Sulfated polysaccharides from *Phaeodactylum tricornutum*: isolation, structural characteristics, and inhibiting HepG2 growth activity *in vitro*

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Microalgae, eukaryotic unicellular plants, are increasing in demand due to their use as nutraceutical and food supplements. They consisted different kinds of biologically active components such as polysaccharides. On the other hand, cancer is the leading cause of death globally. At present, there is no efficient method to cure it. Therefore, in this work, we extracted polysaccharides from *Phaeodactylum tricornutum* (PTP), characterized the chemical composition and structure, and investigated its anticancer activity on HepG2 cells. The results showed that PTP was a sulfated polysaccharide with a high Mw of 4810 kDa, and xylose, fucose, glucose and galactose were the main monosaccharides. PTP has significant anticancer activity in a dose-dependent manner (up to 60.37% at 250 ug/ml) according to MTT assays. Furthermore, cycle analysis was carried out to explain its anticancer activity. The results showed that it exhibited anticancer effect mainly through the induction of apoptosis without affecting the cycle and mitosis of HepG2 cells. This might make it a potential drug for anticancer treatment in the future.

1 **Sulfated polysaccharides from *Phaeodactylum tricornutum*: isolation, structural**

2 **characteristics, and inhibiting HepG2 growth activity *in vitro***

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6 **Abstract:**

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8 nutraceutical and food supplements. They consisted different kinds of biologically active

9 components such as polysaccharides. On the other hand, cancer is the leading cause of death

10 globally. At present, there is no efficient method to cure it. Therefore, in this work, we extracted

11 polysaccharides from *Phaeodactylum tricornutum* (PTP), characterized the chemical

12 composition and structure, and investigated its anticancer activity on HepG2 cells. The results

13 showed that PTP was a sulfated polysaccharide with a high Mw of 4810 kDa, and xylose, fucose,

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22 **1. Introduction**

23 Microalgae are promising source of biomass due to their advantageous features

24 such as their phototropic nature, high growth rate, lack of competition with food crops for arable

25 land, and abundant nutritious components, such as protein, pigments and trace

26 elements(*Hamilton et al., 2014; Wang et al., 2015*). Therefore, it has been used as feedstock,

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27   such as in food, feed, functional foods, biofuels, or chemicals integrated in novel biorefinery

28   concepts(*Zhu L., 2015; Vandamme et al., 2018*).   Unlike terrestrial plants, the biologically

29   active compounds extracted from microalgae have shown unique properties, such as antibacterial,

30   antiviral, antifungal, antioxidative, anti-inflammatory, and anti-tumor properties(*De Jesus*

31   *Raposo et al., 2014; Guzman et al., 2003; Hayashi et al. 1996; Kaji et al., 2004; Challouf et al.,*

32   *2011; De Jesus Raposo et al., 2015*). From economical point of view, polysaccharides from

33   algae are promising products due to their abundance in algae(Kraan S., 2012). Polysaccharides

34   can be extracted from algae by several "green" extraction techniques, such as microwave-

35   assisted extraction(*Rodriguez-Jasso et al., 2011*) and enzyme-assisted extraction methods(*Ko et*

36   *al., 2012*). The characteristics of different polysaccharides from microalgae, including their

37   composition and structure, were discussed(*De Jesus Raposo et al., 2014*). It was reported that *G.*

38   *impudicum* and *C. vulgaris* contained homo galactose(*Yim et al., 2007*) and glucose(Nomoto et

39   al., 1983), respectively. However, the other polysaccharides from microalgae are heteropolymers

40   of galactose, xylose, glucose, rhamnose, fucose and fructose(*Matsui et al., 2003; Talyshinsky et*

41   *al., 2002; Raposo et al., 2014*). Ford and Percival(*1965*) found that the structure of the

42   polysaccharides from *Phaeodactylum tricornutum* was a ramified sulfated flucoronomannan,

43   with a backbone composed of β-(1,3)-linked mannose. Many studies have shown that the

44   polysaccharides from microalgae are characterized by antibacterial, antitumor and antiviral

45   properties(*Michalak et al., 2015*).

