radical scavenging ability in a dose-dependent manner. The pp0 and pp0.4 samples possessed the highest and lowest scavenging abilities.

In the range of test concentration from 5 to 10 mg/ml, the *in vitro* antioxidant activity of pp0 was significant higher *($p < 0.05$)* than pp0.3 and pp0.4. On the other hand, there was no significant difference *($p > 0.05$)* in the DPPH scavenging capacities of pp0.3 and pp0.4 in the concentrations tested from 2.5 to 7.5 mg/ml (Fig. 5). However, the differences between the antioxidant activities of all three samples at the test concentration of 10 mg/ml were significant *($p < 0.05$)*.

The IC50 (half-maximal inhibitory concentration) value of pp0 was 5.625 mg/ml, whereas that of pp0.3 was 9.002 mg/ml and pp0.4 was greater than 10 mg/ml.

The *in vitro* DPPH scavenging activities of the purified polysaccharides were significantly lower than that of ascorbic acid (positive control) as the IC50 value of ascorbic acid is only 24.766 µg/ml. Therefore, it could be safe to state that all of the

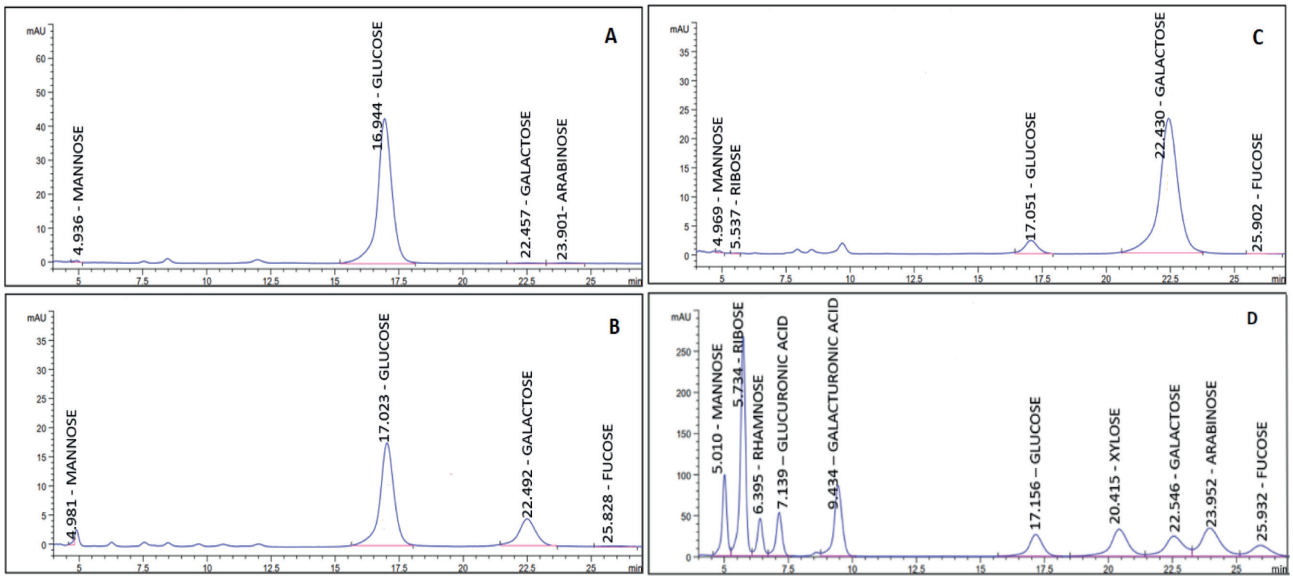

Fig. 3. HPLC-UV chromatograms of monosaccharides present in purified polysaccharide fractions obtained from *P. polycephalum* deproteinated IPSs. **A**: pp0, **B**: pp0.3, **C**: pp0.4, and **D**: standard monosaccharides.

Tab. 3. Monosaccharide composition of different purified polysaccharide fractions obtained from the purification of *P. polycephalum* microplasmodial deproteinated IPSs.

