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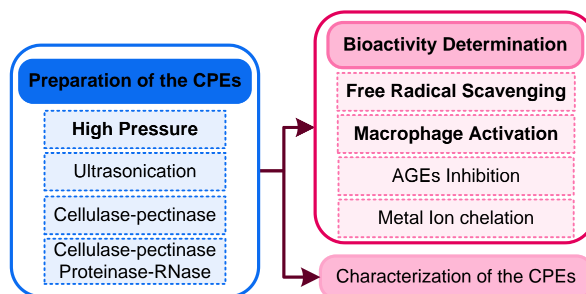

Hot water extracts of *Chlorella pyrenoidosa* prepared under high pressure scavenged free radical and promoted macrophage growth significantly *in vitro*.

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1 **A comparison on preparation of hot water extracts from**
2 ***Chlorella pyrenoidosa* (CPEs) and radical scavenging and**
3 **macrophage activation effects of CPEs**

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16 Running title: Radical scavenging and macrophage activation effects of CPE

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17 **Abstract**

18 Development of hot water extract of *Chlorella pyrenoidosa* (CPE) may help to reduce
19 costs of *C. pyrenoidosa*-based biofuels production, while the extraction conditions of CPE
20 vary greatly, and CPE-related research develops slowly. To find an effective preparation
21 method, the present study prepared CPEs using high pressure, ultrasonication, and
22 enzymolysis, and compared the yields, general ingredients, and bioactivities of those
23 products. The yields of high pressure and enzymolysis methods were more than 25%.
24 CPE-a prepared under an extra 0.1 MPa pressure in an autoclave was rich in protein, and
25 it had the strongest absorbance at 260 and 280 nm, whereas CPE-c and CPE-d prepared
26 under enzymolysis of cellulase and pectinase had higher sugar contents. The CPEs had no
27 inhibitory influence on formation of advanced glycation end products, and their metal
28 chelating activities were poor. However, all the products had significant positive effects
29 on free radical scavenging and macrophage growth promotion *in vitro*. Hydroxyl radicals
30 were scavenged in a concentration-dependent manner by CPE-a, and the cultured
31 macrophage Ana-1 proliferated to 162.98% of the control when CPE-a was administrated
32 at 200 μg mL^{-1}. Furthermore, phagocytic activity and intracellular nitric oxide levels of
33 Ana-1 were significantly enhanced with administration of CPE-a. Taken together, our
34 results suggest that hot water extraction with high pressure is an effective method for
35 preparing high value-added bio-products from *C. pyrenoidosa*, which has strong potential
36 for use in free radical scavenging and macrophage activation.

37 **Key words**: *Chlorella pyrenoidosa*; hot water extracts; radical scavenging; macrophage
38 activation

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39 **1 Introduction**

40 Traditional fossil energy sources are under threat of exhaustion, while liquid fuels are
41 important and hardly to be replaced in the near future, so there is an urgent need for an
42 alternative fuel source. Production of biofuels from microalgae is promising in terms of
43 energy conservation and CO_2 emission reduction, and has attracted widespread attention
44 from the public.[1] However, implementation of biofuels remains commercially challenging
45 because of the large demand for biomass and the high costs. *Chlorella pyrenoidosa* is one
46 of the microalgae that can potentially be used for biofuels production. We previously
47 developed a novel strategy called 'sequential heterotrophy-dilution-photoinduction' for
48 large-scale production of *C. pyrenoidosa* for biomass and lipid production.[2] Now, we are
49 committed to develop high value-added co-products of *C. pyrenoidosa* to lower the costs
50 of biofuels production.

51 *Chlorella* is nutritious and safe,[3] and has become a popular functional food around the
52 world in recent years. *C. pyrenoidosa* is a new resource food announced by the Ministry
53 of Health of the People's Republic of China in late 2012.[4] *Chlorella*-based products such
54 as tablets, drinks, and extracts are sold in the market, one of the most famous being the
55 '*Chlorella* growth factor' (CGF).[5, 6] Reports of health benefits of 'CGF' abound in
56 commercial media or are spread as anecdotal reports from Japan; for example, 'CGF' was
57 found to be effective at promoting growth and extending the lifespan of tested animals.[7, 8]
58 In Japan, 'CGF' is regarded as the substance that makes *Chlorella* a precious and healthy
59 food,[5] and its market price is high. 'CGF' is a mixture of water-soluble substances, which
60 may remain in the non-lipid biomass after lipid extraction. Preparation of 'CGF'-like high

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61 value-added products from the lipid-removed biomass may be a potential way to reduce
62 the costs of *C. pyrenoidosa*-based biofuels production.

63 However, 'CGF' is rarely used in scientific studies; instead, the hot water extract of *C.*
64 *pyrenoidosa* (CPE) or *C. vulgaris* (CVE) is used. CPE is rich in nutrients and contains
65 amino acids, peptides, proteins, vitamins, sugars, and nucleic acids.[9] Numerous
66 commercial CPE-like products are available in the market, but the main functional
67 fractions of the products are still unclear, and there are no suitable product standards. This
68 may be due in part to differences in extraction condition. As described in patents and
69 reports, CPE is extracted with hot water; however, the extraction temperature ranges from
70 80 ℃[10] to 200 ℃,[11] the extraction duration is between 15 min[12] and 5 h,[11] and the
71 *Chlorella* cells may be pretreated with pressure,[11, 12] ultrasonic waves,[13] or enzymolysis[14].
72 Therefore, a standardized CPE preparation method for laboratory use is necessary before
73 the development of high value-added bio-products from *C. pyrenoidosa*.

