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ORIGINAL ARTICLE

Ocotillo-type Ginsenosides from the *Panax vietnamensis* Ha et Grushv Protect H9c2 Cardiomyocytes against H_2O_2-Induced Apoptosis

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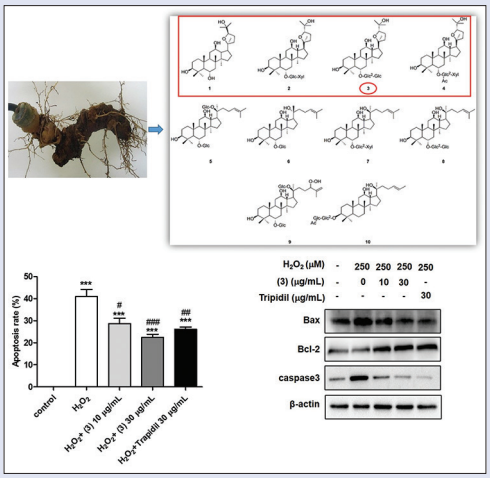
ABSTRACT

Background: The roots of *Panax vietnamensis* Ha et Grushv. (PV) are used in the preparation of local drugs to treat cardiovascular and cerebrovascular diseases. However, it is still not clear if the saponins extracted from PV show a protective effect against myocardial injury, and if so, what are its primary active ingredients. **Materials and Methods:** In this study, a rat model of ischemic myocardial injury induced by isoproterenol was established, and the effect of PV saponin extract (PVS) on the heart rate and changes in the S-T segment were studied. The compounds were isolated through chromatographic techniques. H9c2 damaged cells, after induction by H_2O_2, were established and then treated with ocotillol-type ginsenosides at a concentration of 0.5, 1, 3, 10, and 30 μg/mL. Then, the cell viability, rate of cellular apoptosis, and apoptosis-related protein expression levels were detected by MTT assay, flow cytometry, and Western blot analysis. **Results:** PVS inhibited the elevation in the S-T segment and heart rate in rats with myocardial ischemia induced by isoproterenol. Four ocotillol-type ginsenosides and six dammarane-type ginsenosides were isolated from the PVS. Ocotillol-type ginsenosides compounds 1, 2, and 3 showed the effect of increasing the cell viability of H9c2 cardiomyocytes after induction with H_2O_2, and of them, compound 3 showed the strongest activity. Furthermore, compound 3 significantly inhibited the apoptosis of H9c2 injured cells, decreased the expression level of Bax and caspase 3, and increased the expression level of Bcl-2. **Conclusion:** PVS shows positive effect against myocardial injury. Compound 3 isolated from PVS protected H9c2 cells from damage caused by H_2O_2 by inhibiting apoptosis. These results will provide an important reference for the treatment of coronary heart disease.

Key words: Apoptosis, H9c2 cardiomyocytes, ocotillol-type ginsenosides, *Panax vietnamensis* Ha et Grushv

SUMMARY

- *Panax vietnamensis* Ha et Grushv. saponin has a protective effect on myocardial injury, and majoroside R1 isolated from it is its main active substance.



Abbreviations used: PV: *Panax vietnamensis* Ha et Grushv.; PVS: *Panax vietnamensis* Ha et Grushv. saponin extract; MTT: Methylthiazolyldiphenyl-tetrazolium bromide; CC: Column chromatography; TLC: Thin-layer chromatography; FITC: Fluorescein isothiocyanate; ROS: Reactive oxygen species; PMSF: Phenylmethylsulfonyl fluoride; PVDF: polyvinylidene fluoride.

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INTRODUCTION

Coronary artery disease is a disease characterized by the high rate of morbidity and mortality.[1] According to World Health Organization, in 2016, approximately 17.9 million people have died of cardiovascular disease globally accounting for approximately 31% of the total global deaths (reported on May 17, 2017). In 2030, this number will increase to 23.6 million, of which 12 million will be deaths caused by coronary heart disease.[2] The application of drug thrombolytic therapy, arterial bypass, percutaneous transluminal coronary angioplasty, cardiopulmonary bypass surgery, and other methods can make the ischemic heart regain oxygen-rich blood perfusion in a short time and restore the supply oxygen. However, the sudden reperfusion of blood flow in the ischemic area will aggravate myocardial structural damage and functional damage, which means, it can cause myocardial ischemia/reperfusion injury (MI/RI).[3,4] Therefore, how to effectively reduce this damage and develop low-toxic and high-efficiency anti-MI/RI drugs is an important research direction.

It is known that MI/RI damage mechanisms mainly include the production of free radicals of oxygen, calcium ion overload, activation

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of the inflammatory response by endothelial cells and neutrophils, and cellular apoptosis. At present, globally, the research and development on MI/RI is being conducted on a large scale. The topics mainly include antioxidants, enzymatic and non-enzymatic antioxidants, anti-inflammatory cytokines, and so on.[5-8] At the same time, some medicinal plants and their products have gradually entered the public eye due to their remarkable clinical effects.

P. vietnamensis Ha et Grushv.(PV) was discovered in the mountains at an altitude of 1800 m in Kon Tun Province in central Vietnam in 1973 and was officially named in 1985. It is praised as "divine medicine" by local people and has been used for a long time as a lifesaving medicine for curing diseases and strengthening the body. This plant is cultivated in Jinpin of Yunnan province and is used as a medicinal plant to treat cardiovascular and cerebrovascular diseases.[9-11] Previous studies have shown that PV increases physical strength; reduces fatigue and is hypoglycemic, anticancer agent, and hepatoprotective.[12,13] A previous studies reported that PV mainly contains ginsenosides including dammarane-type, oleanane-type, and ocotillol-type ginsenosides.[14-16] However, to the best of our knowledge, so far, there are no data on cardiovascular and cerebrovascular pharmacological activities of PV.