Monosaccharide composition (%)	pp0	pp0.3	pp0.4
Arabinose	0.09	–	–
Fucose	–	1.28	0.35
Galactose	0.33	23.04	**93.09**
Glucose	**99.49**	**75.47**	6.47
Mannose	0.10	0.22	0.20
Ribose	–	–	0.10

polysaccharide samples, even pp0, had a mild antioxidant activity.

Antiproliferative activities

In this part of the study, the antiproliferative activities of all the samples (pp0, pp0.3, and pp0.4) towards cervical carcinoma HeLa and breast carcinoma MCF-7 cells were evaluated *in vitro* with the test concentrations of 500, 750, and 1000 µg/ml. In addition, the cytotoxicity against a non-transformed cell line (BAEC) of the three polysaccharides was also evaluated. Doxorubicin (50 µg/ml), a multi-anticancer drug, was used as the positive control. The data obtained are presented in Fig. 6.

In general, the purified fractions obtained from *P. polycephalum* microplamodial IPSs, especially pp0 showed a dose-dependent inhibition against the two cancer cell lines. The polysaccharide pp0 showed the highest antiproliferative activity against both HeLa and MCF-7 cells. The remaining cancer cell populations in the treatments with pp0 were significantly lower *($p < 0.05$)* than those with pp0.3 and pp0.4 at the concentration of 1000 µg/ml. After 24 h incubation at 1000 µg/ml, pp0 reduced the two cancer cell populations to 54.54 % and 63.81 %, respectively (Figs. 6A-B). Pp0.4 also exhibited significant antiproliferative activity against the two cancer cell lines compared to the negative control *($p < 0.05$),* but pp0.3 only had significant activity against HeLa cells *($p < 0.05$)* (Fig. 6A). Moreover, the inhibition towards proliferation of HeLa by the pp0.4 sample was significantly higher than pp0.3 *(p value < 0.05)* for all treatments (500, 750, and 1000 µg/ml). The remained viabilities of HeLa cells in the treatments with 1000 µg/ml of pp0.3 and pp0.4 were 79.20 % and 67.71 % *($p < 0.05$)*, respectively.

The effects of three samples on MCF-7 also displayed the same pattern. However, the antiproliferative activity of pp0.4 against MCF-7 was not significantly different from pp0 at the test doses from 500 to 750 µg/ml *($p > 0.05$)*. At the test concentration of 1000 µg/ml, the viable population percentages of MCF-7 cells were 63.81 %, 89.40 %, and