46   As a kind of diatom, *Phaeodactylum tricornutum* has been found in great abundance in

47   coastal and oceanic waters(*Bautista-Chamizo et al., 2018*). It contains approximately 36.4%

48   crude protein, 26.1% carbohydrate, 18.0% lipid, 15.9% ash, and 0.25% neutral detergent fiber on

49   a dry weight (dw) basis(*Rebolloso-Fuentes et al., 2001*). In addition, it can accumulate valuable

50   pigments such as fucoxanthin, triacylglycerols, and omega-3 long-chain polyunsaturated fatty

51   acids, such as eicosapentaenoic acid(EPA; C20:5)(*Kim et al., 2012; Ryckebosch et al., 2012; Yu*

52   *et al., 2009; McClure et al., 2018*). Currently, it is commercialized for its lipids, especially

53   eicosapentaenoic acid (EPA), and several studies have sought to increase the production yield of

54 EPA and biomass(*Grima et al., 1994; Alías et al., 2004; McClure et al., 2018*). In recent years,

55 due to its many therapeutic activities, fucoxanthin has been commercialized from *Phaeodactylum*

56 *tricornutum*. However, there is little research about the polysaccharides extracted from

57 *Phaeodactylum tricornutum*. Therefore, to make full use of the alga, in this paper, we extracted

58 polysaccharides from *Phaeodactylum tricornutum,* characterized its chemical structure, and

59 studied the anticancer activity of the polysaccharides.

60

2. MATERIALS AND METHODS

62 2.1 *Phaeodactylum tricornutum samples and reagents*

63 Dried *Phaeodactylum tricornutum* powder was supplied by the Institute of Oceanology,

64 Chinese Academy of Sciences. All the reagents used were of analytical grade and commercially

65 available unless otherwise stated.

66

67 *2.2 Extraction of polysaccharides from Phaeodactylum tricornutum (PTP)*

68 The extraction diagram was as Fig.1.

69 Fig.1 The extraction diagram of PTP

70 The dried *Phaeodactylum tricornutum* powder was extracted by the Soxhlet method with ethanol

71 to remove pigments and lipids. The residue was then dried in an oven at 50℃, and

72 polysaccharides were extracted by hot distilled water with the assistance of ultrasonic methods.

73 The optimal temperature, times of ultrasonic treatment and extraction time were determined

74 (shown in supplement data). According to the optimal conditions, the residue algal powder was

75 treated by the ultrasonic method for 20 times, 10 seconds working, 10 seconds rest, and 380 W

76 power. Then, it was extracted at 80℃ for 2 h with stirring. The solution produced by filtration

77 was condensed by rotary evaporator and dialyzed for salt removal. The obtained solution was

78 condensed again, and the final solution was freeze-dried to get the purified sulfated

79 polysaccharides, called PTP.

80 *2.3 Chemical characterization*

81   The Mw of PTP was measured by HPLC with a TSK gel G4000PWxl column using 0.05

82   mol/L Na_2SO_4 as the mobile phase on an Agilent 1260 HPLC system equipped with a refractive

83   index detector. The column temperature was 35℃, and the flow rate of the mobile phase was 0.5

84   ml/min. Dextran standards with a Mw of 1KDa, 5KDa, 12KDa, 50KDa, 80KDa, 270KDa and

85   670KDa (Sigma, Mendota Heights, MN, USA) were used to calibrate the column.

86   Total sugars were analyzed by the phenol-sulfuric acid method (*Dubois et al., 1956*) using

87   galactose as the standard. Sulfated content was determined by the barium chloride gelatin

88   method (*Kawai et al., 1969*). The molar ratios of the monosaccharide composition were

89   determined according to Sun et al. (*2017*). 1-phenyl-3-methyl-5-pyrazolone(PMP) pre-column

90   derivation HPLC was used to determine the molar ratio of the monosaccharide composition.

91   Briefly, 10 mg polysaccharide sample was dissolved into 1 ml distilled water. The mixture was

92   hydrolysed in 4mol/L trifluoroacetic acid, followed by neutralization with sodium hydroxide.

93   Then, HPLC was used to determine every monosaccharide composition on a YMC Pack ODS

94   AQ column(4.6mm×250mm). Mannose, rhamnose, fucose, galactose, xylose, glucose and

95   glucuronic acid from Sigma-Aldrich were used as standards. FT-IR spectra of PTP were

96   determined on a Nicolet-360 FT-IR spectrometer between 400 cm^{-1} and 4000 cm^{-1}.

97

98   *2.4 Evaluation of inhibiting HepG2 growth activity in vitro*

99   *2.3.1 Cell culture*

100   HepG2 cells purchased from Kunming Cell Bank, Chinese Academy of Sciences, were

101   cultured in DMEM supplemented with 10% fetal bovine serum solution, 100 U/ml penicillin and

102   100 mg/ml streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2.

103   *2. 3.2 Evaluation of inhibiting HepG2 growth activity in vitro*

104   The cell growth inhibitory activity of PTP with different concentrations(50, 100, 150, 200

105   and 250ug/ml) was assessed by MTT assay. The cells were seeded in a 96-well plate at a

106   concentration of $1×10^4$ cells/ml and incubated with various concentrations PTP for 48h. Then,

107 200ul 0.5mg/ml MTT solution was added to each well. After 4h incubation, the plates were

108 centrifuged for 10min at 8000rpm. MTT solution was removed. And 200ul DMSO was added

109 into each well. The absorbance at 570nm was determined.

