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Original Research Article

Inhibitory effects of total saponins from Ilex pubescens Hook against hydrogen peroxide-induced cardiomyocyte apoptosis

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Abstract

Purpose: *To study the protective effects of total saponins from Ilex pubescens Hook (IPTS) against cardiomyocyte apoptosis.*
Methods: *Response surface methodology (RSM) based on Box-Benhnken Design (BBD) was carried out to optimize the extraction of IPTS. Thereafter, H9c2 cell model prepared by hydrogen peroxide (H_2O_2) treatment was used to investigate the effects of IPTS on cardiomyocyte apoptosis. Cell viability was determined using MTT assay, while the levels of lactate dehydrogenase (LDH), malondialdehyde (MDA), superoxide dismutase (SOD), creatine kinase (CK) and catalase (CAT) were measured as indices of oxidative stress. Expressions of proteins related to apoptosis (caspase-3, Bax and Bcl-2) were measured using Western blot assay.*
Results: *Optimal IPTS extraction was achieved with extraction temperature of 86.6 °C, extraction time of 2.23 h and water: raw material ratio of 10.8 mL/g. IPTS extract, at doses of 200, 400, 600 and 800 µg/mL, significantly increased the viability of H_2O_2-treated H9c2 cells ($p < 0.05$), but significantly decreased LDH and CK activities ($p < 0.01$). It also led to significant increases in SOD and CAT activities, and significant decreases in the levels of MDA in these cells ($p < 0.01$). There were significant down-regulation of the protein expressions of caspase-3 and Bax ($p < 0.01$) in IPTS-treated H9c2 cells, as well as significant up-regulation of Bcl-2 protein expression ($p < 0.01$).*
Conclusion: *These results suggest that IPTS can protect cardiomyocytes against apoptosis via the inhibition of oxidative stress and mitochondria-induced intrinsic apoptosis.*

Keywords: *Ilex pubescens, Total saponins, Cardiomyocytes, Apoptosis, H9c2 cells*

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INTRODUCTION

With the rapid development of social economy, the incidence of cardiovascular disease is rising year by year. And in China, cardiovascular disease has been ranked second only to malignant tumors, amongst the killer diseases [1]. Excessive superoxide anion is considered one of the main factors involved in the etiology of cardiovascular disease; it can neutralize nitric oxide and cause endothelial dysfunction [1]. In addition, a number of studies have shown that oxidative stress is a common mechanism of cardiovascular injury caused by many pathological factors [2]. The increased level of ROS in many cardiovascular diseases could lead to lipid and protein peroxidation, irreversible cell damage and DNA damage [3,4].

Ilex pubescens (IP) Hook is used as a popular folk medicine in southern area of China, for treating coronary heart disease, angina and

myocardial infarction [5]. Studies on the phytochemical composition of IP show that it is rich in saponins, flavones and phenylpropanoid compounds [6]. Saponins possess significant anti-coagulation and anti-thrombotic properties [7]. However, to date, there are no studies on the effects of total saponins from IP on myocardial function. Thus, the current study was aimed at investigating the protective effects of total saponins from Ilex pubescens (IPTS) against apoptosis in myocardial cells, and its underlying mechanism, using H_2O_2-treated cardiomyocyte H9c2 cell line model.

EXPERIMENTAL

Cells culture

The cardiomyoblast H9c2 cell line was obtained from Cell Bank of Chinese Academy of Sciences (Shanghai, China). The cells were maintained in 90 % Dulbecco's modified Eagle's medium (DMEM) with 10 % heat-inactivated fetal bovine serum (FBS) and antibiotics (1 % penicillin and streptomycin). The cells were incubated in an incubator with 5 % CO_2 and 95 % humidity at 37 °C.

