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Full Length Research Paper

Chemical composition of *n*-butanol extract of *Potentilla anserina* L. and its protective effect of EAhy926 endothelial cells under hypoxia

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The protective role of *n*-butanol extract of *Potentilla anserina* roots was measured by 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) method and colorimetric method on human umbilical vein endothelial cells (EAhy926) under hypoxia injury. The extract tested (3 and 1.5 mg/ml) remarkably increased cell viability, the activity of superoxide dismutase (SOD) and the concentration of nitrogen monoxidum (NO), and at the same time reduced the release of lactate dehydrogenase (LDH) and endothelin (ET-1) in cells during hypoxia injury. From this extraction, five compounds were isolated and determined as adenosine (1), daidzin (2), puerarin (3), 3'-methoxypuerarin (4) and daidzein 8-*C*-apiosyl glucoside (5) on the basis of physico-chemical properties and spectroscopic analysis, including 1D and 2D nuclear magnetic resonance (NMR) spectral data. Compound 2 to 5 were isolated from genus *Potentilla* for the first time. Compound 1 was first isolated from the title plant.

Key words: *Potentilla anserine*, chemical constituents, human umbilical vein endothelial cells, isoflavones, *n*-butanol extract, the protective role under hypoxia injury.

INTRODUCTION

Hypoxia of tissues or cells might lead to many diseases, including inflammatory and cardiovascular diseases and cancer (Cogo et al., 2004; Mcloughtin et al., 2005; Rochat et al., 2004; Seema et al., 2007; Laura et al., 2011). There is much evidence that vascular endothelium is a crucial site of hypoxia injury. Data have shown that endothelial cells, under hypoxia conditions, present dysfunction of energy metabolism, over-production of oxygen free radicals which activate endothelial cells themselves and lead to the destruction of the integrity of the vascular endothelium (Eltzschig and Collard, 2004). Such integrity is very necessary for maintaining the normal structure and function of the vessel. Therefore, it is an important work to study and explore novel endothelium-protecting drugs.

Traditional Chinese medicine has over 5000-years of applied history. Many edible and medicinal plants described in classical Chinese Materia Medica provide a potential resource for research and development of ameliorating endothelial cells injury with relatively low toxicity. The Tibetan herb of *Potentilla anserina*, belonging to the genus *Potentilla*, is widely distributed in the western areas of China, especially in Tibet, Gansu and Qinghai provinces. For thousands of years, this Tibetan traditional medicine has been popularly used for replenishing 'Qi' and blood, strengthening the spleen and harmonizing the stomach (Michal and Klaus, 2009). Our previous pharmacological studies indicated that the *n*-butanol extract of the root of *P. anserina* showed a remarkably antioxidant ability by improving the ability of cleaning oxygen free radicals (Wang et al., 2009).

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However few studies were reported about the bioactive components underlying its anti-hypoxia activities of this herb. At the present study, we attempted to investigate the preventative effect of *n*-butanol extract of the root of *P. anserina* in cultured human umbilical vein endothelial cells induced by hypoxia and analyses the related bioactive components for the first time.

MATERIALS AND METHODS

Apparatus and reagents

The cells were incubated in a Thermo Forma 3111 incubator and the absorbance was measured on a BIORAD 680 micro-plate reader. Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 400 spectrometer with tetramethylsilane (TMS) as the internal standard. Electrospray ionization-mass spectra (ESI-MS) and High resolution electrospray mass spectra (HR-ESI-MS) spectra were determined on Bruker Esquire 2000 and Varian QFT mass spectrometer, respectively. Preparative high performance liquid chromatography (HPLC) was carried out using a Venusil XBP C_{18} column (250 × 25 mm) with a HLB-9910 pump and UV-202 ultraviolet visible detector (Tianjin *SCJS* Technology Development Co., Ltd).

