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1 **Antioxidant and Antiproliferative Activities of Polysaccharide Fractions from**
2 **Litchi Pulp**

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18

19 **Abstract:**

20 Three litchi polysaccharide fractions (LPFs), LP-4, LP-6 and LP-8, were obtained by
21 fractional precipitation using 40%, 60% and 80% ethanol, respectively. The

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physicochemical properties, chemical antioxidant, cellular antioxidant and antiproliferative activities of the three polysaccharide fractions were compared. LP-6 contained the highest contents of uronic acid and binding protein among the three fractions, whereas LP-8 contained the least. Amino acid composition analysis of the binding protein revealed that LP-6 contained the most acidic and aromatic amino acids. However, LP-8 contained more galactose and mannose than LP-4 and LP-6. LP-6 exhibited the highest chemical antioxidant activities, with an oxygen radical absorbance capacity of 28.14 μmol TE/g DW. LP-8 exhibited higher cellular antioxidant activity and a greater inhibitory effect on the proliferation of A549, HepG2 and MGC-803 cells at a concentration of 100−800 μg/mL than LP-4 and LP-6. In summary, the different LPFs exhibited different antioxidant and antiproliferative activities with differential physicochemical properties.

Keywords: Litchi pulp; Polysaccharides; Antioxidant activity; Antiproliferative activity

1. Introduction

Oxidative stress can cause oxidative damage to large biomolecules such as proteins, DNA, and lipids, resulting in an increased risk of cancer and cardiovascular disease.[1] The adequate consumption of antioxidants can prevent or reduce oxidative stress induced by free radicals. Fruits and vegetables contain a variety of antioxidant

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43 phytochemicals, such as phenolics, flavonoids and carotenoids, that may help protect
44 cellular systems from oxidative damage and lower the risk of chronic diseases.[2]
45 Important antioxidant compounds found in fruits and vegetables also include
46 polysaccharides, such as the antioxidant polysaccharides found in apple,[3] *Zizyphus*
47 *jujube* [4] and longan.[5]

48 The fruit litchi (*Litchi chinensis* Sonn.) originated in China and is currently
49 distributed in subtropical areas worldwide. Litchi is a traditional Chinese medicine that
50 contains many bioactive polysaccharides.[6-8] Although some studies have reported on
51 the structure and antioxidant activity of litchi polysaccharides fractions,[7, 9] the
52 methods used to assess antioxidant activity remain limited to DPPH radical
53 scavenging and ABTS assays and other chemical methods. The DPPH and ABTS
54 assays are both used to assess the antioxidant activity *in vitro* by using free radicals
55 (DPPH• and ABTS•+). However, these free radicals are not necessarily pro-oxidants
56 and they are of not biologically relevant; hence, their relevance for the assessment of
57 *in vivo* antioxidant efficacy is unknown.[10] In contrast, oxygen radical absorbance
58 capacity (ORAC) and cellular antioxidant activity (CAA) are widely recognised
59 methods for the evaluation of antioxidant activity *in vitro*. ORAC reportedly mimics
60 the antioxidant activity of antioxidants in biological systems better than other
61 chemical methods because it uses biologically relevant free radicals and integrates
62 both the time and the degree of antioxidant activity.[11] The CAA assay is used to
63 screen antioxidants and evaluate the cellular uptake, distribution, and efficiency of

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64 protection against peroxyl radicals under physiological conditions. As a cell-based
65 model, CAA represents a more biologically relevant method for determination of the
66 antioxidant activity than more commonly used "test tube" chemistry methods.[12] Thus,
67 the ORAC and CAA methods may provide more biologically relevant information to
68 elucidate the bioactivities of litchi pulp polysaccharides.

69 Oxidative stress can mediate carcinogenesis via a multistep process involving both
70 mutation and increased cell proliferation. The overproduction of cellular oxidants
71 (reactive oxygen and nitrogen species) has been linked to mutation (as induced by
72 oxidant-induced DNA damage), and the modification of gene expression.[13] Many
73 cellular oxidants alter gene expression through a variety of signalling pathways,
74 including cAMP-mediated cascades, calcium-calmodulin pathways, and intracellular
75 signal transducers such as nitric oxide, resulting in either cell proliferation or selective
76 cell death (apoptosis or necrosis).[14] We have previously demonstrated that crude litchi
77 pulp polysaccharides inhibit the proliferation of tumour cells *in vitro*;[6] however, the
78 relationship between the antioxidant and antitumour cell proliferation abilities of litchi
79 polysaccharides has not been established. Furthermore, the complex composition of
80 the crude polysaccharides has hindered their quantification and functional
81 characterisation. In the present study, we obtained litchi polysaccharides fractions
82 (LPFs) by precipitation using different concentrations of ethanol and evaluated their
83 antioxidant and antiproliferative activities against A549, HepG2 and MGC-803 cells
84 *in vitro*.

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85 The objectives of the present study were the following: (1) to investigate the
86 differences in the physicochemical properties of litchi pulp polysaccharides
87 precipitated using different concentrations of ethanol; (2) to analyse the antioxidant
88 activity of the LPFs by determining the ORAC, CAA and inhibition of tumour cell
89 proliferation; and (3) to elucidate the relationship between polysaccharide structure
90 and bioactivity.

91 **2. Material and Methods**

92 *2.1. Materials and Chemicals*

93 2.1.1. Chemicals and Reagents

94 Standard dextrans, rhamnose, arabinose, glucose, xylose, galactose, mannose,
95 penicillin-streptomycin solution, fluorescein disodium salt,
96 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox),
97 2,2′-azobis-(2-amidinopropane)-hydrochloride (AAPH), RPMI-1640 medium, new
98 bovine calf serum and Hank's balanced salt solution (HBSS) were purchased from
99 Gibco Life Technologies (Grand Island, NY, USA). All other reagents were analytical
100 grade.

