

1 **Antioxidant activity and hepatoprotective potential of**
2 **agaro-oligosaccharides in *vitro* and in *vivo***

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13 **Abstract**

14 **Background:** Agar-oligosaccharides derived from red seaweed polysaccharide have
15 been reported to possess antioxidant activity. In order to assess the live protective
16 effects of agar-oligosaccharides, we did both *in vitro* and *in vivo* studies based on
17 own-made agar-oligosaccharides, and the structural information of this
18 oligosaccharide was also determined.

19 **Method:** Structure of agar-oligosaccharides prepared with acid hydrolysis on agar
20 was confirmed by matrix-assisted ultraviolet laser desorption ionization time of flight
21 mass spectrometry (MALDI-TOF-MS) and NMR. The antioxidant effect of
22 agar-oligosaccharides on intracellular reactive oxygen species (ROS) was assessed
23 by 2', 7'-dichlorofluorescein diacetate. Carbon tetrachloride was used to induce liver
24 injury, some index including SOD, GSH-Px, MDA, AST, ALT were examined to
25 determine the hepatoprotective effect of agar-oligosaccharides.

26 **Results:** Agar-oligosaccharides we got were composed of odd polymerizations with
27 molecular weights ranged from 500 to 2500. Results from intracellular test indicated
28 that agar-oligosaccharides could significantly scavenge the level of oxidants in the
29 hepatocytes, more beneficially, also associated with the improvement of cell viability.
30 *In vivo* studies of the antioxidant effects on tissue peroxidative damage induced by
31 carbon tetrachloride in rat model indicated that agar-oligosaccharides could elevate
32 the activity of superoxide dismutase (SOD), glutathione peroxidase (GSH-Px) and
33 decrease the level of malondialdehyde (MDA), glutamate oxaloacetate transaminase
34 (AST), glutamic pyruvic transaminase (ALT) significantly. At 400 mg/kg, MDA level
35 reduced 44 % and 21 % in liver and heart, SOD and GSH-Px increased to highest in
36 liver and serum, while ALT level decreased 22.16 % in serum.

37 **Conclusion:** Overall, the results of the present study indicate that
38 agar-oligosaccharides can exert their *in vitro* and *in vivo* hepatoprotective effect
39 through scavenging oxidative damage induced by ROS.

40

40 **Background**

41 Liver is the main organ involved in the metabolism of biological toxins and
42 medicinal agents. Such metabolism is always associated with the disturbance of
43 hepatocyte biochemistry and generation of ROS (reactive oxygen species) [1]. Lots of
44 liver damages ranging from subclinical icteric hepatitis to necroinflammatory hepatitis,
45 cirrhosis, and carcinoma have been proved to associate with the redox imbalance and
46 OS (oxidative stress) [2]. Therefore, a potential novel approach, namely developing
47 antioxidant drugs to treat and protect liver injury and liver disease, has been proposed
48 [3]. This strategy is aimed to devise and incorporate antioxidants into the therapeutic
49 for control of viral infections or protecting body from alcohol or other toxin damage.
50 We think antioxidants are able to reduce hepatic inflammation and fibrosis, thus
51 slowing or even preventing progression to cirrhosis. One of such candidates is
52 agaro-oligosaccharides prepared from agar, which was chosen in the present study.

53 Agar was easily extracted from red algae and widely used as food and gelling
54 agent with historic record of more than a thousand years in China and Japan. In recent
55 years, agaro-oligosaccharides, derived from agarose, have been widely investigated in
56 structures and bioactivities [4-8], many beneficial health properties of
57 agaro-oligosaccharides are attributed to their antioxidant activities. For example,
58 agaro-oligosaccharides have been proved to possess antioxidative activities in
59 scavenging hydroxyl free radical, scavenging superoxide anion radical and inhibiting
60 lipid peroxidation in various chemical assays [9-11]. Enoki *et al.* [12] also reported
61 that the agarobiose shows the ability to suppress the expression of iNOS (inducible
62 nitric oxide synthase), an enzyme associated with the production of NO. In our
63 previous work, we also discussed the indirect attenuate effect of
64 agaro-oligosaccharides towards oxidation of human liver cells induced by antimycin
65 A [13]. These reports exhibited the potential prospects of agaro-oligosaccharides as
66 functional ingredient to prevent the ROS related diseases. However, no researches
67 have been done about their antioxidant effect in the *in vivo* system. Therefore, in order
68 to evaluate the ROS scavenging activity of agaro-oligosaccharides as well as possible

69 liver injury protection from OS with the respects of degree of polymerization, we
70 firstly prepared agaro-oligosaccharides with different degrees of polymerizations, then
71 use the compounds to examine the *in vitro* and *in vivo* antioxidant effects depending
72 on hepatocyte cellular assay of H₂O₂ induced damage and experimental rat model of
73 carbon tetrachloride (CCl₄) induced toxic hepatitis.

74

75 **Methods**

76 *Preparation of agaro-oligosaccharides*

77 Agaro-oligosaccharides were prepared by acid hydrolysis. In order to evaluate the
78 difference of DP of oligosaccharides on bioactivity, hydrolysis solution was
79 fractionated by activated carbon column. After loading the hydrolysate onto column,
80 the column was washed with 2 liters water to remove salts and monosaccharides.
81 Followed this step, the agaro-oligosaccharides fraction was eluted sequentially with 8
82 %, 15 % and 25 % hydroalcoholic solution. Each fraction from the column was
83 concentrated under reduced pressure and lyophilized.

84 *Structural information of agaro-oligosaccharides*

85 The average molecular weight of three fractions was measured as described by
86 Somogyi *et al.* [14].

87 The nuclear magnetic resonance (NMR) spectra were acquired on an
88 AVANCEDMX 500 NMR spectrometer. Samples were dissolved in D₂O and ¹³C
89 NMR spectra of 4% (w/v) solutions were recorded at 35 °C under 100.69 MHz.
90 Proton decoupled ¹³C NMR chemical shifts were measured in parts per million. For
91 ¹H-NMR, samples (7–10 mg) were dissolved in D₂O (0.5 ml), and spectra were
92 recorded at room temperature using a spectral width of 5.7 kHz, 90 ° pulse, an
93 acquisition time of 4.4 s for 144 scans.

94 Mass spectrometry analyses were performed on a Bruker Reflex III
95 MALDI-TOF MS (Bruker-Daltonik, Germany), operating in the delayed extraction
96 and positive mode. An accelerating voltage of 20 kV and a reflectron voltage of 22.8
97 kV were used in the measurements. 2, 5-Dihydroxybenzoic acid was used as matrix

98 (20 mg/ml; 3:2 water/MeCN) and approximately 10–100 pg of the DP-H
99 agaro-oligosaccharide mixture was deposited as a mixture together with the matrix on
100 a stainless steel target, and subsequently dried under reduced pressure. During the
101 experiments, the laser power was adjusted to a level just above the threshold for
102 formation of observable ions. The results from 20 to 100 laser shots were summed for
103 sample.

104 ***Measurement of intracellular ROS generation***

105 Intracellular oxidant stress was monitored by measuring changes in fluorescence
106 resulting from intracellular probe oxidation.