Dulbecco's Modified Eagle's Medium (DMEM), dimethylsulfoxide (DMSO), ethylene diamine tetracetic acid (EDTA) and 3-(4, 5-dimethylthiazol-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) were obtained from Sigma. High-glucose DMEM was obtained from Gibco. The reagent kits for measurement of the level of lactate dehydrogenase (LDH), nitric oxide (NO) and superoxide dismutase (SOD) were purchased from Nanjing Institute of Jiancheng Biological Engineering, endothelin-1 (ET-1) kit was from R&D Systems, Inc. Fetal bovine serum (FBS) was purchased from Hangzhou Biologcal Products Co., Ltd (Zhejiang, China). Penicillin and streptomycin were obtained from Amresco Co., Ltd (USA). Column chromatographic separations were performed on silica gel (Qingdao Marine Chemical Co., Ltd, China), Sephadex LH-20 (Pharmacia) and ODS-A (YMC Co., Ltd, Japan). Precoated RP-18 F_{254} plates (Merck) and silica GF_{254} plates (Qingdao Marine Chemical Co., Ltd, China) were used for analytical thin layer chromatography (TLC). All other reagents used were of analytical grade.

Plant

The roots of *P. anserina* were collected from Qinghai province of China in 2003 and were identified by Professor Sun Qishi of Shenyang Pharmaceutical University. A voucher specimen (2003-09-71) is deposited in the Medical College of Chinese People's Armed Police Forces, Tianjin City of China.

Extract preparation

The air-dried roots of *P. anserina* (10 kg) were powdered and refluxed with 8 × 70% EtOH for 3 h (twice) and were concentrated under reduced pressure. The crude extract was suspended in hot water (×10) and partitioned successively with H_2O saturated petroleum ether (×3), EtOAC (×3) and n-butanol (×4) in the same volume. The *n*-butanol extracts were filtered and evaporated under vacuum at 45°C, and were lyophilized.

As positive standard, composite salviae dropping pills were smashed and extracted with 5 × 70% EtOH for 2 h (twice), evaporated under vacuum at 45°C and lyophilized to obtain the composite salviae extract.

Cell culture and induction of hypoxia

Human hybridized endothelial cell (EAhy926) is derived from the fusion of human umbilical vein EC (HUVEC) with the A546 human lung epithelial carcinoma cell line. The cells were maintained in high glucose (4.5 g/L) DMEM, supplemented with 10% FBS, 2 mM L-glutamine, 100 U/ml of penicillin and 100 µg/ml streptomycin solution in a humidified atmosphere of 5% CO_2/95% air at 37°C in 75 cm^2 flasks.

At reaching 80% confluence, cells were passaged by rinsing with Hank's Balanced Salt Solution (12 ml) and adding 4 ml 0.25% trypsin and 0.02% EDTA solution. After 2 min, flasks were gently agitated to detach the remaining cells and culture medium was added (12 ml) to stop trypsin reaction. Harvested cells were diluted to a concentration of 1×10^5 cells/ml using culture medium and the resulting cell suspension was either poured into 96-well (100 µl/well; for cell viability measurement), 6-well (2 ml/well; for observation of cell viability by trypan blue coloration) or 24-well (1 ml/well; for determination of LDH, SOD, ET-1 and NO) microtiter plates and was allowed to attach for 24 h (37°C; 5% CO_2:95% air). The culture medium in the wells was then replaced with FBS-free high glucose DMEM medium that was replenished within 12 h. Then, the culture medium in the wells was changed again with DMEM of glucose-free and FBS-free containing different concentrations of *n*-butanol extract of *P. anserina* or composite salviae extract (previously saturated with 95% N_2/5% CO_2 for 30 min) and incubated in hypoxia condition (95% N_2/5% CO_2) at 37°C for 2 h.