101 2.1.2. Cells

102 The human hepatocellular carcinoma cell line HepG2, human gastric carcinoma
103 cell line MGC-803, and human lung adenocarcinoma epithelial cell line A549 were
104 purchased from the Experimental Animal Laboratory of Sun Yat-Sen University
105 (Guangzhou, China). The cells were cultured in RPMI-1640 medium containing 10%

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106 foetal calf serum, 100 U/mL penicillin and 100 μg/ml streptomycin at 37 °C and 5%
107 CO_2.
108 *2.2. Preparation of the LPFs*
109 The LPFs extraction procedure has been previously described.[6] Fresh litchi pulp was
110 cut into small pieces and soaked in 80% ethanol (the final concentration in the system)
111 at 4 °C for 24 h to remove pigments, monosaccharides and oligosaccharides. After
112 filtering through Whatman No. 1 paper, the residues were homogenised and extracted
113 twice with distilled water (1: 20, g/mL) at 85 °C for 4 h. The aqueous extract was
114 filtered and concentrated to one-fifth of the initial volume in a vacuum evaporator
115 (Eyela, Tokyo, Japan) at 55 °C. Proteins in the extract were removed using Sevag
116 reagent.[15] The deproteinised extract was then successively sub-fractionated by graded
117 precipitation at final ethanol concentrations of 40%, 60% and 80%. The precipitates
118 were subsequently lyophilised to obtain LP-4, LP-6 and LP-8, respectively. The litchi
119 pulp polysaccharides were stored in a desiccator at room temperature until analysis.
120 *2.3. Preliminary Characterisation of the LPFs*
121 2.3.1. Analysis of Chemical Characteristics
122 The neutral polysaccharide content was determined using the phenol-sulphuric acid
123 method[16] and expressed as glucose equivalents. The protein content was measured
124 according to the Bradford method using a bovine serum albumin standard curve.[17] A
125 modified m-hydroxydiphenyl method was used to analyse uronic acid content with
126 galacturonic acid as a standard.[18]

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127 The homogeneity and molecular weights (Mws) of the LPFs were determined by
128 gel permeation chromatography, which was performed using a Sephacryl S-300HR
129 column (1.6 × 70 cm) with a detection limit of 24 μg. Standard dextrans including T-4
130 (molecular mass, 4×10^3 Da), T-10 (1×10^4 Da), T-40 (4×10^4 Da), T-70 (7×10^4 Da),
131 T-500 (5×10^5 Da), and T-2000 (2×10^6 Da) were used as molecular mass markers. The
132 homogeneity of LPFs were further determined by size-exclusion chromatography on a
133 Sephadex G-100 column (1.6×50 cm), and eluted at the flow rate of 0.2 mL/min with
134 distilled water and detected by the phenol-H_2SO_4 colorimetric method. [19]
135 A GC-MS method was employed to identify and quantify the monosaccharides in the
136 LPFs.[6] Briefly, 40 mg of polysaccharides was hydrolysed in a sealed glass tube with 2
137 mol/L H_2SO_4 (10 mL) at 100 °C for 6 h. After neutralising the residual acid with
138 $BaCO_3$, the hydrolysate was passed through 0.2 μm syringe filters (Whatman, Sanford,
139 ME, UK), dried under a N_2 stream and mixed with hydroxylamine hydrochloride (70
140 mg) and pyridine (5 mL) at 90 °C for 60 min. Subsequently, 5 mL of acetic anhydride
141 was added, and acetylation proceeded at 90 °C for 30 min. The acetylated hydrolysates
142 were extracted with trichloromethane and evaporated under a N_2 stream. The final
143 product was analysed by GC-MS (Agilent Technologies Co., Ltd., Colorado Springs,
144 CO, USA) using a DB-1 column and an Agilent 5973 MS detector. The initial column
145 temperature was 100 °C, which was then increased to 280 °C at a rate of 10 °C/min
146 and maintained at 280 °C for 15 min; the injection temperature was 280 °C. The
147 temperature of the mass spectrometer ion source was 230 °C.

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148 The amino acid composition of the LPFs was analysed as previously described.[20]
149 Amino acids were released from the complexes by hydrolysis using 6 M HCl at 110
150 °C for 22 h in a vacuum-sealed tube, and liberated amino acids were determined using
151 an 835-50G automatic amino acid analyser (Hitachi L-8900, Tokyo, Japan).

152 2.3.2. Analysis of Fourier transform-infrared (FT-IR) Spectroscopy

153 FT-IR spectra were recorded on a Nexus 5DXC FT-IR (Thermo Nicolet, Austin, TX,
154 USA) in the frequency range of 4000−400 cm^{-1}. The samples were mixed with
155 potassium bromide (KBr) powder and pressed into a 1 mm thick pellet for FT-IR
156 measurement.

157 2.3.3. Analysis of the Helix Coil Transition

158 The helical structure of the polysaccharides was identified by characterising the
159 Congo red-polysaccharide complex.[21] Briefly, a polysaccharide solution (2 mL, 0.5
160 mg/mL) was mixed with Congo red solution (2 mL, 50 μmol/L) in a tube, and NaOH
161 solution (1 mL, final concentrations of 0, 0.05, 0.10, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40,
162 0.45 or 0.50 mol/L) was then added. Distilled water (2 mL), Congo red solution (2
163 mL) and NaOH solution (1 mL) were mixed as a control. After 10 min at room
164 temperature, the maximum absorption wavelength (λmax) of the mixture was scanned
165 at 400—600 nm.

166 *2.4. Oxygen Radical Absorbance Capacity Assay*

167 The ORAC assay was conducted as previously described by Ou *et al.*[11] with some
168 modifications. The LPFs were diluted in 75 mM phosphate buffer (pH = 7.4). The assay
169 was performed in black-walled 96-well plates (Corning Scientific, Corning, NY, USA).

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170 Each well contained 20 μL of each sample or 20 μL of the Trolox standard or a blank
171 and 200 μL of fluorescein (at a final concentration of 0.96 μM). The plates were
172 incubated at 37 °C for 20 min on an Infinite M200 Pro plate reader (Tecan Austria
173 GmbH, Salzburg, Austria). Following incubation, 20 μL of 119 mM AAPH, which was
174 freshly prepared for each run, was added to each well with the exception of the F well,
175 which was treated with 20 μL of 75 mM phosphate buffer. The fluorescence conditions
176 were as follows: excitation at 485 nm and emission at 520 nm for 35 cycles at intervals
177 of 4.5 min. The ORAC results are reported as micromoles of Trolox equivalents (TE)
178 per g of dry weight (DW). The ORAC values are presented as the means ± SD for
179 triplicate sets of data obtained from three replicates.