74 Preparation of CPE directly from untreated *C. pyrenoidosa* is difficult and inefficient
75 because of its tough cell wall.[15] Pressure,[11, 12] ultrasonic waves,[13] and enzymolysis[14] are
76 commonly used to disrupt the cell wall of *Chlorella*. In the present study, we prepared
77 CPEs using these methods with some modifications, and determined *in vitro* bioactivities
78 by measuring their free radical scavenging and macrophage promoting capacity.[11, 16]
79 Effects of CPEs on advanced glycation end products (AGEs) formation and redox active
80 metal ion chelation, the bioactivities of lipophilic extracts of *Chlorella* were also
81 examined.[17, 18]

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82 **2 Materials and methods**

83 **2.1 Chemicals and Materials**

84 Spray-dried *C. pyrenoidosa* cells were offered by Jiaxing Zeyuan Bio-Products Co.,Ltd.
85 (Zhejiang, China). Cellulase and pectinase were purchased from Yakult (Tokyo, Japan).
86 Proteinase K was from Calbiochem (Germany). RNase A, bovine serum albumin (BSA,
87 fraction V), pyrocatechol violet (PV), ferrozine (FZ), neutral red (NR), and dimethyl
88 sulfoxide (DMSO) were from Sigma (St. Louis, MO, USA). Dulbecco's modified Eagle
89 medium (DMEM, high glucose), RPMI-1640 medium, trypsin, and penicillin
90 streptomycin combination were from Hyclone Thermo Fisher (Beijing, China). Fetal
91 bovine serum was from Biosera (Kansas City, MO, USA). Thiazolyl blue (MTT) was
92 purchased from Genview (USA). 3-amino,4-aminomethyl-2',7'-difluorescein diacetate
93 (DAF-FM DA) was from Beyotime Institute (Haimen, Jiangsu, China). Hydroxyl radical
94 scavenging activity detection kit was from Jiancheng Bioengineering Institute (Nanjing,
95 Jiangsu, China).

96 **2.2 Preparation of CPEs**

97 *C. pyrenoidosa* cells were suspended in distilled water (10%, w/v), and CPEs were
98 prepared from aliquots of the suspension as shown in Table 1. For high pressure
99 extraction, the *C. pyrenoidosa* suspension was kept at 121 ℃ (0.1 MPa) for 40 min in an
100 autoclave. After separating at 10,000 rpm for 30 min, the residue was extracted again
101 under the same condition. The supernatants of the two extractions were combined, filtered,
102 and subsequently freeze-dried to yield CPE-a. For the ultrasonic assay, the *C. pyrenoidosa*
103 suspension was treated with ultrasonic disintegration for 30 min in an ultrasonic cleaner

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104 and then extracted at 95 ℃ for 30 min. After separating at 10,000 rpm for 30 min, the
105 residue was extracted again under the same condition, and the supernatants were
106 combined, filtered, and freeze-dried to yield CPE-b. The *C. pyrenoidosa* suspension
107 treated with ultrasonic disintegration was enzymolyzed using cellulase (80 mg mL^{-1}) and
108 pectinase (10 mg mL^{-1}) at 50 ℃ for 2 h, and terminated at 100 ℃ for 30 min. After
109 centrifugation at 10,000 rpm for 30 min, the supernatant was filtered and freeze-dried to
110 obtain CPE-c. CPE-d was produced by incubating the *C. pyrenoidosa* suspension at 70 ℃
111 for 10 h followed by a sequence of enzymolysis: the incubated suspension was treated
112 with cellulase (80 mg mL^{-1}) and pectinase (10 mg mL^{-1}) at 50 ℃ for 12 h first, and
113 proteinase K (100 μg mL^{-1}) and RNase A (30 μg mL^{-1}) at 50 ℃ for 2 h later. After
114 enzymolysis was terminated by boiling, the mixture was separated at 10,000 rpm for
115 30 min. Finally the supernatant was freeze-dried to yield CPE-d. CPE-e was prepared by
116 incubating the *C. pyrenoidosa* suspension twice at 80 ℃ for 1 h each, and the
117 supernatants were treated in the same way as the others to get the final product.

118 **2.3 Characterization of the extracts**

119 All the samples were dissolved in water and UV absorbance was measured at
120 wavelengths of 260 and 280 nm. The Bradford and the Biuret assays were used to detect
121 protein using BSA as standard, whereas sulphuric acid-phenol assay and dinitrosalicylic
122 acid assay were used to assess the total sugar and the reducing sugar contents,
123 respectively, using glucose as standard.

124 **2.4 Effect on AGEs formation**

125 AGEs were prepared as described by Zhuang *et al* with some modifications.[19] In brief,

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126 10 mg mL^{-1} BSA was incubated with 500 M glucose in 20 mM phosphate-buffered saline
127 (PBS, pH 7.4) as a glycation mode solution. Effects of CPEs on AGEs formation were
128 determined by adding these extracts to the mode solutions and incubating at 37 ℃ for
129 28 d. BSA was similarly incubated in the absence of glucose and the CPEs. After
130 incubation, the solutions were extensively dialyzed and diluted to 1 mg mL^{-1} protein. All
131 the samples were examined using a microplate reader (EnSpire® Multimode Reader,
132 PerkinElmer, Finland) at excitation/emission wavelengths of 370/440 nm and
133 335/385 nm,[20] whereas the fluorescence intensity (FI) values of each group were obtained
134 by normalizing fluorescence values to that of the BSA control.

135 **2.5 Hydroxyl radical scavenging activity**

136 Our preliminary study indicated that CPEs were efficient at scavenging free radicals
137 such as hydroxyl radical, but the extracts had negative impact on catalase activity,
138 superoxide dismutase activity, and total anti-oxidation competence (data not shown).
139 Therefore, the present study investigated the influence of the extracted sample on
140 hydroxyl radical scavenging. Because hydroxyl radical generated from the Fenton
141 reaction might react with the Griess reagent and the coloured products could be detected
142 at wavelength of 550 nm, chromatogenic reaction was carried out at 37 ℃ for exactly
143 1 min according to the manufacturer's instructions. Distilled water was considered as
144 control, and radical scavenging ratios of the CPEs were evaluated according to Equation
145 (1).

146 $$\text{Radical scavenging/Metal chelating ratio} = \frac{A_{H_2O} - A_{CPEs}}{A_{H_2O}} \times 100\% \quad (1)$$

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147 **2.6 Metal chelating activity**

148 The metal chelating potential of the sample was investigated according to the method
149 described by Custódio *et al* with some modifications.[18] In brief, 20 μL of the sample and
150 20 μL of 2.5 mM ferrous ion were mixed in 200 μL of 100 mM sodium acetate buffer
151 (pH 4.9) and incubated at room temperature for 30 min for ferrous ion chelating activity
152 assay. Adding 20 μL of 50 mM FZ triggered the FZ-ferrous chromatogenic reaction. The
153 reaction tubes were then shaken vigorously and kept at room temperature for 30 min.
154 Then, absorbance at 562 nm was measured in succession, and ferrous ion chelating ratios
155 of the CPEs were calculated according to Equation (1).