Therefore, in this study, we aimed to evaluate the protective effect of PV saponins against myocardial injury and isolate, purify, and identify the active substances in it.

MATERIALS AND METHODS

Chemical and reagents

Dimethyl sulfoxide (DMSO) was purchased from Shanghai Acmec Biochemical Co., Ltd (Shanghai, China). Sulfuric acid ethanol was purchased from ANPEL Laboratory Technologies (Shanghai, China). Analytical-grade EtOH, MeOH, $CHCl_3$, and EtOAc were purchased from Aladdin (Shanghai, China). MTT was obtained from Sigma-Aldrich Co (St. Louis, MO, USA). Trapidil and isoproterenol were purchased from Zrbiorise (Shanghai, China). Hydrogen peroxide (1000 μmol/L) was purchased from PythonBio Technologies Inc.(Guangzhou, China). Nifedipine was obtained from Solarbio (Beijing, China). RIPA lysate solution was purchased from Beyotime Biotechnology (Shanghai, China).

General experimental procedure

The column chromatography (CC) was performed using silica gel (100–200 or 200–300 mesh, Qingdao Marine Chemical, Inc., Shandong, China), AB-8 macroporous resin (Tianjing Hongbo resin Inc., Tianjing, China), and Sephadex LH-20 (Amersham Pharmacia Biotech, Uppsaia, Sweden). Thin-layer chromatography (TLC) was performed using GF254 silica gel on glass plates (Qingdao Marine Chemical, Inc., Shandong, China) with different proportion of solvents. The spots were revealed by spraying 5% sulfuric acid in ethanol. ^{1}H-NMR and ^{13}C-NMR were conducted using AVANCE III-600 spectrometers with TMS which was used as an internal standard (Bruker Optics, Ettlingen, Germany).

Plant material

The plants of PV were collected in Jingpin, Yunnan province, which is in the southwest of China, in June 2018. The plants were identified by Prof. Shenchao Yang, and a voucher specimen (Accession no: PV20180601) was deposited in the Key Laboratory of Medicinal Plant Biology, Yunnan Agricultural University. The plant collection and experiments were reviewed and approved by the National and Local Joint Engineering Research Center on Germplasm Innovation and Utilization of Chinese Medicinal Materials in southwestern China.

Extraction and isolation of *Panax vietnamensis* saponins

PV powder (10 kg) was extracted thrice with 70% ethanol. The extracts were combined, and the concentrated extract was dried using a rotary evaporator. The concentrate (2 kg) was chromatographed on an AB-8 macroporous resin column with elution with distilled water until the Molisch reaction was negative, and then with elution with 80% ethanol (10 L). The eluent was concentrated to obtain the total saponin extract (2.8 kg).

The total saponin extract was passed through a CC on silica gel (with solvent systems $CHCl_3$:CH_3OH: H_2O, 10:1:0-5:5:1), and the eluting fractions were identified using TLC to yield five fractions (Fr. A–Fr. E). Fr. C (300 g) was passed through a silica-gel column ($CHCl_3$: CH_3OH: H_2O, 8:2:0.2-7.5:2.5:0.25) to yield four sections: Fr. C-1 to Fr. C-4. Fr. C-1 was passed through a Rp-18 column (H_2O: CH_3OH, 90:10) and a Sephadex LH-20 column (CH_3OH) to yield compounds 2 (10 g), 3 (2.5 g), and 4 (246 mg), respectively. Fr. C-2 was passed through a silica gel column ($CHCl_3$: EtOAc: MeOH: H_2O, 8:3:2:0.2) and then purified with an Rp-18 column (H_2O: CH_3OH, 60:40) to obtain compound 5 (10 mg). Fr. C-3 was separated using silica gel column ($CHCl_3$:CH_3OH: H_2O, 8:2:0.2; $CHCl_3$: EtOAc: CH_3OH: H_2O, 8:3:2:0.2) to yield compounds 1 (10 mg), 7 (9 mg), 8 (10 mg), and 9 (10 mg). Fr. C-4 was passed through a silica gel column ($CHCl_3$: CH_3OH: H_2O, 8:1:0.1) and Rp-18 column (CH_3OH: H_2O, 50:50–80:20) to yield compounds 6 (11 mg) and 10 (18 mg).

Cell culture

H9c2 cardiomyocytes were purchased from the National Cell Bank of Shanghai Institute of Cell Research. The cells are cultured in Dulbecco's modified Eagle's medium (HyClone, CA, USA), which contained 10% fetal bovine serum (Gibco BRL, Grand Island, NY, USA) and 1% streptomycin/penicillin (Solarbio, Beijing, China). The culture conditions are 37°C under 5% CO_2.

MTT assay

MTT assay was performed as previously described.[17] H9c2 cardiomyocytes were seeded into 96-well plates (5×10^3 cells/well). After 24 h, the cell culture medium was replaced with serum-free medium. After 4 h, the cells were either treated with vehicle (0 μg/mL), saponin extract (5, 10, 30, 100, 300 μg/mL), four ocotillol-type ginsenosides (0.5, 1, 3, 10, and 30 μg/mL), or trapidil (30 μg/mL) for 4 h. Then, 20 μL of 5 mg/mL MTT solution was added and incubated for 4 h. Then, the supernatant was discarded, and 200 μL of DMSO was added to dissolve the formazan crystals formed. Next, the absorbance of the color developed was read at 568 nm on a microplate reader (Thermo Scientific™ Multiskan™ FC, Waltham, MA, USA).