110 *2.3.3Apoptosis assessment*

111 The apoptosis states of HepG2 cells were determined by an Annexin V-FITC/PI apoptosis kit.

112 Cells were collected and washed with ice-cold PBS twice. Then, the cells were resuspended and

113 diluted to 1×10^6 cell/mL with binding buffer. The suspended cells were dyed by 10 μL of

114 Annexin V-FITC for 30 min at room temperature and then stained with 5 μL of PI for 5 min.

115 After incubation, the apoptosis of cells was determined by flow cytometry with Guava®

116 easyCyte 6-2L (Millipore, Billerica, MA, USA).

117 *2.3.4 Analysis of the cell cycle*

118 A cell cycle analysis kit (Beyotime, China) was used to analyze the cell cycle according to

119 the manufacturer's instructions. Briefly, cells were plated in DMEM with different

120 concentrations of sample for 24 h. Then, both the suspension and the adherent cells were

121 collected and placed into the flow cytometry tube and centrifuged at 1500 rpm for 5 min to

122 obtain cell pellets. After that, the cell pellets were washed with precooling PBS and fixed in ice-

123 cold 70% ethanol overnight at 4 °C. Fixed cells were rewashed with PBS and incubated with

124 propidium iodide (PI) staining solution (0.5 mL of staining buffer, 25 μL of PI staining solution,

125 and 10 μL of RNAase A) for 30 min at 37 °C in the dark. Cell cycle analysis was carried out

126 with Guava® easyCyte 6-2L (Millipore, Billerica, MA, USA) using 10,000 counts per sample.

127 The percentage of cells distributed in the different phases of G0/G1, S and G2/M were recorded

128 and analyzed.

129 2.4 Statistical analysis

130 All data are shown as means ± SD (standard deviation) of three independent

131 experiments to ensure the reproducibility of the results. Statistical analysis was performed using

132 SPSS. The difference among groups was analyzed by one-way ANOVA.

133

3. Results

3.1 Chemical characterization

PTP was extracted and purified from *Phaeodactylum tricornutum*, with a yield of 1.5%(% dry weight). It was further characterized regarding Mw, total sugars, sulfate content and monosaccharide composition (Table 1).

According to Table 1, the total sugar and sulfate contents were 29.94% and 20.36%, respectively, which indicated that PTP was a type of sulfated polysaccharide. The Mw of PTP was higher (4810 kDa). The results of the monosaccharide composition showed that the most common monosaccharide of PTP was xylose, followed by fucose, glucose and galactose, with a small amount of rhamnose. The glucuronic acid content of PTP (0.68) was higher. These results indicated that PTP was a hybrid and acidic polysaccharide.

To further characterize the chemical structure of PTP, the corresponding FT-IR spectrum was examined (Fig. 2). The O-H stretching vibration appeared at 3272 cm^{-1}, and the C-H stretching vibration appeared at 2926 cm^{-1}. The adsorption at 1632 cm^{-1} and 1408 cm^{-1} represented the asymmetric and symmetric stretching vibration of C=O, respectively. The adsorption at 1226 cm^{-1} and 1038 cm^{-1} corresponded to the S=O stretching vibration and C-O-H deformation vibration, respectively. These results further indicated that PTP was an acidic and sulfated polysaccharide, which chelated with other positive ions.

Fig.2 FT-IR spectra of PTP

3.2 Evaluation of inhibiting HepG2 growth activity in vitro

Fig.3 The effect of different concentration PTP on the inhibition rate of HepG2 by MTT assay for 48h

Fig. 3 shows the inhibitory effect of different concentrations of PTP on HepG2 tumor cells. The results indicated that PTP had an antiproliferative effect on HepG2 cells in a dose-dependent manner. With concentration increasing, PTP had higher inhibitory activity, and the inhibition rate was up to 60.37% when the concentration was 250 ug/ml. However, the manner of PTP

161   inhibiting HepG2 growth was not clear. To analyze the main cause, we determined the cell

162   apoptosis and cell cycle by flow cytometry.

163

164   *3.3 Induction of apoptosis according to cell cycle analysis*

165   **Fig.4 The cell apoptosis rate under different concentration of PTP**

166   **Fig.5 The cell cycle rate under different concentration of PTP**

167   Fig. 4 shows the results of flow cytometry, when the HepG2 cells were treated with different

168   concentrations of PTP. From the results, we deduced the apoptosis rate under different

169   concentrations of PTP (shown in Fig. 4). From Fig. 4, when HepG2 cells were treated with PTP,

170   the apoptosis rate increased in a dose-dependent manner, although it decreased slightly under

171   200 ug/ml PTP. When the concentration of PTP was 250 ug/ml, 30% apoptosis of cells was

172   induced. Double negative PI-Annexin V cells accounted for about 63%. The above results were

173   consistent with those of the MTT assay. They indicated that PTP could significantly induce cell

174   apoptosis. Then, we determined the HepG2 cell cycle rate under three different concentrations

175   (50 ug/ml, 150 ug/ml and 250 ug/ml) of PTP, as shown in Fig. 5. From Fig. 5, the treatment of

176   different concentrations of PTP did not influence the HepG2 cell cycle rate, which might indicate

177   that PTP's anticancer effect occurred mainly through induction of apoptosis without affecting the

178   mitosis of HepG2 cells.