180 *2.5. Cellular Antioxidant Activity Assay*

181 The CAA assay was conducted as previously described by Wolfe *et al.*[12] In brief,
182 HepG2 cells were seeded at a density of 6×10^4/well in a black 96-well microplate in
183 100 μL of growth medium (DMEM medium containing 10% foetal bovine serum). The
184 growth medium was removed after 24 h, and triplicate wells were then treated for 1 h
185 with 100 μL portions of different concentrations of quercetin or litchi polysaccharides
186 plus 25 μM DCFH-DA in DMEM. The cells were then treated with 100 μL of 600 μM
187 AAPH in phosphate-buffered saline (PBS), and the fluorescence was measured in a
188 microplate reader (Tecan Infinite Pro 200, Männedorf, Switzerland) at an excitation
189 wavelength of 485 nm and an emission wavelength of 520 nm for 12 cycles at 5 min
190 intervals. The CAA results are reported as micromoles of quercetin equivalents (QE)

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191 per g of DW. The CAA values are presented as the means ± SD for triplicate sets of
192 data obtained from three replicates.

193 *2.6. Assay of In Vitro Inhibition of Tumour Cell Proliferation by LPFs*

194 2.6.1. Tumour Cell Cytotoxicity Assay

195 The cytotoxicities of the litchi polysaccharides were determined using a methylene
196 blue assay.[22] In brief, when each cancer cell line was adjusted to an appropriate
197 concentration, 100 μL of the cell suspension (4×10^4 cells/mL) was plated in 96-well
198 plates. Cells were allowed to attach for 6 h, and the medium was then replaced with
199 litchi polysaccharides dissolved in RPMI-1640 medium, followed by incubation at 37
200 °C in 5% CO_2 for an additional 24 h. The medium was then aspirated, and each well
201 was gently rinsed twice with PBS. The cells were stained and fixed by the addition of
202 50 μL of methylene blue solution (HBSS + 1.25% glutaraldehyde + 0.6% methylene
203 blue) to each well. After 1 h of incubation, the plates were rinsed by gently submerging
204 them in distilled water six times. The plates were drained and air-dried prior to the
205 addition of 100 μL of elution solution (50% ethanol + 49% PBS + 1% acetic acid) to
206 each well, followed by homogenisation for 15 min to completely dissolve the stained
207 materials. The plates were read in a microplate reader at 570 nm.

208 2.6.2. Inhibition of Tumour Cell Proliferation Assay

209 The inhibitory effects of the polysaccharides on the growth of tumour cells were
210 evaluated by a methylene blue method as previously described. HepG2, MGC-803
211 and A549 cells were plated at a density of 2×10^4 cells/mL in 96-well plates and
212 allowed to attach for 6 h. The medium was then replaced with medium containing

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213 litchi polysaccharides at final concentrations of 100, 200, 400 and 800 μg/mL. After
214 72 h of incubation, the cells were stained with methylene blue solution for 1 h. The
215 cells were then rinsed with water and dried. The methylene blue stain was eluted with
216 elution solution by agitating the plates at room temperature for 1 h. The absorbance
217 was measured at 570 nm in a microplate reader. The inhibition rate (%) was
218 calculated as follows: (1 − ODs/ODc) × 100, where ODs and ODc represent the OD
219 values of the samples and control group, respectively.

220 *2.7. Statistical Analysis*

221 Data were expressed as the means ± SD. Significant differences were evaluated by
222 one-way ANOVA followed by the Student-Newman-Keuls test using SPSS 19.0
223 software. A p-value of 0.05 was used as the threshold for significance.

224 **3. Results and Discussion**

225 *3.1. Preliminary Characterisation of the LPFs*

226 3.1.1. Chemical Composition of the LPFs

227 The yields of LP-4, LP-6 and LP-8 were 49.6%, 23.42% and 19.43%, respectively,
228 of the total mass yield of all LPFs. The homogeneity of LPFs has been evaluated by gel
229 filtration chromatography and high-performance gel-permeation chromatography
230 (HPGPC) (Fig. 1). The figures showed LPFs had single symmetrically and
231 concentrated sharp peaks, indicating that they were homogeneous polysaccharides.[19, 23]
232 The neutral sugar, uronic acid and protein contents and the Mws of each fraction are
233 summarised in Table 1. LP-6 contained a greater content of uronic acid and protein
234 than LP-4 and LP-8 ($p < 0.05$). Moreover, as the ethanol concentration increased from

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235 40% to 80%, the Mws of the extracted polysaccharides decreased, consistent with
236 previous results obtained for polysaccharides extracted from *Asparagus officinalis*[24]
237 and longan seed.[25]
238 Monosaccharide analysis revealed that the LPFs (Table 2) were all
239 heteropolysaccharides. The major monosaccharide in LP-4 and LP-6 was glucose,
240 whereas galactose was more abundant in LP-8. The percentage contributions of
241 galactose, rhamnose, arabinose and mannose increased with increasing ethanol
242 concentration. However, the glucose content gradually decreased with increasing
243 ethanol concentration.
244 Amino acid composition analysis of the LPFs (Table 3) revealed that all fractions
245 contained 17 amino acids within the binding proteins. Acidic amino acids (aspartate
246 and glutamate) were more abundant in LP-6 than in LP-4 and LP-8. In addition, LP-6
247 had the highest content of aromatic amino acids (tyrosine and phenylalanine) among
248 the three fractions.
249 3.1.2. Spectroscopic Characteristics of the LPFs
250 FT-IR spectroscopy analysis revealed that the LPFs exhibited similar IR absorption
251 profiles (Fig. 2), with characteristic polysaccharide bands such as hydroxyl group
252 bands at 3357.8 and 1048.3 cm^{-1}, alkyl group bands at approximately 2929.1 cm^{-1}, and
253 carboxyl group bands at 1654.6 and 1424.3 cm^{-1} (which are characteristic of uronic
254 acid). The absorption bands between 1100 cm^{-1} and 1000 cm^{-1} are characteristic of
255 C-O-C glycosidic bond and ring vibrations overlapping with stretching vibrations in the

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256 side groups of the C-O-H bonds. The absorption peaks at approximately 3357.8 and
257 1654.6 cm^{-1} are typical of protein IR peaks.[26]