Determination of cell viability by MTT assay

Either the *n*-butanol extract of *P. anserina* or composite salviae extract were dissolved in double-distilled water. Cell viability was determined by a colorimetric assay with MTT (Tansuwangwong et al., 2006). Briefly, cells were seeded in a 96-well plate for 24 h, then replenished with the FBS-free medium for 12 h and exposed to hypoxia condition for 2 h in the absence or presence of composite salviae extract and various concentrations of *n*-butanol extract of *P. anserina*, followed by the addition of MTT solution (0.5 mg/ml) to each well. The plates were then incubated for a further 4 h at 37°C. The insoluble formazan product was dissolved in dimethyl sulfoxide. The absorbance (OD) in each well was then measured at 490 nm.

Observation of cell viability by trypan blue coloration

The method used to assess cellular viability in hypoxia was derived from the *in vitro* model of cellular ischemia developed by Vander as modified by Sato (James and Tewin, 2011). After incubation of hypoxia condition, every wells were mixed with 10 µl trypan blue solution (0.4% Trypan Blue, T8154, Sigma-Aldrich, St. Louis MO, China) and placed in cell counting chamber. Viable cells were detected under inverted light microscopy. In this technique, trypan blue can penetrate only dead cell, therefore every dyed cell (blue color) is dead.

Measurement of LDH, NO and ET- 1 release, as well as intracellular SOD

The supernatant was collected from 24-well plates after incubation and the LDH content was determined using LDH assay kit according to the manufacturer's instructions. Enzyme activity was

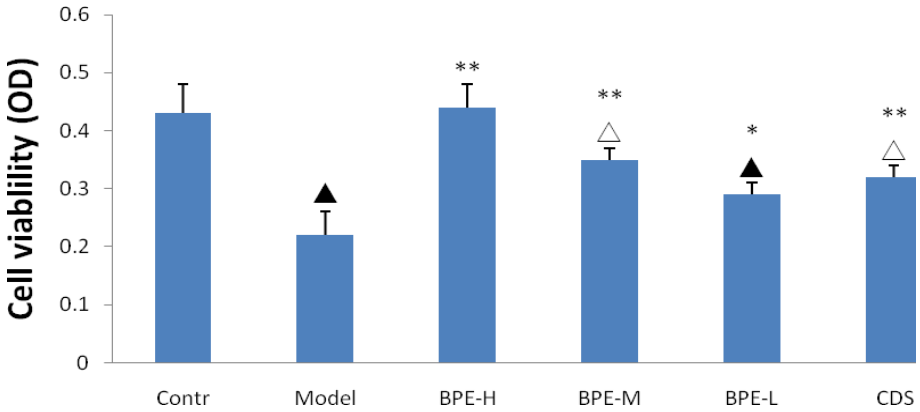

Figure 1. Cell viability in EAhy926 cell by MTT method. Contr, control: EAhy926 Cells from normal control group; Model: EAhy926 Cells from hypoxia injury control group; BPE-H: EAhy926 Cells from hypoxia injury + *n*-butanol extract of *P. anserina* (3 mg/ml) group; BPE-M: EAhy926 Cells from hypoxia injury + *n*-butanol extract of *P. anserina* (1.5 mg/ml) group; BPE-L: EAhy926 cells from hypoxia injury + *n*-butanol extract of *P. anserina* (0.75 mg/ml) group; CDS: EAhy926 cells from hypoxia injury + composite salviae extract (3 mg/ml) group. $^{\Delta}p < 0.05$ compared with control group; $^{\blacktriangle}p < 0.01$ compared with control group; *p < 0.05 compared with model group; **p < 0.01 compared with model group.

expressed as units per liter and the absorbance was read at 440 nm. At the end of incubation, the medium in the 24-well plates was collected, and the amount of NO released by cells was determined by using a NO assay kit according to the manufacturer's protocol. The method involved measuring amount of NO metabolites (nitrite and nitrate), which were more stable than NO. The absorbance of samples was read at 550 nm.