179

180   **4.   Discussion**

181   Cancer is the leading threat to the world population, and it is the first-leading cause of death

182   worldwide. The current cancer treatments often cause side effects(*Samarakoon et al., 2014;*

183   *Boopathy et al., 2010*). Recently, due to their favorable properties, polysaccharides from

184   microalgae have been given increased attention. Polysaccharides from *Spirulina platensis* have

185   been shown to have antitumor functions on human HT-29 cells(*Xu et al., 2012a*), MB-231

186   cells(*Xu et al., 2012b*), HeLa cells(*Yu et al., 2003*) and HepG2 cells(Di et al., 2013).

187   Polysaccharides from *Platymonas subcondigoramis* inhibited melanoma(*Liu et al., 2007*).

188 Umemura et al. (*2003*) showed that polysaccharides from the dinoflagellate *Gymnodinium* sp.

189 exhibited significant cytotoxicity against a variety of cancer cells, which meant that the

190 polysaccharides might be a potential anticancer chemotherapeutic agent. For *Phaeodactylum*

191 *tricornutum*, some references reported antioxidant(*Agustini et al., 2017*), anti-obesity(*Kim et al.,*

192 *2016*), anti-inflammatory and immunomodulatory activities(*Kim et al., 2013; Guzmán et al.,*

193 *2003*). A novel fatty alcohol ester isolated from *Phaeodactylum tricornutum* showed apoptotic

194 anticancer activity(*Samarakoon et al., 2014*). Few studies have addressed polysaccharides

195 isolated from *Phaeodactylum tricornutum*. Agustini et al.(2017) extracted endo-

196 exopolysaccharide and determined its antioxidant activity on DPPH. The composition of the

197 polysaccharides included xylose, glucose and galactose. Similarly, the monosaccharide

198 composition of PTP mainly included xylose, fucose, glucose and galactose. Fucose was not

199 reported by Agustini et al.(2017), which may be due to the different origins of the algae. In this

200 paper, we determined not only the monosaccharide composition but also the total sugar, sulfate

201 contents and Mw (29.94%, 20.36% and 4810 kDa, respectively) and found that PTP is a

202 complicated sulfated polysaccharide. A type of lipopolysaccharide extracted from

203 *Phaeodactylum tricornutum* exhibited anti-inflammatory activity by blocking the activation of

204 nuclear factor-κB and phosphorylation of p38 mitogen-activated protein kinases, extracellular

205 signal-regulated kinases 1 and 2 and c-Jun N-terminal kinase(Kim et al., 2013). However, there

206 was no related information about the lipopolysaccharide. To our knowledge, no anticancer

207 activity has been reported for PTP. In this paper, we determined the anticancer activity of PTP on

208 HepG2 cells. Significant anticancer activity (up to 60.37% under 250 ug/ml) by MTT assays,

209 which was much better than for polysaccharides isolated from *Spirulina platensis*(Xu et al.,

210 2012a; Xu et al., 2012b).

211 In addition, several studies reported that polysaccharides isolated from *Spirulina platensis*

212 exhibited anticancer activity by blocking G0/G1 phase of cancer cells, which induced the mitosis

213 of cancer cells and led to apoptosis of the cells(*Xu et al., 2012a; Xu et al., 2012b; Yu et al., 2003;*

214 *Di et al., 2013*). However, in this paper, although the apoptosis rate of HepG2 cells increased,

215 cell cycle analysis indicated that PTP's anticancer effect occurred mainly through induction of

216 apoptosis without affecting the cell cycle and mitosis of HepG2 cells. This result might differ

217 according to the chemical components and structure. It needs further investigation. In addition,

218 some references reported that microalgae polysaccharides have the capacity to modulate the

219 immune system so that they display anticancer activity in vivo(*Andrade et al., 2018*). For this

220 study, only in vitro cell experiments were carried out, and it is necessary to explore the

221 anticancer activity in vivo. Further research will address this issue.

