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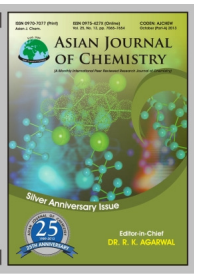

Extraction and Antioxidant Activities of Polysaccharides from *Gynostemma pentaphyllum* Makino

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Crude polysaccharides (GPMPP) and purified polysaccharides (GPMP) were prepared from *G. pentaphyllum* Makino. An orthogonal design [$L_9(3^4)$] was applied to optimize the effects of processing parameters of GPMPP on the yield of polysaccharides. Based on the orthogonal test, optimum conditions were determined. The antioxidant activities of GPMPP and GPMP were investigated and evaluated using various *in vitro* assays. Among these assays, GPMP showed the best ability on the scavenging of hydroxyl radicals, while GPMPP showed the best ability on the inhibition of lipid spontaneous peroxidation, which was higher than that of ascorbic acid. Furthermore, GPMPP showed higher activity of reducing power and scavenging ability on 1,1-diphenyl-2-picryhydrazyl (DPPH) radical but lower activity of inhibitory ability on lipid peroxidation induced by Fe^{2+}-H_2O_2 than that of GPMP in the concentration of 0.1-5.0 mg/mL. The polysaccharides from *G. pentaphyllum* Makino should be explored as a potential antioxidant.

Key Words: *Gynostemma pentaphyllum* Makino, Polysaccharides, Antioxidant activity, Hydroxyl radicals, Lipid spontaneous peroxidation.

INTRODUCTION

Gynostemma pentaphyllum Makino, a perennial liana, is a Chinese herbal medicinal which grows wildly in southwestern China (southern Shaan Xi and areas south of the Yangtze River), Japan, India and Korea[1]. In China, *G. pentaphyllum* Makino is called Jiaogulan and usually used as a herbal tea[2]. Recently several pharmacological effects of *G. pentaphyllum* Makino have been reported, such as antitumor, cholesterol-lowering, immunopotentiation, anti-gastric ulcer, anti-irradiation, treatment of inflammation, hyperlipidemia and atherosclerosis[3-5].

Except for those, more and more researches showed that *G. pentaphyllum* had antisenescent and antioxidant activities. Gypenoside (15.6-500 mg/L) had inhibitory effects on MDA formation in rat hepatic microsomes (including spontaneous, Fe^{2+}-cysteine, vitamin C-NADPH and CCl_4-induced peroxidation of hepatic microsomal lipid in rats). Gypenoside (2.5-160 mg/L) had protective effects on damaged hepatic microsome and mitochondria membrane fluidity[6]. *G. pentaphyllum* also showed scavenging effects where it decreased O_2^- and OH radicals[7]. To date, the research results indicated that the effective antioxidant activities of *G. pentaphyllum* were mainly attributed to gypenoside[2,8]. However, recent studies have suggested that the polysaccharide components of *G. pentaphyllum* Makino also exhibit significant bioactivities, such as immunomodulating activity, anti-exercise fatigue activity, antioxidant activity, *etc*[9-11]. Wang and Luo[11] fractionated and purified polysaccharides from *G. pentaphyllum* Makino and investigated the antioxidant activities of three polysaccharide fractions (GMA, GMB and GMC) on the basis of superoxide radical assay, hydroxyl radical assay and self-oxidation of 1,2,3-phentriol assay. They found that GMC had the higher scavenging effects on superoxide radicals and inhibitory effects on self-oxidation of 1,2,3-phentriol and so should be explored as a novel potential antioxidant. But there is a paucity of more detailed data regarding the antioxidant activity based on other *in vitro* assays.

Accordingly, we attempted to investigate the extraction conditions and the antioxidant activity of the polysaccharides, employing various *in vitro* assay systems, such as reducing power, DPPH/hydroxyl radical scavenging, inhibition of lipid spontaneous peroxidation and lipid peroxidation induced by Fe^{2+}-H_2O_2.