Chemicals

Ilex saponin B1 was purchased from Shanghai biochemical reagent Co., Ltd. (Shanghai, China). Methyl-thiazdyldiphenyl-tetrazolium bromide (MTT), H_2O_2 and dimethyl sulfoxide (DMSO) were products of Sigma-Aldrich Co. (St. Louis, MO, USA). The DMEM media and FBS were obtained from Gibco Co. (Grand Island, NY, USA). LDH, MDA, SOD, CK and CAT assay kits were obtained from the Jiancheng Bioengineering Institute (Nanjing, China). IP Cell Lysis Buffer, BCA Protein Assay Kit, and Caspase-3, Bcl-2 and Bax monoclonal primary antibodies were obtained from the Beyo time Co. (Haimen, China). All other chemicals used in this study were of analytical reagent grade.

Preparation of LPTS

Ilex pubescens (IP) was purchased from Bozhou Chinese herbal medicine market (Anhui, China) in August 2014, and authenticated at the International Medical Center of the Chinese PLA General Hospital. A voucher specimen (PLA no. 2014-119) was deposited in the herbarium of the Department. Dried LP was processed to powder and exhaustively extracted with 50 % ethanol by hot reflux. The extract was concentrated, dried at 50 °C, dissolved with distilled water and re-extracted with ether and water-saturated butanol. The resultant extract was evaporated under vacuum, and dried at 50 °C to obtain the crude IPTS. The crude IPTS was dissolved in distilled water, and purified using the macro porous adsorption resin method [8].

The IPTS content was determined by vanillin-perchloric-acid method, with Ilex saponin B1 as standard [8]. The relationship between absorbance and concentration can be expressed as A= 0.00315C + 0.0079 (R=0.9920), where A = absorbance, and C = concentration. The LPTS yield (%) was calculated from this equation.

Experimental design and statistical analysis

Results of previous single factor experiments showed that the major factors which influence the extraction of IPTS were extraction temperature (A), extraction time (B) and ratio of water to raw material (C). To obtain optimal extraction of IPTS, RSM based on BBD was used in the present studies. As shown in Table 1, seventeen experiments were performed in random order.

All the tests were replicated three times, and analysis of variance (ANOVA) was used to analysis the BBD results. Design Expert (version 8.0.6, Stat-Ease, Inc., Minneapolis, MN, USA) software was used to estimate the response of independent variables. Response surfaces were obtained to determine the individual and interactive effects of the experimental variable. Subsequently, additional confirmatory experiments were carried out to verify the validity of the statistical experimental design.

MTT assay

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium (MTT) assay was carried out to determine cell viability according to the method reported previously[9]. The H9c2 cells (5×10^4/0.2 mL) were seeded in 96-well plates and treated with LPTS (100, 200, 400, 600 and 800 μg/mL) with the presence of H_2O_2 (100 μmo1/L) for 24 h at 37 °C [10]. Absorbance (A) was measured at 570 nm using a 96-well plate reader (BioTek Instruments, Inc, Burlington, VT, USA). Cell viability (V) was calculated using Eq 1.

$$V\ (\%) = (At/Ac)100 \quad \ldots\ldots\ldots\ldots\ldots\ (1)$$

where At and Ac are the absorbance values of treated and control samples respectively.

Table 1: BBD experimental design with the independent variables

Run	A (°C)	B (h)	C	Yield (%)
1	60.00	1.00	10.00	3.41
2	100.00	3.00	10.00	5.36
3	80.00	1.00	15.00	4.79
4	80.00	2.00	10.00	6.06
5	60.00	2.00	5.00	4.08
6	100.00	2.00	15.00	5.12
7	80.00	2.00	10.00	5.98
8	60.00	2.00	15.00	3.63
9	80.00	2.00	10.00	5.79
10	100.00	1.00	10.00	4.14
11	80.00	2.00	10.00	6.09
12	80.00	2.00	10.00	5.86
13	80.00	3.00	5.00	4.76
14	100.00	2.00	5.00	4.78
15	60.00	3.00	10.00	3.52
16	80.00	3.00	15.00	5.04
17	80.00	1.00	5.00	4.01

Note: BBD = Box-Behnken Design

Determination of LDH, CK, SOD, CAT and MDA in H9c2 cells

After 24 h of treatment, supernatants from the H9c2 cells were collected for the assay of LDH and CK activities using commercial kits according to the manufacture's instruction. Subsequently, total proteins were extracted from the H9c2 cells with IP cell lysis buffer. Then protein concentration was determined using a BCA protein assay kit. The contents of SOD, CAT and MDA were determined with commercial kits.