107 Human hepatocyte L-02 purchased from Chinese Institute of Biochemistry and
108 Cell Biology was cultured in RPMI-1640 medium with 20 % fetal bovine serum.
109 Viable cells (10^5 /ml) were plated into a 96-well for 1 day. On the day of the
110 experiments, after removing the medium, the cells were washed with PBS for three
111 times and then incubated with different doses of agaro-oligosaccharides in 5 % CO₂ at
112 37 °C for 2 h. After incubation, 20 μM DCFH-DA was added for another 45 min. The
113 DCFH-DA was removed by washing the cells with PBS. 100 μM H₂O₂ were added
114 into cells for 45 min and the fluorescence change was monitored by fluorescence
115 spectrophotometer at $\lambda_{\text{ex}}=475$ nm, $\lambda_{\text{em}}=525$ nm [15].

116 ***Cell viability and cytotoxicity assessment***

117 The cell viability was quantified using MTT assay. Briefly, 1×10^4 cells were
118 seeded in each well of microtiter plate and allowed to attach overnight. Cells were
119 treated with various doses of agaro-oligosaccharides for different period according to
120 the experiment purpose. For cytotoxicity test, the hepatocyte L-02 was treated for 48 h.
121 But for the detection of protective effect of agaro-oligosaccharides on H₂O₂ damage,
122 the L-02 was only treated for 2 h, and then 100 μM H₂O₂ was added for another 2 h.
123 MTT in PBS was added to each well, followed by incubation for 4 h at 37 °C. The
124 formazan crystals were dissolved in DMSO. The optical density was determined with
125 a microculture plate reader at 492 nm [16].

126 ***Animals model***

127 Mature Wistar rats weighing 150 ± 20 g were supplied by the animal center of
128 Hangzhou, China. The animals were housed in a room with a 12 h light/dark cycle at
129 about 22 °C and fed on standard diet with ad libitum access to drinking water. All
130 treatments were conducted between 9:00 am and 10:00 am to minimize variations. In
131 this study, rats were randomly divided into six groups. Group 1 (control, n=8): water
132 for 10 days followed by administration of liquid paraffin only; group 2 (CCl₄, n=8):
133 water for 10 days followed by administration of CCl₄ on the final day; group 3
134 (positive control, n=8): vitamin C (200 mg/kg) + CCl₄; group 4 to 6 (n=8):
135 agaro-oligosaccharides (200, 400, 600 mg/kg, respectively) + CCl₄. Rats were
136 injected i.p. with vitamin C or agaro-oligosaccharides for ten consecutive days. On the
137 final day, all animal except control group were administered with 20 % CCl₄ in liquid
138 paraffin at a dose 5 ml/kg to induce hepatotoxicity. Previous studies demonstrated that
139 the OS indexes could reach a maximum at 48 h after CCl₄ i.p. administration [17],
140 therefore, in this work rats were sacrificed by collecting the blood from the carotid
141 artery after 48 h of administration. Two organs (liver and heart) were excised
142 immediately.

143 ***Biochemical assays***

144 Serum was separated by centrifugation at $1000 \times g$ at 4 °C for 10 min. 10 %
145 organ homogenates including liver and heart were prepared in ice-cold isotonic
146 physiological saline. The GSH-Px, MDA, SOD, AST and ALT levels of tissue and
147 serum were measured by spectrophotometric methods as described in the assay kits.

148 ***Statistical analysis***

149 All data are expressed as mean \pm SD. In cell based assay, the control and
150 agaro-oligosaccharides treated cells were compared by student *t*-test. In animal assay,
151 the statistical tests were one-way ANOVA followed by post-hoc Newman-Keuls
152 multiple comparisons test. A probability level of 0.05 was considered statistically
153 significant.

154 **Results**

155 *Preparation and structure analysis of agaro-oligosaccharides*

156 Activated charcoal column has been performed as saccharide isolation tool for
157 decades. Depending on this technology, we successfully achieved to isolate three
158 fractions of agaro-oligosaccharides with average molecular weight of 619, 1126 and
159 1631, respectively, eluted by 8 %, 15 % and 25 % aqueous alcoholic solution. We use
160 these three fractions for the following experiments, designated as DP-L, DP-M and
161 DP-H, according to their differences in molecular weight.

162 Since agar is a linear copolymer of galactose (G), alternated with 3,
163 6-anhydrogalactose (A), the structural difference of agaro-oligosaccharides are mainly
164 related with the degree of polymerization. In this report, the $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$
165 spectra of agaro-oligosaccharides was studied using acid hydrolyzed fragments, and
166 typical deshielded $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ signals corresponding to the anomeric
167 hydrogens and carbons were obtained and presented in Fig. 1. Assignments were
168 based on the close similarity with literature values, and the interpretation of these
169 signals was indicated in Table 1. The spectra give out twelve distinctive major
170 anomeric carbon signals which were expected for the major disaccharide repeat unit.
171 The presence of these signals demonstrates the presence of floridean starch in this
172 fraction, because all of the signals illustrated the galactose ring structures present in
173 seaweed galactans [18, 19]. The ^{13}C NMR spectrum of oligosaccharide are very
174 consistent with those previously published for neoagarose series, with chemical shifts
175 of carbons of unit G'-1 α and G'-1 β appeared at identically 92.4 and 96.4 ppm, having
176 intensities in the ratio of 1 : 2. While, from the result, we didn't observe any signal for
177 3,6-anhydrogalactose at the non reducing ending (a peak at 91.4 ppm) [20-22]. These
178 results reflect the presence of galactose units at the reducing ends of the reaction
179 products, but no 3,6-anhydrogalactose at non-reducing end.

180 We applied MALDI-TOS-MS in order to know the structural information of
181 composition and DP in the agaro-oligosaccharide fractions which were obtained from
182 hydrolysis. The results shown in Fig. 2 indicated that a large number of well-regulated

183 peaks are present, and these agaro-oligosaccharide ions could be identified as series of
184 sodium molecular ions with relatively high intensities corresponded to 509, 815, 1121,
185 and so on. It can be seen, by comparing these ions, that the molecular mass difference
186 between every two adjacent ion is the same as 306 Da. This molecular mass
187 difference of 306 Da is the exact molecular mass of agaro-biose (GA), the basic
188 structural unit, which is 324 Da, minus 18, which is the number of H₂O's molecular
189 weight, therefore, it is clearly observed that the agaro-oligosaccharides had very
190 regular molecular structures with gradient increase of its chain length with the
191 polymerization unit of agarobiose. Further calculation for m/z 509, the lowest high
192 intensity ion observed in the mass spectrum, found that m/z 509 corresponds to the
193 sodium adduct of agarotriose (GAG) [M_{tri}+Na]⁺. Based on this information, the ion at
194 m/z 815, 1121, 1427.... corresponds to agaro-oligosaccharides for n=5, 7, 9...,
195 respectively with galactose at both reducing end and non-reducing end. For
196 agaro-oligosaccharides, two forms of saccharides exist depending on the end sugar
197 moiety, namely, neoagaro-series with 3, 6-anhydro-galactose at the non-reducing end
198 and agaro-series with galactose at the non-reducing end. The results obtained here
199 indicated that our sample obtained belong to agaro-series with odd numbers of sugar
200 unit.