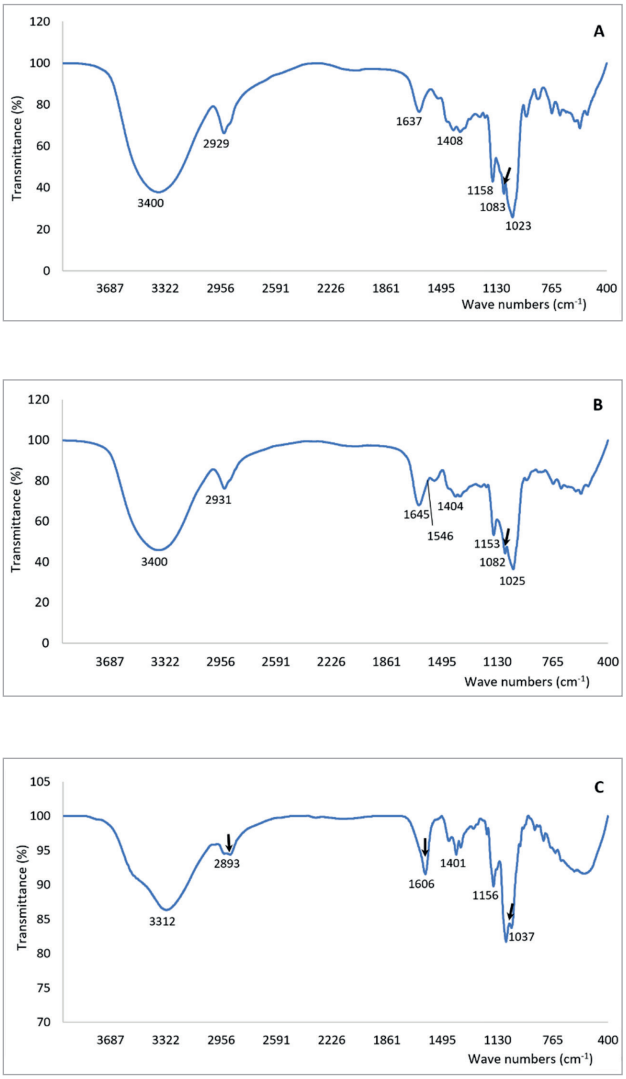

Fig. 4. FTIR spectra of purified polysaccharides obtained from *P. polycephalum* microplasmodial deproteinated IPSs. **A**: pp0, **B**: pp0.3, and **C**: pp0.4.

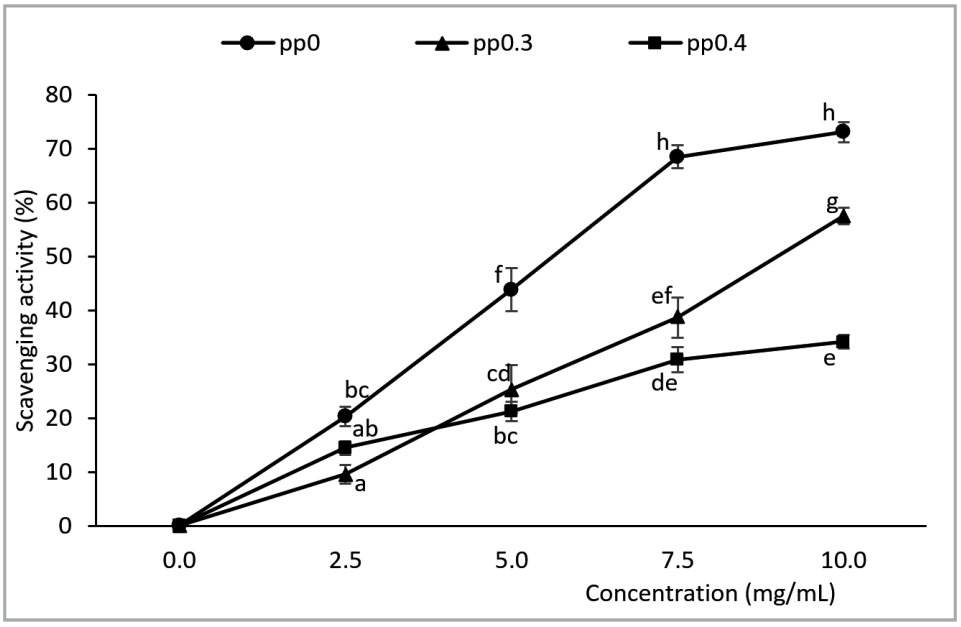

Fig. 5. DPPH scavenging activity of the purified polysaccharides (pp0, pp0.3 and pp0.4) obtained from *P. polycephalum* microplasmodial deproteinated IPSs. Different letters (a, b, c, d, e, f, g, and h) indicate significant differences *($p < 0.05$)*.

75.55 % *($p < 0.05$)*, for treatments with pp0, pp0.3, and pp0.4, respectively.

Interestingly, all three samples showed no inhibition towards non-transformed BAE cells (Fig. 6C) at every test concentration. The viability of BAECs in all treatments with pp0, pp0.3, and pp0.4 at any test concentration were insignificantly different *($p > 0.05$)* from the negative control (without polysaccharides).