Assays were also conducted in the presence of H_2O_2 using a method similar to that described above. Briefly, H9c2 cardiomyocytes were seeded into 96-well plates (5×10^3 cells/well). After 24 h, the cells were cultured in a serum-free medium. After 4 h, the cells were treated with vehicle, ocotillol-type ginsenosides, or trapidil for 4 h. Then, the supernatant was discarded, and 100 μL of the normal medium containing 125 μM H_2O_2 was added. After 6 h, 20 μL of 5 mg/mL MTT solution was added and incubated for 4 h. Then, the supernatant was removed, and 200 μL of DMSO was added. Absorbance was read at 568 nm on a microplate reader (Thermo Scientific™ Multiskan™ FC, Waltham, MA, USA).

In vivo experiment

Acute MI rat model was induced in accordance with the method as described previously.[18] A total of 50 male 8-week-old Sprague–Dawley rats (Changzhou Cavans Laboratory Animal Co., Ltd) were adaptively fed for 1 week. Then, the mice were randomly divided into five groups: control

group (distilled water); positive control group with –nifedipine (12.5 mg/kg); and saponin extract low-dose (50 mg/kg), medium-dose (100 mg/kg), and high-dose (200 mg/kg) groups. Each group of rats was fed via intragastric gavage, and 5 min later, isoproterenol (20 mg/kg) was injected subcutaneously. After gavage on the third day, 40 mg/kg 1% sodium pentobarbital was injected intraperitoneally for anesthesia. Then, the anesthetized rats were fixed and connected the BL-420E + biological function experiment system, and the changes electrocardiogram (ECG) were observed. After subcutaneous injection of isoproterenol, the changes of S-T segment and heart rate were recorded at the following time points: 1 min, 2 min, 3 min, 4 min, 5 min, 10 min, and 15 min. The standard voltage and paper speed were 10 mm·mV^{-1} and 25 mm·s^{-1}, respectively.

Observation of cell morphology

H9c2 cardiomyocytes were seeded in 60 mm culture plates (1×10^6 cells/plate). After 24 h of incubation, the medium was replaced with serum-free medium and incubated for 4 h. Then, the cells were treated with either vehicle (0 μg/mL), compound 3 (0.5, 1, 3, 10, and 30 μg/mL), or trapidil (30 μg/mL) for 4 h. Then, the supernatant was discarded, and 100 μL of the normal medium containing 125 μM H_2O_2 was added. After 6 h, fresh culture medium was subsequently added, and the cells were observed under an inverted microscope with × 100 magnification (Olympus, Tokyo, Japan) and photographed.

Cell apoptosis assay

Flow cytometry was performed as previously described.[19] Briefly, H9c2 cardiomyocytes were seeded into 6-well plates (1×10^6 cells/well) and incubated for 24 h. Then, the cell culture medium was replaced with serum-free medium. After 4 h, the cells were incubated with vehicle (0 μg/mL), compound **3** (10 and 30 μg/mL), or trapidil (30 μg/mL) for 4 h. Then, the supernatant was discarded, and 100 μL of the normal medium containing 125 μM H_2O_2 was added. After 6 h, the rate of cell apoptosis was detected according to the manufacturer's protocol (Sigma–Aldrich, Germany) and analyzed within 1 h on a flow cytometry (BD, FACSCalibur, CA, USA).

Western blot analysis

Western blot analysis was performed as previously described.[20] Briefly, H9c2 cardiomyocytes were seeded into 6-well plates (1×10^6 cells/well) and incubated for 24 h. Then, the cell culture medium was replaced with serum-free medium. After 4 h, different samples of the cells were treated with vehicle (0 μg/mL), compound **3** (10 and 30 μg/mL), or trapidil (30 μg/mL) for 4 h, and then the supernatant was discarded, and 100 μL of the normal medium containing 125 μM H_2O_2 was added to each plate. After 6 h, the total protein of H9c2 cardiomyocytes was extracted using RIPA lysate buffer containing phenylmethylsulfonyl fluoride (PMSF) (RIPA: PMSF = 100: 1). The concentration of protein was measured, and the samples were prepared for Western blot analysis. The protein sample was loaded onto the electrophoresis device through a micropipette, and then the protein was transferred onto PVDF membrane (Merck Millipore, Billerica, MA, USA). The membrane was blocked with 5% bovine serum albumin at room temperature for 1 h to prevent nonspecific binding. After 1 h, the primary antibodies (caspase-3 (1:1000, Santa Cruz, CA, USA), Bax, Bcl-2 (1:1000, Abcam, CA, USA), and β-actin (1:2000, Cell Signaling Technology, MA, USA)] were added and incubated at 4°C overnight. Then, an appropriate peroxidase-labeled secondary antibody (IgG; 1:5000, R and D Systems, USA) was added and incubated at room temperature for 1 h, the membranes were visualized using a chemiluminescence system and analyzed using Image-J software (Media Cybernetics, Silver Springs, MD, USA) and GraphPad Prism 5 (La Jolla, CA, USA).

Statistical analysis

All data are represented as mean ± standard error of mean. Unpaired Student's *t*-test and one-way analysis of variance were performed using GraphPad Prism 5 software. The acceptable level for statistical significance was $P < 0.05$.

