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Effect of acetylation on antioxidant and cytoprotective activity of polysaccharides isolated from pumpkin (*Cucurbita pepo*, lady godiva)

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ABSTRACT

Acetylation of pumpkin (*Cucurbita pepo*, lady godiva variety) polysaccharide using acetic anhydride with pyridines as catalyst under different conditions was conducted to obtain different degrees of acetylation on a laboratory scale. Furthermore, antioxidant activities and cytoprotective effects of pumpkin polysaccharide and its acetylated derivatives were investigated employing various established *in vitro* systems. Results showed that addition of pyridine as catalyst could increase the degree of substitution, whereas volume of acetic anhydride had little effect. The acetylated polysaccharides in DPPH scavenging radical activity assay, superoxide anion radical activity assay and reducing power assay exhibited higher antioxidant activity than that of unmodified polysaccharide. H_2O_2-induced oxidative damages on rat thymic lymphocyte were also prevented by pumpkin polysaccharide and its acetylated derivatives and the derivatives presented higher protective effects. On the whole, acetylated polysaccharide showed relevant antioxidant activity both *in vitro* and in a cell system.

1. Introduction

Lady godiva pumpkins (*Cucurbita pepo* lady godiva), released by United States Department of Agriculture (USDA) in 1972, are grown only for their seeds, which are hulless. Lady godiva pumpkins have been accepted as a dietary constituent in China and received considerable attention in recent years because of the nutritional and health protective value of the proteins and oil, from the seeds as well as the polysaccharides from the fruits (Murkovic, Piironen, Lampi, Kraushofer, & Sontag, 2004; Siegmund & Murkovic, 2004).

Polysaccharides isolated from plants have attracted much attention in the biomedical field because of their therapeutic properties and relatively low toxicity (Schepetkin & Quinn, 2006). The high potential for exploiting polysaccharides with their broad range of structural, functional and physicochemical properties has provided the stimulus for the search for new polysaccharides and their application. Some plant polysaccharides even have been commercially developed into the important components of drugs and skin care products (Deters, Dauer, Schnetz, Fartasch & Hensel, 2001). It is well known that chemical modification of polysaccharides is an effective method to modify the bioactivities through partial derivatization of functional groups such as hydroxyl groups (Ouk, Thiebaud, Borredon, Legars, & Lecomte, 2002). Thus, chemical modification could provide an opportunity to obtain new pharmacological agents with possible therapeutic uses (Alban, Schauerte, & Franz, 2002), for example, antioxidants.

Antioxidants are important for bodily protection against oxidative stress. Lipid oxidation by reactive oxygen species (ROS) such as super oxide anion, hydroxyl radicals and hydrogen peroxide also causes a decrease in nutrition value of lipids, in their safety and appearance. In addition, it is the predominant cause of qualitative decay of foods, which leads to rancidity, toxicity and destruction of biochemical components important in physiologic metabolism. Polysaccharides from various sources have emerged as an important class of antioxidants, for instance, *Porphyra haitanensis* (Zhang et al., 2010), *Ulva pertusa* (Qi et al., 2005) and *Laminaria japonica* (Wang, Liu, Zhang, Zhang, & Qi, 2009).

The antioxidant activity of polysaccharide mainly depends on several structural parameters such as degree of substitution, the molecular weight, type of sugar and functional groups (Melo, Feitosa, Freitas, & de Paula, 2002). Our previous work has investigated the structure and hypoglycemic activity of pumpkin polysaccharide (Li, Fu, Rui, Hu, & Cai, 2005; Song, Li, Hu, Ni, & Li, 2011), but the antioxidant activity of the polysaccharide and its derivatives still remains unknown. The central goal of this study

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was to investigate the structural changes of the acetylated pumpkin polysaccharides and evaluate the antioxidant and cytoprotective activities *in vitro*, which may represent a new approach for inhibiting the harm caused by excessive free radicals.

2. Materials and methods

2.1. Plant materials and chemicals

Fresh lady godiva pumpkin (*C. pepo*, lady godiva variety) fruits at the commercially mature stage were purchased from a local commercial market in Beijing, China. Fruits were selected for their uniformity in shape, weight and color.

DPPH, NBS, NADH, NBT, PMS and MTT were purchased from Sigma Chemical Co. (St. Louis, MO). All the other chemicals used purchased from Sinopharm Chemical Reagent Beijing Co. Ltd and were all of analytical grade.