The *in vitro* anticancer activities of all three polysaccharides were significantly weaker than the positive control, doxorubicin. In the treatments with 50 µg/ml doxorubicin, the remaining viable HeLa and MCF-7 cell percentages were 26.90 % and 48.83 %, which were significantly lower than those of treatments with pp0, pp0.3, and pp0.4 at any tested concentration from 500 to 1000 µg/ml. However, the cytotoxicity of doxorubicin against normal cells (BAECs) was also significantly higher than all the purified polysaccharides, with only 46.03 % of the cell population remaining viable. The repression effects of doxorubicin on MCF-7 and BAECs were comparable, with insignificant difference in cell viability values ($p > 0.05$) (Figs. 6B-C).

Discussion

Three polysaccharide molecules were obtained when the deproteinated IPSs sample from *P. polycephalum* microplasmodia was subjected to DEAE cellulose-52 chromatography. The PI values of these purified polysaccharides varied from 1.283 to 1.976 (Tab. 2), which are lower than the PI value of the deproteinated IPS sample (3.160) prior to ion exchange chromatography (IEC) (Do et al. 2021). Lower PI value indicates higher level of homogeneity of the polysaccharide (Villay et al. 2012, Yun et al. 2018) and when the PI value is equal to 1.0, the particles are monodispersed (Ge et al. 2023). This suggested the fractionation by IEC resulted in more homogenous polysaccharides than the initial sample. Among the purified polysaccharide fractions, pp0 showed the highest homogeneity since its PI value was the lowest (1.283).

The total mass recovery of three fractions were 74.44 % of the deproteinated IPSs weight before fractionation. This value is smaller than 100 %, indicating some unbound or loosely associated substances (e.g., amino acids, peptides, organic acid or microelements) that are not structurally components of the polysaccharides have been removed after the chromatography treatment. However, it should be noted that even purified polysaccharides do not consist of 100 % carbohydrate as their backbones also contain other constituents including amino acids, organic acids or metal ions (Delattre et al. 2016). This would explain why the carbohydrate content of three purified IPS fractions (pp0, pp0.3, and pp0.4) varied from 72.83 % to 92.54 %.