RESULTS

The protective effect of *Panax vietnamensis* saponin extract on myocardial injury

To investigate whether PV saponin extract (PVS) shows protective effect against myocardial injury, a rat model of ischemic myocardial injury was established. We found that isoproterenol significantly caused changes in the ECG-ST in rats. However, the extract group and the positive drug group significantly improved the ST changes induced by isoproterenol when compared with the control group [Table 1]. In addition, isoproterenol significantly increased the heart rate of rats; however, the 100 mg/kg extract and the positive drug significantly decreased the heart rate of the rats when compared with the control group [Table 2]. These results indicated that the PVS has the effect of protecting rats from ischemic myocardial injury.

Separation, purification, and identification of *Panax vietnamensis* saponin extract

To determine the active substances in the saponin extract, we isolated, purified, and identified the active components of the extract. The compounds were separated and purified by repeated CC on silica gel, Sephadex LH-20, and high-performance liquid chromatography. Their structures were determined by spectroscopic analyses of ^{1}H-NMR and ^{13}C-NMR data. Four of the isolated compounds were identified as ocotillol-type ginsenosides: 20(*S*), 24(*S*)-epoxydammarane-3 β, 6α, 12 β, 25-tetrol (1);[16] majonoside R_2 (2);[21] majonoside R_1 (3);[21] and vina-ginsenoside R_2 (4).[15] The other six compounds were identified as dammarane-type ginsenoside: ginsenoside Rg_1 (5),[22] 20 (R)-ginsenoside Rh_1 (6),[23] notoginsenoside R_2 (7),[24] ginsenoside R_f (8),[25] floralginsenoside A (9),[26] and ginsenoside Rs_3 (10)[27] [Figure 1].

Compound 3 protects H2c9 cardiomyocytes from H_2O_2-induced injury

To determine whether the four compounds identified in this study, which are ocotillol-type ginsenosides, are cytotoxic to H2c9 cardiomyocytes, we evaluated their effects on the survival rate of H2c9 cardiomyocytes through MTT assay. We found that any of the four compounds had no effect on cell viability [Figure 2a] indicating that they are nontoxic to H2c9 cardiomyocytes.

Then, we used the H_2O_2-induced H2c9 cardiomyocyte injury model to study the inhibitory effects of ocotillol-type ginsenosides on cardiomyocyte injury. The results showed that compared with the cell viability in the control group (i.e., the group including neither H_2O_2 nor any ginsenoside), the cell viability in the H_2O_2 group (i.e., the group including H_2O_2 but no ginsenoside) was significantly decreased. In addition, compared with the H_2O_2 group, inclusion of any of the compounds (1–4) (each at 0.5, 1, 3, 10, and 30 μg/mL) showed significant increase in the growth of H9c2 cardiomyocytes in a dose-dependent manner. Quantitatively, the cell viability increased from 57.59% ± 2.37% for the H_2O_2 group to higher levels (significantly so in most cases) for the groups containing the compounds together with H_2O_2: specifically to 61.91% ± 3.16%, 72.65% ± 2.31%, 64.02% ± 2.78%, 66.88% ± 3.20% ($P < 0.05$), and 75.42% ± 3.24% ($P < 0.01$), respectively, for the compound (1) group to 59.81% ± 3.98%, 68.29%

Table 1: Effect of *Panax vietnamensis* Ha et Grushv. saponin extract on S-T segment changes in myocardial ischemia rats

Groups	Dosage (mg/kg)	Changes of S-T segment after intravenous administration of isoproterenol (mV)						
		1	2	3	4	5	10	15 (min)
Control		0.11±0.06	0.12±0.05	0.14±0.05	0.14±0.05	0.13±0.07	0.11±0.04	0.13±0.05
Extract	50	0.07±0.05	0.09±0.05	0.06±0.03**	0.09±0.04	0.12±0.07	0.08±0.05	0.08±0.07
	100	0.09±0.07	0.09±0.05	0.08±0.06	0.07±0.05	0.09±0.04	0.09±0.05	0.07±0.05*
	200	0.10±0.04	0.07±0.03	0.07±0.05*	0.08±0.06	0.10±0.04	0.10±0.09	0.10±0.06
Nifedipine	12.5	0.08±0.04	0.11±0.04	0.08±0.03*	0.08±0.03	0.06±0.04*	0.07±0.04*	0.09±0.05

Compared with the control group, *P<0.05, **P<0.01

Table 2: Effect of *Panax vietnamensis* Ha et Grushv. saponin extract on the heart rate in myocardial ischemia rats

Groups	Dosage (mg/kg)	Heart rate (times/min)							
		0	1	2	3	4	5	10	15 (min)
Control	-	440.6±52.4	408.4±56.0	414.9±48.4	405.3±45.7	400.8±49.7	403.0±46.0	399.6±132.6	438.6±22.8
Extract	50	388.3±29.9	397.7±114.8	348.7±80.0	368.1±64.0	376.3±111.6	349.4±98.3	343.7±75.9	346.9±91.8
	100	426.6±45.8	358.4±45.0▲	363.1±45.0▲	363.1±37.6▲	354.7±30.7*,▲	357.7±30.7*,▲	357.7±35.6*,▲	354.0±70.9▲
	200	408.5±55.6	380.0±55.6	380.0±55.6	380.0±55.6	380.0±52.2	377.3±52.2	377.3±52.2	377.3±103.5
Nifedipine	12.5	399.4±44.5	323.1±39.3**,▲	328.7±43.1**,▲	326.2±37.9**	332.6±40.1**	331.4±43.8**	324.7±43.8**,▲	322.9±99.1*,▲