201 *The antioxidant action of agaro-oligosaccharides in cell based assay*

202 We firstly investigated the antioxidant activities of agaro-oligosaccharides in the
203 cellular system. DCFH-DA, which can be converted from non-fluorescence into
204 fluorescence through oxidation, was used as fluorescent probe to monitor the changes
205 of oxidative stress in hepatocyte L-02 induced by addition of H₂O₂. In our experiment,
206 all the measurements were carried out at the steady stage (incubation time, 60 min) in
207 order to minimize variations, because it has been reported that treatment of H₂O₂ will
208 lead to the abruption of ROS in few minutes, and then decrease to a steady stage [23].

209 Fig. 3 showed that addition of agaro-oligosaccharides caused
210 concentration-dependent attenuation of DCF fluorescence. Three groups of
211 agaro-oligosaccharides showed almost no inhibitory activity at the 125 µg/ml. When

212 the concentration increased, DP-H expressed highest activity, followed by DP-M,
213 much weaker for DP-L group, which indicating that the antioxidant bioactivity *in vitro*
214 improves with the higher degree of polymerization of agaro-oligosaccharide. Fig. 4 is
215 a typical fluorescent microscopic picture of the DCF fluorescence in hepatocyte L-02
216 treated with DP-H. It is obvious that H₂O₂ lead to the production of ROS, which
217 transformed the DCFH into DCF (Fig. 4 D), showing more fluorescent cells than
218 untreated cells (Fig. 4 A). DP-H additions decreased the free radical formation. Fig. 4
219 B clearly illustrated that DP-H at concentration of 1 mg/ml could inhibit the oxidation
220 of DCFH significantly. While with the concentration decreased to 125 µg/ml (Fig. 4
221 C), the number of fluorescent cells was also increased, and which means that the
222 antioxidant activity of agaro-oligosaccharides acts in a concentration-dependent
223 manner.

224 ***Protective effect of agaro-oligosaccharides on oxidative stress injury***

225 Oxidative stress is an important factor to induce the cell death. Cell viability
226 assay showed that the presence of H₂O₂ (100 µM) resulted in cell death ratio
227 increasing to 60 % after 2 h of treatment (Fig. 5). Compared to H₂O₂ alone, cell death
228 was reduced obviously when exposed to each agaro-oligosaccharide group at the
229 higher concentrations (from 500 µg/ml to 1 mg/ml). The cell viability significantly
230 increased to 64.26 % for DP-M treated cells at the concentration of 1 mg/ml (Fig. 5).
231 At low concentrations (125 µg/ml to 500 µg/ml), there was almost no variation
232 observed between the agaro-oligosaccharide treated cells and the control, except
233 DP-M treated group showing some weak cell protective effect. These results
234 demonstrated that the antioxidant activities of agaro-oligosaccharides were positively
235 correlated with the improvement of the cell viability.

236 In order to test whether agaro-oligosaccharides affected the growth of human
237 hepatocyte L-02 without H₂O₂ treatment, cell proliferation was assessed by direct
238 MTT assay. The cells were incubated with various amounts of agaro-oligosaccharides
239 for 48 h, and the change in cell number was determined (Fig. 6). From result, we
240 found that the agaro-oligosaccharides exhibited very slight effects on the cell growth.

241 After 48 h of treatment, the growth is slightly inhibited as of 14.18 % for DP-H at 1
242 mM, while for DP-M and DP-H, the corresponding cell proliferation ratio was >100
243 % with concentration at 250 μ M. Therefore, the cell survival effect of
244 agaro-oligosaccharide alone in the antioxidation cellular assay can be considered
245 almost naught because the cells were only treated for 2 h, so we concluded that
246 agaro-oligosaccharides can effectively protect the cells from oxidation induced death
247 through scavenging intracellular oxidative damage induced by ROS.

248 ***Effect of agaro-oligosaccharides on an acute CCl₄ oxidative damage***

249 We further studied the *in vivo* antioxidant effects of agaro-oligosaccharides. It
250 was not uncommon that compounds possessing *in vitro* activity, however, fail to
251 maintain the activity when administrated into body. We established an oxidative
252 animal model by CCl₄ injection. Considering the proliferation and antioxidant effects
253 of agaro-oligosaccharides on hepatocyte, we used the mixture of DP-M and DP-H as
254 our sample for animal test. The effects of agaro-oligosaccharides on oxidative stress
255 in rats were estimated by determining the activities of MDA, SOD, GSH-Px, ALT and
256 AST in serum and tissues.

257 MDA level is a main marker of endogenous lipid peroxidation [24]. In CCl₄
258 treated group, the MDA level increased significantly in liver (F=2.087, P<0.05), but
259 little difference was observed in serum, which confirmed that the toxicity of CCl₄ is
260 focused in the liver. By contrast, MDA level in the agaro-oligosaccharides treated
261 groups decreased significantly compared with CCl₄ treated group. At 400 mg/kg, the
262 MDA level reduced at least 44 % and 21 % in liver (F=4.274, P<0.05) and heart,
263 respectively, versus the CCl₄ treated group. Actually, the MDA level of
264 agaro-oligosaccharides treated groups showed almost the same as the blank control
265 group (Table 2). It provided the information that exhibiting a very successful block of
266 lipid oxidation.

267 SOD and GSH-Px are intracellular antioxidant enzymes that protect against
268 oxidative process [25]. As show in Table 3 and 4, a single high dose injection of CCl₄
269 induced severe oxidative damage and the SOD and GSH-Px level decreased markedly.

270 While various concentrations of agaro-oligosaccharides could effectively normalize
271 the enzyme activities and the two indexes were even higher than Vitamin C group. In
272 liver and serum, the SOD level reached to highest at 400 mg/kg (F=3.878, P<0.05;
273 F=9.363, P<0.05). Similar results were obtained in case of the GSH-Px activities.

274 Serum levels of transaminases (ALT, AST) were used as indicators to evaluate
275 the attribution of agaro-oligosaccharides to the structure damage of the liver [26, 27].
276 In this experiment, the enzyme assays of serum transaminases showed that a toxic
277 dose of CCl₄ significantly raised the levels of ALT and AST to 687 (F=3.761, P<0.05)
278 and 415 U/l (F=4.204, P<0.05). Agaro-oligosaccharides could inhibit the enzyme
279 activities effectively. The ALT level reached to minimum when the sample
280 concentration was 400 mg/kg (22.16 % less than the control group). For AST, the
281 agaro-oligosaccharides reduce it in a dose dependent manner. At the highest
282 concentration (600 mg/kg), AST level decreased to 222 U/l. However, it is strange to
283 find that Vitamin C didn't reduce AST but raised it to 32 % versus the control without
284 CCl₄ treatment. (Fig. 7).