Western blot analysis

Proteins were extracted from the H9c2 cells treated with IPTS (200, 400 and 800 μg/mL) were extracted with IP cell lysis buffer, and protein concentration was measured using BCA protein assay kit. Equal amounts of cell proteins (35 μg) were separated by 12 % sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinyl idene difluoride (PVDF) membrane and probed with various primary antibodies (caspase-3,Bax and Bcl-2). The resultant protein bands were detected after incubating with secondary antibodies and visualized by enhanced chemiluminescence reagent (Pierce-ECL western blotting substrate, Thermo Scientific). To normalize for protein loading, antibodies directed against GAPDH were used. In addition, immune blotting signals were quantitated using a Bio-Rad Chemi Doc XRS gel imaging system (Hercules, CA, USA).

Statistical analysis

All data are presented as mean ± standard deviation (SD). The RSM data analysis was performed by using Design Expert Version 8.0.6 software (Stat-Ease, Inc., USA). Group comparison was performed by ANOVA (SPSS 16.0, SPSS Inc., USA). Statistical significance was assumed at $p < 0.05$.

RESULTS

RSM data

Multiple regression analysis of the experimental data revealed the predicted response Y (extraction yield of IPTS, %) could be obtained from the second-order polynomial equation:

$$Y = - 165.936 + 0.401\ A + 2.633\ B + 0.320\ C + 0.014\ AB + 0.002\ AC - 0.025\ BC - 0.003\ A^2 - 0.801\ B^2 - 0.020\ C^2 \quad \ldots\ldots\ldots\ldots\ldots\ldots (2)$$

The results of response surface analysis are shown in Table 2 and Figure 1. The determination coefficient ($R^2 = 0.9804$) and the adjusted determination coefficient ($R^2adj = 0.9551$) showed a high degree of correlation between the observed and predicted values. The experiment possessed a very low *p*-value (0.0001) and a very high F-value (F = 38.85), which showed that the proposed model was highly significant. In addition, F-value (4.09) and *p*-value (0.1036) of lack-of-fit showed that it was not significant relative to the pure error, indicating that the model equation was adequate for predicting the IPTS yield under any combination of variables values.

The 2D contour plots and the 3D response surface were presented on Figure 1, which indicated the effects of independent variables and mutual interaction on the IPTS yield.

Table 2: Analysis of variance (ANOVA) data obtained from BBD

Source		Sum of squares	df	Mean square		*F*-value	*P*-value
Model		13.43	9	1.49		38.85	< 0.0001
A		2.83	1	2.83		73.73	< 0.0001
B		0.68	1	0.68		17.67	0.0040
C		0.11	1	0.11		2.94	0.1303
AB		0.31	1	0.31		8.02	0.0253
AC		0.16	1	0.16		4.06	0.0837
BC		0.063	1	0.063		1.63	0.2428
A^2		4.62	1	4.62		120.39	< 0.0001
B^2		2.70	1	2.70		70.24	< 0.0001
C^2		1.08	1	1.08		28.01	0.0011
Residual		0.27	7	0.038			
Lack of fit		0.20	3	0.068		4.09	0.1036
Pure error		0.066	4	0.017			
Cor total		13.70	16				
Standard deviation	Mean	C.V.%	Press	R^2	R^2_{Adj}	R^2_{Pred}	Adequate precision
0.20	4.85	4.04	4.40	0.9804	0.9551	0.7557	16.347

Note: BBD = Box-Behnken Design

The predicted optimum condition for IPTS yield was obtained: extraction temperature of 86.6 °C, extraction time of 2.23 h and water:raw material ratio of 10.77 mL/g. And the theoretical highest LPTS yield was 6.10 %. Verificatory experiments were carried out at the modified conditions. The average yield of LPTS obtained from real experiments is 6.08 %, which demonstrated the validation of the RSM model.

