

Lycium barbarum polysaccharide exhibits cardioprotection in an experimental model of ischemia-reperfusion damage

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Abstract. Cardiovascular diseases are a major cause of mortality and disability worldwide. The present study investigated the cardio-protective effects of polysaccharides extracted from *Lycium barbarum* (LB), the fruit of which is traditionally used in Chinese medicine. Polysaccharides were characterized using Fourier transform infrared spectroscopy and high-performance liquid chromatography techniques. The present study demonstrated that LB polysaccharides are composed of glucose and fructose monosaccharides in a molar ratio of 1:2. A total of 36 rats were divided into three groups plus a control group, with nine animals in each group, and were used for studying the cardioprotective effects of LB polysaccharides. The low-dose group received 150 mg/kg body weight (BW) polysaccharides and the high-dose group received 300 mg/kg BW polysaccharides. The results demonstrated that the LB polysaccharides reduced the levels of myocardial lactate dehydrogenase and increased the sodium-potassium ATPase and calcium ATPase activities in rats with heart ischemia-reperfusion injury. In addition, there was a decrease in the myocardial Bax-positive expression and the rate of myocardial cell apoptosis, along with a dose-dependent increase in Bcl-2-positive expression. Therefore, it was concluded that LB polysaccharides are able to halt the progression of cardiovascular diseases.

Introduction

Cardiovascular disorders claim approximately 3.6 million lives each year worldwide, and among cardiovascular disorders, myocardial infarction is the main cause of mortality (1). The rapidly increasing incidence of

metabolic disorders including diabetes, obesity and metabolic syndrome, combined with more aggressive treatment of hypertension, is a causative factor for cardiovascular diseases. The classical approach to the treatment of acute myocardial infarction includes the use of primary percutaneous coronary intervention or thrombolytic therapy, which aim at limiting the size of the infarct. However, the process of reinstating blood flow to the ischemic myocardium can cause further injury to heart tissue and is referred to as ischemia-reperfusion damage. Even with the most advanced medical treatment, ~10% of the patients die following ischemia-reperfusion procedure and ~25% die of heart failure as a consequence of ischemia-reperfusion (1). Experimental studies have validated that oxidative stress generated from ischemia-reperfusion can also lead to myocardial injury (1,2).

Lycium barbarum (LB; also known as goji berry or wolfberry) is a member of the family Solanaceae, and its fruit is well-known in traditional Chinese herbal medicine. At present, it is widely consumed as food, with a number of beneficial effects, including free radical scavenging, lipid lowering, antidiabetic, immuno-modulating and anticancer activities (3-7). Although LB has been traditionally been used in Chinese medicine, its efficacy has been validated recently by numerous studies from western countries (1,2). The beneficial effects of LB fruit have been attributed to its bioactive polysaccharide-protein complex (LBP) (8-12). The LBP fractions were demonstrated to contain six monosaccharides, galactose, rhamnose, glucose, mannose, arabinose and xylose, and 18 different amino acids (8-10). The efficacy of LBP has also been validated in various preclinical models of liver damage, immune dysfunction, diabetes and cancer (9,10,13-15). Taurine, one of the 18 amino acids identified in LB, is recommended as a complementary therapeutic agent for the management of type II diabetes complications and is also known to inhibit caspase-3 in animal models of ischemia-reperfusion (16,17).

To the best of our knowledge, there has been no organized research study on the prevention of cardiovascular disorders using LB at present. Thus, the current study aimed to characterize the extracted polysaccharides of LB and then evaluate the level of biomarkers such as lactate dehydrogenase (LD) and nitric oxide (NO) and the activities of sodium-potassium ATPase and calcium ATPase in the heart tissue in a rat model of ischemia-reperfusion injury.

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Materials and methods

Materials. Commercially available heat-dried wolfberries were purchased from Rich Nature Nutraceutical Labs (Mukilteo, WA, USA).

Polysaccharide extraction. Dry *LB* fruits (100 g) were treated with 300 ml CHCl_3 -MeOH (3:1 v/v) at 70°C under reflux for 2 h, and the extract was passed through an acetone extraction while maintaining the same environment. The lipid-free substance was removed with the aid of 400 ml of ethanol at 70°C and then introduction to 500 ml of water at 100°C for 3 h both under reflux.