222

223 **5. Conclusion**

224 In this paper, a sulfated polysaccharide (PTP) was extracted from *Phaeodactylum*

225 *tricornutum* with a high Mw (4810 kDa). The monosaccharide composition of PTP was mainly

226 xylose, fucose, glucose and galactose. MTT assays showed that PTP has significant anticancer

227 activity (up to 60.37% under 250 ug/ml). Furthermore, the anticancer effect occurred mainly

228 through induction of apoptosis without affecting the cell cycle and mitosis of HepG2 cells. Thus,

229 PTP may be a potential drug for anticancer treatment.

230

231

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355

Figure 1

Extraction process

The extraction diagram of PTP
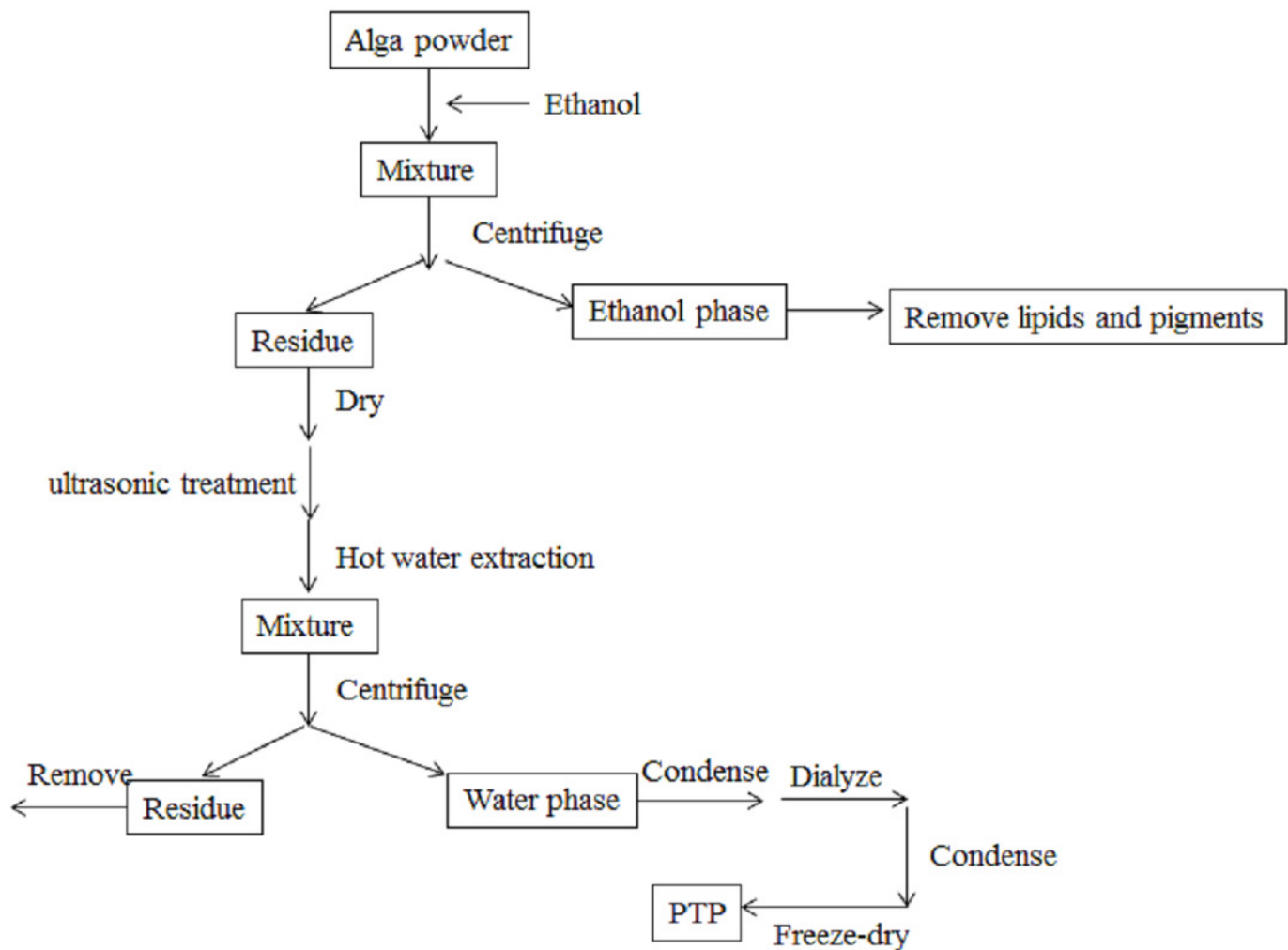
Fig.1 The extraction diagram of PTP