Cell viability

The results of MTT assay are shown in Figure 2. It can be seen that H9c2 cell viability was significantly reduced after H_2O_2 treatment, when compared to the normal H9c2 cells ($p < 0.01$). However, the cell viability was significantly increased after IPTS treatment (200, 400, 600 and 800 µg/mL), when compared to the H9c2 cells treated with H_2O_2 ($p < 0.05$, $p < 0.01$, $p < 0.01$, $p < 0.01$, respectively). These results clearly demonstrate that IPTS enhanced the viability of H_2O_2 treated cardiomyocytes.

Changes in LDH and CK activities

The results in Figure 3 show that the LDH and CK contents of H9c2 cells exposed to H_2O_2 increased significantly ($p < 0.01$), when compared with normal cells. After treating with IPTS at dose of 200, 400 and 800 µg/mL, the contents of LDH and CK were significantly decreased ($p < 0.01$, $p < 0.01$, $p < 0.01$, respectively), relative to the control group. Therefore, the results indicate that LPTS reduces the levels of LDH and CK from cytoplasm of H_2O_2-induced.

SOD, CAT and MDA levels

The effects of IPTS on the SOD, CAT and MDA levels in H_2O_2-exposed H9c2 cells are presented on Table 3. After H_2O_2 stimulation, the experiment levels of SOD and CAT in H9c2 cells significantly decreased ($p < 0.01$), whereas the MDA level was sharply increased ($p < 0.01$), when compared to normal H9c2 cells. Interestingly, IPTS increased the levels of SOD and CAT ($p < 0.01$) in the H9c2 cells and significantly decreased MDA levels, relative to control group.

Effect of IPTS on apoptosis-related proteins

The results of expressions of apoptosis related proteins are shown in Figure 4. It can be seen that the expressions of caspase-3 and Bax were significantly up-regulated in H9c2 cells treated with H_2O_2, whereas the expression of Bcl-2 was down-regulated, relative to normal group. However, IPTS at dose of 200, 400 and 800 µg/mL brought about significant down-regulation of the expressions of caspase-3 and Bax in H9c2 cells ($p < 0.01$), while significantly up-regulating the expression Bcl-2 ($p < 0.05$), relative to the control group.

DISCUSSION

Cardiovascular diseases have become one of the major causes of death all over world [11]. Myocardial infarction leads to myocardial ischemia and even irreversible myocardial necrosis if blood supply is not timely restored [11,12].