Monosaccharide composition analyses. The monosaccharide composition was determined using the acid digestion method. High-performance liquid chromatography (HPLC) was used to examine the produced hydrolyzates. The procedures were performed following the method of Fu and Oneill (18).

Infrared spectrum analysis of *LB* polysaccharides. With the aid of a Fourier transform infrared spectrophotometer (Shimadzu Corporation, Kyoto, Japan) running OPUS software, version 3.1 (Bruker Corporation, Billerica, MA, USA), the infrared spectrum of *LB* polysaccharides was ascertained. For transformation infrared spectrum analysis, 5 mg *LB* polysaccharides was mixed with powdered KBr and pressed into pellets. Measurements were made in a frequency range of 400–4,000 cm^{-1} (19).

Animals and operative procedures. For the present study, 36 adult Wistar male rats were obtained from the Experimental Research Section of Linyi Peoples Hospital (Shandong, China). Each rat weighed 250 ± 10 g. They were kept in cages and were maintained under a constant room temperature and 12/12 h light/dark cycle. The rats were fed with the commercially available rat chow and tap water *ad libitum*. All the experimental procedures performed were approved by the Shandong University Ethical Committee of School of Medicine.

The rats were randomly divided into a control group and three surgical groups. The hearts of the rats were perfused as follows: The rats were anesthetized with 5% napental [30 mg/kg body weight (BW); Shanghai Kefeng Chemical Reagent Co., Ltd., Shanghai, China] and their hearts were immediately excised and placed in cold (4°C) Krebs-Henseleit solution containing 118 mM NaCl, 4.8 mM KCl, 1.2 mM KH_2PO_4 , 1.6 mM CaCl_2 , 1.2 mM MgSO_4 , 25 mM NaHCO_3 and 11.5 mM glucose. Within 2 min of thoracotomy, the hearts were mounted on an experimental setup and retrogradely perfused with Krebs-Henseleit solution (37°C) through the aorta at 15 ml/min (Minipuls-3 peristaltic pump; Gilson SAS, Villiers-Le Bel, France) and aerated with 95% O_2 + 5% CO_2 to maintain normal pH, pO_2 , and pCO_2 levels.

A polyethylene catheter with a small latex balloon at the tip (size 3; Enove Precision Plastics Catheter Co., Ltd., Jiangsu, China) was inserted in the left ventricular cavity through the mitral valve opening to record the pressure in the left ventricle. Hearts that did not reach the peak left ventricular systolic pressure of 85–90 mmHg with left ventricular end-diastolic pressure of 5–6 mmHg (contractile performance 8–10%) were

removed ($n=3$). Left ventricular developed pressure was calculated as the difference between peak left ventricular systolic pressure and ventricular end-diastolic pressure.

The three treatment groups had nine rats each and were as follows: Model control, low-dose and high-dose groups. The low-dose group received 150 mg/kg BW polysaccharides and the high-dose group received 300 mg/kg BW polysaccharides. The rats were anesthetized with 5% napental (30 mg/kg BW; Shanghai Kefeng Chemical Reagent, Co., Ltd.). The hearts were rapidly removed and stored at -70°C for biochemical measurements.

Biochemical analysis. The function of sodium-potassium ATPase was assayed as described previously (20) with certain changes. Briefly, the assay mixture was prepared by mixing microsome (50 μg) and a buffer containing 50 mM Tris-HCl pH 7.4, 100 mM NaCl, 20 mM KCl, 1 mM EGTA, 5 mM ATP and 5 mM MgCl_2 to a final volume of 1 ml. Controls contained the similar reaction mixture together with 1 mM ouabain (Nanjing Chemlin Chemical Co., Ltd, Nanjing, China). ATP was added to the tubes to initiate the reaction and they were incubated at 37°C for 10 min. To stop the reaction, 500 μl 20% trichloroacetic acid (TCA) was added to the tubes, and the quantity of inorganic phosphate was determined. The sodium-potassium ATPase function (percent inhibition against the 100% value) was determined by measuring the difference between the activity in the presence and absence of 1 mM ouabain.