Figure 2

FTIR

Fig.2 FT-IR spectra of PTP
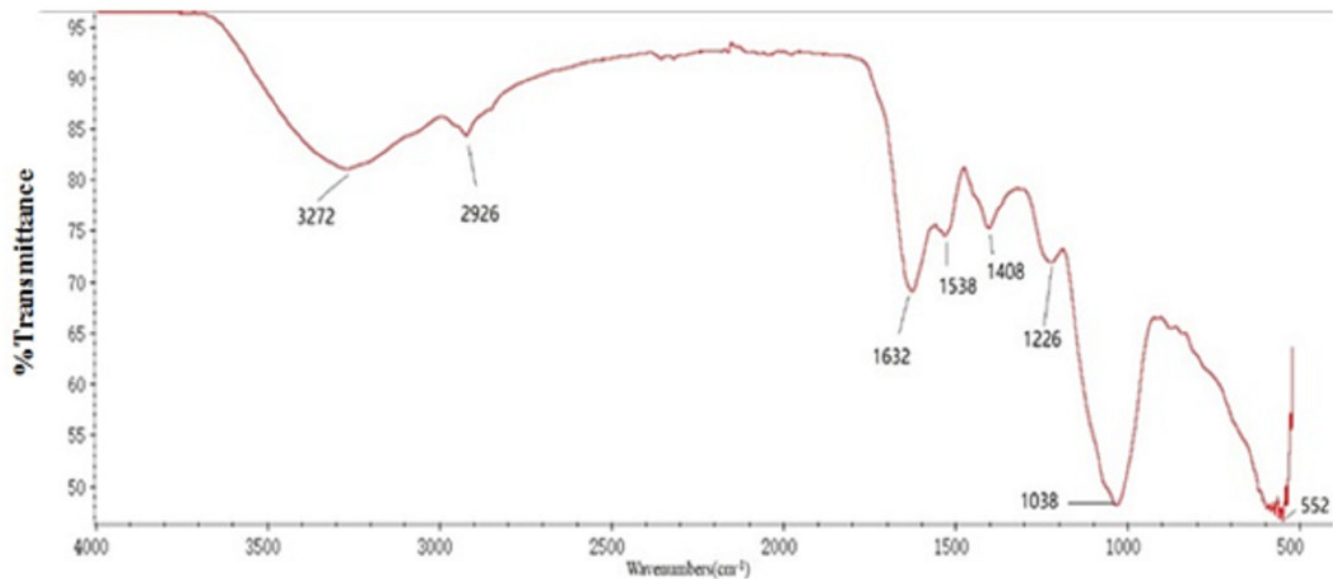
Fig.2 FT-IR spectra of PTP

Figure 3

inhibition rate of HepG2 by MTT assay

Fig.3 The effect of different concentration PTP on the inhibition rate of HepG2 by MTT assay for 48h
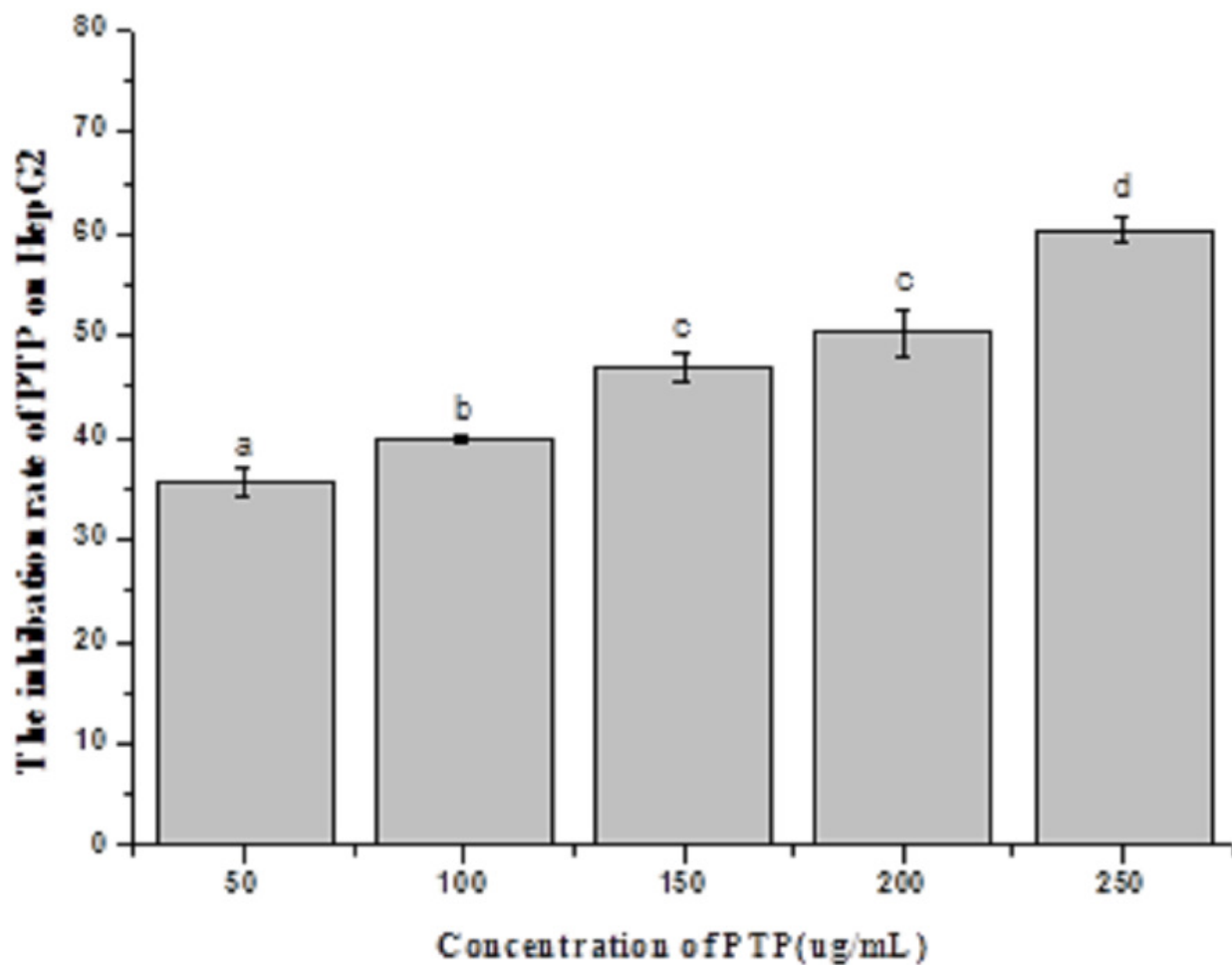
Fig.3 The effect of different concentration PTP on the inhibition rate of HepG2 by MTT assay for 48h