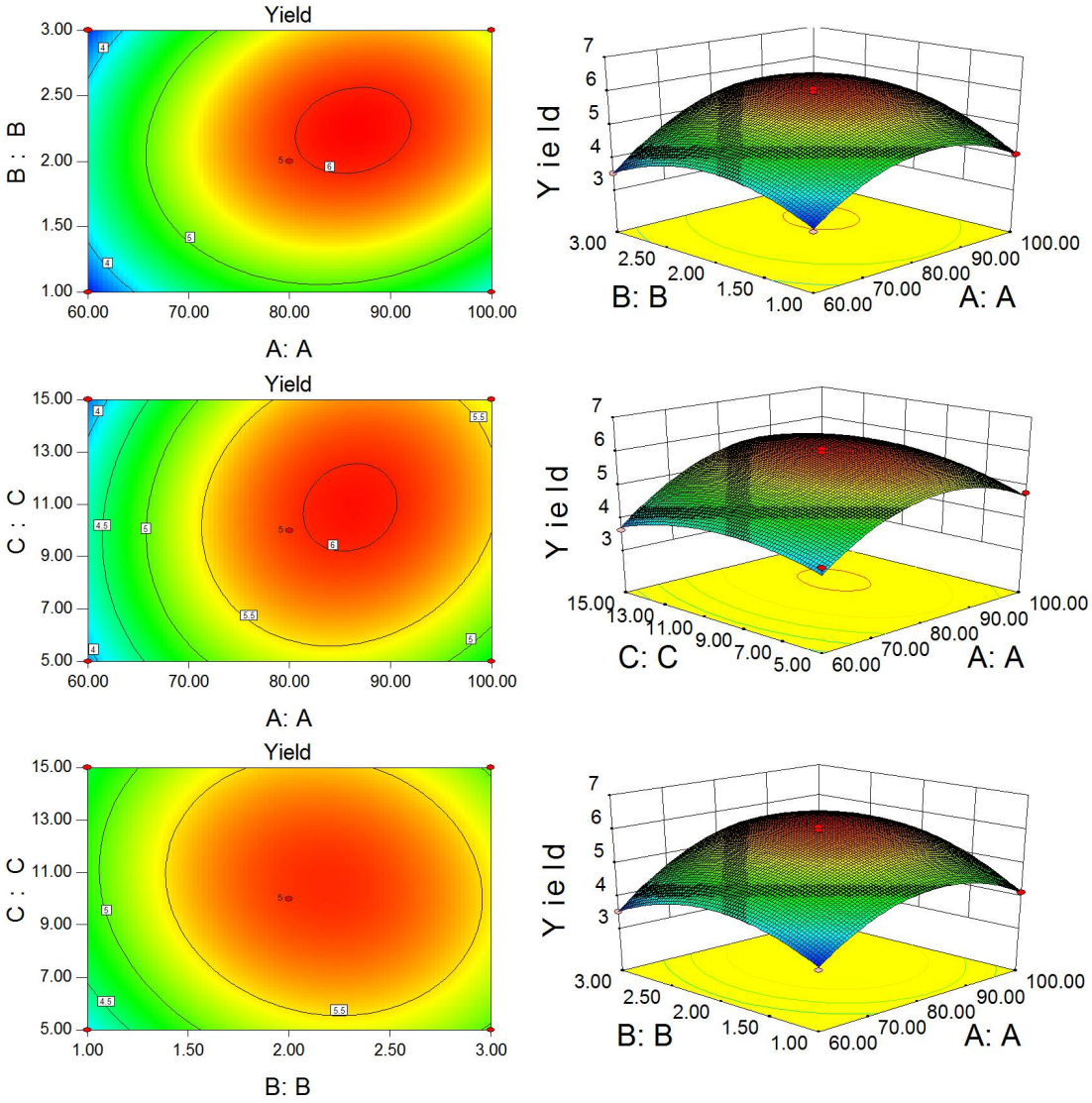

Figure 1: Results from response surface methodology

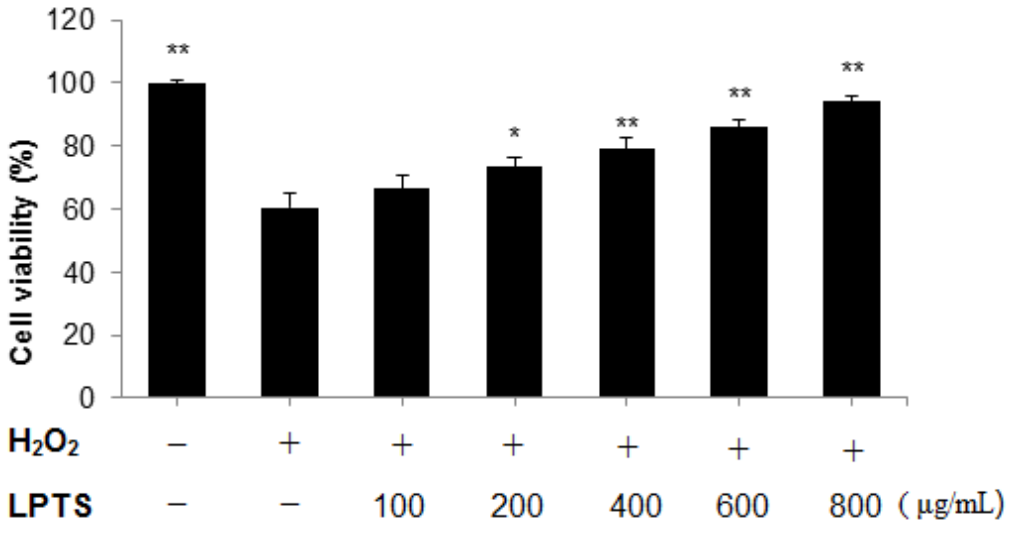

Figure 2: Effect of LPTS on the cell viability rate of H_2O_2 stimulated H9c2 cells. Data are expressed as mean ± SD (n = 6); *$p < 0.05$, **$p < 0.01$, *vs* control group