2.2. Extraction and purification of pumpkin polysaccharide

Pumpkin polysaccharides were extracted by hot water following the method of Song et al. (2011). Briefly, the pumpkins were first treated with water of 85 °C, and then extracted with anhydrous ethanol to the final alcohol concentration of 80%. The polysaccharides were obtained by applying to a DEAE Sepharose Fast Flow gel column and furthered purified by a column of Sephacryl S-300 High Resolution.

The total sugar content of the polysaccharide was estimated by the phenol-sulfuric method (Dubois, Gillis, Hamilton, Rebers, & Smith, 1956).

2.3. Acetylation of pumpkin polysaccharide

Acetylation of the polysaccharide was carried out by the method of Xu, Leppänen, Eklund, Holmlund, and Sjöholm (2010) with some modifications. The dried polysaccharide powder (1 g) was dissolved in acetic anhydride of predetermined amount and 1% *N*-bromosuccinimide (NBS). Pyridine (3 mL) used as catalyst was then added. After reacted for 6 h at 80 °C, distilled water was added to terminate reaction. The sample was cooled to ambient temperature and precipitated with 75% ethanol for 12 h. The precipitate was then dissolved in distilled water and neutralized with 1 M NaOH. The acetylated polysaccharide was concentrated at low pressure by a rotary evaporator and then lyophilized.

The acetyl content was determined by hydroxylamine–ferric trichloride method described by Notari and Munson (1969). Acetyl content (%A) was obtained as the following equation:

$$A\% = \frac{[(V_0 - V_n) \times \mathrm{N} \times 43]}{M} \times 100\% \quad (1)$$

where V_0 is the volume of hydrochloric acid consumed for the sample in liters, V_n represents the volume of hydrochloric acid consumed for the blank in liters, N stands for the normality of the hydrochloric acid, 43 is the molecular weight of acetyl group and M is the mass of the sample in grams.

Degree of substitution was calculated as follows:

$$DS = \frac{162 \times A\%}{(4300 - 42 \times A\%)} \quad (2)$$

2.4. Infrared spectrum analysis

Fourier-transform infrared spectra of the polysaccharide were recorded with a Nicolet 6700 FT-IR spectrometer (Madison, WI, U.S.A) using the KBr disk method.

2.5. Assay for antioxidant activity

2.5.1. Assay of DPPH radical scavenging activity

The DPPH radical scavenging activity was measures by the method of Giri, Osako, Okamoto, Okazaki, and Ohshima (2011). The acetylated polysaccharide was precisely weighted and dissolved in distilled water to obtain a final concentration of 100 μg/mL. Two milliliters of 0.2 mM DPPH in ethanol was added to 1 mL of the sample solution. The absorbance was measured at 517 nm after 20 min of incubation at 25 °C. Ethanol instead of DPPH was used for the control while distilled water instead of sample was used for the blank. The DPPH radical scavenging activity of the sample was calculated by the following equation:

$$\text{DPPH radical scavenging activity}(\%) = \left[1 - \frac{(A_{\text{sample}} - A_{\text{control}})}{A_{\text{blank}}}\right] \times 100 \quad (3)$$

2.5.2. Assay of superoxide anion radical scavenging activity

Superoxide anion radical scavenging activity was determined by the method of Song, Mei, Hu, Zhang, and Chai (2012) with some modifications. The samples were dissolved in distilled water to obtain a final concentration of 100 μg/mL. Then the polysaccharide was mixed with Tris–HCl (16 mM, pH 8.0), NADH (338 μM), NBT (72 μM) and PMS (30 μM). The reaction solution was incubated at room temperature for 5 min and the absorbance was then measured at 560 nm. The reaction mixture without sample was used as blank. The superoxide anion radical scavenging activity of the sample was calculated by the following equation:

$$\text{superoxide anion radical scavenging activity}(\%) = \left(1 - \frac{A_{\text{sample}}}{A_{\text{blank}}}\right) \times 100 \quad (4)$$

where A_{sample} was the absorbance of the test sample mixed with reaction solution; A_{blank} was the absorbance of the control (deionized water).

2.5.3. Assay of reducing power

The reducing power of all the samples was measured by the method of Sun, Bai, Zhang, Liao, and Hu (2011). Samples of 100 μg/mL were mixed with 2.5 mL of 0.2 M sodium phosphate buffer (pH 6.6) and 2.5 mL of potassium ferricyanide (1%). The mixture was incubated for 20 min at 50 °C. The reaction terminated by adding TCA solution (10%, w/v) and the mixture was centrifuged at 3000 rpm for 10 min. The supernatant was mixed with distilled water and ferric chloride (0.1%, w/v) solution and the absorbance was measured at 700 nm against blank.