Compared with the control group, *P<0.05, **P<0.01; compared with 0 min, ▲P<0.05

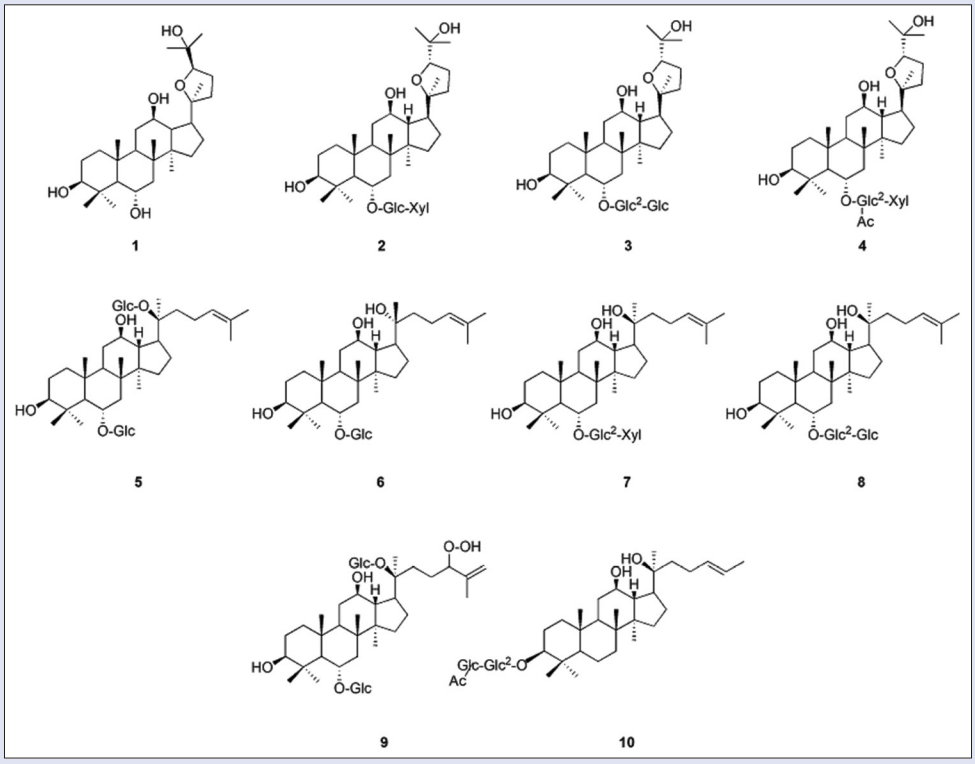

Figure 1: The structure of Compound 1-10. Glc, β-D-glucopyranosyl; Xyl, β-D-xylopyranosyl

± 3.29%, 72.07% ± 2.56%, 81.25% ± 6.15% (P < 0.01), and 85.90% ± 2.32% (P < 0.01), respectively, for the compound (2) group; to 59.78% ± 2.98%, 70.09% ± 2.73%, 79.86% ± 5.14% (P < 0.01), 82.69% ± 2.92% (P < 0.01), and 85.52% ± 3.72% (P < 0.001), respectively, for the compound (3) group; and to 58.76% ± 4.28%, 65.38% ± 3.01%, 66.45% ± 6.16% (P < 0.01), 66.67% ± 3.66%, and 69.82% ± 2.28%, respectively, for the compound (4) group; as well as to 75.05% ± 4.16% (P < 0.05) for the trapidil group [Figure 2b]. These results indicated that compounds 1, 2, and 3 each have a protective effect against H_2O_2-induced H9c2 cardiomyocyte injury; compound 3 showed the strongest activity. Therefore, we conducted further experiments using compound 3.

Compared with the control cells, which displayed polygonal shapes and were tightly arranged, the H2c9 cells treated with H_2O_2 were small in size, round in shape, and less in number. However, compound 3 effectively reversed these signs of damage of H_2O_2 on the cells [Figure 2c]. These results showed the compound 3 is effective in protecting H2c9 cardiomyocytes from H_2O_2-induced injury.