The concentration of ET-1 was measured with a radioimmunoassay kit according to the manufacturer's instructions. The activities of SOD in EAhy926 cells were determined by using xanthine oxidase method. At the end of incubation, the cells were washed with PBS for three times. Then, they were trypsinized with 0.25% trypsin and ultrasonicated in 1 ml 0.01 M PBS followed by centrifugation at 1000 g for 10 min at 4°C. The supernatant was evaluated for SOD with an assay kit according to the manufacturer's protocol, and the absorbance of samples was read at 550 nm.

Statistical analysis

All values are expressed as the mean ± SD. Inter-group comparisons were performed with 1-way ANOVA to test for difference. Statistical Package for the Social Science software (SPSS Inc.) was used for all analyses. Differences were considered to be significant at a value of $P < 0.05$.

Isolation of chemical constituents

The n-butanol extract (80 g) was subjected to column chromatography over silica gel eluted with $CHCl_3$-MeOH gradiently (99:1 to 0:100). The 68 to 75th fractions were combined according to TLC results to afford 1.05 g extract. This extract was further separated by ODS column chromatography eluted with MeOH-H_2O mixtures (20 to 40%), Sephadex LH-20 eluted with 50% MeOH-H_2O solvent and finally, purified by preparative HPLC with 15 and 25% MeOH-H_2O, respectively to yield compound 1 (42 mg), compound 2 (27 mg), compound 3 (12 mg), compound 4 (19 mg) and compound 5 (14 mg).

RESULTS

Cell viability in EAhy926

As shown in Figure 1, relative to normoxia, hypoxia resulted in a rapid and sustained reduction in EAhy926 cell viability ($p < 0.01$). However, treatment with different *n*-butanol extract of *P. anserina* or composite salviae extract improved cell viability under hypoxia ($p < 0.01$).

Observation of cell viability by trypan blue coloration

The effects of hypoxia on EAhy926 cells were visualized using contrast microscopy. As shown in Figure 2, the change in cell morphology can be seen in Figure 2B, where the cells have been subjected to hypoxia. The cells appear round and shriveled, having lost their elongated neuron shape after hypoxia, whereas the cells that have been pretreated for 30 min with 3, 1.5 and 0.75 mg/ml *n*-butanol extract of *P. anserina* have maintained their shape and prevented cells dead as compared to the normoxia control cells in a dose-dependent manner as shown in Figure 2D, E and F.

The effects of different extracts on LDH and SOD in EAhy926 induced by hypoxia injury