156 For copper ion chelating activity, 20 μL of the samples and 20 μL of 2.5 mM copper
157 ion were mixed in 200 μL of 50 mM sodium acetate buffer (pH 6.0), and incubated at
158 room temperature for 30 min. The PV-copper chromatogenic reaction was triggered by
159 adding 5 μL of 20 mM PV. After being shaken vigorously and kept at room temperature
160 for 30 min, the mixture was tested at 632 nm, and copper ion chelating activities of the
161 CPEs were evaluated according to Equation (1).

162 **2.7 Cell culture and treatment**

163 The murine macrophage cells Ana-1 and Raw264.7 were cultured in RPMI-1640
164 medium or DMEM medium at 37 ℃ in 5% CO_2, supplemented with 10%
165 heat-inactivated fetal bovine serum (FBS) and 1% penicillin streptomycin combination.
166 In cell viability and phagocytosis assays, 100 μL of the cell suspensions were distributed
167 into 96-well plates at a density of 2×10^4 cells mL^{-1}. When the cells reached about 50%
168 confluence density, the CPE solutions at different concentrations were added, followed by

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169 further incubation at 37 ℃ for 24 h.

170 **2.8 Cell viability**

171 MTT was used to detect cell viability. The treated cells were incubated with 0.5 mg
172 mL^{-1} MTT at 37 ℃ for 4 h, after which 100 μL of the lysis solution (consisting of 10%
173 SDS, 5% isobutanol, and 12 mM HCl) was added into each well to dissolve formazan.
174 Absorbance values were detected at 492 nm with a reference wavelength at 630 nm.[21] All
175 values were expressed as percentages of the controls.

176 **2.9 Phagocytic activity**

177 Phagocytic activity of the macrophage was measured using NR as a probe. The treated
178 Ana-1 cells were incubated with 20 μL of 1 mg mL^{-1} NR at 37 ℃ for 3 h. After
179 incubation, the cells were gently rinsed with PBS to remove the dye and dissolved in a
180 liquid consisting of 50% ethanol and 50% acetic acid for 2 h. Absorbance values were
181 detected at 535 nm with a reference wavelength at 630 nm. The phagocytic activity of the
182 cells was likewise calculated as percentage of the control.

183 **2.10 Intracellular nitric oxide (NO) generation**

184 Cells were harvested and washed with physiological PBS and incubated with 5 μM of
185 DAF-FM DA at 37 ℃ for 20 min. After washing, the cells were seeded into black 96-well
186 plates (Greiner, Germany) and incubated with 100 μg mL^{-1} of the CPE-a solution at 37 ℃.
187 The cells were observed on a microplate reader (GENios Pro, Tecan, Austria) at
188 excitation/emission wavelength of 485/535 nm for 0, 4, 6, and 24 h. CPE-a solution filled
189 into a blank well was used as reference to subtract the fluorescent background.

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190 **2.11 Statistical analysis**

191 Results were analysed with one-way analysis of variance, and differences between
192 groups were determined by Duncan or Games-Howell multiple tests. $P < 0.05$ was
193 considered statistically significant. Each assay was performed at least thrice and all data
194 were expressed as mean ± SD.

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195 **3 Results**

196 **3.1 Yield and general ingredients of CPEs**

197 *Chlorella* hot water extract prepared at 80 ℃ for 1 h (CPE-e) was considered as
198 control. Extraction yields of CPE-a (25.17%), CPE-c (27.93%), and CPE-d (25.89%)
199 were significantly increased compared with the 12.56% extraction yield of CPE-e (Fig.
200 1A, $p < 0.05$). However, no significant differences were found among the three products.

201 UV absorbances at 260 and 280 nm varied greatly. The samples were dissolved in
202 water and then diluted to 500 μg mL^{-1}. UV absorbances of CPE-a and CPE-b at both 260
203 and 280 nm were similar to that of CGF1 (Fig. 1B, $p > 0.05$), whereas absorbances of
204 CPE-c and CPE-d at 260 nm were lower than that of CGF1 ($p < 0.05$). CGF1, along with
205 CGF2, is a commercial product obtained from Taiwan. Among the four CPEs, the
206 absorbances of CPE-c and CPE-d were significantly lower than that of CPE-a ($p < 0.01$).

207 Sugar contents of CGF1 and CGF2 were higher than their protein contents (Table 2).
208 The CGF1 protein content was 39.03 mg g^{-1} as determined by the Bradford assay, but
209 232.10 mg g^{-1} according to the Biuret assay. These data suggested that the protein of the
210 extracts differed greatly from that of BSA, and that a large amount of peptide bonds must
211 be present in the products. Similar results were observed for sugar contents: total sugar
212 content of CGF1 was 440.81 mg g^{-1} but the reducing sugar content was much higher at
213 601.41 mg g^{-1} (equivalent to glucose). On the other hand, total sugar and reducing sugar
214 contents of the possibly carbohydrate-in-essence CGF2 were 1,028.42 mg g^{-1} and
215 1,363.88 mg g^{-1}, respectively. Thus, the protein content of CPE-a was significantly higher
216 than that of CGF1 according to the results of Bradford and Biuret assays ($p < 0.01$),

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217 whereas the total sugar content of CPE-a was lower than those of CPE-c and CPE-d
218 ($p < 0.05$). However, all CPEs had lower sugar contents when compared to the CGFs
219 ($p < 0.01$); reducing sugar could not be detected in CPEs except in CPE-c.
220 Cellulase-pectinase and proteinase-RNase treatments were likely responsible for the
221 altered protein and sugar levels of CPE-c and CPE-d.

222 **3.2 Effect on AGEs formation**

223 The effects of CPEs on AGEs formation at excitation/emission wavelengths of
224 370/440 nm and 335/385 nm, the most popular wavelengths used for determining total
225 AGEs and pentosidine, respectively, were analysed. Incubation was maintained for 28 d
226 but CPEs showed hardly any inhibitory impact on AGEs formation. In contrast, total
227 AGEs and pentosidine levels were enhanced when CPE-d was supplemented at 1,000 μg
228 mL^{-1} (Fig. 2, $p < 0.05$).

229 **3.3 Hydroxyl radical scavenging activity**

230 As shown in Fig. 3, the hydroxyl radical scavenging ratios of the CPEs increased in a
231 concentration-dependent manner, and CPE-a exhibited the most pronounced activity with
232 scavenging ratio of 74.31% at 1,000 μg mL^{-1} (Fig. 3A). The hydroxyl radical scavenging
233 abilities of CGF1 and CGF2 differed greatly: the former increased in a
234 concentration-dependent manner but the latter seemed to be non-existent. Interestingly,
235 there were no significant differences between the hydroxyl radical scavenging abilities of
236 CGF1 and CPE-a (Fig. 3B, $p > 0.05$).