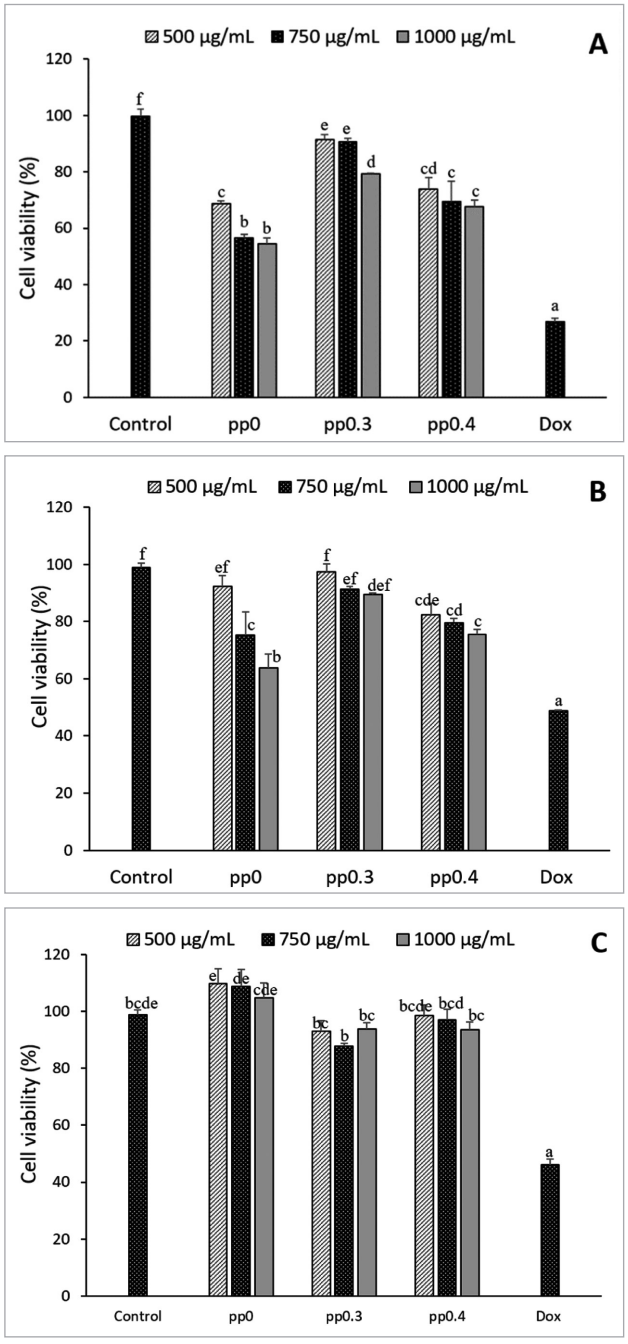

Fig. 6. Percentages of viable HeLa (**A**), MCF-7 (**B**), and BAE (**C**) cells treated with 500, 750, and 1000 µg/mL polysaccharide samples for 24 hours. Doxorubicin (50 µg/mL) and phosphate-buffered saline (pH = 7.4) were used as positive and negative controls, respectively. Different letters (a, b, c, d, and e) indicate significant differences *($p < 0.05$)*.

In this study, protein and sulfate contents were the other two chemical compositions (besides carbohydrate) that were analyzed because they often existed in the polysaccharide structure and correlate to the bioactivities of interest. The total percentage of three parameters (carbohydrate, protein, and sulfate contents) of three purified IPSs varied from 73.47 % to 92.93 %, indicating that there were unknown components present in the IPSs (Leung et al. 2009).

Regarding FTIR spectrum, a broad and intense peak occurred at 3312–3400 cm^{-1} (Fig. 4) indicating the presence of O-H stretching vibration (Gan et al. 2011). Moreover, similar peaks in the region of 1200–1000 cm^{-1} typically illustrate the vibration of C-O-H and C-O-C glycosidic bonds, typical for polysaccharides (Cui et al. 2014, Wang et al. 2015) which appeared in the spectra of all samples (Fig. 4). Among three purified IPS fractions by DEAE cellulose-52 chromatography, the pp0 polysaccharide was ascribed to be a neutral polysaccharide since it was eluted by the Tris-HCl buffer (20 mM, pH 8.0) without NaCl, while the pp0.3 and pp0.4 were attributed to be acidic as they were eluted by NaCl solutions (Gan et al. 2011). This conclusion was also further supported by the FTIR analysis since the spectra of the latter two presented the more intense typical peaks of COO^- groups (at around 1606–1645 cm^{-1}), which was not the case for pp0 (Fig. 4). Some studies have suggested that the presence of uronic acids accounts for the acidic characteristic in polysaccharides (Yang & Zhang 2009, Cui et al. 2023). However, other studies have reported that no uronic acid was detected in the composition of acidic polysaccharides (Shao et al. 2014, Min et al. 2019) when using colorimetric methods such as Dische's carbazole and m-hydroxydiphenyl (Dische 1947, Filisetti-Cozzi & Carpita 1991). Hence, the question of whether the acidic property of polysaccharides is due to the presence of uronic acids and, if that is the case, if these can be detected by regular techniques (e.g., colormetric methods) still remain unanswered.

All of the polysaccharide samples had protein in their composition based on the results of Bradford assay (Tab. 1) and FTIR analysis. The pp0.3 had the highest protein content and also exhibited the clearest peak of amino groups at 1546 cm^{-1} (Zheng et al. 2014) (Fig. 4B) in its FTIR spectrum. The deproteination step applied in the extraction of IPS might have removed the unbound proteins but not the protein content associated with the polysaccharides. Proteins were also found to exist in the composition of purified polysaccharides from mushrooms with different contents. A polysaccharide named AAP, obtained from *Auricularia auricula-judae* (Bull.) Quél. fruiting bodies, had a protein content of 0.26 % after being introduced to depro-