As shown in Figure 3, LDH release in the culture medium

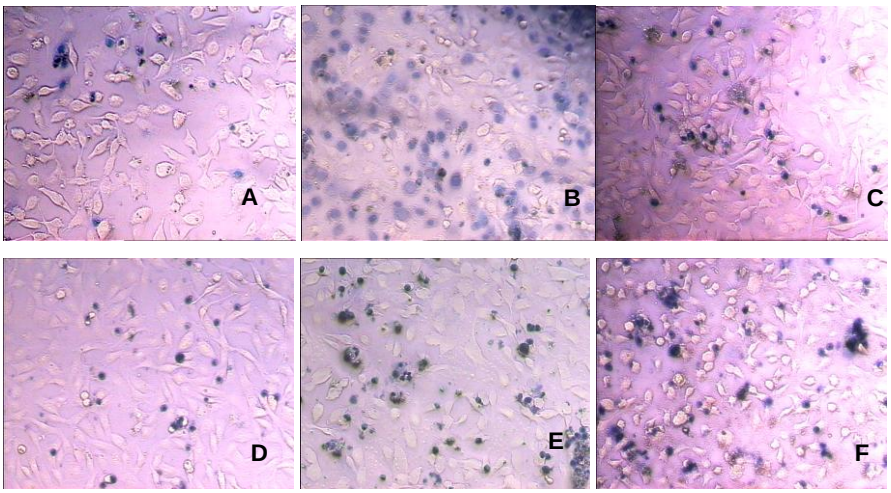

Figure 2. The protective effects of *n*-butanol extract of *P. anserine* against cell death caused by hypoxia. EAhy926 cell cultured in 6 well culture plates at a density of 10^5 cells per well and photographed using a soligor microscope adaptor tube for Canon A650 with the Austria micros microscope 10×objective and 6×zoom on the camera. (A) EAhy926 cells subjected to normoxic conditions cells; (B) Untreated EAhy926 cells subjected to hypoxia 2-h; (C) EAhy926 cells pretreated with composite salviae extract (3 mg/ml) subjected to 2-h of hypoxi; (D) EAhy926 cells pretreated with *n*-butanol extract of *P. anserine* (3 mg/ml) subjected to 2-h of hypoxia; (E) EAhy926 cells pretreated with *n*-butanol extract of *P. anserine* (1.5 mg/ml) subjected to 2-h of hypoxia; (F) EAhy926 cells pretreated with *n*-butanol extract of *P. anserine* (0.75 mg/ml) subjected to 2-h of hypoxia.

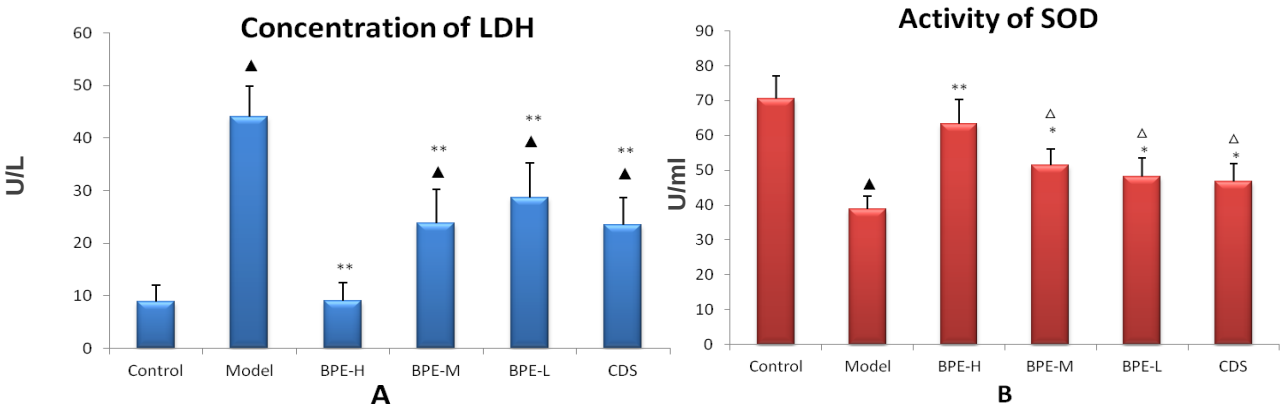

Figure 3. The effects of different extracts on LDH and SOD in EAhy926 induced by hypoxia injury. A: The concentration of LDH in EAhy926 cells induced by hypoxia injury cultured with different extracts previously. B: The activity of SOD in EAhy926 cells induced by hypoxia injury cultured with different extracts previously. $^{\Delta}$, $p < 0.05$ compared with control group; $^{\blacktriangle}$, $p < 0.01$ compared with control group; *, $p < 0.05$ compared with model group; **, $p < 0.01$ compared with model group.

of hypoxia model group were increased significantly ($p < 0.01$) when compared with the normal control group, SOD activity was significantly decreased ($p < 0.01$). The *n*-butanol extract of *P. anserina* could reduce LDH release and improve SOD activity of the EAhy926 cells during hypoxia incubation when compared with the hypoxia injury control group (Model) with a significant difference in a concentration-dependent manner ($p < 0.05$/$p < 0.01$).