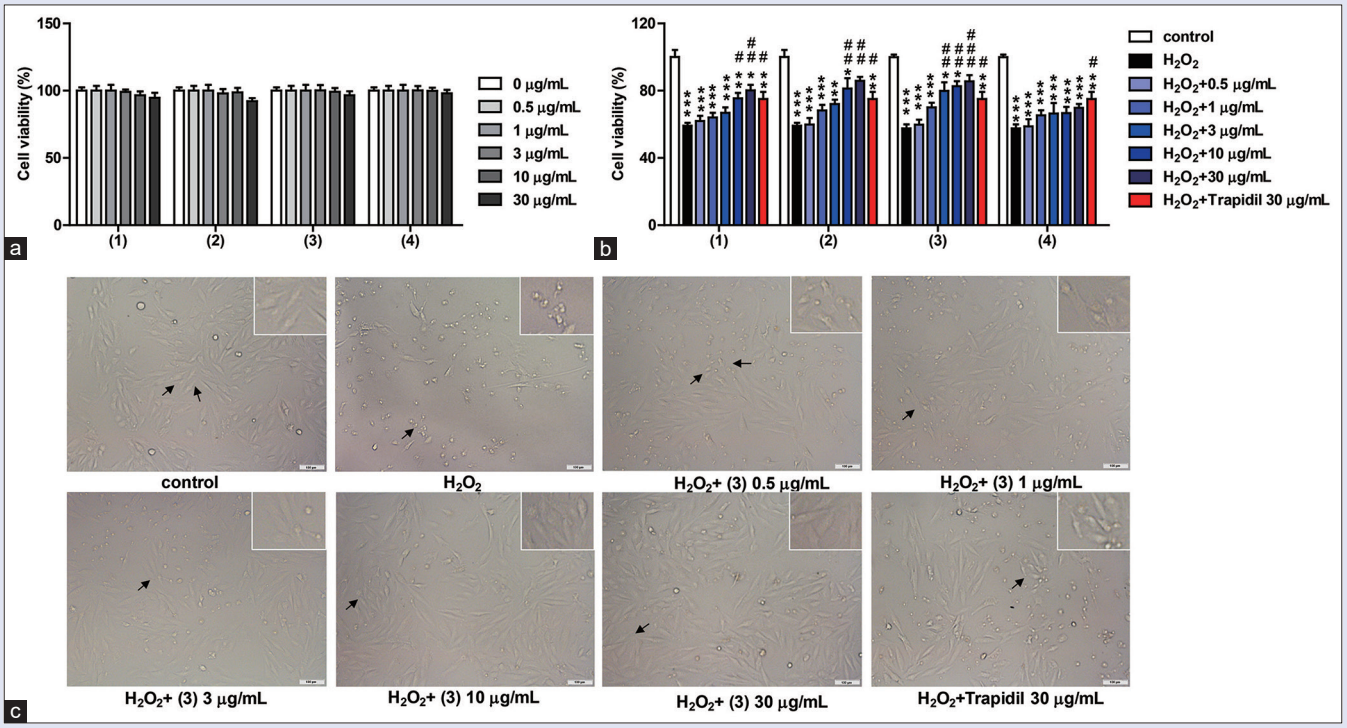

Figure 2: The effect of ocotillol type ginsenosides on H_2O_2-induced H9c2 cardiomyocyte injury (a) MTT assay results showing cytotoxicity levels of four ocotillol-type ginsenosides toward H9c2 cardiomyocytes. (b) MTT assay results showing the protective effects of four ocotillol-type ginsenosides toward H_2O_2-induced H2c9 cardiomyocyte injury. (c) The effect of compound (3) concentration on the growth state of H9c2 cardiomyocytes exposed to H_2O_2. Mean ± standard error of mean ($n = 3$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$, and $^{\#\#\#}P < 0.001$ compared with H_2O_2 group

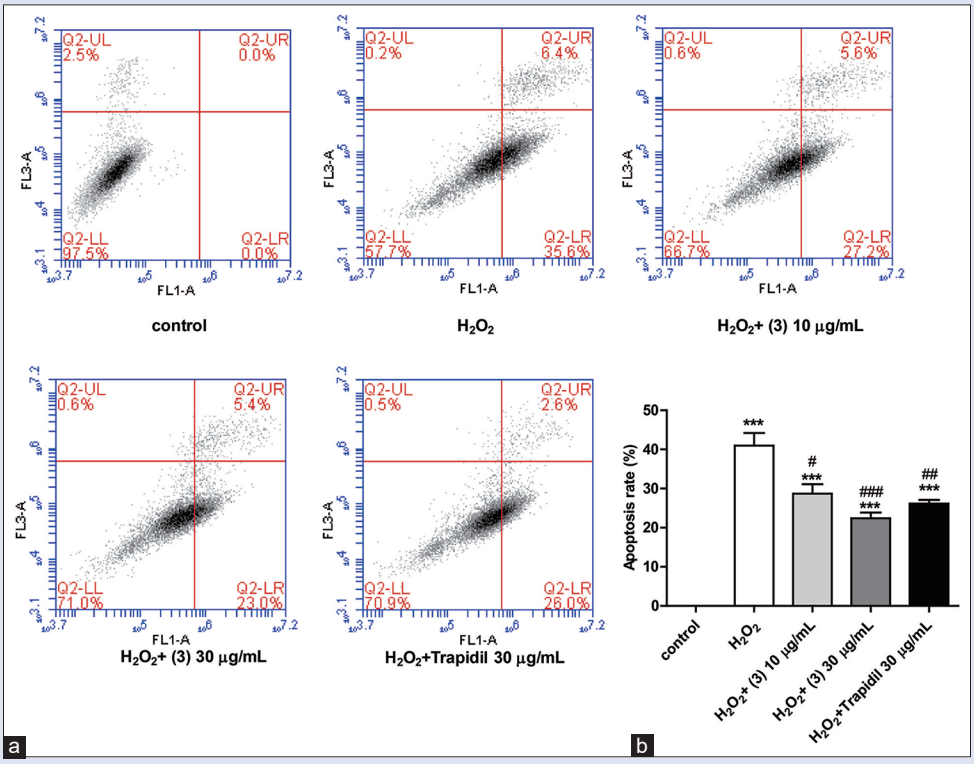

Figure 3: The effect of compound (3) on H9c2 cardiomyocyte apoptosis induced by H_2O_2. H9c2 cardiomyocytes was treated with compound 3 (0, 10, and 30 μg/mL) and another with trapidil (30 μg/mL), each for 4 h, and then treated with 125 μM H_2O_2 for 6 h.(A) Flow cytometry-based assessment of apoptosis in H9c2 cardiomyocytes. (b) The diagram showed the total apoptosis cells percentage of (a). Mean ± standard error of mean ($n = 3$). $^{***}P < 0.001$ compared with control group; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ and $^{\#\#\#}P < 0.001$ compared with H_2O_2 group