285

286 **Discussion**

287 Among therapeutics for liver diseases, protective drugs have been attracted more and
288 more attentions, such as antioxidant prevention approaches. In this paper, we focused
289 on the *in vitro* and *in vivo* antioxidative activities of agaro-oligosaccharides with the
290 model related with liver disease.

291 Agaro-oligosaccharides are linear oligomers cleaved from agar which is built of
292 1,4-linked 3,6-anhydro- α -L-galactose alternating with 1,3-linked β -D-galactopyranose.
293 When agar is attacked by degradation reagents, such as hydrolysis enzyme, acid or
294 alkali, numerous possibilities for combination, viz., the repetition of AG, GA, AGA, or
295 GAG, etc will exist. In this research, depending on NMR and MALDI-TOF-MS
296 analysis, we detected the precise structural features of our hydrolysate. NMR results
297 give us information that our product is agarose structure, furthermore, there was no
298 signal of A at reducing end. In the spectrum of MALDI-TOF-MS, the first high

299 intensity peak observed at m/z 509 was assigned to $(M_{tri}+Na)^+$ containing two
300 galactopyranose (Galp) residues and one 3,6-anhydrogalactopyranose (AnGalp)
301 residues, followed by a series of agaro-oligosaccharides: agaropentaose,
302 agarohexaose, agaroseptaose, agarooctaose, and so forth. In our case, the agaro-oligosaccharides
303 with odd polymerization degree were dominant.

304 For the *in vitro* antioxidant studies, we noticed that agaro-oligosaccharides expressed
305 different antioxidant abilities with different ranges of DPs. In them, the fraction of
306 DP-H with average MW of 1631 showed highest free radical scavenging activity
307 which agrees well with the result obtained by Zhao *et al.* [10]. However, Enoki *et al.*
308 [12] found, in a different assay system, that agarobiose possessed the highest ability to
309 inhibit the expression of iNOS. Therefore, comparison of structure-bioactivity *in vitro*
310 for different studies should be careful bearing different assays in mind.

311 It is quite significant that the *in vivo* animal experiment for agaro-oligosaccharides is
312 quite consistent with the *in vitro* assays. Besides successful protection of liver damage
313 by efficiently inhibiting MDA formation and decreasing AST and ALT,
314 agaro-oligosaccharides enhance the activities of antioxidant enzyme system of the
315 host, including SOD, GSH-Px. We also notice that vitamin C only slightly reduced
316 AST and ALT level in rats in our experiment, although it prevented MDA formation
317 effectively (Fig. 7). The result indicates that agaro-oligosaccharides have better
318 impact to improve the hepatoprotective ability. Since antioxidant enzymes such as
319 SOD and GSH-Px are considered to be a primary defense system for oxidative
320 damage prevention, agaro-oligosaccharides exert antioxidant not only through its own
321 radical scavenging activity, but also, by boost the host antioxidant enzyme system. On
322 the other hand, we found that when the sample concentration increased from 400
323 mg/kg to 600 mg/kg, several indexes showed a different change. At concentration of
324 600 mg/kg, the MDA level increased slightly and SOD, GSH-Px and AST activities
325 reduced a little. This result implied that excessive administration of
326 agaro-oligosaccharides will decrease their antioxidant ability with unknown reasons.

327 In conclusion, by carefully examining the antioxidant protective effects of

328 agaro-oligosaccharides both *in vitro* and *in vivo*, the agaro-oligosaccharides prepared
329 via solid acid hydrolysis showed consistent and concentration-dependent
330 antioxidation activities, as well as significant protection against liver injury.

331 Conclusion

332 **Conclusion**

333 These results support a beneficial relationship between antioxidant activity and
334 hepatoprotective effect of agaro-oligosaccharides which belong to agaro-series with
335 odd numbers of sugar unit as their dominant composition.

336

336 **Abbreviations**

337 **A:** 3, 6-anhydrogalactose;

338 **DP:** degree of polymerization;

339 **DP-H:** Degree of Polymerization-High, representing the experiment group of the
340 agaro-oligosaccharides with average molecular weight of 1631, eluted by 25 %
341 ethanol from the charcoal column;

342 **DP-L:** Degree of Polymerization-Low, representing the experiment group of
343 agaro-oligosaccharides with average molecular weight of 619, eluted by 8 % ethanol
344 from the charcoal column;

345 **DP-M:** Degree of Polymerization-Middle, representing the experiment group of
346 agaro-oligosaccharides with average molecular weight of 1126, eluted by 15 %
347 ethanol from the charcoal column;

348 **G:** galactose;

349 **MDA:** malondialdehyde;

350 **MW:** molecular weight;

351 **OS:** oxidative stress;

352 **ROS:** reactive oxygen species

353

354 **Author's contribution**

355 HMC have been involved in drafting the manuscript.

356 XJY have made substantial contributions to conception and design.

357 ZP carried out the animal experiment.

358 LJ participated in the cell biology research.

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363

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432 **Figure Legends**

433 **Figure 1. ¹H-NMR and ¹³C-NMR spectra of solid acid hydrolysate.**

434 **Figure 2. MALDI-TOF mass spectrum of agar hydrolysate. (m/z 400-2700)**

435 **Figure 3. Effect of agaro-oligosaccharides on DCF fluorescence in hepatocytes.**

436 Values expresses as mean ± SD. n=6. * P<0.05, ** P<0.01, vs control cells without
437 sample

438 **Figure 4. Inhibition of intracellular oxidant by agaro-oligosaccharides.**

439 (A) Control without H₂O₂, (B) 1 mg/ml 25 % ethanol eluted fraction, (C) 125 µg/ml
440 25 % ethanol eluted fraction, (D) Positive control.

441 **Figure 5. Effect of agaro-oligosaccharides on cell survival during H₂O₂
442 exposure.**

443 Values expresses as mean ± SD. n=6, * P<0.05, ** P<0.01, vs control

444 **Figure 6. Effects of different concentrations of agaro-oligosaccharides on cell
445 proliferation after exposure of cells for 48 h.**

446 Values expresses as mean ± SD. n=3

447 **Figure 7. Effect of agaro-oligosaccharides on AST and ALT activity in serum.**

448 Values expresses as mean ± SD. n=8, # P<0.05, vs Normal group. G4, G5 and G6:
449 group 4, 5, 6 which administrated with sample of 200mg/kg, 400mg/kg and 600mg/kg,
450 respectively

451

451 **Table 1 Chemical shift assignments for ¹H-NMR and ¹³C-NMR spectra of**
 452 **agaro-oligosaccharides**

Unit		Chemical shifts (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Carbon	G ^a	102.3	70.6	82.3	68.7	75.2	61.2
	A ^b	98.1	69.9	79.7	77.1	75.5	69.4
Proton	G	4.39	3.79	3.6	4.12	3.55	3.63 ^c /3.67 ^d
	A	4.97	3.96	4.37	4.48	4.4	3.84 ^e /4.06 ^f

453 a galactose.

454 b anhydrogalactose.