237 **3.4 Metal chelating activity**

238 Both iron and copper are redox active metal ions. Considering the efficient radical

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239 scavenging capacity of the CPEs, the ferrous and copper ion chelating activities of the
240 extracts were tested. Figures 4A and B show the absorption spectra of the chelated
241 products. The chelated product FZ-ferrous had maximum absorption at 562 nm, whereas
242 the PV-copper had maximum absorption at 632 nm. The ferrous and copper chelating
243 ratios of EDTA at 5 mM were 99.12% and 95.23%, respectively. The CPEs were able to
244 chelate ferrous and copper ions at 10 mg mL^{-1}; the ferrous ion chelating ratio of CPE-c
245 was 45.87% and copper ion chelating ratio of CPE-d was 53.92% (Fig. 4C and D,
246 $p < 0.05$ compared with the CPE-a).

247 **3.5 Cell viability**

248 The CPEs were supplemented to murine macrophage cells at increasing concentrations.
249 Viability of Ana-1 was significantly elevated in a dose-dependent manner. Compared with
250 CPE-a, which enhanced cell viability of Ana-1 up to 162.98% at 200 μg mL^{-1}, the effects
251 of the other three extracts were visibly faint (Fig. 5A, $p < 0.05$ compared with the CPE-a).
252 Raw264.7 cells proliferated with the addition of CPEs as well; CPE-a was the most
253 effective extract which raised the viability of Raw264.7 to 123.29% at 50 μg mL^{-1} (Fig.
254 5B, $p < 0.01$).

255 **3.6 Phagocytic activity and intracellular NO generation**

256 The influence of CPE-a on macrophage phagocytic activity was also examined owing
257 to its excellent proliferation effect on murine macrophages. The cellular morphological
258 alteration of Ana-1 exposed to CPE-a was photographed with or without NR staining as
259 shown in Fig. 6A and B. The total cell number decreased and a majority of the cells were
260 greatly enlarged by CPE-a treatment, which indicated that the macrophages were

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261 activated. The macrophages, especially the enlarged cells (Fig. 6C and D), treated with
262 CPE-a phagocytosed much of the dye and the phagocytic ratio was 116.20% of the
263 control when CPE-a was supplemented at 100 μg mL^{-1} for 24 h (Fig. 6E, $p < 0.01$).
264 Moreover, macrophage intracellular NO production was strongly induced by CPE-a
265 treatment; NO levels significantly increased when the cells were incubated with CPE-a at
266 100 μg mL^{-1} for 6 h, and later reached 123.51% of the control when the incubation time
267 was prolonged to 24 h (Fig. 6F, $p < 0.05$).

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268 **4 Discussion**

269 Product yield is an important metric for evaluating methods of preparing high
270 value-added products. The CGF content in raw *C. pyrenoidosa* has been reported to be
271 approximately 5%,[6] whereas Kralovec *et al* prepared CPE at 80 ℃ for 1 h with a yield of
272 10.2%.[22] In this study, CPE-e was obtained at a yield rate of 12.56% by incubating at
273 80 ℃ for 1 h, which indicates the superiority of *C. pyrenoidosa* produced with the
274 'sequential heterotrophy-dilution-photoinduction' method. The tough cell wall of *C.*
275 *pyrenoidosa* prevents the release of intracellular substances.[6, 15] It is believed that
276 breaking the cell wall helps in absorption of nutrients from *Chlorella*.[6] The same problem
277 exists in the preparation of CPE. Song *et al* prepared CVE at 121 ℃ for 15 min with a
278 yield 17.4%.[12] Plaza *et al*[23] and Kitada *et al*[11] found that the product yield rose as the
279 extraction pressure, time, and temperature increased constantly. The yield of CPE-b was
280 obviously lower than that of the other three products, which indicated the poor efficiency
281 of ultrasonic and ordinary hot water extraction. With the addition of an extra enzymolysis
282 step, the yield of CPE-c rose drastically from 14.84% to 27.93%. The high yield of CPE-a,
283 CPE-c, and CPE-d observed in the present study might be due to rupture of the cell wall
284 by pressure and enzymes. Unlike the method of Song *et al* at 121 ℃ for 15 min,[12] the *C.*
285 *pyrenoidosa* suspension was treated twice at 121 ℃ for 40 min in this study, and a yield
286 rate of 25.17% was obtained for CPE-a in the end. These results suggest that increases in
287 the extraction duration and frequency improved the extraction efficiency under high
288 pressure. CPE-d was prepared by enzymolysis, but its yield was not increased compared
289 to those of CPE-a and CPE-c.

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290 Since enzymes are expensive and difficult to remove completely, and reactions
291 involving them are complicated and time consuming, enzymolysis methods appear
292 impractical based on our findings. In contrast, the high pressure assay required just water
293 and an autoclave, and the extraction period was much shorter. In terms of yield, hot water
294 extraction at 121 ℃ for 40 min appears to be the most efficient way of preparing CPE.
295 Aside from differences in yield, the general ingredients of the CPEs were also
296 obviously different. The protein and sugar contents of the extracts required further
297 investigation because they could not be simply calculated based on standard materials.
298 The hydroxyl radical scavenging capability and the metal chelating activity of the four
299 CPEs, as well as their influences on AGEs formation and macrophage activation, were
300 compared to learn the properties of the high value-added extracts.
301 Reportedly, CPE can inhibit the activity of α-glucosidase *in vitro*,[24] while oral
302 administration of *Chlorella* can lower plasma glucose levels and improve insulin
303 resistance status in fructose-rich chow-fed rats, and increase the sensitivity to exogenous
304 insulin in streptozotocin-induced diabetic rats.[25] The accumulation of AGEs due to
305 increase in blood glucose levels is believed to be one of the contributing factors in the
306 pathogenesis of diabetic complications, and inhibition of AGEs formation may be an
307 effective strategy in the prevention and remedy of diabetes and its complications.
308 However, CPEs had hardly any inhibitory impact on AGEs formation in our study,
309 indicating that CPEs are not potential inhibitors of AGEs formation. Considering their
310 glucose-reducing effects, CPEs might improve diabetes and its complications by
311 hypoglycemia-related mechanisms rather than by inhibiting AGEs formation.