teination and fractionation by DEAE-52 cellulose and Sephadex G-100 columns (Bao et al. 2020). Similarly, purified polysaccharides from the gray oyster mushroom (*Pleurotus sajor-caju* (Fr.) Singer) also contained 1.08 % to 3.85 % protein (Sermwittayawong et al. 2018). The purified samples were derived from processes similar to those used in the present study, which included deproteination and fractionation by ion exchange chromatography. However, not every polysaccharide has protein in its composition. For example, purified polysaccharides from *Lentinus velutinus* and *Trichoderma* sp. were reported to have no proteins (Li et al. 2017, Udchumpisai & Bangyeekhun 2020). Thus, the presence of proteins in purified polysaccharides depends upon the nature of the polysaccharides and only free proteins might be eliminated in deproteination process as mentioned above.

With respect to the sulphated groups content, only pp0.3 contained sulfated groups with a low content (0.066 %). There have been some reports on the presence of sulphated groups in the composition of purified fungal polysaccharides. The purified samples from *Auricularia auricula-judae* (Bao et al. 2020) and *Pleurotus eous* (Berk.) Sacc. (Gunasekaran et al. 2021) contained 1.35 % and 3.02 % of sulphated groups, respectively. The presence of sulphate groups has been suggested to generate beneficial anticancer activities (Matsuda et al. 2003, Kamble et al. 2018). Jiang et al. (2015) stated that introducing sulphate groups into polysaccharides could lead to changes in their physical characteristics, chemical composition, and chain conformation, which would subsequently enhance their antitumor activities. However, in the present study, the content of sulphated groups in pp0.3 (0.066 %) might be too low to bring about noticeable anticancer activities since this sample exhibited the lowest cytotoxicity against HeLa and MCF-7 cells compared with other purified polysaccharides (Figs. 6A-B). On the other hand, two other IPS fractions (pp0 and pp0.4) did not possess sulfate content in their compositions, though their antiproliferative activities were significantly higher than pp0.3 in almost all treatments. This might suggest that sulfate groups would not be the factor contributing to the activity of the two IPSs.

In terms of monomer composition, pp0 was composed mostly of glucose (99.49 %). In addition, the absorption at 1083 cm^{-1} is a characteristic of β-glucans according to Morales et al. (2020). These clues suggested the existence of β-glucan structure in pp0. The FTIR spectrum of pp0.3 shared some similarity with that of pp0, especially the typical band at 1082 cm^{-1} (relative to β-glucans). The pp0.3 also contained a relatively high content of glucose (75.47 %); however, other monomers also occurred with significant values, especially galactose (23.04 %) (Tab. 3). Hence, this sample might also have β-glucan structure in its composition, although there also might be other types of glycan present. Also, this sample is the one that had the highest polydispersity index (PI) value (1.976, Tab. 2), which suggested that pp0.3 may not be a homogenous fraction, given that a lower PI value signifies a more homogeneous polysaccharide (Villay et al. 2012, Yun et al. 2018) and when the PI value is equal to one, the particles are monodispersed (Ge et al. 2023). In our previous study, results from ATR-FTIR and monosaccharide analysis suggested the existence of β-glucan in the structure of partially purified *P. polycephalum* IPS (ppIPS) (Do et al. 2021). Hence, it would be appropriate when considering the β-glucan content that it might have come from pp0 and pp0.3, fractions of partially purified *P. polycephalum* IPS.

As for the pp0.4 sample, the high content of galactose (93.09 %) as well as the similar FTIR spectrum to that from potato pectic galactan (González-Ayón et al. 2019), especially in the range of 3312–1401 cm^{-1} and the peak around 1037 cm^{-1} (Fig. 4C), indicated that pp0.4 was a galactan.

With respect to DPPH scavenging activity, there is an inverse correlation between molecular weight and antioxidant activity. The high antioxidant activities observed in low MW polysaccharides have been reported in several studies (Sun et al. 2009), which indicates that low MW polysaccharides have more reductive hydroxyl group terminals (in a mass unit) than high MW polysaccharides (Wang et al. 2016). The hydroxyl groups take part in the antioxidant activities by accepting and eliminating free radicals (Wang et al. 2016). In our study, the pp0 sample with highest DPPH scavenging capacity had the lowest MW (306 kDa), while the pp0.4 with the largest MW (of 1342 kDa) displayed the weakest activity (Fig. 5).