455 c A-6exo proton

456 d A-6'endo proton

457 e G-6 proton

458 f G-6' proton

459

460

460 **Table 2 Effect of agaro-oligosaccharides on MDA activity in different organs of**
 461 **CCl₄ induced rats ^a**

Groups	Liver (nmol/mg prot)	Heart (nmol/mg prot)	Serum (nmol/ml)
Normal control	2.75±0.51	0.56±0.08	4.20±0.22
CCl ₄ control	4.62±0.77 [#]	0.68±0.05	4.44±0.64
Vitamin C	2.59±0.02 [*]	0.67±0.11	3.53±0.74
G4 (200mg/kg)	3.45±0.77	0.54±0.11	3.33±0.11
G5 (400mg/kg)	2.71±0.18 [*]	0.53±0.14	3.36±0.63
G6 (600mg/kg)	2.99±0.47	0.45±0.02	2.82±0.66

462 ^a n=8. Each value represents the mean ± SD.

463 Significant values: *P < 0.05 (vs CCl₄ group); [#]P < 0.05 (vs Normal group).

464 G4, G5 and G6: group 4, 5, 6 which administrated with sample of 200mg/kg,
 465 400mg/kg and 600mg/kg, respectively.

466

466 **Table 3 Effect of agaro-oligosaccharides on SOD activity in different organs of**
 467 **CCl₄ induced rats ^a**

Groups	Liver (U/mg prot)	Heart (U/mg prot)	Serum (U/ml)
Normal control	34.18±2.45	46.80±2.84	313.77±24.01
CCl ₄ control	26.97±6.69 [#]	27.71±2.26 [#]	306.89±19.29
Vitamin C	33.11±2.79 [*]	32.75±1.73	318.35±16.39
G4 (200mg/kg)	33.45±2.87 [*]	34.37±1.34	361.08±9.37 ^{*#}
G5 (400mg/kg)	38.64±8.44 [*]	37.33±2.45	365.53±21.13 ^{*#}
G6 (600mg/kg)	35.42±2.86 [*]	40.49±2.21 [*]	364.92±14.21 ^{*#}

468 ^a n=8. Each value represents the mean ± SD.

469 Significant values: *P < 0.05 (vs CCl₄ group); [#]P < 0.05 (vs Normal group).

470

470 **Table 4 Effect of agaro-oligosaccharides on GSH-Px activity in different organs**
 471 **of CCl₄ induced rats ^a**

Groups	Liver (NU/mg prot)	Heart (NU/mg prot)	Serum ($\times 10^3$ NU)
Normal control	159.17 \pm 6.97	200.20 \pm 15.46	12.50 \pm 1.44
CCl ₄ control	91.60 \pm 3.97 [#]	191.81 \pm 36.90	10.38 \pm 1.48 [#]
Vitamin C	119.41 \pm 9.86	204.86 \pm 17.11	11.02 \pm 0.66
G4 (200mg/kg)	120.50 \pm 17.05	203.63 \pm 25.01	12.18 \pm 1.95
G5 (400mg/kg)	127.19 \pm 12.17 [*]	217.40 \pm 10.82	13.13 \pm 1.21
G6 (600mg/kg)	118.92 \pm 17.56	248.47 \pm 39.28	12.26 \pm 1.30

472 ^a n=8. Each value represents the mean \pm SD.

473 Significant values: *P < 0.05 (vs CCl₄ group); [#]P < 0.05 (vs Normal group).

474

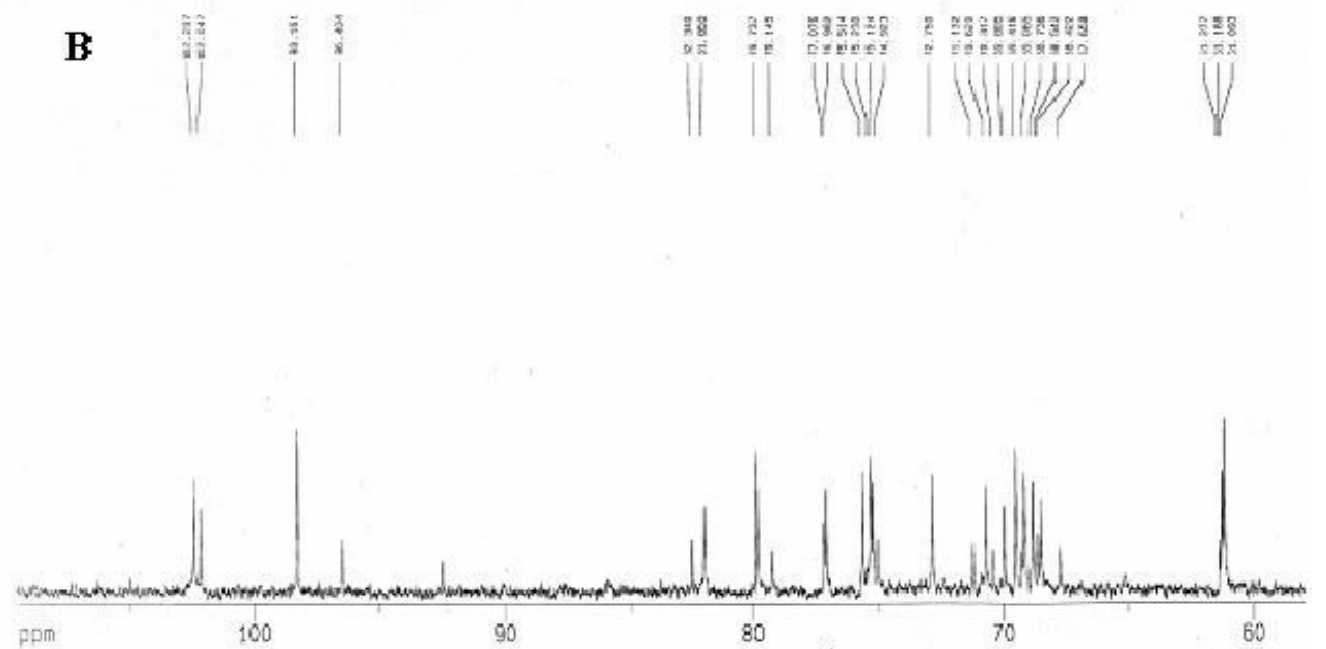
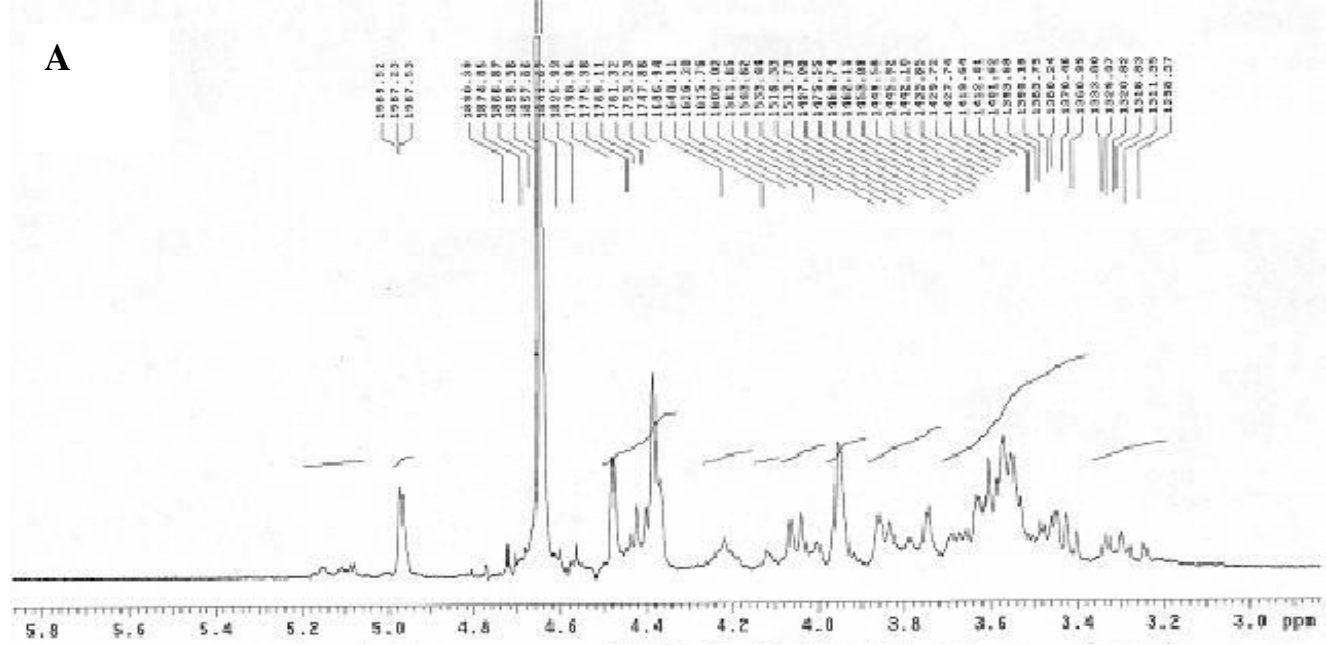