EXPERIMENTAL

1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), ascorbic acid (Vc), thiobarbituric acid (TBA) were purchased from Sigma Co. (St. Louis, USA). All other chemical reagents were analytical grade and purchased from Xi'an Chemical Reagent Co., Ltd. (Xi'an, China). Freshly prepared deionized and redistilled water was prepared in our laboratory. Dried *Gynostemma pentaphyllum* Makino was purchased from Market of Chinese medical materials (Xi'an, Shaanxi Province, China).

Preparation of crude polysaccharides: The *G. pentaphyllum* Makino (200 g) was pretreated with 95 % ethanol at 50 ºC for 3 times to remove some coloured materials, oligosaccharides and some small molecule materials. The organic solvent was volatilized at room temperature and pretreated dry *Gynostemma pentaphyllum* Makino was obtained. The pretreated dry *Gynostemma pentaphyllum* Makino was extracted with distilled water. An orthogonal design [$L_9(3^4)$] was applied to optimize the extraction conditions. Four variables including solid: liquid ratio (A), extraction time (B), extraction temperature (C), times of extraction (D) were investigated. Nine extractions were carried out. The polysaccharide extraction parameters are shown in Table-1. After each extraction, the solid removed by centrifugation and the supernatant was collected. The supernatant was concentrated and precipitated with ethanol (1: 4, v/v). The mixture was kept at 4 ºC for 12 h to precipitate the polysaccharides. The precipitate that formed was collected by centrifugation at 12,000 × g and repeatedly washed sequentially with possibly less amounts of ethanol, acetone and ether, respectively. Then the precipitate was dried at reduced pressure, the dried powder obtained named as GPMPP.

TABLE-1
FACTORS AND LEVELS FOR ORTHOGONAL TEST

Levels	Main parameters			
	A Solid: liquid ratio (g/mL)	B Extraction time (h)	C Extraction temperature (ºC)	D Times of extraction
1	1:5	0.5	75	2
2	1:10	1.0	85	3
3	1:15	1.5	95	4

Preparation of purified polysaccharides: The GPMPP (10 g) was dissolved in distilled water and then decoloured by hydrogen peroxide and excluded protein with Sevag method[12] and dialyzed against distilled water for 96 h (firstly, dialyzed with reversely flowing distilled water for 48 h. Secondly, dialyzed with still distilled water for 48 h, changing the distilled water every 4 h) with dialysis tubing (molecular weight cut-off, 8000 Da) to remove low-molecular weight matters (*e.g.*, chromones and anthranoids) and then concentrated and precipitated with 4-fold volumes of 95 % ethanol to obtain the polysaccharides. The precipitate that formed was collected by centrifugation at 12,000 × g and repeatedly washed sequentially with possibly less amounts of ethanol, acetone and ether, respectively. Then the precipitate was dried at reduced pressure, the dried white powder obtained named as GPMP.

Detection method

Reducing power: The reducing power was determined as described by Tseng and Yang[13]. Each polysaccharide powder (0.1-5 mg/mL, 2.5 mL), in deionized water, was mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of 1 % potassium ferricyanide and the mixture was incubated at 50 ºC, for 20 min. After 2.5 mL of 10 % trichloroacetic acid (w/v) were added, the mixture was centrifuged at 200 g for 10 min. The upper layer (5 mL) was mixed with 5 mL of deionized water and 1 mL of 0.1 % ferric chloride and the absorbance was measured at 700 nm against a blank (water instead of GPMPP and GPMP solution). A higher absorbance indicates a higher reducing power. Ascorbic acid and BHT were used for comparison.

Scavenging ability on 1,1-diphenyl-2-picrylhydrazyl (DPPH) radicals: Each polysaccharide powder (0.1-5 mg/mL, 4 mL) in deionized water was mixed with 1 mL of methanolic solution containing DPPH radicals, resulting in a final concentration of 0.2 mM DPPH. The mixture was shaken vigorously and left to stand for 0.5 h in the dark and the absorbance was then measured at 517 nm, against a blank (water instead of GPMPP and GPMP solution). The scavenging ability was calculated as follows: scavenging ability (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. Ascorbic acid and BHT were used for comparison.