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312 It was reported that CPE may act as an antioxidant by scavenging free radicals. Plaza
313 *et al* found that the antioxidant activity of CPE rose as the extracting temperature
314 increased,[23] Kitada *et al* reported that the 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical
315 scavenging activity of CPE rose when the extraction temperature increased at a pressure
316 of 2 MPa,[11] whereas another investigation found that the DPPH radical scavenging
317 activity of 1 g CPE was equivalent to that of 2.47 mg ascorbic acid.[26] In the present study,
318 the hydroxyl radical scavenging ratios of CPEs were found to increase in a
319 concentration-dependent manner. CPE-a showed the best antioxidant capability, as the
320 scavenging ratios of other three CPEs were significantly lower than that of CPE-a at 100
321 and 200 μg mL^{-1} (Fig. 3A).

322 Although *Chlorella* is believed to eliminate toxic substances such as heavy metals[27],
323 the heavy metal chelating activity of CPE has been rarely reported aside from the ferrous
324 and copper ion chelating activities of *Chlorella* lipophilic extracts. Wang *et al* reported
325 that the ferrous chelating ratio of ultrasonic extraction products in 50% aqueous ethanol
326 reached 46.69% at a concentration of 0.83 mg mL^{-1}.[28] Tested at 10 mg mL^{-1}, ferrous ion
327 chelating ratio of CPE-c was 45.87% while copper ion chelating ratio of CPE-d was 53.92%
328 in the present study. The low ferrous and copper chelating ratios of CPEs in the present
329 study suggest that lipophilic compounds may possess high metal chelating activity but not
330 hydrosoluble compounds.

331 The immunoregulatory effect of *Chlorella* is an important research topic. Han
332 prepared a CPE product similar to CPE-d in the present study, which was reported to
333 increase physiological function in mice; the phagocytic function of macrophages was

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334 evidently boosted after CPE administration at 75 and 150 mg kg^{-1} d^{-1} for 30 d.[14] The
335 results of the present study demonstrate the proliferation effect of CPEs on incubated
336 murine macrophages *in vitro*. Cell viability of Ana-1 was significantly raised to 162.98%
337 by CPE-a at 200 μg mL^{-1}, and to 122.34% by CPE-d at the same concentration (Fig. 5A).
338 Furthermore, CPE-a exhibited significant effects on macrophage promotion. The majority
339 of macrophages were greatly enlarged and much of the dye was phagocytosed by the cells.
340 Appropriate elevation on NO generation is the hallmark of the activated
341 immunoregulation of macrophage in general. The results of the present study suggest that
342 intracellular NO production of macrophage might be efficaciously induced by CPE-a
343 treatment. Taken together, the observed effects of CPE-a on macrophage cell viability,
344 phagocytic activity, and intracellular NO generation suggest that CPE-a has strong
345 immunoregulatory influence on macrophages.

346 The positive influences of CPE-a on hydroxyl radical scavenging and macrophage
347 activation indicate that the procedure involving incubation under high pressure described
348 in the present study is highly effective for preparation of CPE.

349 Development of CPE may lower the costs of *C. pyrenoidosa*-based biofuels production
350 and help to alleviate the energy crisis around the world. The present study improved the
351 procedure for CPE extraction, which is only the beginning work of our long-term research.
352 We shall study functional components of CPE and their mechanisms in further
353 investigations, and establish product standards according to functional components. Then
354 the present CPE production method ought to be improved in the light of product standards
355 to realise the large-scale production of CPE.

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356 **5 Conclusion**

357 In the present study, we prepared CPEs from *C. pyrenoidosa* by adding high pressure,
358 ultrasonication, or enzymolysis steps to existing methods. The yields, ingredients, and
359 bioactivities of the products were compared, especially their free radical scavenging
360 activities, metal chelating activities, and macrophage activation effects. With a yield rate
361 of 25.17%, CPE-a prepared under an extra 0.1 MPa pressure in an autoclave showed
362 higher free radical scavenging activity and macrophage promotion effects *in vitro*. Hot
363 water extraction assisted with high pressure is thus proposed as an effective method of
364 producing CPE.

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365 **6 Acknowledgments**

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411 **8 Tables**

412 **Table 1.** Conditions for CPEs preparation.

Process	Details	CPE-a	CPE-b	CPE-c	CPE-d	CPE-e
Pretreatment	Method	-	Ultrasonication	Ultrasonication	-	-
	Time	-	30 min	30 min	-	-
Extraction	Temp	121 ℃	95 ℃	95 ℃	70 ℃	80 ℃
	Time	40 min	30 min	30 min	10 h	1 h
Enzymolysis	Enzyme	-	-	Cellulase-pectinase	[1] Cellulase-pectinase [2] Proteinase-RNase	-
	Temp	-	-	50 ℃	50 ℃	-
	Time	-	-	2 h	2 h	-

413 [1] The first enzymolysis process; [2] the second enzymolysis process following [1].

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414 **Table 2.** Protein and sugar contents of the CPEs.

	Protein contents (mg g^{-1} CPE)		Sugar contents (mg g^{-1} CPE)	
	Bradford assay	Biuret assay	Total sugar	Reducing sugar
CGF1	39.03 ± 0.30	232.10 ± 1.77	440.81 ± 13.64	601.41 ± 78.37
CGF2	22.98 ± 0.50^{**}	115.47 ± 6.16^{**}	1,028.42 ± 14.28^{**}	1,363.88 ± 106.80^{**}
CPE-a	44.97 ± $1.42^{**\#\#}$	307.93 ± $5.18^{**\#\#}$	143.64 ± $7.65^{**\#\#}$	^{n}
CPE-b	35.25 ± $0.81^{**\#\#++}$	369.52 ± $8.50^{**\#\#++}$	114.43 ± $6.24^{**\#\#}$	^{n}
CPE-c	17.17 ± $0.23^{**\#\#++}$	304.08 ± $6.01^{**\#\#}$	208.32 ± $3.23^{**\#\#+}$	289.39 ± $84.40^{**\#\#+}$
CPE-d	10.39 ± $0.19^{**\#\#++}$	362.59 ± $7.12^{**\#\#++}$	207.65 ± $0.00^{**\#\#+}$	^{n}

415 Values are expressed as mean ± SD; ^{n} not detectable; ** $p < 0.01$ compared with the CGF1; $^{\#\#}$ $p < 0.01$
416 compared with the CGF2; $^{+}$ $p < 0.05$, $^{++}$ $p < 0.01$ compared with the CPE-a.