Calcium ATPase activity was determined as indicated in a previous study (21). The incubation mixture was prepared by mixing 40 mM Tris-HCl buffer (pH 7.0), 50 mM KCl, 5 mM MgCl_2 , 2 mM ATP (neutralized with potassium hydroxide), 0.1 mM ouabain, microsomal protein (0.5–0.8 mg/ml) with either 2 mM EGTA or 20 mM CaCl_2 . The enzyme reaction was initiated by adding microsomes, and the tubes were incubated at 37°C for 10 min. Ice cold TCA (10%) was then added to stop the reaction, and the amount of inorganic phosphate released in terms of nmol/min/mg protein was recorded. Calcium ATPase activity was measured by subtracting the values obtained with EGTA from those obtained with CaCl_2 .

Immunohistochemistry was performed by the avidin-biotin-peroxidase method (Thermo Fisher Scientific, Inc., Waltham, MA, USA) (22) on the 300- μm thick sections of heart tissue adjoining those used for terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) detection. The sections were first dewaxed (using xylene) and rehydrated (using distilled water), and then placed into 0.3% H_2O_2 solution. The sections were then incubated with 1.5% normal goat serum to prevent nonspecific binding and fulfill the endogenous peroxidase function. Rat anti-Bcl-2 (cat no. #15071, Cell Signalling Technologies, Danvers, MA, USA) and anti-Bax (cat no. #AF820, R&D Systems, Inc., Minneapolis, MN, USA) antibodies were separately diluted 1:200 and 1:300, respectively, and the sections were then incubated with these primary antibodies in a humidified chamber for 24 h at 4°C. Negative control sections were incubated without the primary antibody. After washing with phosphate-buffered saline (PBS), the sections were individually incubated with biotinylated secondary antibody, goat-anti-rat immunoglobulin G (IgG; 1:200 dilution) (cat no. #ab97057, Abcam, Cambridge, UK),

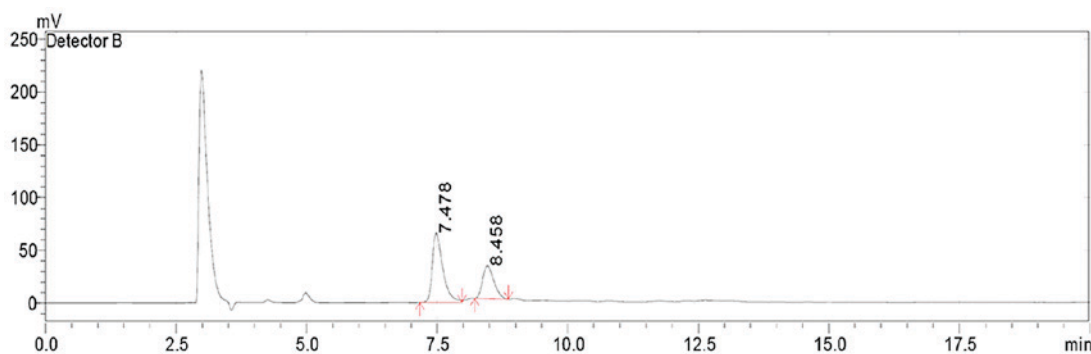


Figure 1. High-performance liquid chromatography analysis of *Lycium barbarum* polysaccharides.

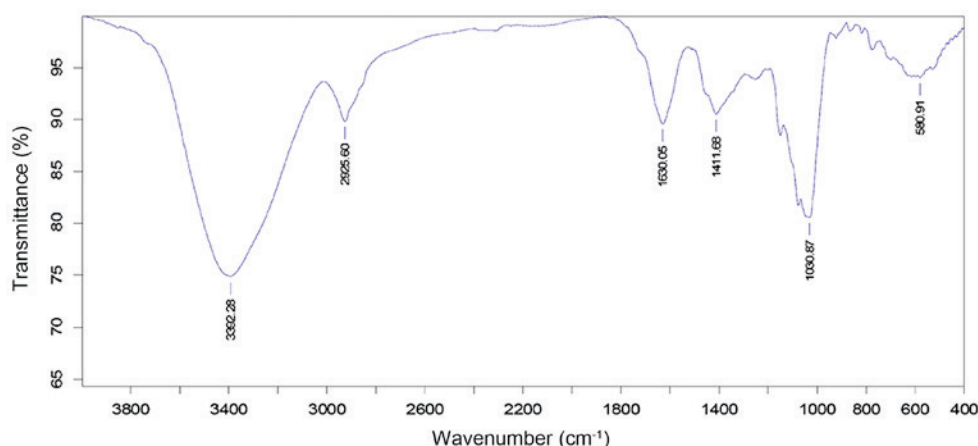


Figure 2. Fourier transform infrared spectroscopy analysis of *Lycium barbarum* polysaccharides.