Compound 3 inhibits the apoptosis of H_2O_2-induced H2c9 cardiomyocytes

We performed flow cytometry to investigate the regulatory effect of compound 3 on H_2O_2-induced apoptosis in H2c9 cardiomyocytes. According to the results, compared with the control cells (0% H_2O_2), the rate of apoptosis of H_2O_2-induced H2c9 cardiomyocytes significantly increased to 40.97% ± 3.24% ($P < 0.001$). However, compared with the H_2O_2 group, compound 3 (10 and 30 μg/mL) significantly decreased the rate of apoptosis of H_2O_2-induced H9c2 cardiomyocytes in a dose-dependent manner (28.70 ± 2.40% [$P < 0.05$] and 22.40% ± 1.47% [$P < 0.001$], respectively). In addition, the inhibitory effect of compound 3 was found to be equivalent to that of the same dose of the positive-control drug trapidil (26.13% ± 0.98%) [Figure 3a and b].

To further determine the regulatory effect of compound 3 on cellular apoptosis, the expression levels of apoptosis-related proteins were detected through Western blot analysis. According to our results, compared with the control group, H_2O_2 significantly increased the expression levels of Bax ($P < 0.05$) and caspase-3 ($P < 0.01$) and increased the expression levels of Bax/Bcl-2 ratio ($P = 0.07$) [Figure 4a, b, d, and e]. However, compared with the H_2O_2 group, compound 3 (30 μg/mL) significantly decreased the expression levels of Bax ($P < 0.05$) and caspase-3 ($P < 0.01$), increased the expression of the Bcl-2 ($P < 0.05$), and decreased the Bax/Bcl-2 ratio [Figure 4]. In addition, the rate of inhibition of apoptosis was similar for both compound 3 and trapidil. These results indicated that compound 3 protects H9c2 cardiomyocytes from H_2O_2-induced injury by inhibiting cell apoptosis.

DISCUSSION

PV is an important biological resource, which contains both the chemical components of *Panax ginseng* and *Panax notoginseng*, and more abundant ocotillol-type saponins, which has a wide range of research and utilization value. These herbs have been well researched in the aspects of pharmacology, phytochemical components, ginsenoside biosynthesis, and so on. However, although it has a powerful pharmacological effect, the mechanism of action at the molecular level is still unclear. Therefore, it is necessary to conduct a comprehensive scientific study in future.

At present, the incidence and mortality of coronary heart diseases are increasing year by year, and the age of onset is getting younger and younger. Coronary artery recanalization is the main method for the treatment of coronary heart disease, but it is easy to cause MI/RI after coronary artery recanalization. Therefore, it is of great significance to develop drugs that can combat MI/RI. Our research shows that PVS can significantly inhibit myocardial injury in rats with myocardial ischemic injury. In addition, ten ginsenosides were isolated from the extract of PV, with four of them being ocotillol type ginsenosides and the other six being of the dammarane type ginsenosides. Among them, ocotillol type