Figure 4

cell apoptosis rate

Fig.4 The cell apoptosis rate under different concentration of PTP
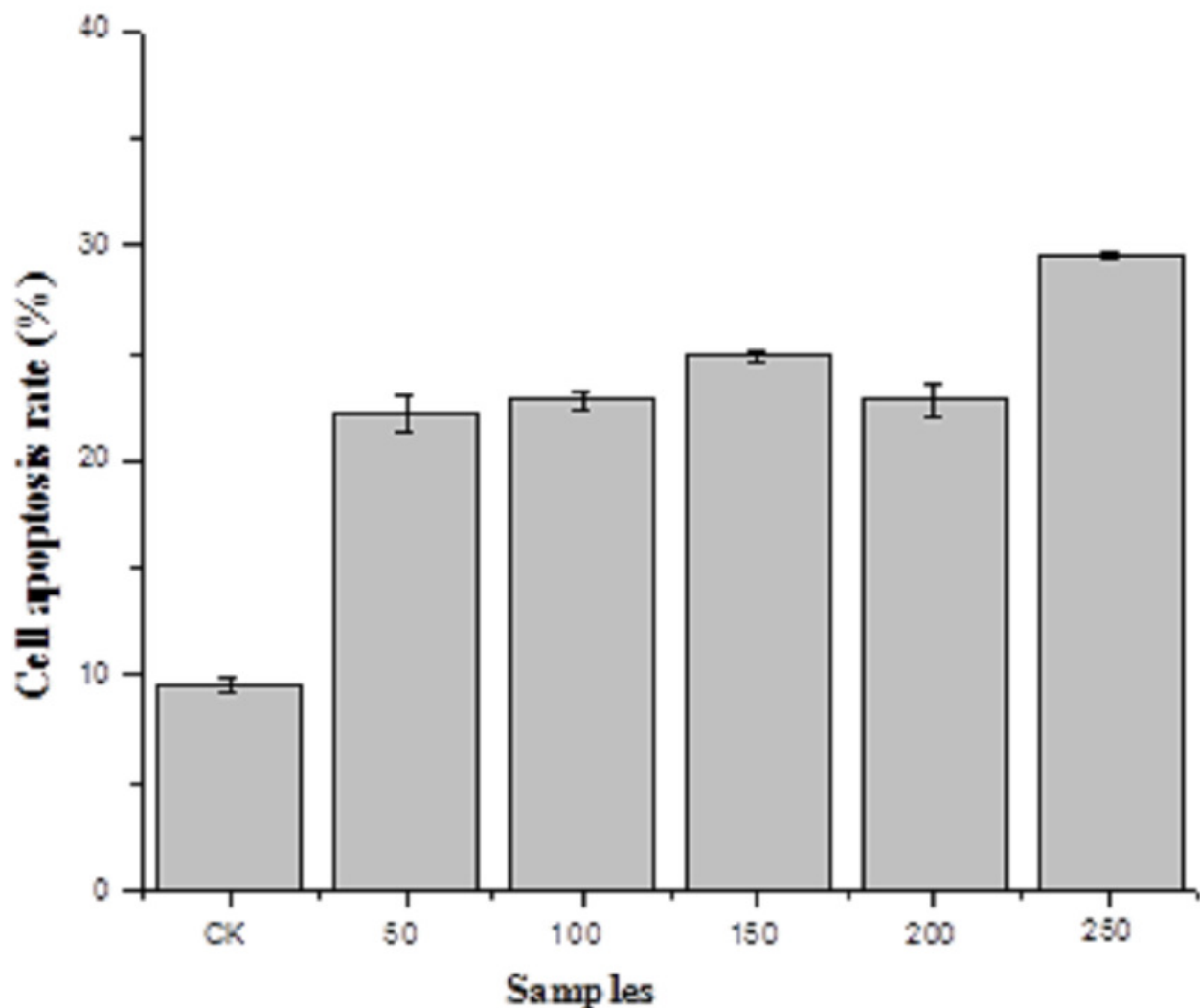
Fig.4 The cell apoptosis rate under different concentration of PTP