Scavenging ability on hydroxyl radicals: Hydroxyl radical scavenging activity was measured using a modified Smimoff and Cumbes' method[14]. The reaction mixture, containing different samples (0.1-5.0 mg/mL), was incubated with 2 mM EDTA-Fe (0.5 mL), 3 % H_2O_2 (1 mL) and 360 µg/mL crocus in 4.5 mL sodium phosphate buffer (150 mM, pH 7.4) for 0.5 h at 37 ºC and hydroxyl radical was detected by monitoring absorbance at 520 nm. In the control, sample was substituted with distilled water and sodium phosphate buffer replaced H_2O_2. The scavenging ability was calculated as follows: scavenging ability (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. Ascorbic acid and BHT were used for comparison.

Inhibiting ability on lipid spontaneous peroxidation: The assay was performed by using the method described by Sun *et al.*[15] with some modifications. The liver tissue was separated rapidly and 20 % homogenates were formed with iced Tris-HCl buffer (20 mmmol/L, pH 7.4). The reaction mixture that contained polysaccharides of different molecular weight (0.1-5.0 mg/mL, 0.2 mL) and liver homogenate (2.5 %, 1 mL) was incubated at 37 ºC for 1 h. The reaction was terminated by adding 1 mL of 20 % (w/v) trichloroacetic acid. The reaction mixture was shaken and heated at 100 ºC for 15 min to remove protein after adding thiobarbituric acid (TBA) (0.67 %, 1.5 mL) and then centrifuged at 6000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm. The inhibition of lipid peroxidation was calculated as follows: inhibition effect (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the control and A_1 is the absorbance of the sample. Ascorbic acid and BHT were used for comparison.

Inhibiting ability on lipid peroxidation induced by Fe^{2+}-H_2O_2: The reaction mixture that contained polysaccharides of different molecular weight (0.1-5.0 mg/mL, 0.4 mL), liver homogenate (2.5 %, 1 mL), $FeSO_4$ (6 mmol/L, 0.1 mL) and H_2O_2 (60 mmol/L, 40 µL) was incubated at 37 ºC for 1 h. The reaction was terminated by adding 1.0 mL of 20 % (w/v) trichloroacetic acid. The reaction mixture was shaken and heated at 100 ºC for 15 min to remove protein after adding thiobarbituric acid (TBA) (0.67 %, 1.5 mL) and then centrifuged at 6000 rpm for 10 min. The absorbance of the upper layer was measured at 532 nm. The inhibition of lipid peroxidation was calculated as follows: inhibition effect (%) = $[(A_0 - A_1)/A_0] \times 100$, where A_0 is the absorbance of the

control (water instead of GPMPP and GPMP solution) and A_1 is the absorbance of the sample. Ascorbic acid and BHT were used for comparison.

Statistical analysis: All the data were expressed as means ± standard deviations (SD) from three independent replicates. Student's *t*-test was used for the statistical analysis. Difference was considered significant when *p*-value was < 0.05.

RESULTS AND DISCUSSION

An orthogonal design [$L_9(3^4)$] was applied to optimize the solid: liquid ratio, extraction time, extraction temperature and the times of extraction. Table-2 shows the yields of the extraction of polysaccharide under the indicated conditions. The results of experiments presented in Table-2 indicated that the maximum extraction yield of the crude extract was 7.73 %. According to the R values, we can find that the orders of impact of the different factors on the yield are: A > C > D > B. The solid: liquid ratio was found to be the most important determinant of the yield. The times of extraction had the slighter effect on the yield of polysaccharide comparing with the solid: liquid ratio and extraction temperature. The extraction time had the slightest impacts on the overall yield of polysaccharide. Furthermore, the influence of the solid: liquid ratio on the yield corresponded to that of the extraction temperature. According to the results of the orthogonal test, the optimization of the extraction of the polysaccharides was $A_3B_3C_3D_3$. In other words, the maximum yield of the polysaccharides was obtained when solid: liquid ratio of 1:15, extraction temperature of 95 ºC, times of extraction of 4, extraction time of 1.5 h.