The effects of different extracts on NO and ET-1 in EAhy926 induced by hypoxia injury

As shown in Figure 4, background NO release was decreased and ET-1 production was increased significantly after hypoxia incubation ($p < 0.01$ versus the normal control group). However, they were inhibited by *n*-butanol extract of *P. anserine* in a dose-dependent

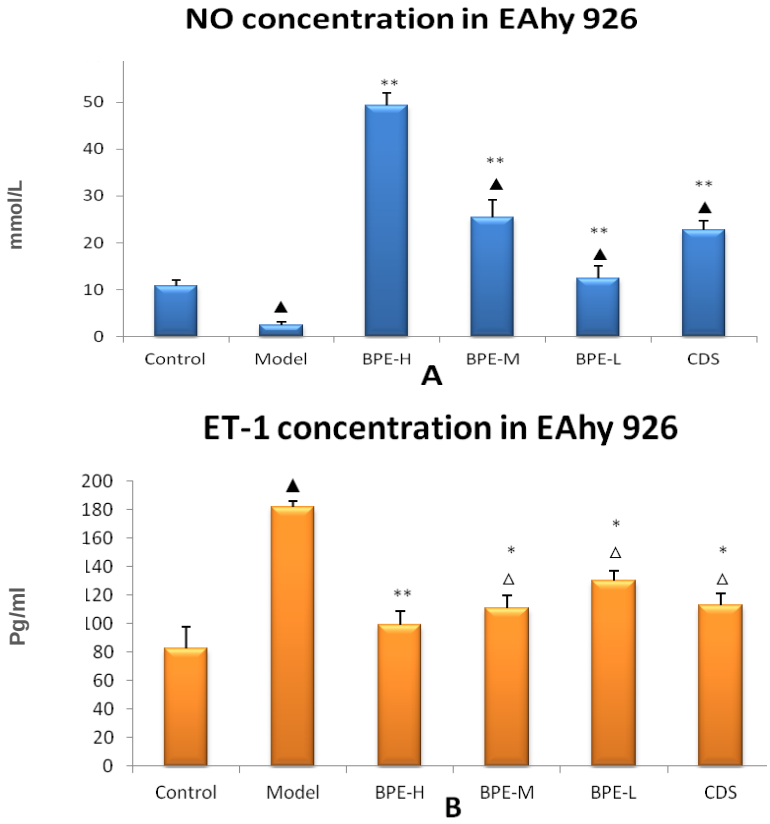

Figure 4. The effects of different extracts on NO and ET-1 in EAhy926 cells induced by hypoxia injury. A: The concentration of NO in EAhy926 cells induced by hypoxia injury cultured with different extracts previously. B: The concentration of ET-1 in EAhy926 cells induced by hypoxia injury cultured with different extracts previously. $^{\Delta}$, $p < 0.05$ compared with control group; $^{\blacktriangle}$, $p < 0.01$ compared with control group; *, $p < 0.05$ compared with model group; **, $p < 0.01$ compared with model group.

manner through administration previously ($p < 0.05/p < 0.01$).

Structure elucidation of chemical constituents

Five compounds were isolated from the *n*-butanol extract of *P. anserina* and their structures were elucidated as followed (Figure 5).

Compound 1

Compound 1 is a white amorphous powder, HR-ESI-MS (positive) m/z: 268.1045 $[M+H]^+$ (calculated for $C_{10}H_{14}N_5O_4^+$, 268.10403), ^{1}H-NMR (DMSO-d_6, 400 MHz): 8.34 (1H, s, H-8), 8.14 (1H, s, H-2), 7.32 (2H, s, NH_2); ^{13}C-NMR (DMSO-d_6, 100 MHz): 152.3(C-2), 149.1(C-4), 119.3(C-5), 156.1(C-6), 139.9(C-8), 87.9(C-1'), 73.5(C-2'), 70.6(C-3'), 85.9(C-4'), 61.6(C-5'). Compound 1 was characterized as adenosine by comparison of the physical and spectral data with literature (Xue et al., 2005).