along with an avidin-biotin-peroxidase complex (Thermo Fisher Scientific, Inc.) for 30 min. The sections were then rinsed in PBS and exposed to diaminobenzidine and H_2O_2 in 50 mM Tris-HCl (pH 7.6) for 3 min. Following a final bath, the immunostained sections were dehydrated in a graded ethanol series. Bcl-2- and Bax-positive cells were quantitatively analyzed using an image analyzer system (Aperio ePathology eIHC IVD System, Leica Biosystems, Beijing, China). The number of cells/mm of the CA1 pyramidal cell layer was calculated for each rat, and the average value from the adjoining two sections was used.

Nitric acid (NO^-) and lactic acid concentrations were calculated using a fluorometric nitric oxide assay kit (cat. no. #K252-200, BioVision, Inc., Milpitas, CA, USA). The apoptotic rate was determined using flow-cytometry (FACSCalibur; BD Biosciences, Franklin Lakes, NJ, USA).

Histopathological analysis. The triphenyl tetrazolium chloride method was used to assess the injuries to the myocardium (23). Myocardial tissue was fixed in 10% paraformaldehyde for 24 h and embedded in paraffin wax. Sections were cut at 3 μm thick and were stained with hematoxylin and eosin following standard procedure and examined under a light microscope (highest magnification, x400). The myocardial damage was graded as follows: Grade III, severe injury to the myocardium characterized by severe edema and predominant contraction bands; grade II, moderate injury to the myocardium characterized by regular presence of contraction bands and significant

interstitial and cellular edema; grade I, minor injury to the myocardium characterized by hydropic cardiomyocytes, few contractions, and low grade interstitial edema; and grade 0, myocytes appear normal without hydrops, cell disruption or interstitial edema. Myocardial damage was measured using the semi-quantitative scale reported by Miller *et al* (24).

Data analysis. Statistical analysis was performed using one-way analysis of variance, Mann-Whitney U-test and Duncan's multiple range test, using SPSS for Windows, version 11.5 (SPSS, Inc., Chicago, IL, USA). Results were expressed as the mean \pm standard error, and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Chemical analysis of LB polysaccharides. HPLC analysis identified that the LB polysaccharides comprised two types of monosaccharides, glucose and fructose in the molar ratio 1:2 (Fig. 1). The infrared spectrum of LB polysaccharides depicted a dominant, broad and stretching peak at 3,392 cm^{-1} for the hydroxyl group and a weak band at 2,925 cm^{-1} showing C-H stretching vibration. A band at 1,000 cm^{-1} referred to the sugar units in the polysaccharide, and a band at 1,630 cm^{-1} to the bound water (Fig. 2).

Effect of LB polysaccharides on LD and NO levels and sodium-potassium ATPase and calcium ATPase activities.

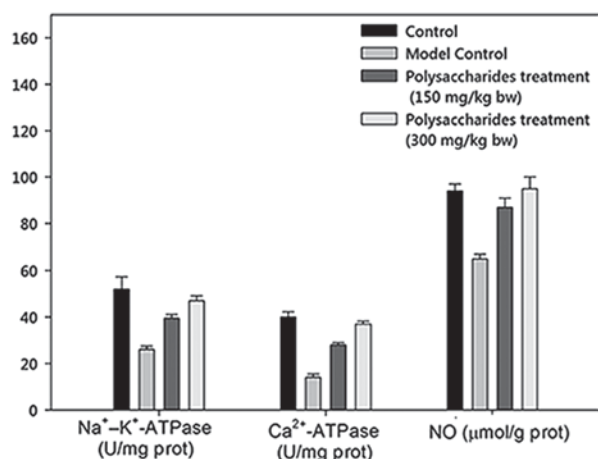


Figure 3. Effect of *Lycium barbarum* polysaccharides on NO levels and sodium-potassium and calcium ATPase activities. NO, nitric oxide.