TABLE-2
ANALYSIS OF $L_9(3^4)$ TEST RESULTS

No.	A Solid: liquid ratio (g/mL)	B Extraction time (h)	C Extraction temp. (ºC)	D Times of extraction	Extraction yield (%)
1	A_1	B_1	C_1	D_1	3.98
2	A_1	B_2	C_2	D_2	5.31
3	A_1	B_3	C_3	D_3	7.29
4	A_2	B_1	C_2	D_3	6.61
5	A_2	B_2	C_3	D_1	7.13
6	A_2	B_3	C_1	D_2	5.93
7	A_3	B_1	C_3	D_2	7.73
8	A_3	B_2	C_1	D_3	7.15
9	A_3	B_3	C_2	D_1	6.86
I	16.58	18.32	17.06	17.97	–
II	19.67	19.59	18.78	18.97	–
III	21.74	20.08	22.15	21.05	–
K_1	5.527	6.107	5.687	5.990	–
K_2	6.557	6.530	6.260	6.323	–
K_3	7.247	6.693	7.383	7.017	–
R^a	1.720	0.586	1.696	1.024	–

[a]Refers to the result of extreme analysis.

Generally, the reducing power of samples is stronger and their antioxidant activity is higher. The absorbance value of the produce of each sample at 700 nm reflects its antioxidant capability, as higher absorbance value means stronger reducing power of samples.

Fig. 1 showed that the polysaccharides from *G. pentaphyllum* Makino show some valuable reducing power, but can't compare with that of ascorbic acid and BHT. Fig. 1 also showed that the reducing power of polysaccharides exhibited a dose-dependent activity within the test concentration range of 0-5 mg/mL. With increasing the sample concentration, the reducing powers (absorbance at 700 nm) of crude polysaccharide (GPMPP) tended to increase. At the concentration of 1 mg/mL, the reducing power of GPMPP was 0.642. When the concentration increased to 5 mg/mL, the reducing power increased to 1.618. In addition, GPMPP showed higher activity than that of GPMP, but less than that of ascorbic acid and BHT. Various mechanisms result in the antioxidant activities of antioxidants, including binding of transition metal ion catalysts, decomposition of peroxides, prevention of chain initiation and continued hydrogen abstraction, reductive capacity and radical scavenging[16]. The reducing capacity of a certain compound may serve as a significant indicator of its potential antioxidant activity[17]. Furthermore, during the course of excluding protein, hydrogen bond in crude polysaccharides breaking caused to the transform of polysaccharide structure, which resulted in the weaken reducing power of GPMP.