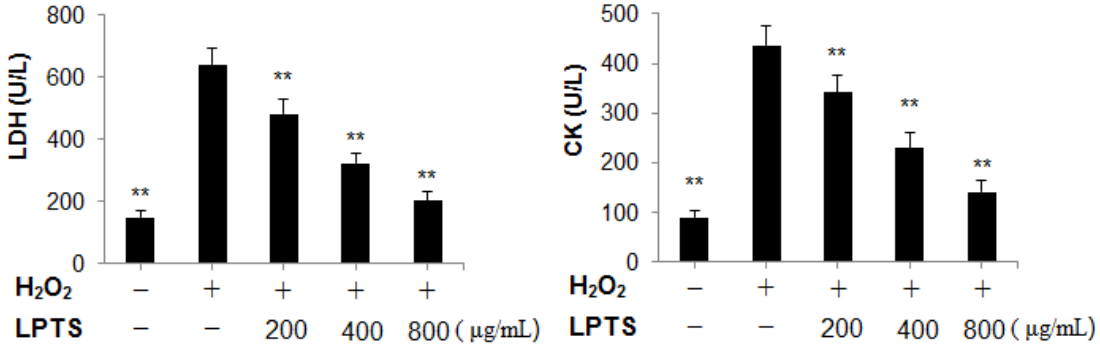

Figure 3: Effect of LPTS on LDH and CK contents in H_2O_2 stimulated H9c2 cells. Data are expressed as Mean ± SD (n = 6); *$p < 0.05$, **$p < 0.01$, *vs* control group

Table 3: Effect of LPTS on SOD, CAT and MDA in H_2O_2 stimulated H9c2 cells

Group	SOD/U/mg	CAT/U/mg	MDA/μmol/g
Normal	97.39±9.54**	70.17 ± 6.47**	14.93 ± 1.54**
Control	46.29±6.21	21.05 ± 4.06	35.21 ± 3.18
200 μg/mL	63.12±7.06**	24.18 ± 4.63	26.67 ± 3.03*
400 μg/mL	82.08±8.92**	47.93 ± 5.54**	19.33 ± 2.16**
800 μg/mL	96.83±10.02**	62.53± 6.72**	15.64 ± 1.31**

*Data are expressed as mean ± SD (n = 6);*p < 0.05, **p < 0.01, vs control group*