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417 **9 Figure Captions**

418 Fig. 1 Yield and UV absorbances of the CPEs. (A) Yield of the CPEs derived from
419 different methods were expressed as percentage of the dried *Chlorella* powder; (B) UV
420 absorbances of the CPEs at 500 μg mL^{-1} were detected using a UV spectrophotometer. All
421 results were expressed as means ± SD (n=3). * $p < 0.05$, ** $p < 0.01$ compared with the
422 CPE-e (A) or CGF1 (B); # $p < 0.05$, ## $p < 0.01$ compared with CGF2; + $p < 0.05$, ++
423 $p < 0.01$ compared with the CPE-a.

424 Fig. 2 Effect of the CPEs on AGEs formation. Effects of the CPEs on total AGEs and
425 pentosidine formation were tested at excitation/emission wavelengths of (A) 370/440 nm
426 and (B) 335/385 nm in increasing concentration of 10, 100, and 1,000 μg mL^{-1}. All data
427 were normalized to BSA and presented as means ± SD (n=4). # $p < 0.05$ compared with
428 AGEs.

429 Fig. 3 Hydroxyl radical scavenging activity of the CPEs. (A) The four extracts were
430 diluted to 40, 100, 200, 400, and 1,000 μg mL^{-1} and their hydroxyl radical scavenging
431 activity was detected by a kit using distilled water as control. (B) Effects of CGF1, CGF2,
432 and CPE-a at different concentrations were also compared. All results were expressed as
433 means ± SD (n=4). n, not detectable, * $p < 0.05$, ** $p < 0.01$ compared with control; +
434 $p < 0.05$, ++ $p < 0.01$ compared with the CPE-a at the same concentration.

435 Fig. 4 Ferrous and copper ion chelating activities of the CPEs. (A) FZ-ferrous and (B)
436 PV-copper absorption spectra were scanned. Fe-1−Fe-5 (A) and Cu-1−Cu-5 (B) indicated
437 the chelating products of the increasing concentrations of the two metal ions at 0, 0.625,
438 1.25, 1.875, and 2.5 mM. (C) Ferrous ion chelating activity and (D) copper ion chelating

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439 activity of the CPEs at 10 mg mL^{-1} and EDTA at 5 mM were detected at 562 nm and
440 632 nm. All results were expressed as means ± SD (n=3). ** $p < 0.01$ compared with
441 EDTA; + $p < 0.05$, ++ $p < 0.01$ compared with the CPE-a.

442 Fig. 5 Effect of the CPEs on cell proliferation. Macrophage cell lines (A) Ana-1 and (B)
443 Raw264.7 were administrated with increasing concentrations of the CPEs for 24 h, and
444 cell viability was then tested by MTT assay. All results were expressed as means ± SD
445 (n=6). * $p < 0.05$, ** $p < 0.01$ compared with control; + $p < 0.05$, ++ $p < 0.01$ compared
446 with the CPE-a.

447 Fig. 6 Effects of the CPE-a on phagocytic activity and intracellular NO production of
448 Ana-1. Morphological images of Ana-1 exposed to (A) control or (B, C, and D) CPE-a for
449 24 h were observed (B, 100 μg mL^{-1} of the CPE-a) before or (C and D, 100 and 200 μg
450 mL^{-1} of the CPE-a, respectively) after NR staining. (E) The altered phagocytic activiry
451 undergone CPE-a treatment were detected at 550 nm (n=6), whereas (F) intracellular NO
452 production of the cells supplemented with 100 μg mL^{-1} of the CPE-a for 4, 6, and 24 h
453 were monitored at excitation/emission wavelengths of 485/535 nm (n=4), NO levels of
454 0 h were considered as control. Results were expressed as means ± SD. * $p < 0.05$, **
455 $p < 0.01$ compared with control.