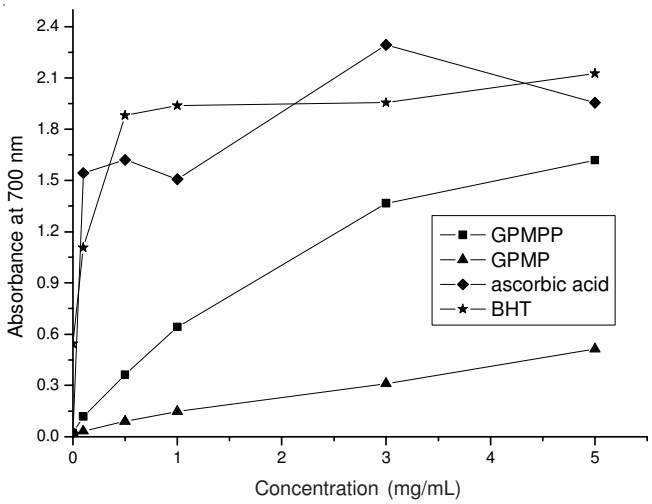

Fig. 1. Reducing power of the polysaccharides from *G. pentaphyllum* Makino

In the experiment, the purple colour of test solution changes into yellow colour induced by antioxidants. Fig. 2 illustrated the scavenging activity of the polysaccharide samples on the DPPH radical. The scavenging ability increased with the increase of concentration up to 5 mg/mL. At the concentration of 0.1 mg/mL, the DPPH radical scavenging activity was 23.66 and 2.2 % for GPMPP and GPMP, respectively. At the concentration of 1 mg/mL, the DPPH radical scavenging activity was 62.88 and 19.79 % for GPMPP and GPMP, respectively. At the concentration of 5 mg/mL, the DPPH radical scavenging activity increased to 77.5 and 63.41 % for GPMPP and GPMP, respectively.

In the concentration ranged from 0.1-5.0 mg/mL, the scavenging ability on DPPH radical of GPMPP was much higher than that of GPMP, but can't be comparable with that of ascorbic acid and BHT. The order of the antioxidant activity is BHT > ascorbic acid > GPMPP > GPMP. We found GPMPP had the higher DPPH radical scavenging activity than GPMP, which proved the results of reducing power. The crude polysaccharides had the higher scavenging ability maybe due to the protein

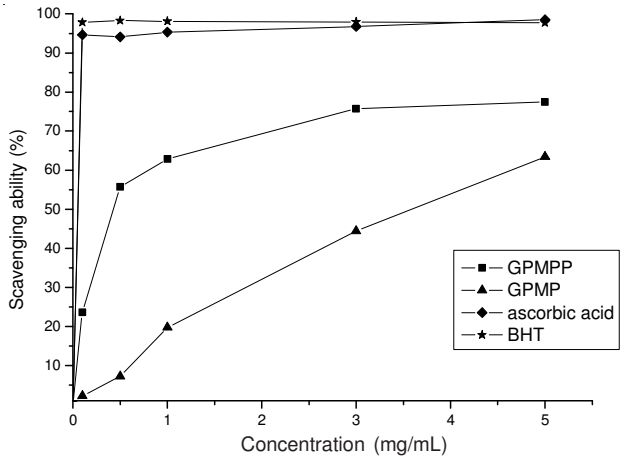

Fig. 2. Scavenging ability of the polysaccharides from *G. pentaphyllum* Makino on DPPH radicals

and pigments in the crude polysaccharides. The protein and pigments had the DPPH radical scavenging activity, thus the synergistic action of them caused to the high scavenging activity.

Hydroxyl radical can easily cross cell membranes and can readily react with most biomolecules such as carbohydrates, proteins, lipids, DNA in cells and cause issue damage or cell death. Thus, for the protection of living systems, removing hydroxyl radical is very important[18]. Fig. 3 shows the scavenging activity of the polysaccharide samples on hydroxyl radical. As shown in Fig. 3, GPMPP and GPMP exhibited obvious scavenging activity on hydroxyl radical in a concentration-dependent manner. The scavenging ratios were improved with the increase of polysaccharide concentration. As the concentration beyond 3 mg/mL, the scavenging ratios increased lower, tending to steady. At the concentration of 1 mg/mL, the hydroxyl radical scavenging activity of GPMPP was close to 50 %. But when the scavenging activity of GPMP was 50 %, the concentration was just 0.5 mg/mL, indicating that the scavenging activity of GPMP was higher than GPMPA at the same time.

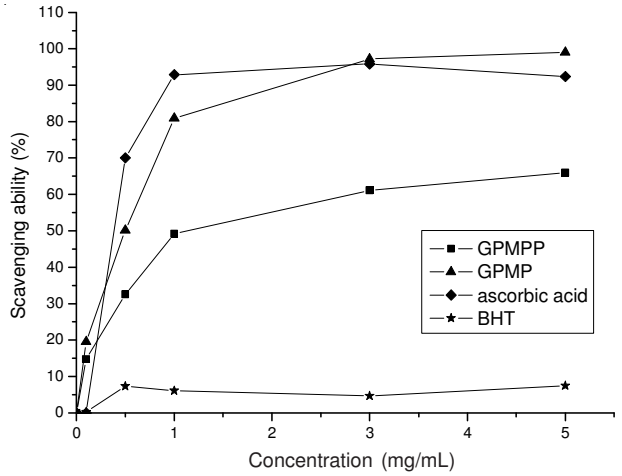

Fig. 3. Scavenging ability of the polysaccharides from *G. pentaphyllum* Makino on hydroxyl free radicals