Compound 2

Compound 2 is a white needles, showing a positive reaction to $FeCl_3$ reagent. ESI-MS (positive) m/z: 439$[M+Na]^+$, 417$[M+H]^+$, 255$[M+H-Glc]^+$; ESI-MS(negative) m/z: 415$[M-H]^-$, 253$[M-H-Glc]^-$. ^{1}H-NMR (DMSO-d_6, 400 MHz): 8.38(1H, s, H-2), 8.05(1H, d, J=8.8 Hz, H-5), 7.14(1H, dd, J=8.8, 2.2 Hz, H-6), 7.23(1H, d, J=2.2 Hz, H-8), 7.41(2H, d, J=8.5 Hz, H-2'/6'), 6.82(2H, d, J=8.5 Hz, H-3'/5'), 9.53(1H, s, 4'-OH), 5.08(1H, d, Glc-H1). ^{13}C-NMR (DMSO-d_6, 100 MHz): 153.2(C-2), 122.3(C-3), 174.7(C-4), 118.4(C-4a), 126.9(C-5), 115.5(C-6), 161.4(C-7), 103.4(C-8), 157.2(C-8a), 123.7(C-1'), 130.0(C-2'/6'), 114.9(C-3'/5'), 157.0(C-4'), 100.0(Glc-1), 73.1(Glc-2), 76.4(Glc-3), 69.6(Glc-4), 77.2(Glc-5), 60.6(Glc-6). The NMR spectral data were in agreement with those of daidzin reported (Yuan et al., 2008; Kingo et al., 1987), so compound 2 was determined

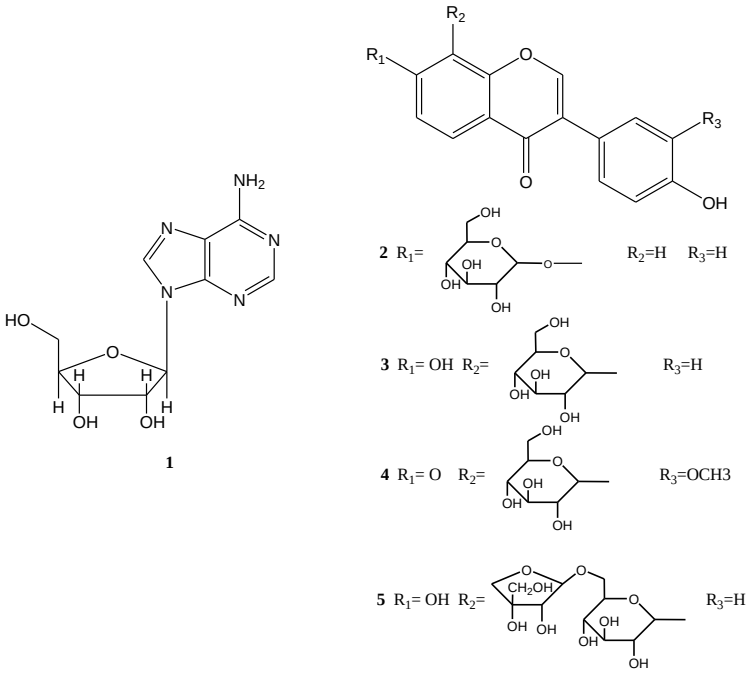

Figure 5. The structures of isolated compounds.

as daidzin.