2.6. Evaluation of cell viability

Thymus was aseptically removed from sacrificed mice and forceps in cold phosphate-buffered saline (PBS, pH 7.2), homogenized with loose Teflon pestle, and passed through a sterilized mesh to obtain single cell suspensions, according to the method by Yuan et al. (2006). Thymic lymphocytes were grown in RPMI 1640 supplemented with 5% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2/95% air incubator.

To evaluate the protective effect of acetylated polysaccharide on H_2O_2 induced oxidative stress to thymic lymphocytes, 2×10^4 cells were pretreated with acetylated polysaccharide of different DS for 24 h. After two wash with PBS (pH 7.2), the cells were exposed to

200 μM H_2O_2 for 24 h, and control cells were also incubated under the same condition.

The cell viability was determined by MTT assays according to Cetin and Bullerman (2005). Briefly, cells were transferred into a 96-well plate and 20 μL of MTT tetrazolium salt solution (5 mg/mL) was added into each well with the final concentration at 0.5 mg/mL and incubated at 37 °C for 4 h. Aliquots were measured by absorbance at 490 nm. All the experiments were conducted in triplicate.

2.7. Analysis of cell apoptosis

Flow cytometric analysis of apoptotic cell death was performed by FITC-Annexin V and propidiumiodide (PI) double labeling as described by Zamal, Falcierim, Marhejka, and Vital (1996). Cells treated with H_2O_2 in the presence or absence of polysaccharide for 24 h were harvested, washed with PBS, and then stained with FITC-Annexin V and PI followed by flow cytometry analysis.

2.8. Statistical analysis

Data were expressed as means ± standard deviations of three replicated determinations. One way of variance analysis was applied for determining significant difference at $P < 0.05$ between the results.

3. Results and discussion

3.1. Extraction and chemical analysis

Hot water was used to extract the crude water-soluble polysaccharides from lady godiva pumpkin fruits. The chemical analysis of all the samples was given in Table 1. Relatively to the control, the total sugars of the derivative decreased after modified. The acetyl content of the derivatives was higher than the control, which indicated the acetylation was successful (Aburto et al., 1997; Xu et al., 2010).

Pyridine was used as a catalyst by adding a different amount from 0 to 3 mL. The DS value increased with the utilization of pyridine as seen by comprising Samples 2–5 (Table 1). Acetylation of carbohydrates with various heterogeneous and homogeneous methods has been carried out using different acetylating agents, such as acetic anhydride, acetyl chloride and ketene with acidic and basic catalysts. It has been observed that no remarkable degradation was observed in the acetylation of spruce galactoglucomannan, glucuronoxylan and konjac glucomannan when pyridine was used as catalyst (Fringant, Desbrières, & Rinaudo, 1996; Gröndahl, Teleman, & Gatenholm, 2003; Xu et al., 2010). Although some operating methods are still far from being feasible for application in actual production, pyridine as a conventional catalyst was still utilized.

A variation of acetic anhydride amount from 10 mL to 30 mL showed that DS value was slightly higher at a higher amount of acetic anhydride by comparing Samples 3–5. It might because the amount of acetic anhydride using in the experiment approached the actual consumption.

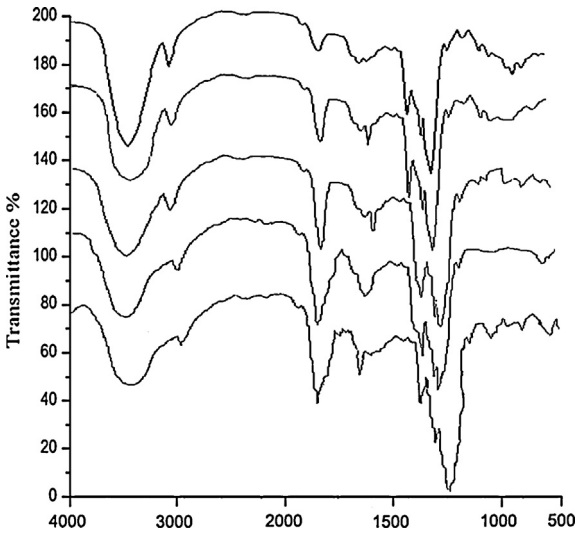

Fig. 1. FT-IR spectrums of unmodified and the acetylated pumpkin polysaccharides.