Figure 1

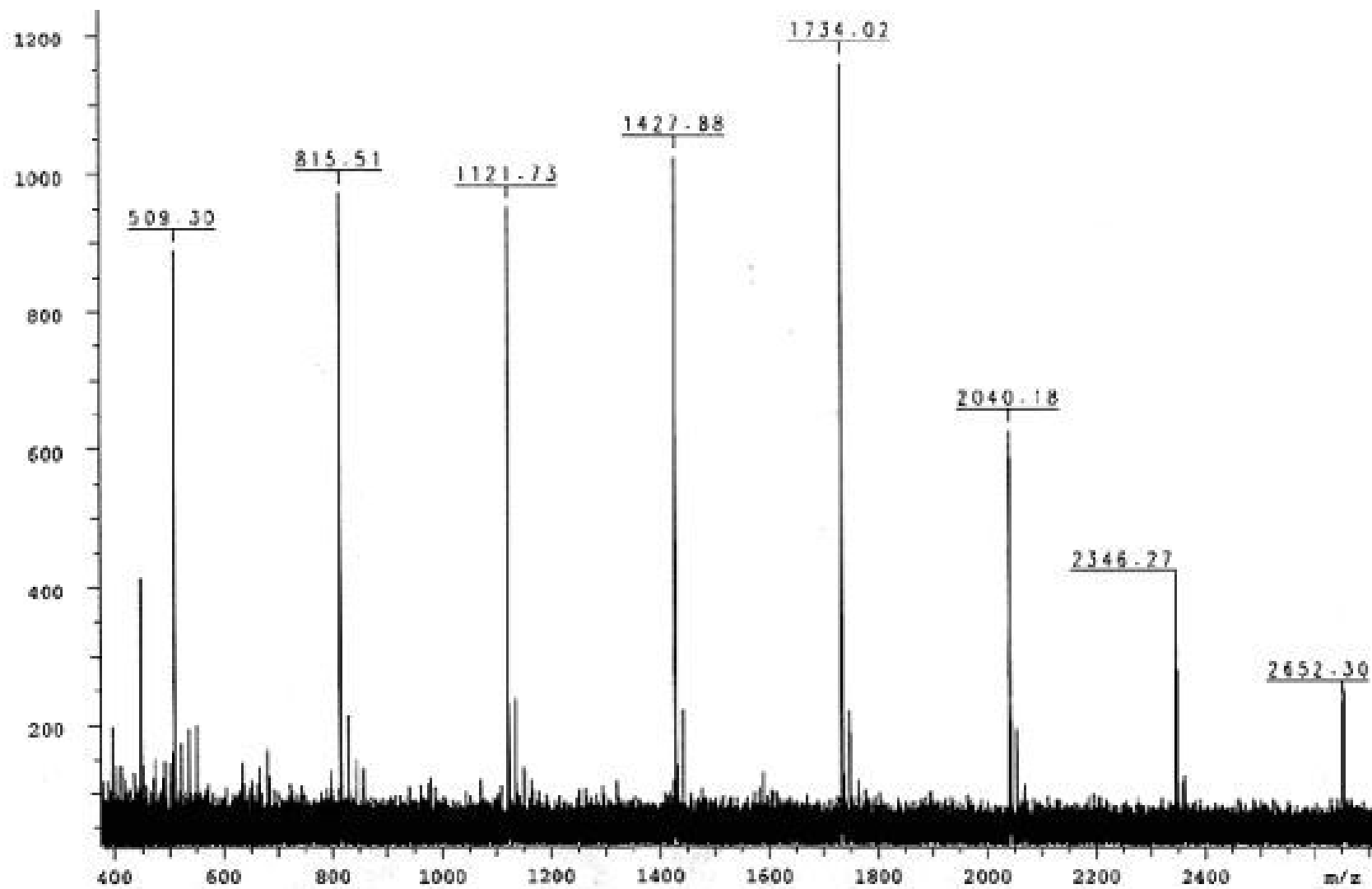


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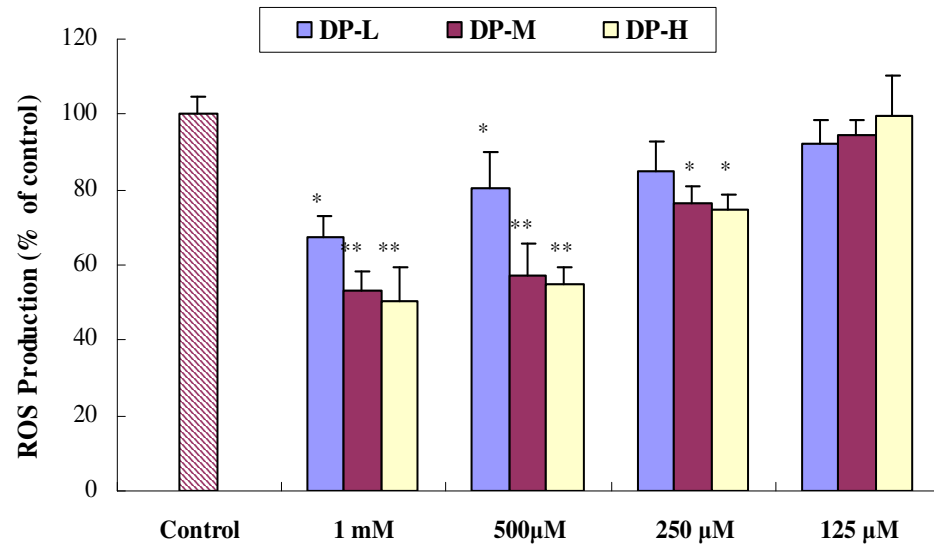