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Figure 1

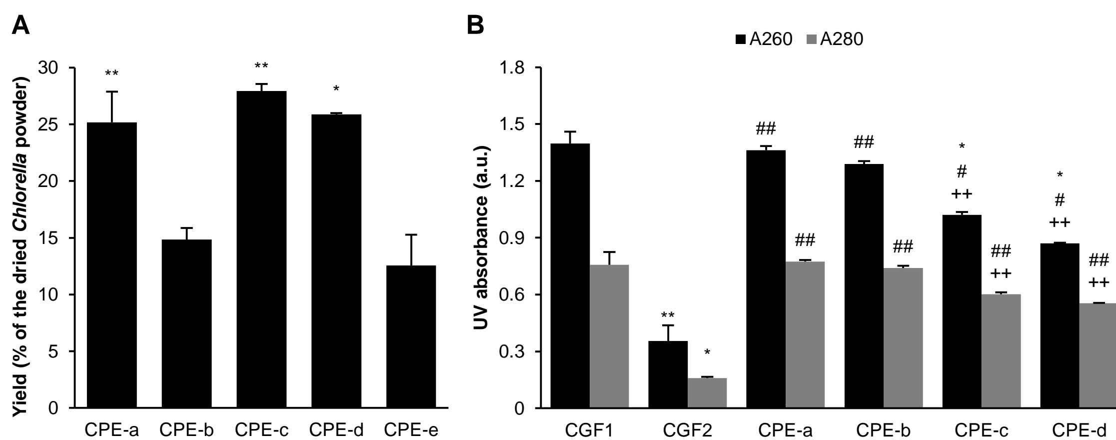

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Figure 2

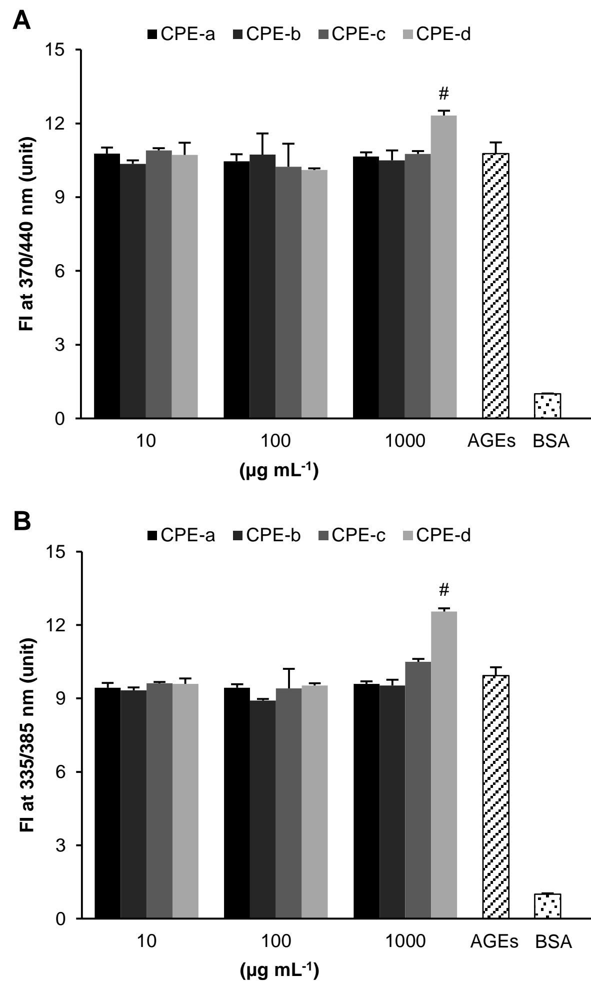

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Figure 3

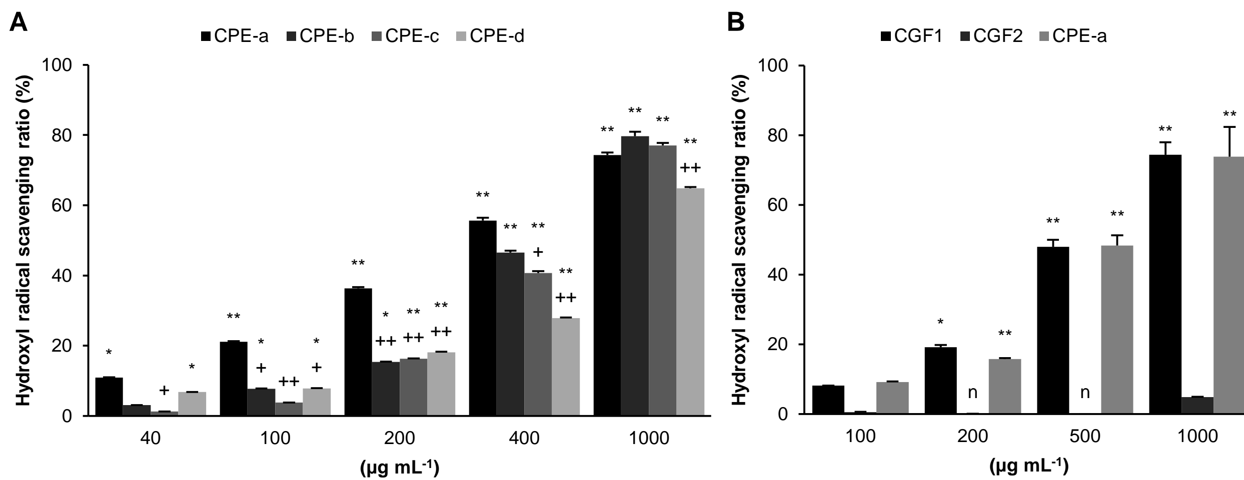