258 3.1.3. Helical Structures of the LPFs

259 The dye Congo red can combine with helical polysaccharides, particularly
260 single-helical polysaccharides, to cause λmax redshift.[21, 27] The λmax values of
261 the litchi polysaccharide-Congo red complexes within a NaOH concentration
262 range of 0−0.5 mol/L are shown in Fig. 3. The λmax values of the complexes and
263 Congo red alone gradually decreased with increasing NaOH concentration. The
264 λmax of the LP-8-Congo red complex was nearly constant between 0 and 0.05
265 mol/L NaOH, corresponding to depolymerisation from a triple helix to single
266 helix, whereas the subsequent decreases in λmax corresponded to the change
267 from a single helix to a random coil.[28] In contrast, the λmax values of the other
268 polysaccharides and the Congo red complexes decreased continuously with
269 increasing NaOH concentration and were clearly lower than that of the
270 LP-8-Congo red complex at the same NaOH concentration, indicating that LP-4
271 and LP-6 had less organised conformations without a triple helix structure.[29]
272 The less-organised LP-4 and LP-6 fractions precipitated earlier than the
273 triple-helix LP-8 fraction, which is consistent with the tendency of
274 exopolysaccharides from the fungus *Cordyceps sinensis* fungus with an
275 expanded chain to precipitate earlier than triple-helix and spherical
276 exopolysaccharides.[30]

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277 *3.2. Oxygen Radical Absorbance Capacity of the LPFs*

278 The antioxidant activities of the litchi polysaccharides were first evaluated using
279 ORAC assays (Fig. 4). The ORAC values of the LPFs ranged from 22.08 to 28.14 μmol
280 TE/g DW, which are comparable to the ORAC values of polysaccharides from Lapins
281 cherries, cranberries[31] and *Rabdosia serra* (MAXIM.),[32] but are lower than those of
282 polysaccharides from *Tricholoma matsutake*,[33] North American ginseng[34] and
283 *Ganoderma lucidum*.[35] This discrepancy is attributable to differences in the structural
284 characteristics of these polysaccharides derived from different raw materials. The
285 ORAC values of LP-6 were higher than those of LP-4 and LP-8 ($p < 0.05$). The
286 differences in the ORAC values among the LPFs are attributable to differences in
287 their chemical features. Antioxidant activity is positively influenced by the uronic
288 acid and binding protein contents of natural polysaccharides.[36, 37] The antioxidant
289 activities of polysaccharides have also been related to their monosaccharide
290 compositions. Glucose, mannose and galactose play an important role in the
291 antioxidant activity of polysaccharides from North American ginseng.[34] The
292 differential antioxidant capacities of the LPFs can also be attributed to the amino acid
293 compositions of the binding protein, which exhibits antioxidant activity by donating
294 protons to electron-deficient radicals.[38] Aromatic amino acids, such as tyrosine and
295 phenylalanine, exhibit radical-scavenging properties by donating electrons to convert
296 radicals to stable molecules.[39] Carboxyl and amino groups in the side chains of acidic
297 amino acids exert antioxidant activities by functioning as metal-ion chelators and

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298 hydrogen donors.[40] In addition, molecular weight is also an important factor
299 influencing the antioxidant activity of polysaccharides. For example, the
300 polysaccharide fraction AOP-4 with higher molecular weights showed better hydroxyl
301 radical-scavenging activity than other fractions from *Asparagus officinalis.*[24] However,
302 Zhang *et al.* found that lower molecular weights polysaccharide from *Inonotus*
303 *obliquus* had a stronger inhibitory effect on lipid peroxidation in liver.[41] Moderate
304 molecular weight polysaccharides fraction (8–10 kDa) prepared from *Ganoderma*
305 *lucidum* showed relatively higher antioxidant activity than the other fractions with
306 lower or higher molecular weights (>10 kDa or 2.5–8 kDa).[35] It can be concluded that
307 the effects of molecular weights of polysaccharides on their antioxidant activity are not
308 consistent. In other words, we cannot rank their antioxidant activity according only to
309 their molecular weights. In this study, LP-6 (87697 Da) showed better chemical
310 antioxidant activity than LP-4 (93214 Da) and LP-8 (87062 Da). The molecular
311 weights of these 3 fractions are approximate. Especially for LP-6 and LP-8, their gap
312 between their molecular weights is less than 1 kD. Such a tiny gap is not enough to
313 account for their difference in antioxidant activity. Therefore, we do not think
314 molecular weight play an important role in resulting different antioxidant activities of 3
315 litchi polysaccharides fractions. Of the three LPFs, LP-6 contained the highest uronic
316 acid and protein contents and was rich in glucose, mannose and galactose as well as
317 acidic and aromatic amino acids. The specific composition of LP-6 may be
318 responsible for its superior antioxidant activity.

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319 *3.3. Cellular Antioxidant Activity of the LPFs*

320 The CAA assay is considered to be more physiologically relevant than chemical
321 antioxidant activity assays for the determination of antioxidant activity.[12] Therefore,
322 we used the CAA assay to further evaluate the antioxidant activity of the LPFs (Fig. 5).
323 The CAA value of LP-8 (10.79 μmol QE/g DW) was higher those of LP-4 (9.66 μmol
324 QE/g DW) and LP-6 (4.72 μmol QE/g DW), which is in contrast to the results of the
325 ORAC assays. This discrepancy is attributable to the different reaction characteristics
326 and mechanisms of the two assays. In chemical assays, the antioxidant activity of
327 polysaccharides depends on their ability to donate a hydrogen atom to a radical. The
328 hydrogen atoms provided by the polysaccharides can terminate radical chain reactions
329 and convert free radicals to non-harmful products. The presence of additional
330 electron-withdrawing groups, such as carboxyl or carbonyl groups, in a
331 polysaccharide decreases the dissociation energy of the O–H bond, resulting in the
332 increased release of hydrogen atoms and enhanced antioxidant activity.[42] For the
333 CAA assay, antioxidants must be bound to the cell membrane and/or pass through the
334 membrane to enter the cell and exert their antioxidant effects.[12] As biological
335 macromolecules, polysaccharides mediate free radical activity by interacting with
336 different receptors and/or modulating various postreceptor intracellular signalling
337 pathways.[43] The monosaccharide composition of polysaccharides has been correlated
338 with the recognition of cell surface receptors, such as the mannose receptor which
339 binded mannosyl/fucosyl ligands.[44] The ability of the hydrogen atom-donating