Figure 3

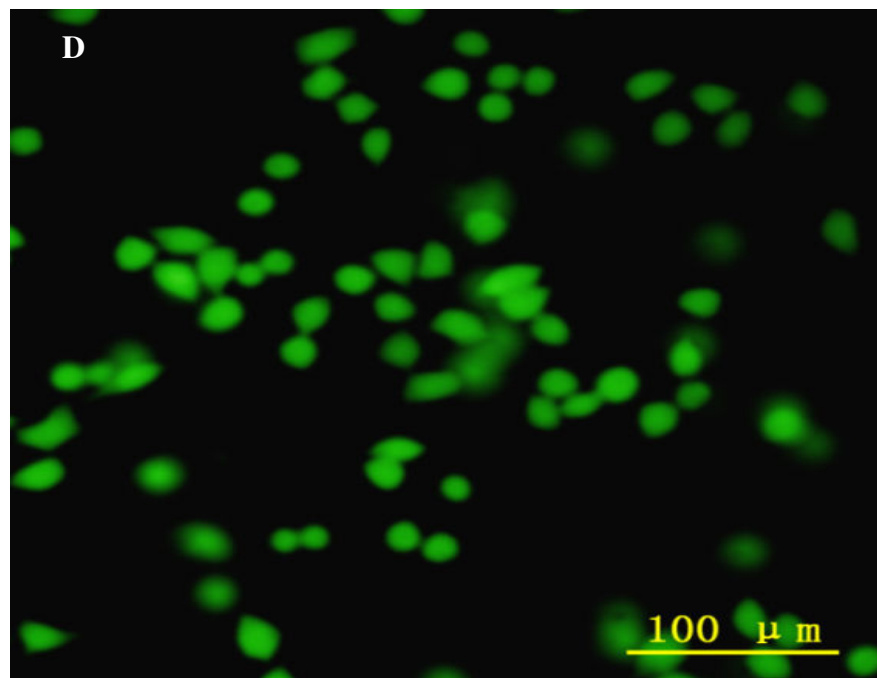
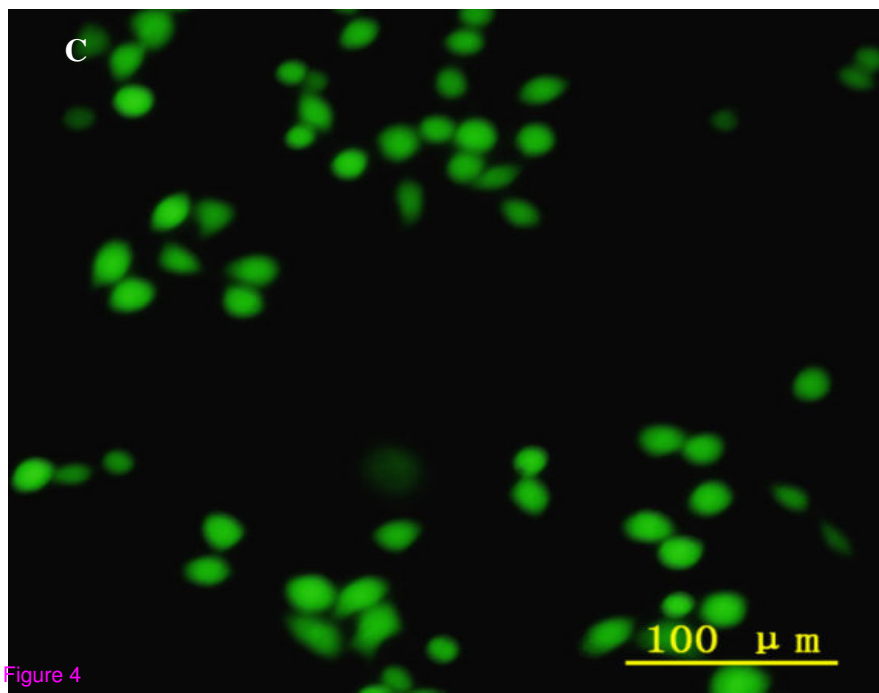
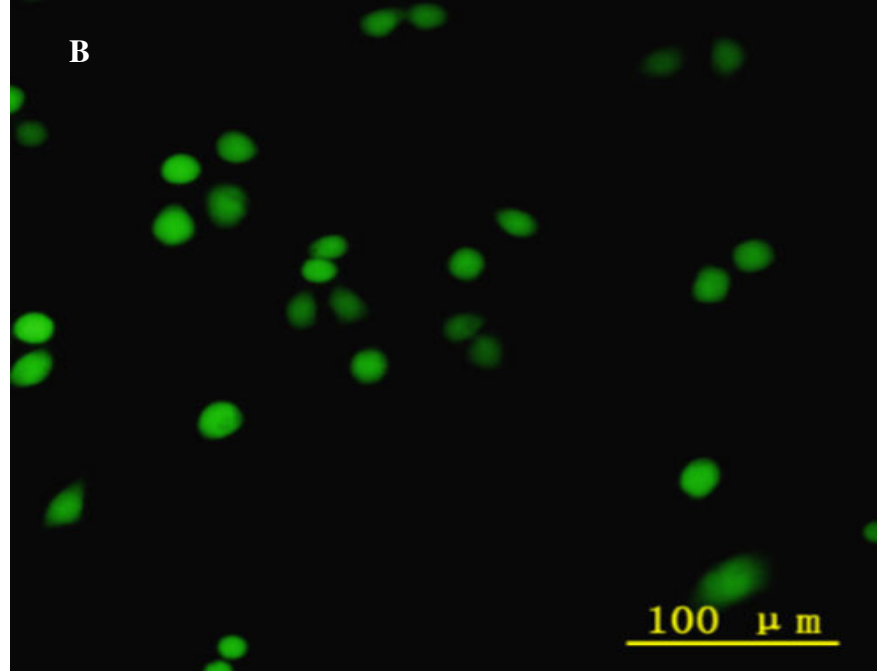
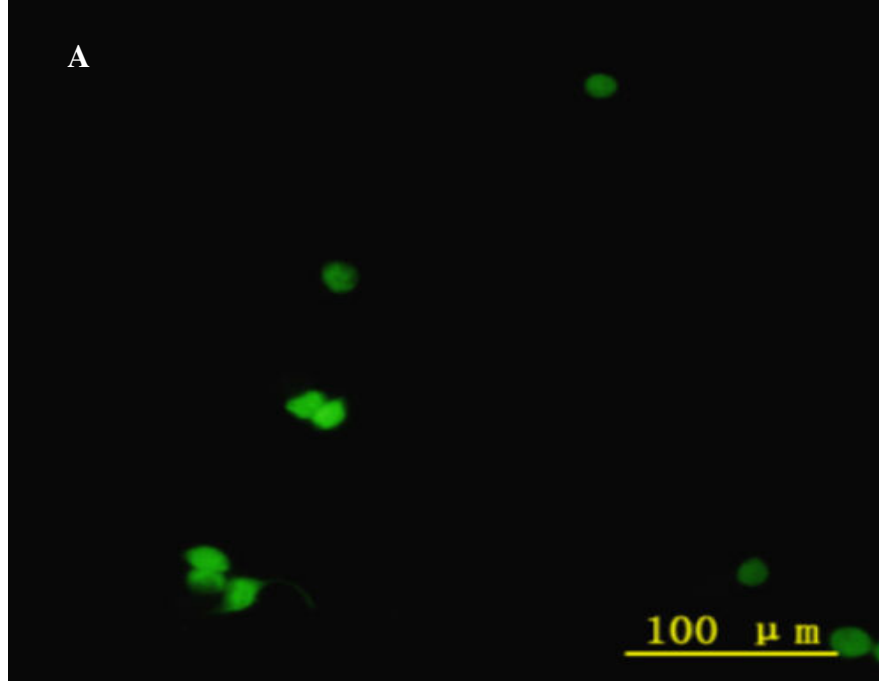