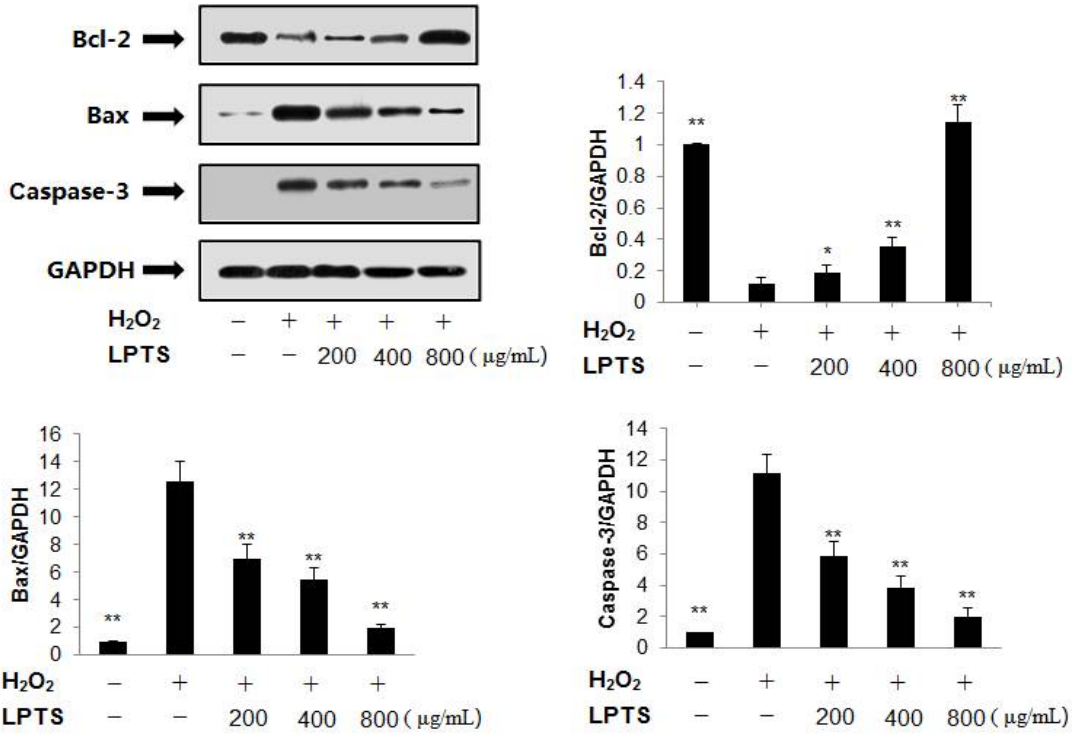

Figure 4: Effect of LPTS on expressions of Caspase-3, Bcl-2, and Bax in H_2O_2 stimulated H9c2 cells. Data are expressed as mean ± SD (n = 6); *$p < 0.05$, **$p < 0.01$, *vs* control group

It has been reported that myocardial ischemia results from the imbalance between the supply of oxygen to the heart and oxygen demand by the heart [13,14]. In addition, oxidative stress leads to over-production of oxygen free radicals, which can induce cell apoptosis by damaging cell membrane and enzymes [2]. Hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) enhance intracellular oxidative stress and eventually lead to cell apoptosis and necrosis [15]. H_2O_2 may also induce the generation of ROS at mitochondria. The myocardial apoptosis model of

H2O2-treated H9c2 cell line is a convenient model for studying the molecular mechanisms of myocardial ischemic injury. The results of the present investigation demonstrated that LPTS alleviates the H_2O_2-induced myocardial apoptosis. In addition, LPTS also decreases the contents of CK and LDH from cytoplasm.

MDA, one of the main end products of lipid peroxidation, is reported to be used as an indicator of tissue damages. CAT and SOD are the important anti-oxidative enzymes, and commonly considered as the antioxidant defense system in body which plays crucial roles in suppression of oxidative stress [17]. The present results demonstrated that LPTS could increase the CAT and SOD levels in H_2O_2 induced H9c2 cell, whereas the MDA level decreased. The results above showed that LPTS inhibits the oxidative stress level in H9c2 cell induced by H_2O_2.

Caspase proteins, a group of aspartate specific cysteine proteases, play key roles in regulating apoptosis induced by different stimuli. Caspase-3 is the most important effect or in the apoptotic process [18]. Bax is the pro-apoptotic protein, which can translocate to the mitochondria [19]. In contrast, Bcl-2 is an anti-apoptotic protein, which inhibits apoptosis by protecting mitochondrial function [20]. In the present investigation, IPTS down-regulated the levels of caspase-3 and Bax, and up-regulated the expression of Bcl-2. This demonstrated that IPTS can protect myocardial cells from mitochondria-mediated intrinsic apoptosis.

CONCLUSION

The results of this study demonstrate that the total saponins from *Ilex pubescens* Hook. (IPTS) exert protective effects of the LPTS on myocardial cells and is a potential new drug for the management of cardiovascular diseases. The mechanism involved in this protective effect might be related to the inhibition of oxidative stress and mitochondria-mediated intrinsic apoptosis

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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