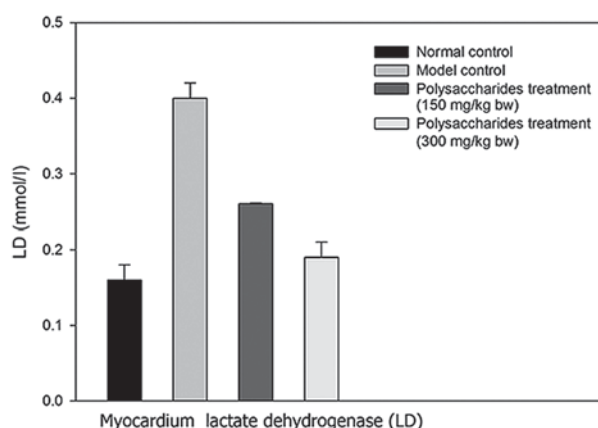


Figure 4. Effect of *Lycium barbarum* polysaccharides on LD activity. LD, lactate dehydrogenase.

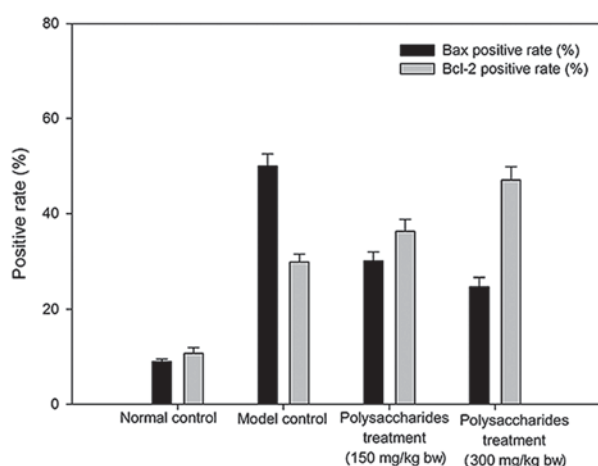


Figure 5. Effect of *Lycium barbarum* polysaccharides on Bax and Bcl-2.

The pharmacological actions of the natural medicines have been demonstrated previously (25-28). There was a marked increase in the myocardial LD concentration in the model group rats in contrast to the healthy control rats (Figs. 3 and 4). Notably, there was a significant increase in myocardial LD

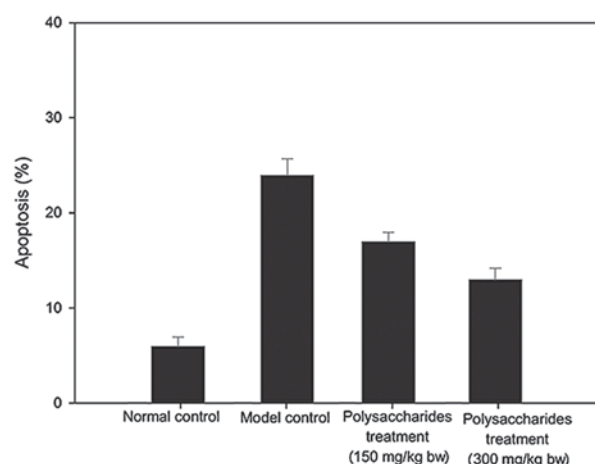


Figure 6. Effect of *Lycium barbarum* polysaccharides on apoptosis (%).

levels in rats receiving 150 and 300 mg/kg BW *LB* polysaccharides. Sodium-potassium ATPase and calcium ATPase activities were significantly decreased in the model controlled group than in healthy controlled rats. Comparatively, there was a significant increase in the myocardial sodium-potassium and calcium ATPase activities in rats receiving 150 and 300 mg/kg BW *LB* polysaccharides.

Effect of *LB* polysaccharides on Bax and Bcl-2. The model group rats demonstrated markedly greater Bax and Bcl-2 positive rates than the healthy group rats. Rats that were administered *LB* polysaccharides demonstrated comparatively higher Bcl-2 positive rates and significantly higher Bax positive rates ($P < 0.01$) when compared with model control rats (Fig. 5).