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340 carboxyl group in uronic acid to function as electron donors increased the antioxidant
341 activity of LP-6 in the chemical assays. LPF-6 contained more acidic amino acids such
342 as aspartate and glutamate in the binding protein than LPF-4 and LPF-8. The carboxyl
343 and amino groups in the side chains of acidic amino acids as a hydrogen donor and a
344 chelator of metal ions increased the antioxidant activity of LP-6.[38] The higher aromatic
345 amino acids (tyrosine and phenylalanine) content in LPF-6 promoted its chemical
346 antioxidant activity by converting radicals to stable molecules for donating electron.[39]
347 In contrast, LP-8 contained a higher content of galactose and mannose, which may be
348 beneficial for polysaccharides binding to cell galactose and/or mannose receptors to
349 exert antioxidant activity.[44, 45] Therefore, LPF-6 with more uronic acid, acidic and
350 aromatic amino acids exhibited better antioxidant activity in the chemical assays, while
351 LPF-8 containing more galactose and mannose showed better antioxidant activity in the
352 cellular assays.

353 3.4. *Antiproliferative Activities of the LPFs*

354 The inhibitory activities of the LPFs on the proliferation of the tumour cell lines
355 A549, HepG2, and MGC-803 are shown in Fig. 6. No cytotoxicity against the three cell
356 lines was observed at concentrations ≤1000 μg/mL LPFs (data not shown). The
357 inhibitory effects of the LPFs on the proliferation of the three tumour cell lines
358 generally increased with increasing dose at 100−800 μg/mL. The rates of inhibition of
359 the LPFs against A549 cells ranged from 10.69% to 38.1%, which are comparable to
360 the rates of inhibition observed for polysaccharides from *Angelica sinensis* (Oliv.)

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361 Diels[46] and longan.[21] The rates of inhibition of the LPFs against HepG2 cells ranged
362 from 9.34% to 40.96%, which are comparable to the rates of inhibition observed for
363 polysaccharides from *Tricholoma matsutake*[33] but are inferior to those observed for
364 polysaccharides from *Bullacta exarata* Philippi.[47] The rates of inhibition of the LPFs
365 against MGC-803 cells ranged from 4.8% to 58.86%, which are superior to the rates
366 of inhibition observed for polysaccharides from *Hedysarum polybotrys* Hand-Mazz[48]
367 but are inferior to the rates of inhibition observed for polysaccharides from the brown
368 seaweed *Sargassum pallidum.*[49]

369 The antiproliferative effects of LP-8 were significantly greater than those of LP-4
370 and LP-6 against all three cell lines at all evaluated concentrations ($p < 0.05$). The
371 greater activity of LP-8 is likely attributable to its Mw, monosaccharide composition
372 and helical structure. The ASP-3 polysaccharide from *Amomum villosum,* which
373 contains more galactose and mannose than ASP-1 and ASP-2, exhibits greater
374 antitumour cell line activity than ASP-1 and ASP-2.[50] The LBP-a4 polysaccharide
375 from *Lycium barbarum,* which has a lower Mw than LBP-a8, exhibited stronger
376 antitumor activity.[51] The antitumour activity of polysaccharides from schizophyllan[52]
377 and lentinan[53] is dependent on their triple-helical conformation and decreases with
378 progressive denaturation of the tertiary structure. LP-8, which possesses a triple-helix
379 structure, the lowest Mw, and more galactose and mannose, exhibited the strongest
380 antitumour activity among the three LPFs, which is consistent with these previous
381 observations.

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382 The level of antioxidant correlates well with the generation and malignant
383 transformation of cancer cells. Compounds that enhance the level of antioxidant
384 activity and eliminate reactive oxygen species in cancer cells may inhibit cell
385 growth.[54] LP-8 exhibited the highest cellular antioxidant activity; and also
386 demonstrated the greatest antitumour activity, which was consistent with the activity
387 of polysaccharides from *Tricholoma matsutake*[33] and *Cyclina sinensis*.[55] The
388 correlation between cellular antioxidant activity and antitumour activity against each of
389 the three tumour cell lines may indicate a similar mechanism of interaction between the
390 polysaccharides and cells for each cell line. The results of the CAA assay suggested that
391 the different polysaccharide fractions exerted their antioxidant effects through
392 interaction with different receptors and/or the modulation of different postreceptor
393 intracellular signalling pathways.[43] Similarly, polysaccharides from *Inonotus*
394 *obliquus*[56] and *Ganoderma atrum*[57] act by recognising the receptor on the cell surface
395 to trigger the MAPK cascade and mitochondrial apoptotic pathways to induce tumour
396 cell apoptosis. Ouchi *et al.* reported that galactomannan is specifically recognised by a
397 galactose receptor on the HepG2 cellular surface, and thus polysaccharides that are rich
398 in galactose exhibit high antitumour cell line activities.[45] Therefore, litchi
399 polysaccharides may trigger intracellular signalling pathways via interactions with
400 galactose receptors on the HepG2 cellular surface to exert their antioxidant and
401 apoptotic effects. Litchi polysaccharides may recognise other receptors on A549 and

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402 MGC-803 cells to induce apoptosis. The mechanism by which LPFs exert their
403 antioxidant and antitumour activities requires further research.

404 **4. Conclusions**

405 Gradient ethanol precipitation is a simple, feasible method for the isolation and
406 fractionation of litchi polysaccharides with different physicochemical properties and
407 biological activities. Compared to LP-4 and LP-8, LP-6 had the highest contents of
408 uronic acid, protein, and acidic and aromatic amino acids and exhibited the highest
409 antioxidant activities when chemically assayed. LP-8 had higher contents of galactose
410 and mannose than LP-4 and LP-6 and exhibited greater cellular antioxidant and
411 antiproliferative activities. The optimum concentration of ethanol to isolate and
412 precipitate litchi polysaccharides with possessing biological antioxidant and
413 antiproliferative activities is 80%.

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Table 1. The chemical compositions of the LPFs

	LP-4	LP-6	LP-8
Neutral sugar (%)	82.53±5.09 [b]	65.73±4.56 [a]	64.31±0.86 [a]
Protein (%)	3.34±0.15 [b]	4.95±0.09 [c]	2.10±0.16 [a]
Uronic acid (%)	6.19±0.24 [b]	7.88±0.72 [c]	1.68±0.06 [a]
Molecular weight (Da)	93214	87697	87062

Each value is expressed as the mean ± standard deviation (n = 3). Means with different letters within a row are significantly different ($p < 0.05$).