The scavenging activity of GPMP on hydroxyl radical was so strong that can be comparable with ascorbic acid. At the concentration of 1 mg/mL, the scavenging activity of GPMP had exceeded 80 %. But at 5 mg/mL, the scavenging activity reached up to 99 %, which was higher than that of ascorbic acid. The antioxidant mechanism of polysaccharides from *G. pentaphyllum* Makino may be due to the degradation of the main component by hydroxyl radical[19].

Lipid peroxidation is a typical free radical oxidation and proceeds *via* a cyclic chain reaction, some of its end-products such as lipid hydroperoxides and various species have unpaired electrons or the ability to attract electrons from other molecules. All of the end-products mentioned above can damage DNA directly or indirectly[20]. The effects of GPMPP and GPMP on liver lipid spontaneous peroxidation were shown in Fig. 4. GPMPP and GPMP inhibited lipid spontaneous peroxidation obviously and the inhibitory rates were improved as the polysaccharide concentration increased.

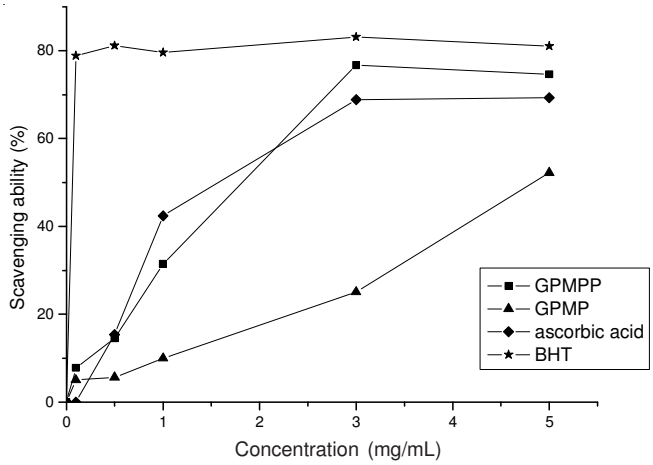

Fig. 4. Effects of the polysaccharides from *G. pentaphyllum* Makino on liver lipid peroxidation

In the concentration range of 0.1-5.0 mg/mL, the inhibitory rate of lipid spontaneous peroxidation of GPMPP was higher than that of GPMP. At the concentration of 0.1 mg/mL, the inhibitory rates were 7.86 and 5.05 % for GPMPP and GPMP, respectively. At the concentration of 5 mg/mL, the inhibitory rates were 74.63 and 52.23 % for GPMPP and GPMP, respectively. When the concentration of GPMPP was above 3 mg/mL, the inhibitory rates tended to stability. But to GPMP, the inhibitory rates increased with the concentration (0.1-5.0 mg/mL) increased. The mechanism of inhibiting effect may be relative to the polysaccharide-membrane interactions[21]. Especially for the crude polysaccharide, the inhibitory rate can be comparable with that of ascorbic acid, which is one of the most important finds in our research as well.

Fe^{2+} and H_2O_2 are strong free radical revulsivums. The MDA quantity produced by autoxidation increased after adding free radical revulsivums into liver homogenate of mice. The effects of GPMPP and GPMP on liver lipid peroxidation induced by Fe^{2+}-H_2O_2 were shown in Fig. 5. The inhibitory rates were improved as the polysaccharide concentration increased. At the concentration of 0.5 mg/mL, the inhibitory rates were 13.45 and 11.05 % for GPMPP and GPMP, respectively. At the concentration of 3 mg/mL, the inhibitory rates were 32.73 and 52.47 % for GPMPP and GPMP, respectively.