Figure 5

cell cycle

Fig.5 The cell cycle rate under different concentration of PTP
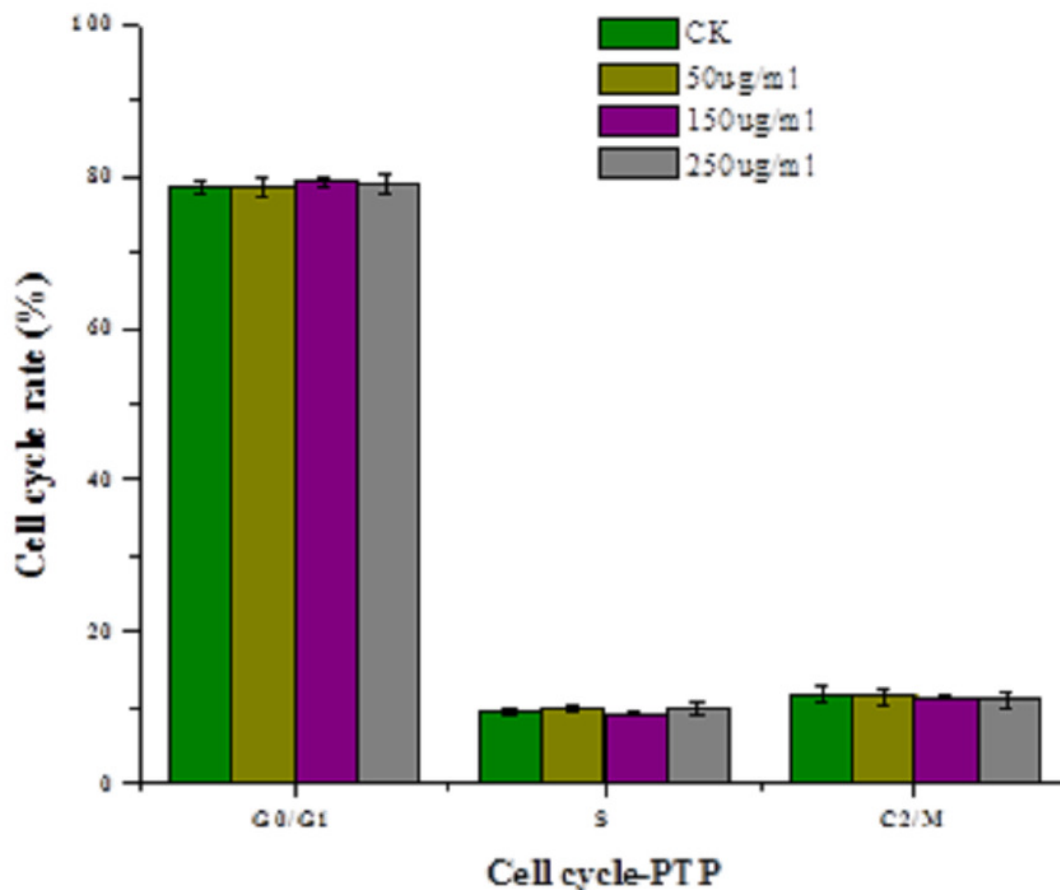
Fig.5 The cell cycle rate under different concentration of PTP

Chemical composition

Chemical composition of PTP(%w/w dry weight)

1 **Table:**

2 **Table 1 Chemical composition of PTP(%w/w dry weight)**

Sample	Total sugar/%	Sulfate/%	Mw/kDa	Monosaccharides Composition (Molar Ratio)						
				Man	**Rha**	**Glc A**	**Glc**	**Gal**	**Xyl**	**Fuc**
PTP	29.94	20.36	4810	0.00	0.25	0.68	0.53	0.56	1.00	0.75

3 Man: mannose; Rha: rhamnose; Glc A: glucuronic acid; Gal: galactose; Glc: glucose;

4 Xyl:xylose; Fuc: fucose