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Table 2. The monosaccharide composition of the LPFs

Monosaccharide composition (%)	LP-4	LP-6	LP-8
Ribose	0.83	1.37	0.86
Rhamnose	0.29	0.68	1.02
Arabinose	1.18	7.16	8.48
Xylose	0.75	2.93	2.89
Mannose	17.25	20.89	23.66
Glucose	75.44	43.23	29.94
Galactose	4.26	23.74	33.15

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Table 3. The amino acid compositions of the LPFs

Amino acid composition (g/100 g)	LP-4	LP-6	LP-8
Aspartate[#]	0.33	0.69	0.22
Glutamate[#]	0.59	1.18	0.31
Tyrosine[*]	0.02	0.04	0.03
Phenylalanine[*]	0.07	0.15	0.1
Histidine	0.19	0.18	0.1
Threonine	0.26	0.31	0.12
Serine	0.24	0.31	0.11
Glycine	0.16	0.28	0.11
Alanine	0.27	0.38	0.18
Valine	0.22	0.21	0.14
Cysteine	0.07	0.09	0.02
Methionine	0.22	0.21	0.14
Isoleucine	0.02	0.1	0.02
Leucine	0.09	0.05	0.08
Lysine	0.13	0.08	0.14
Arginine	0.27	0.24	0.19
Proline	0.11	0.19	0.08

represents acidic amino acids; * represents aromatic amino acids

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Figure captions

Fig. 1. Sephadex G-100 column chromatogram of LPFs from distilled water stepwise elution (A) and GPC elution profiles of the LPFs with refractive index detector (B).

Fig.2. FT-IR spectra of the LPFs. The FT-IR spectra of the LPFs were acquired over a frequency range of 4000-400 cm^{-1}.

Fig.3. The maximum absorption wavelengths of litchi polysaccharide-Congo red complexes at NaOH concentrations between 0 and 0.5 mol/L.

Fig.4. The oxygen radical absorbance capacities of the LPFs. Bars labelled with different letters are significantly different at $p < 0.05$.

Fig. 5. The cellular antioxidant activities of the LPFs. Bars labelled with different letters are significantly different at $p < 0.05$.

Fig. 6. The inhibition of proliferation of cancer cells by the LPFs: (a)A549, (b)HepG2, and (c)MGC-803. Bars labelled with different letters are significantly different at $p < 0.05$.