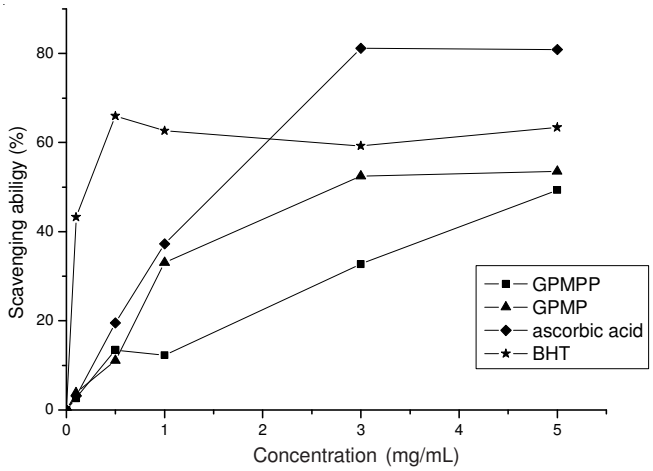

Fig. 5. Effects of the polysaccharides from *G. pentaphyllum* Makino on liver lipid peroxidation induced by Fe^{2+}-H_2O_2

At the concentration of 5 mg/mL, the inhibitory rates were 49.35 and 53.53 % for GPMPP and GPMP, respectively. GPMPP inhibited lipid spontaneous peroxidation in a concentration-dependent manner. When the concentration was above 3 mg/mL, the inhibitory rates of GPMP increased lower. Furthermore, GPMPP and GPMP possessed weaker lipid peroxidation induced by Fe^{2+}-H_2O_2 inhibiting ability than that of ascorbic acid and BHT (3 mg/mL, 81.14 and 59.28 %, respectively). Compared with GPMP, GPMPP showed little higher inhibitory activities on lipid peroxidation. In the assay of iron-induced lipid peroxidations, the presence and involvement of metal ions and oxygen induced generation of free radicals[22]. The results suggested that there was a combination between GPMPP or GPMP and metal ions, which could interfere with the free radical reaction chains, although this combination was not very strong. Liu *et al.*[22] reported that the antioxidant activity of polysaccharides may have originated from their hydrogen atom-donating capacity. The polysaccharides appear to function as good hydrogen atom donors and therefore should be able to terminate radical chain reactions by converting free radical to more stable products.

Conclusion

In this study, we prepared polysaccharides from *G. pentaphyllum* Makino by boiling-water extraction. The optimum conditions obtained by orthogonal test for extraction of the polysaccharides from *G. pentaphyllum* Makino include the following parameters: solid: liquid ratio of 1:15, extraction temperature of 95 ºC, times of extraction of 4, extraction time of 1.5 h. The polysaccharides exhibited strong antioxidant properties, especially scavenging ability on hydroxyl radicals and inhibiting ability on lipid spontaneous peroxidation. In the assay of reducing power and scavenging ability on DPPH radical, GPMPP showed higher activity than that of GPMP in the concentration ranged from 0.1-5.0 mg/mL, but less than that of ascorbic acid and BHT. However, in the assay of inhibitory activities on lipid peroxidation induced by Fe^{2+}-H_2O_2, GPMP showed higher activity than that of GPMPP in the concentration of 0.1-5.0 mg/mL. GPMPP showed much higher inhibiting ability on lipid spontaneous peroxidation than that of GPMP. Especially in the concentration above 3 mg/mL, the inhibiting ability on lipid spontaneous peroxidation was so high that can be comparable with BHT even beyond ascorbic acid. Furthermore, GPMP showed so high scavenging ability on hydroxyl radical that can be comparable with ascorbic acid and better than that of GPMPP. The results suggest that polysaccharides from *G. pentaphyllum* Makino have direct and potent antioxidant activities and should be explored as a novel potential natural antioxidant. Further works on the mechanisms of antioxidant activity and structure characteristics of the purified polysaccharides isolated from *G. pentaphyllum* Makino are in progress.

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