Regarding antiproliferative property, among three purified fractions, at 1000 µg/ml pp0 had significantly higher activities against both HeLa and MCF-7 than others. Notably, the antiproliferative activities towards HeLa and MCF-7 cells of three purified fractions displayed the same trend as those of the partially purified IPSs (the sample where the three fractions were obtained when it was subjected to the IEC treatment). Specifically, at 1000 µg/ml, the partially purified IPSs exhibited the stronger inhibition towards the proliferation of HeLa than

MCF-7, as it reduced the viable cell percentages of two cancer cells to 38.42 % and 81.27 %, respectively, compared with the negative control (Do et al. 2021). In the present study, all three purified fractions also showed the stronger activities towards HeLa than MCF-7 (Figs. 6A–B). The viability of HeLa and MCF-7 in the treatments with 1000 µg/ml of pp0 reduced to 54.54 and 63.81 %; 79.20 and 89.40 % for pp0.3; and 67.71 and 75.55 % for pp0.4, respectively. The purification had enhanced the antiproliferative activity against MCF-7 as the pp0 possessed the significantly higher inhibition against this cancer cell line than the initial sample. However, the toxicity towards HeLa was reduced as all the purified polysaccharides exhibited the lower activities than the sample prior to IEC purification. The purification processes were recorded to have both positive and negative impacts to the bioactivities (Liu et al. 2009, Gan et al. 2011, Li et al. 2014). Usually, the increase of bioactivities through purification processes is the expected result, since the procedures help to obtain the polysaccharide with higher purity and enhance the effect caused by the corresponding polysaccharide. However, sometimes some components that just exist in the unpurified polysaccharides were the elements contributing to the bioactivities of the polysaccharides (Liu et al. 2009), and the purifying steps had eliminated them from the polysaccharide matrix. In the present study, there might be some unknown small particles (like salts, metal ions or amino acids) that stayed in the partially purified IPS and participated in the HeLa inhibiting activity and were excluded through the purification by IEC. As such, the purification process with IEC is still needed to evaluate the bioactivities that truly come from polysaccharide structures.

Several studies have indicated that the antitumor effects of polysaccharides depend on molecular weight, chemical composition, structure of polymeric backbone, and degree of branching, along with other factors (Jin et al. 2003, Cui et al. 2007). High MW is suggested to be a necessary trait for polysaccharides to exert immunological and antitumor activities since they have more of a chance to collide and bind more receptors on the surface of immune cells (Chen et al. 2009, Ren et al. 2013). A large number of fungal polysaccharides with MW values in the range of 10^5–10^6 have been shown to exhibit antitumor activities (Ren et al. 2013). Interestingly, in this study, the MW of all polysaccharides are in the mentioned range. Hence, this might be the reason for the significant *in vitro* antiproliferative activities of almost all polysaccharides towards HeLa and MCF-7 cancer cell lines by the means of lower remaining viable cancer cell population than the negative control (Fig. 6A-B). Pp0.3 was the only case that did not show any inhibition towards MCF-7 at any concentration tested (Fig. 6B). Furthermore, pp0 was considered as the best sample, among the obtained fractions, in terms of antiproliferative activity. The recognizable difference of pp0 from the two others was the existence of a significantly higher percentage of glucose (99.49 %). The FTIR analysis even suggested the existence of β-glucan, a structure that usually exists in many antitumor polysaccharides (Meng et al. 2016). Geller et al. (2019) reported that glucans are well-established natural immunomodulators with remarkable anticancer properties. Vetvicka & Vetvickova (2020) examined anticancer activities of β-glucans from a number of different sources, including yeast, algae, bacteria, and higher fungi (mushrooms). Their results showed that almost all β-glucans expressed anticancer activities by either directly inhibiting cancer cell growth or exerting a control on cells such as natural killer cells or neutrophils to kill cancer cells once they bind with glucan receptors. In another study, Udchumpisai & Bangyeekhun (2020) isolated a polysaccharide (LVP) from the mushroom *Lentinus velutinus*. The LVP (2 mg/ml) showed an inhibition against HeLa cervical cancer and HepG2 liver cancer cells with the percentages of 22.44 % and 21.92 % after 24 h treatment, respectively. This polysaccharide shared some similar characteristics with pp0 in our research, such as the LVP being composed of only glucose subunits with a MW of 336 kDa (Udchumpisai & Bangyeekhun 2020). The mechanisms behind the antiproliferative activity of β-glucan towards cancer cells is not clearly understood. However, previous studies have suggested that β-glucan polysaccharides would strengthen phagocytosis or induce the expressions of cytokines (Meng et al. 2016) or possibly enhance cancer cell apoptosis through up-regulating of Bax and down-regulating of Bcl-2 expressions (Ma et al. 2010). Nevertheless, it should be noted that pp0.3 might also contain β-glucan in its composition, although the *in vitro* anticancer activity of this sample was the weakest among the three samples. Perhaps the monomer content of pp0.3 (galactose, fucose, and mannose) might build other unclarified structures that have negative effects on its antiproliferative activities, a mechanism which cannot be fully understood in the scope of this study. In addition, the pp0.4 sample that exhibited the mild antiproliferative activity against two cancer cell lines was considered to have a galactan structure in its