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Figure.1

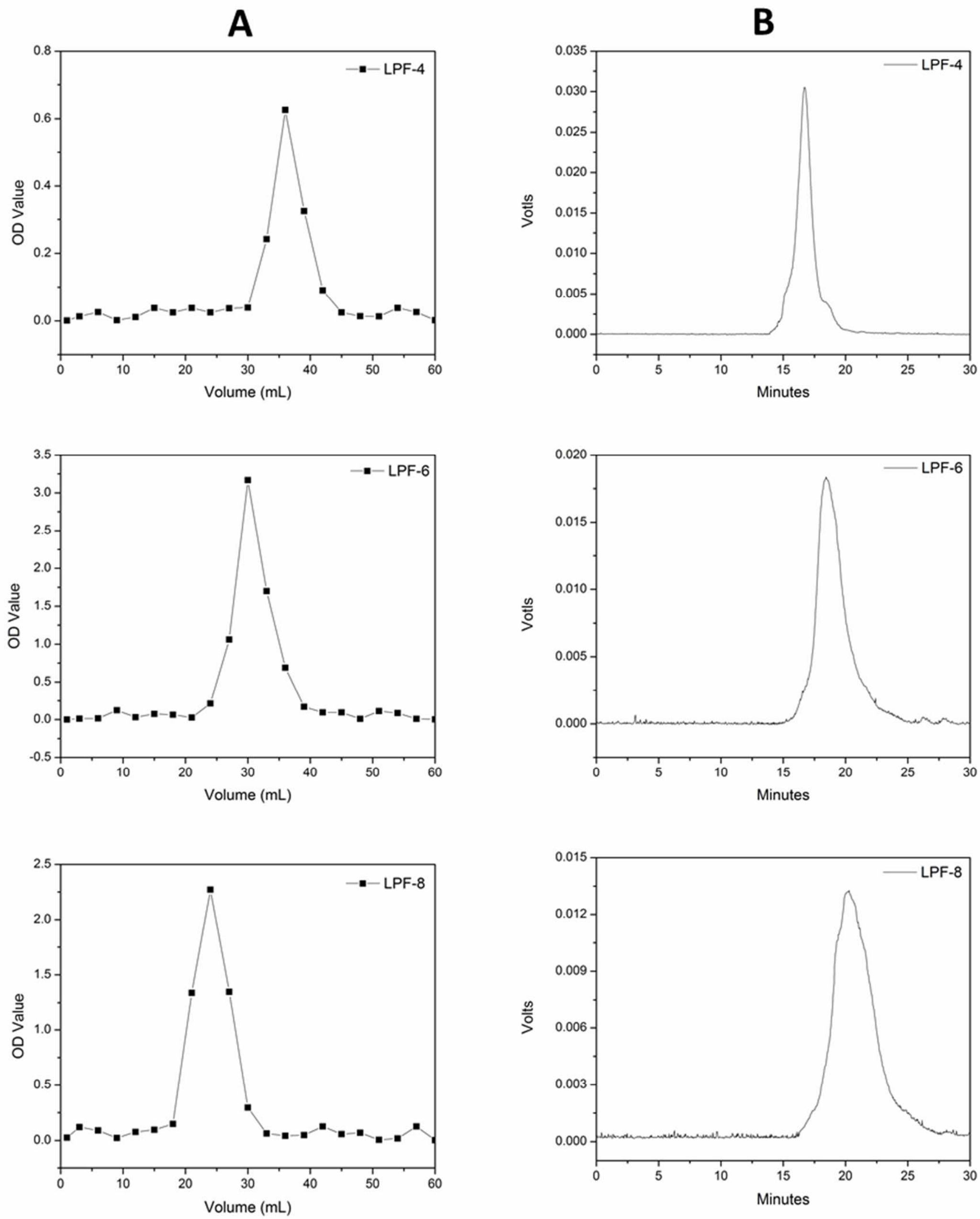

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Figure.2

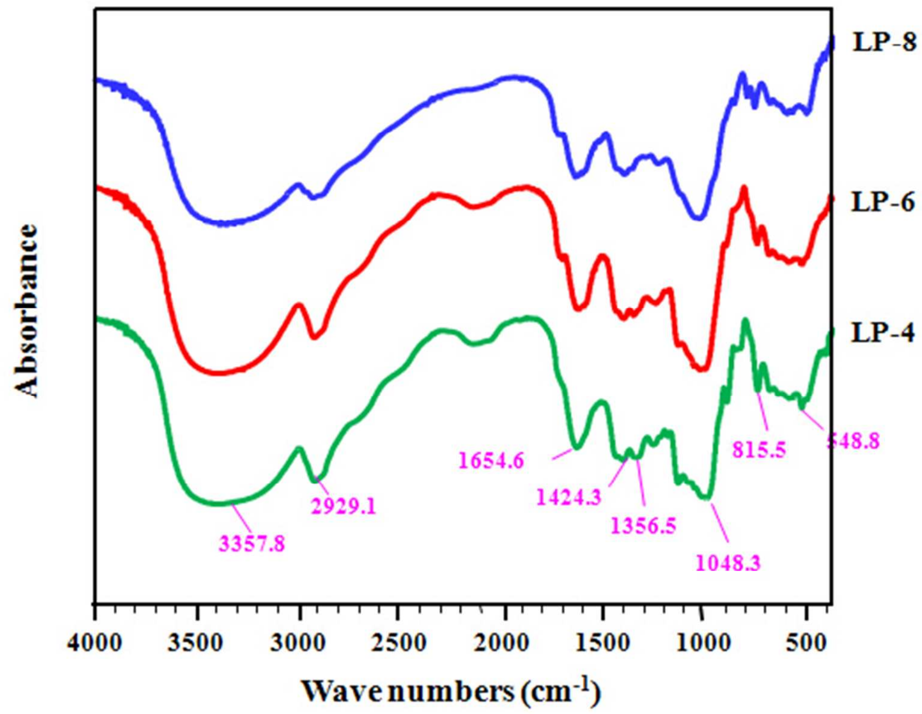

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Figure.3

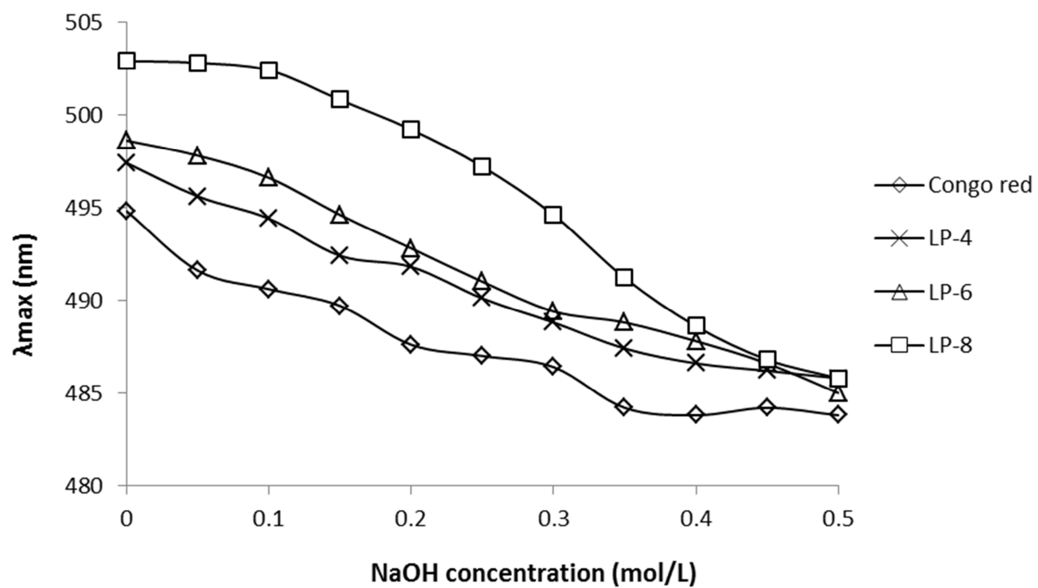

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Figure.4