3.2. Analysis of FT-IR spectrum

FT-IR spectrum of acetylated pumpkin polysaccharide was shown in Fig. 1. After acetylation, the hydroxyl stretching band around 3368 cm^{-1} became considerably smaller. The band around 2931 cm^{-1}, representing the symmetric C-H vibration decreased too. The absorption at 1745 cm^{-1}, which was assigned to stretching vibration of the C=O in carbonyl groups, remarkably increased with the increase of DS value (Lin et al., 2012). Concomitant with the increase of the carbonyl stretching vibration, the absorptions at 1238 cm^{-1} assigned to stretching vibration of the C—O—C in carbonyl groups, was also increased with an increase of DS value (Ren, Sun, Liu, Cao, & Luo, 2007). So did as for the absorption at 1375 cm^{-1} which was assigned to the CH_3 symmetric deformation vibration. These results indicated that acetylation of the polysaccharide proceeded and degree of acetylation was enhanced with increase of substitution degree.

3.3. Antioxidant activities of the acetylated polysaccharide

The DPPH radical scavenging activity is widely used to evaluate antioxidant ability. This method is based on the reduction of ethanolic $DPPH^-$ solution in the presence of a hydrogen donating antioxidant, leading to the formation of non-radical form DPPH-H. As shown in Fig. 2a, the DPPH radical scavenging activity of the purified polysaccharide was 37.36%, which was lower than all the acetylated samples. Furthermore, the highly acetylated polysaccharide presented a significantly higher ($P < 0.05$) scavenging activity than lowly acetylated polysaccharide, for the reason that the highly acetylated derivatives appear to function as good hydrogen atom donors and be able to convert more free radicals to stale products (Jayaprakasha & Patil, 2007; Singh & Rajini, 2004).

Superoxide anion is one of the precursors of the singlet oxygen and hydroxyl radicals, and indirectly initiates lipid per-oxidation (Wickens, 2001). The superoxide anion radical scavenging

Table 1
Chemical analysis of unmodified and acetylated polysaccharides.

Samples	Acetic anhydride (mL)	Pyridine (mL)	Total sugar (%)	Acetyl (%)	DS
Sample 1	–	–	97.42	–	–
Sample 2	30	0	74.33	6.95	0.28
Sample 3	10	3	71.25	11.84	0.41
Sample 4	20	3	75.36	14.77	0.64
Sample 5	30	3	75.21.	15.15	0.67