Fig. 6 presents the myocardial apoptotic rates. Despite the comparatively significant increase in apoptosis of the myocardium cells in the model group in comparison with the healthy group, these variations were remedied with an *LB* polysaccharides supplement.

Histopathological changes. There were significant histological differences ($P = 0.022$) between the control and study groups. The control group demonstrated higher grade II damage, and the study group demonstrated decreased myocardial edema ($P < 0.05$; Fig. 7). Student's *t*-test for independent groups was performed to compare the myocardial damage in each group.

Discussion

LB fruits have been used in Chinese traditional medicine, however there have been limited reports on their efficacy in ischemia-reperfusion damage. The glucose lowering and anti-apoptotic activity of *LB* has been well documented (29) and there is increasing evidence of its efficacy in age-associated macular degeneration (30). The present study demonstrated that fructose and glucose were the most abundant monosaccharides. The fruits of *LB* contained 18 different amino acids, the most abundant of which was taurine (31). Although, taurine is a partially essential amino acid in humans (32), it has demonstrated efficacy in type II diabetes and ischemic

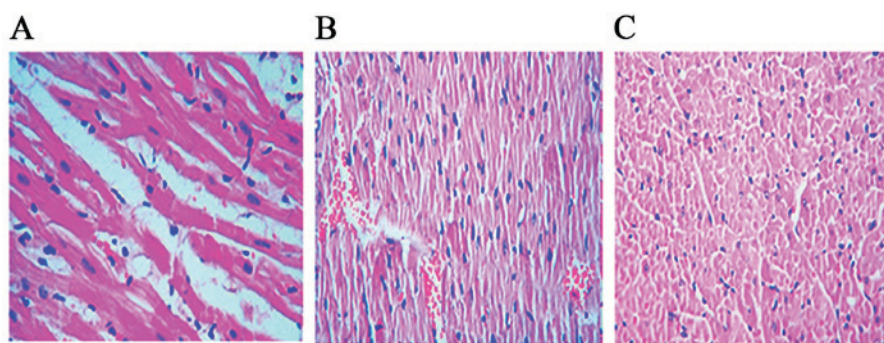


Figure 7. (A) Myocardial morphology of normal rats. (B) Mildly injured myocardium in the *LB* polysaccharides-treated group. (C) Severely injured myocardium following ischemic reperfusion without *LB* polysaccharide treatment (hematoxylin and eosin stain; magnification, x400). *LB*, *Lycium barbarum*.

cardiomyocytes (16). The present study also confirmed that the cardioprotective properties of *LB* polysaccharide was via increased sodium-potassium ATPase and calcium ATPase activities.

The present study also demonstrated that the administration of *LB* polysaccharides significantly decreased myocardial LD levels. Sharikabad *et al* (33) demonstrated that Ca^{2+} and Na^{+} alterations in heart failure decrease the tolerance to ischemia by increasing Ca^{2+} overload. It has been established that an increase in the sodium ion causes excessive Ca^{2+} entry via reverse (Ca^{2+} influx mode) $\text{Na}^{+}/\text{Ca}^{2+}$ exchange during ischemia-reperfusion (34,35). The $\text{Na}^{+}/\text{Ca}^{2+}$ exchange is increased in myocardium disease (36,37). Sjaastad *et al* (37) reported that cardiomyocytes from rats with chronic heart failure demonstrated higher Ca^{2+} influx in an experimental model of Na^{+} -loaded cells. It has also been reported that intracellular Na^{+} concentration is increased in chronic heart disease (38), which causes an increase in Ca^{2+} influx through the $\text{Na}^{+}/\text{Ca}^{2+}$ exchanger (39).

Calcium overload is the determinant to ischemia-reperfusion damage of the myocardium. Increased concentration of intracellular Ca^{2+} also increases the activity of degrading enzymes (40,41), which damages mitochondria and causes arrhythmias (35,42). Therefore, alterations in Na^{+} and Ca^{2+} may render the myocardium prone to ischemia-reperfusion damage.

In conclusion, the data collected from the ischemia-reperfusion model confirmed that *LB* polysaccharides prevent damage to heart tissue by decreasing the myocardium LD and NO levels and increasing sodium-potassium ATPase and calcium ATPase activities. The present study requires further validation in a clinical setting and its efficacy in patients with myocardial infarction remains to be established.

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