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Figure 4

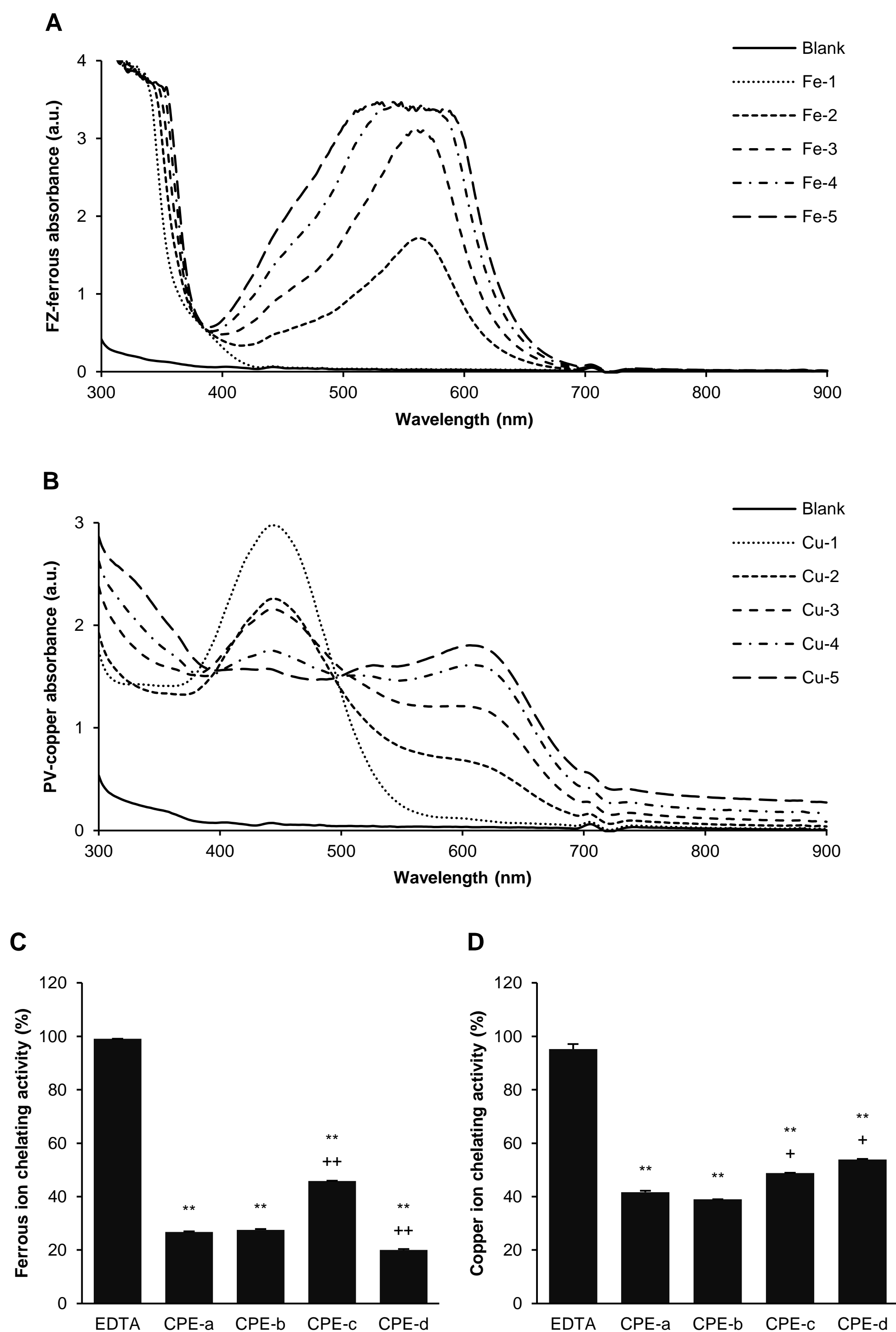

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Figure 5

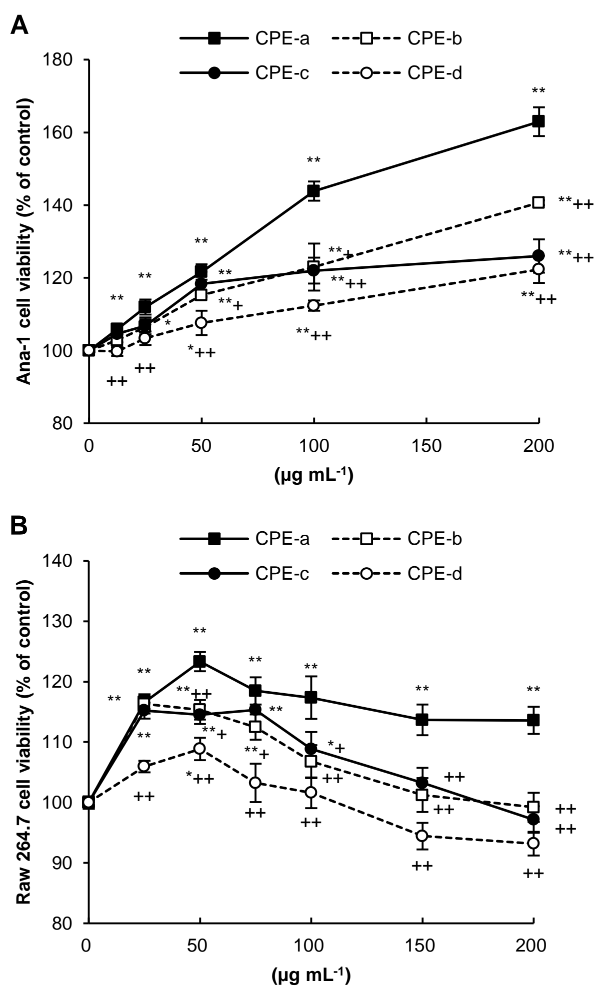

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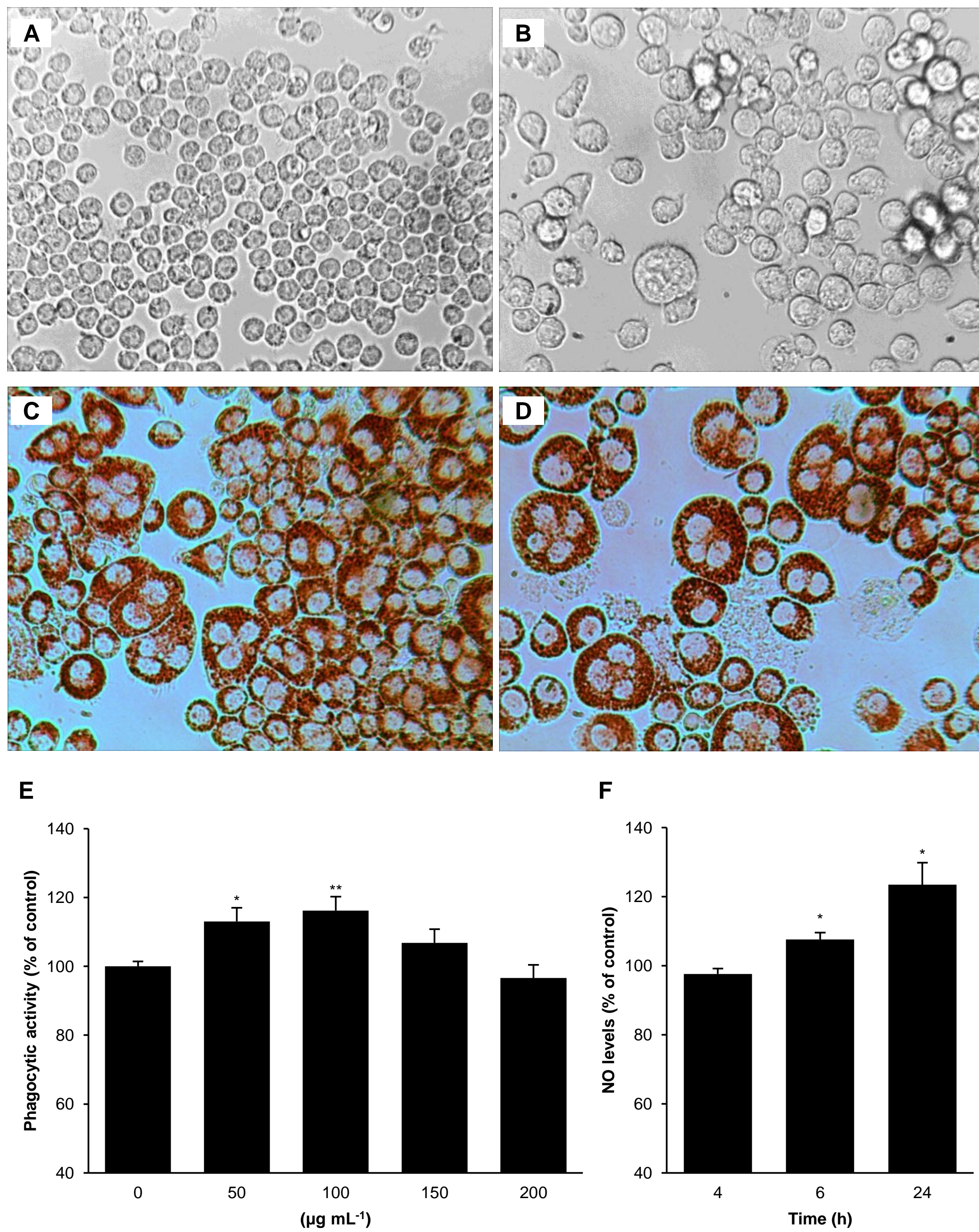
Figure 6



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