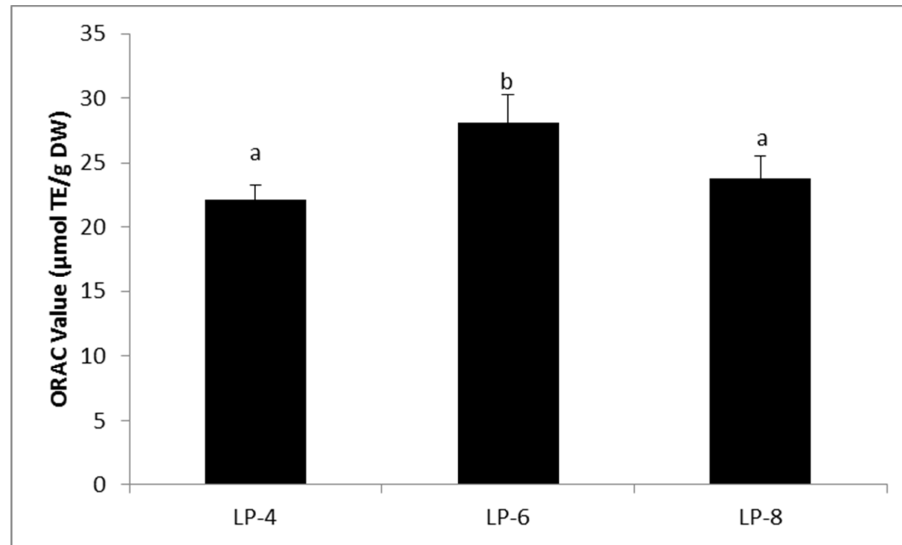

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Figure.5

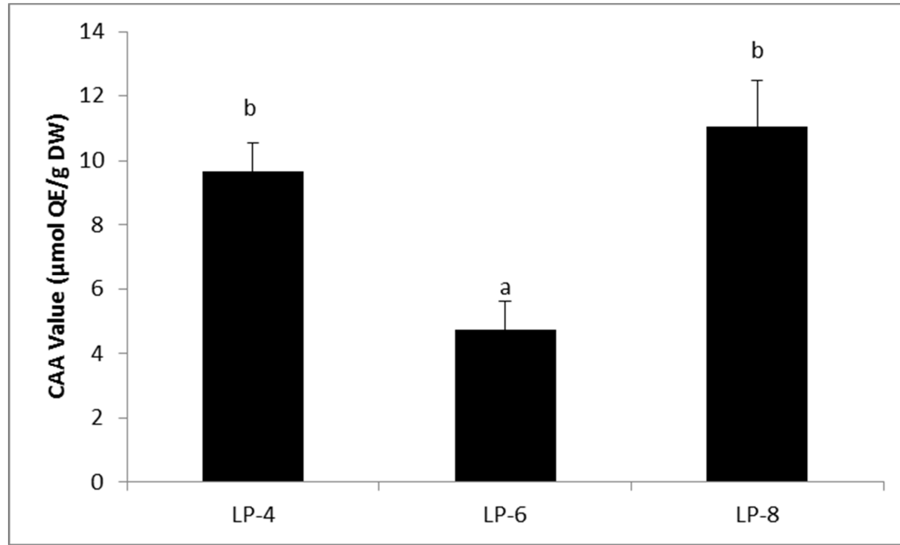

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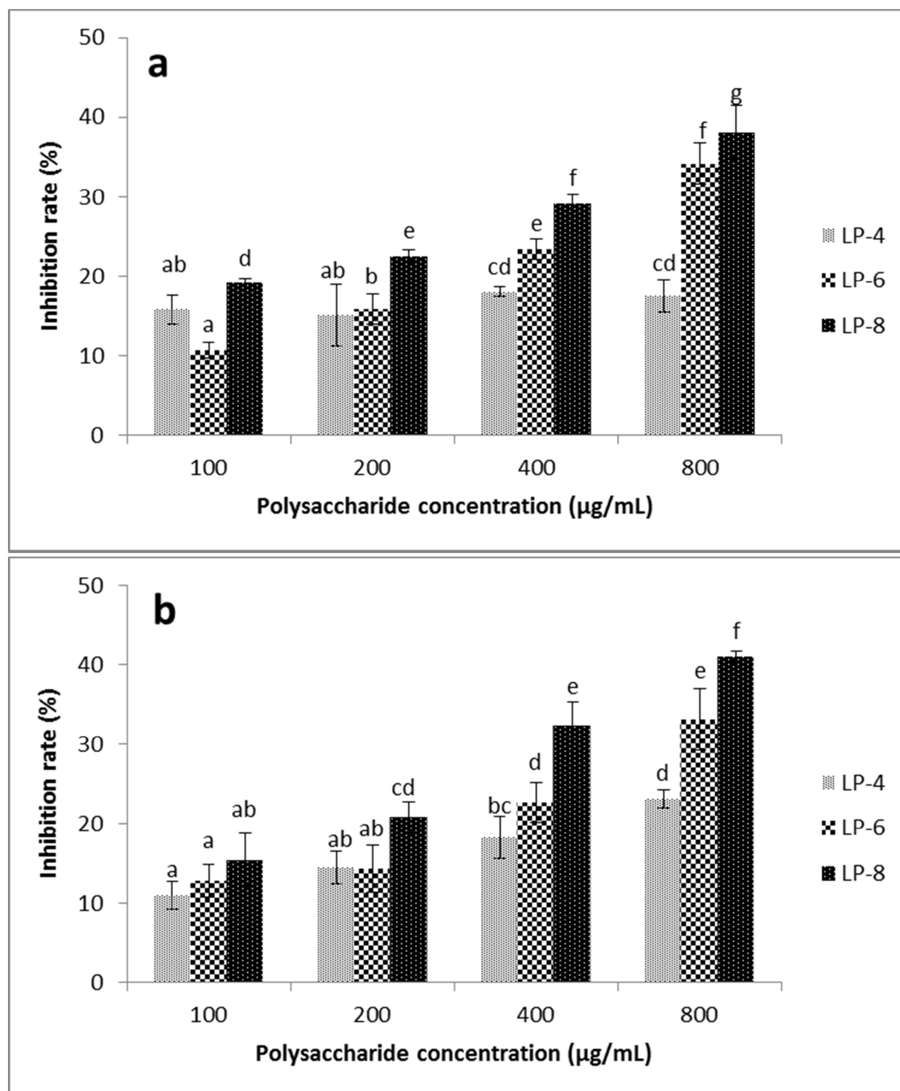
Figure.6



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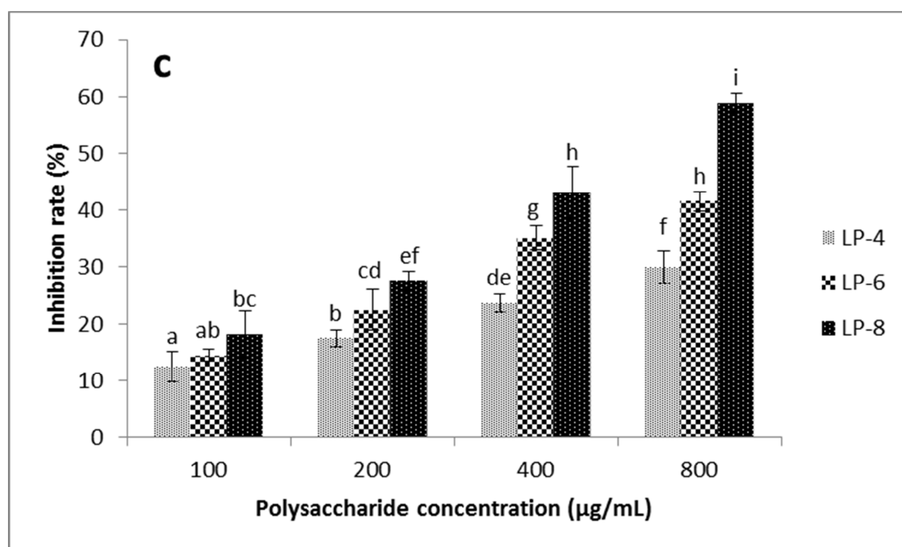

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