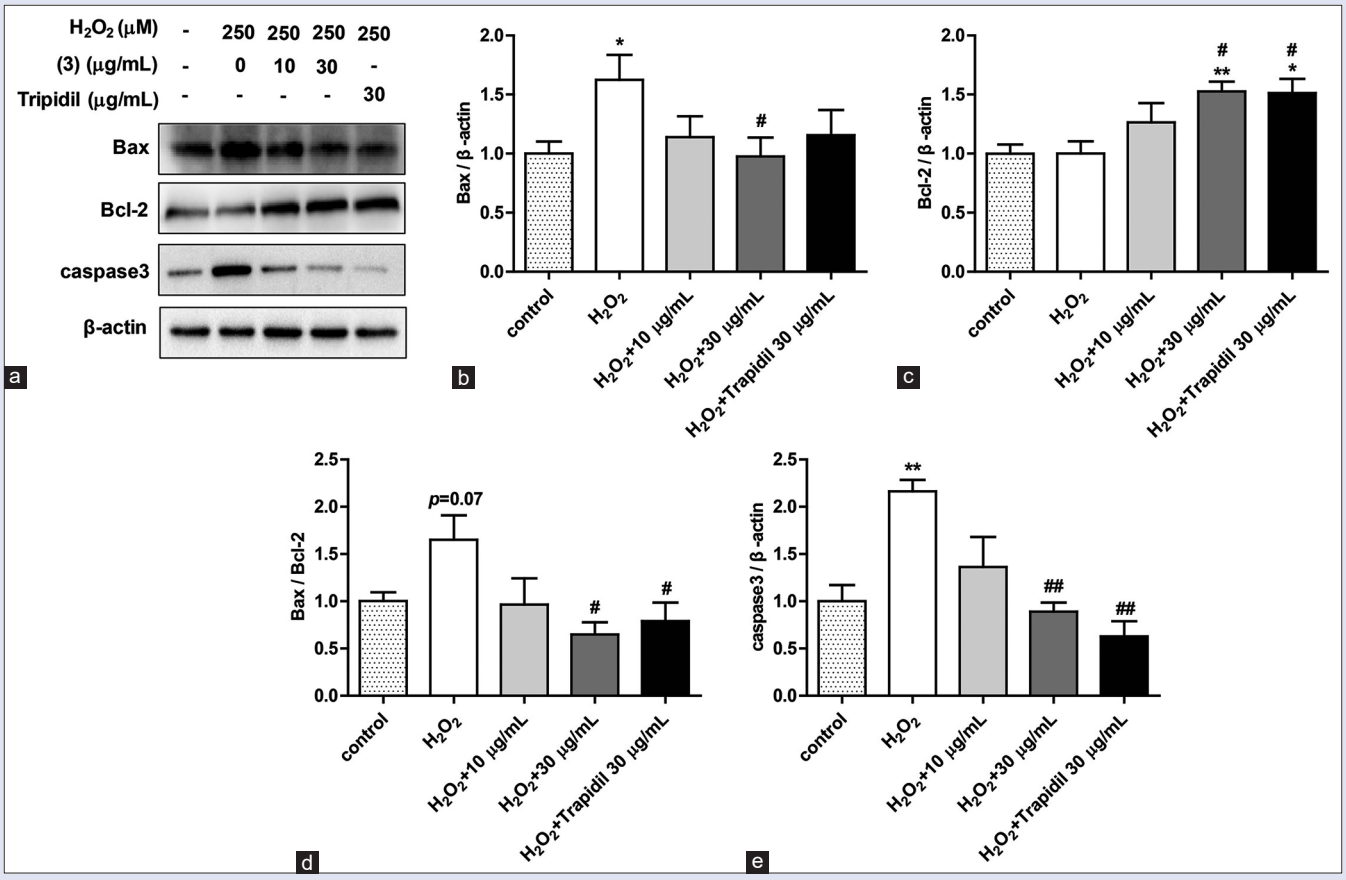

Figure 4: The effect of compound 3 on the expression levels of apoptosis proteins in H9c2 cardiomyocytes. One set of H9c2 cardiomyocytes was treated with compound (3) (0, 10 and 30 μg/mL) and another with trapidil (30 μg/mL), each for 4 h, and then treated with 125 μM H_2O_2 for 6 h. (a) Western blot results showing the levels of caspase-3, Bax, and Bcl-2 of H9c2 cardiomyocytes. (b, c, e) Quantifications of the relative protein levels of (b) Bax, (c) Bcl-2, and (e) caspase-3, and (d) the ratio of Bax to Bcl-2. Mean ± standard error of mean ($n = 3$). *$P < 0.05$ and **$P < 0.01$ compared with control; #$P < 0.05$ and ##$P < 0.01$ compared with H_2O_2 group

ginsenoside, compound 3, showed the strongest protective effect against myocardial injury by inhibiting cellular apoptosis, which will provide an important reference for the treatment of coronary heart disease.

Ocotillol type saponins are tetracyclic triterpenoids with furan rings in their side chains. Ocotillol type saponins are found in less quantity in plants, yet they show strong biological activities including protection against myocardial injury, enhancement of neuronal activity, and antitumor activity.[28,29] At the same time, studies on the *in vitro* metabolism of some C-20 ginsenosides without carbonyl group suggest that their effect on microsomal enzymes in animal liver predisposes to the generation of ocotillol type metabolites.[30,31] Some scholars speculate that the trace quantity of ocotillol ginsenosides is responsible for the actual activity in the body.[32] At present, studies have confirmed that ginsenosides have cardiocerebral vascular protection. However, these results have not been reported for PV saponins. Therefore, we studied the protective effect of PVS against myocardial injury, as well as separated, purified, and identified the main active components in PVS.

Apoptosis is the typical manifestation and main pathological mechanism of MI/RI.[33] Previous studies have shown that injury to cardiomyocytes caused by apoptosis is an important cause of various heart diseases.[34] Therefore, preventing the process of cellular apoptosis is important in preventing MI/RI-induced myocardial damage, thereby slowing down or even preventing the occurrence of heart failure.[35] In this study, we evaluated the regulatory effect of compound 3 on the apoptosis of H_2O_2-induced H2c9 cardiomyocytes. We found that compound 3 significantly decreased the apoptosis induced by H_2O_2, and its inhibitory effect was comparable to that of positive drugs.

Caspase-3 is an enzyme that plays an important role in the process of cellular apoptosis.[36] Bcl-2 and Bax are two important members of the Bcl-2 family of apoptosis-regulating genes. Both can regulate cellular apoptosis by forming homodimers or heterodimers.[37] In this study, we determined the regulatory effect of compound 3 on the expression level of apoptotic protein in H_2O_2-induced H9c2 cardiomyocytes. Our results showed that compound 3 significantly inhibited the increase in the ratio of Bax/Bcl-2 and the increase in the expression level of caspase3 in cells induced by H_2O_2. These results were consistent with the effects of *P. notoginseng* saponins, i.e., antagonizing free radical damage, decreasing the expression levels of caspase-3 and Bax, and increasing the expression levels of Bcl-2 to inhibit neuronal apoptosis.[38-40]

CONCLUSION

PVS exhibits a positive effect against myocardial injury. In this study, a total of ten ginsenosides were isolated from the PVS; of them, four were ocotillol-type ginsenosides and the other six were dammarane-type ginsenosides. Ocotillol-type ginsenosides, that is., compounds 1, 2, and 3, protected H9c2 cardiomyocytes against H_2O_2-induced injury. Compound 3 showed the strongest effect through inhibition of cellular apoptosis.

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Statement of ethics

The present investigation conforms to the standard ethical procedures approved by Institutional Animal Care and Use Committee (IACUC), Yunnan Agricultural University (IACUC-20200514-23), in compliance with the ARRIVE guidelines.[41]

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Conflicts of interest

There are no conflicts of interest.

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