Figure 4

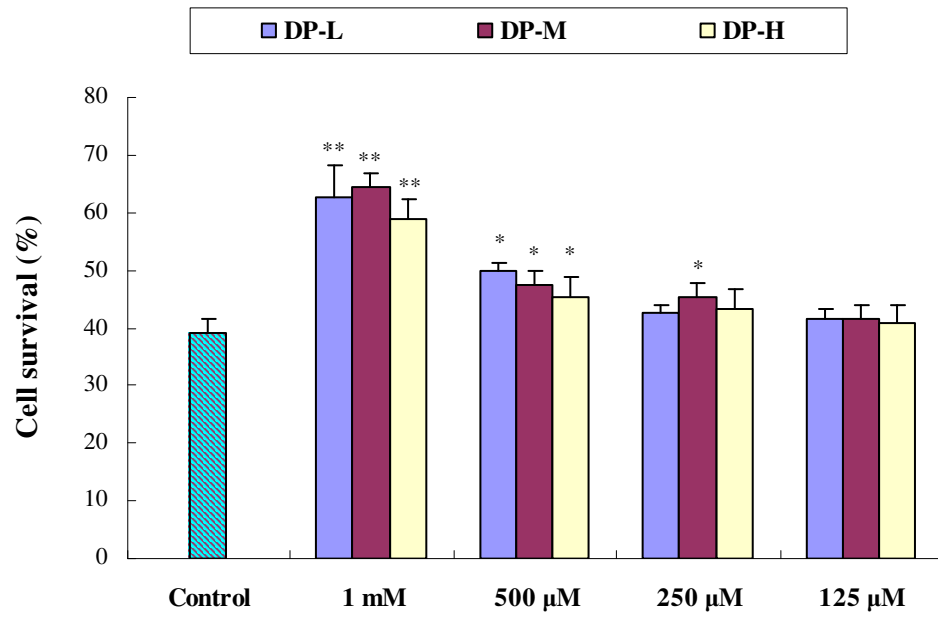


Figure 5

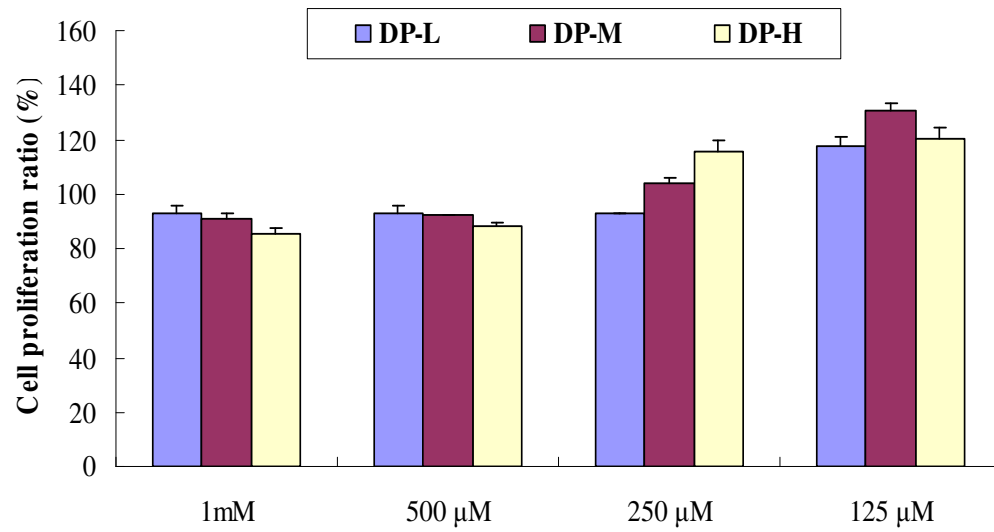


Figure 6

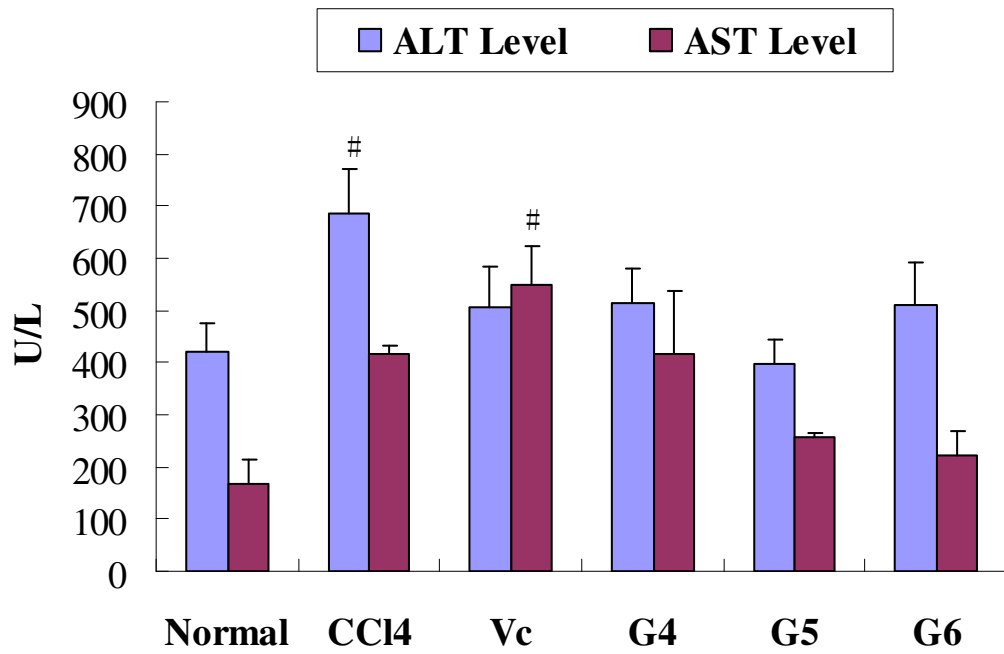


Figure 7