composition. There have been a few studies that have evaluated *in vitro* anticancer activities of different galactans derived from mushroom fruiting bodies against melanoma (Meng et al. 2019, Zavadinack et al. 2021). A galactan from *Amanita muscaria* (L.) Lam. at 1 mg/ml showed inhibiting capacity of 34.4 % against murine melanoma B16-F10 proliferation. Interestingly, a glucan from the same source also exhibited a similar antiproliferative activity against B16-F10 (Zavadinack et al. 2021). There is not a lot of information on the anticancer pathway of galactan. Nevertheless, a study carried out by Meng et al. (2019) pointed out that a *Cantharellus cibarius* Fr. galactan had the ability to inhibit B16 melanoma growth through its immune-regulating function.

Generally, pp0 had a broad spectrum of antiproliferative activity toward cancer cells as it could significantly inhibit both the cervical and breast cancer cells. Notably, this polysaccharide is significantly safer for normal cells when compared to doxorubicin, a multi anticancer drug. Therefore, pp0 could be a potential sample for follow-up research in cancer treatment, in which the mechanism and toxicity (if any) would be further investigated. Apart from this, as mentioned above, *P. polycephalum* microplasmodia have no cell walls and they are capable of fast growing, thus, large scale production and extraction of IPSs from this microbe is much easier than others with the presence of rigid cell walls.

Conclusions

This is the first study to use ion exchange chromatography for the purification of the IPSs obtained from *P. polycephalum* microplasmodia and to characterize the purified polysaccharide fractions. In addition, the antioxidant and antiproliferative activities of each purified polysaccharide fraction were individually evaluated. Three fractions (pp0, pp0.3 and pp0.4) which accounted for 74.44 % of the total weight of the IPSs were obtained. These purified polysaccharides had different monosaccharide compositions, and different antioxidant and antiproliferative activities. Among them, the pp0 fraction, a neutral polysaccharide with MW of 305 kDa, exhibited significantly higher antioxidant and antiproliferative activities toward cancer cells than the other fractions. Notably, the sample was non-toxic to the normal cells. This is a significant advantage compared to the positive control (doxorubicin) since this drug displayed remarkably higher toxicity toward both Hela and MCF-7 cells, but it also had a significantly higher level of toxicity toward the normal cells.

FTIR analysis suggested that pp0 would have a β-glucan structure. This sample, with its broad antiproliferative activity toward cancer cells while being nontoxic to normal cells, is worth being investigated further for its potential application in cancer treatment.

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