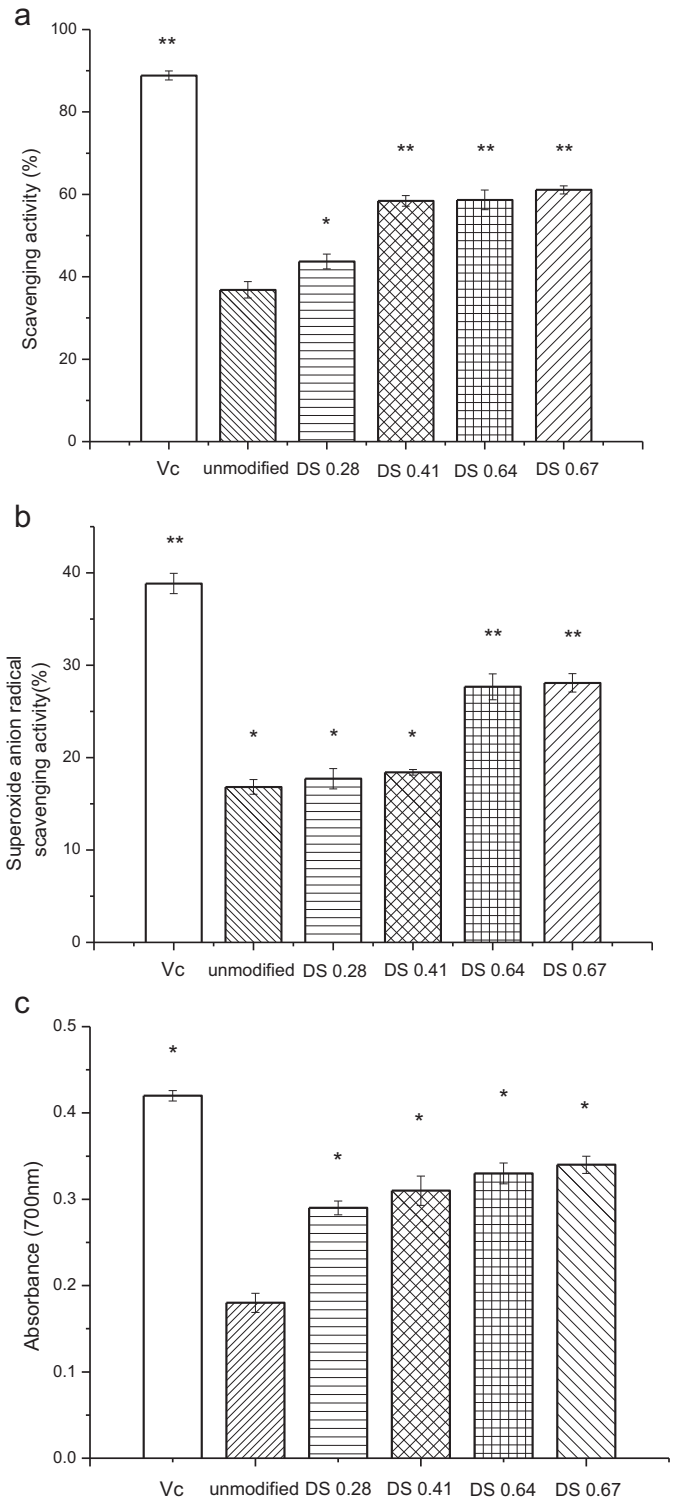

Fig. 2. (a) DPPH radical scavenging activities of the unmodified and acetylated pumpkin polysaccharides. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* vitamin C. (b) Superoxide anion radical scavenging activities of the unmodified and acetylated pumpkin polysaccharides. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* vitamin C. (c) Reducing power of the unmodified and acetylated pumpkin polysaccharides. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* vitamin C.

activity of the polysaccharide was shown in Fig. 2b. The activity of acetylated was 16.83–28.09% with increasing sample DS, whereas that of vitamin C was 38.84%. The acetylated polysaccharide exhibited weaker scavenging effect than vitamin C, but comparing to

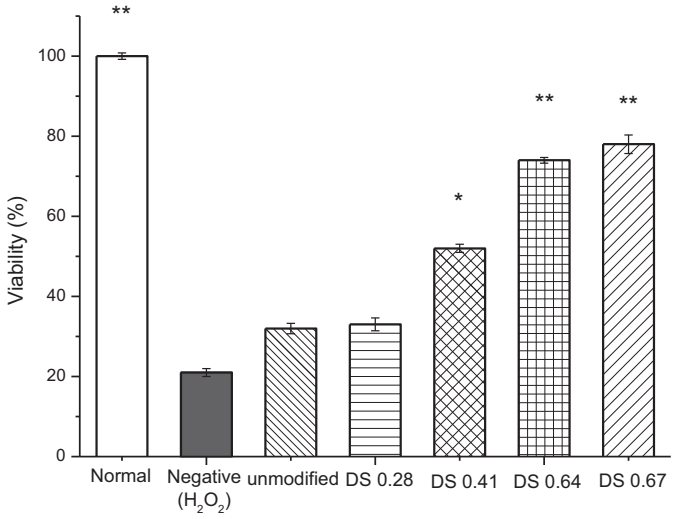

Fig. 3. Protective effect of the unmodified and acetylated pumpkin polysaccharides on H_2O_2-induced cytotoxicity in cells. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* negative control.

non-acetylated polysaccharide, the scavenging activity was higher, especially when the sample was highly acetylated.

The antioxidant activity has been reported to have a direct and positive correlation with reducing power (Osman, Nasarudin, & Lee, 2004). Fig. 2c depicted the reducing power of acetylated polysaccharides. In this assay, the yellow color of the test solution changed to various shades of green and blue depending on the reducing power of each sample with different DS, presenting the reductive potential of the polysaccharides and their acetylated derivatives. The acetylated polysaccharides showed a higher reducing power than the polysaccharide, but compared to vitamin C, the reducing capacity was still lower. The reducing properties are always associated with the presence of reductones, whose antioxidant action is based on the breaking of the free radical chain by donating a hydrogen atom. Reductones also react with certain precursors of peroxide, thus preventing peroxide formation. The results in this experiment showed that acetylated polysaccharides tended to donate more electrons and terminate radical chain reactions.

Chemical modification of polysaccharide plays an important role in obtaining new antioxidants. Many derivatives have been proved to have the ability to scavenge radical, which is related to their chemical structure (Rao & Muralikrishna, 2006; Tsai, Song, Shih, & Yen, 2007; Yuan et al., 2005). The results obtained in this study revealed that, the antioxidant activities of polysaccharide could be improved after acetylated. Acetylated derivatives of high DS exhibited more excellent antioxidant activities than that of little acetyl groups. In general, DS is an essential parameter influencing antioxidant activity of polysaccharide. Besides DS, molecular weight is another important parameter influencing antioxidant activities. Higher antioxidant activities were found when molecular weight decreased (Hou, Wang, Jin, Zhang, & Zhang, 2012; Zhao et al., 2006), which might due to the reaction of acidic surroundings.

3.4. Protective effect against cell death induced by H_2O_2

MTT assay was carried out to measure the cell viability of thymic lymphocyte cells against H_2O_2-induced cell death, cells were exposed to H_2O_2 for 24 h or pretreated with acetylated polysaccharides for 24 h prior to H_2O_2 exposure. As seen in Fig. 3, cell viability significantly decreased after H_2O_2 exposure, but it significantly increased when pretreated with polysaccharide and its acetylated derivatives, compared to H_2O_2 alone. Especially when

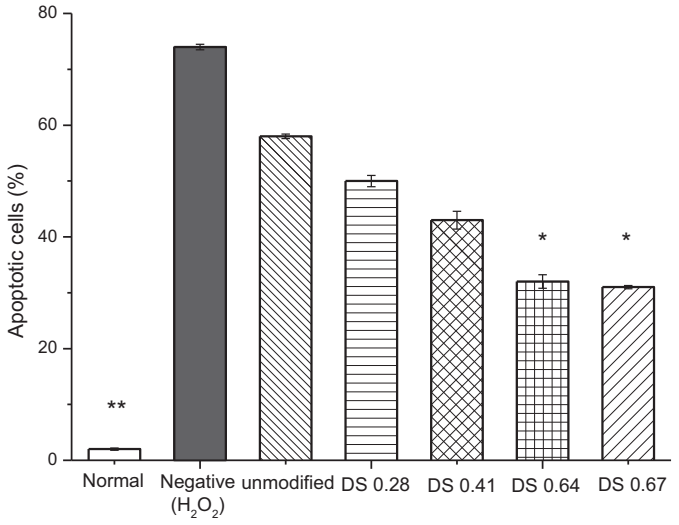

Fig. 4. Inhibition effect of the unmodified and acetylated pumpkin polysaccharides on H_2O_2-induced apoptosis in cells. $^*P < 0.05$, $^{**}P < 0.01$ *vs.* negative control.

the DS reached 0.64 and 0.67, over 70% viability was observed during the test interval.

3.5. Protective effect against cell apoptosis induced by H_2O_2

Significant cell apoptosis was induced in thymic lymphocyte cells after challenge with H_2O_2. However, pretreatment with acetylated polysaccharide of different DS significantly protect cells from H_2O_2-induced oxidative stress as compared with negative control (Fig. 4).

The different derivatives of pumpkin polysaccharide exhibited higher antioxidant and cytoprotective effect than the pumpkin polysaccharide in certain antioxidant systems *in vitro*, which indicated that the chemical modification could enhance their antioxidant and cytoprotective effect. The acetyl groups could activate the hydrogen atom of the anomeric carbon. The higher activated capacity of the group, the stronger hydrogen atom-donating capacity the acetyl derivatives have. Meanwhile, the derivatives showed effective protection against H_2O_2 induced oxidative damage on rat lymphocytes, which may be due to the improvement in the reduced activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Mou, Jiang, & Guan, 2003).

4. Conclusions

Addition of pyridine as catalyst could increase the degree of substitution, whereas volume of acetic anhydride had little effect. The acetylated polysaccharides showed higher antioxidant activity than that of unmodified polysaccharide. Pumpkin polysaccharide and its acetylated derivatives could also inhibit H_2O_2-induced cell death and cell apoptosis on rat thymic lymphocyte and the derivatives presented higher protective effects. Taken together, acetylated polysaccharide exhibited relevant antioxidant activity both *in vitro* and in a cell system.

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