Compound 3

Compound 3 is a white amorphous powder, showing a positive reaction to $FeCl_3$ reagent. ESI-MS (positive) m/z: 439$[M+Na]^+$, 417$[M+H]^+$; ESI-MS(negative) m/z: 415$[M-H]^-$. ^{1}H-NMR (DMSO-d_6, 400 MHz): 8.22(1H, s, H-2), 7.83 (1H, d, J=8.0 Hz, H-5), 6.85 (1H, d, J=8.0 Hz, H-6), 7.38(2H, d, J=8.6 Hz, H-2'/6'), 6.79(2H, d, J=8.6 Hz, H-3'/5'), 4.83(1H, d, J=9.0Hz, Glc-H1). ^{13}C-NMR (DMSO-d_6, 100 MHz): 151.9(C-2), 122.9(C-3), 174.6(C-4), 116.7(C-4a), 125.7(C-5), 114.9(C-6), 160.9(C-7), 112.3(C-8), 156.7(C-8a), 122.7(C-1'), 129.9(C-2'/6'), 114.9(C-3'/C-5'), 157.0(C-4'), 73.9(Glc-1), 70.7(Glc-2), 79.0(Glc-3), 70.1(Glc-4), 81.4(Glc-5), 61.6(Glc-6). Compound 3 was elucidated as puerarin by comparison of the NMR spectral data reported (Kingo et al., 1987). In addition, compound 3 showed the same equal R_f value with the standard puerarin using TLC, eluted with two different developing solvents [$CHCl_3$-MeOH-H_2O (70:30:5) and *n*-BuOH-EtOAC-H_2O (4:1:5)].

Compound 4

Compound 4 is a white amorphous powder, showing a positive reaction to $FeCl_3$ reagent. ESI-MS (positive) m/z: 469$[M+Na]^+$, ESI-MS (negative) m/z: 445$[M-H]^-$. ^{1}H-NMR (DMSO-d_6, 400MHz): 8.38(1H, s, H-2), 7.94 (1H, d, J=8.8 Hz, H-5), 6.99(1H, d, J=8.8 Hz, H-6), 7.17(2H, d, J=1.8 Hz, H-2'), 6.81(2H, d, J=8.2 Hz, H-5'), 7.03(1H, d, J=1.8,8.2 Hz, H-6'), 4.81(1H, d, J=9.8 Hz, Glc-H1), 3.80(3H, s, -OCH_3). ^{13}C-NMR (DMSO-d_6, 100 MHz): 152.7(C-2), 123.0(C-3), 174.7(C-4), 116.5(C-4a), 126.1(C-5), 115.1(C-6), 161.5(C-7), 112.5(C-8), 156.1(C-8a), 122.9(C-1'), 113.1(C-2'), 147.1(C-3'), 146.4(C-4'), 115.1(C-5'), 121.4(C-6'), 73.5(Glc-1), 70.8(Glc-2), 78.7(Glc-3), 70.4(Glc-4), 81.7(Glc-5), 61.3(Glc-6), 55.6(-OCH_3). Compound 4 was determined as 3'-methoxypuerarin by comparison of the NMR spectral data with literature (Kingo et al., 1987).

Compound 5

Compound 5 is a white amorphous powder, showing a positive reaction to $FeCl_3$ reagent. ESI-MS (positive) m/z: 587$[M+K]^+$, 571$[M+Na]^+$; ESI-MS(negative) m/z: 547$[M-H]^-$. ^{1}H-NMR (DMSO-d_6, 400MHz): 8.29(1H, s, H-2), 7.90 (1H, d, J=8.8 Hz, H-5), 6.97 (1H, d, J=8.8 Hz, H-6), 7.39(2H, d, J=8.5 Hz, H-2'/6'), 6.80(2H, d, J=8.5 Hz, H-3'/5'), 4.80(1H, d, Glc-H1), 4.78(1H, d, J=3.0 Hz, Api-H1). ^{13}C-NMR (DMSO-d_6, 100 MHz): 151.9(C-2), 122.9(C-3), 174.7(C-4), 116.0(C-4a), 126.0(C-5), 114.9(C-6), 162.2(C-7), 112.3(C-8), 156.3(C-8a), 122.6(C-1'), 129.9(C-2'/6'), 114.9(C-3'/5'), 157.0(C-4'), 73.5(Glucose-1), 70.7(Glc-2), 78.7(Glc-3), 70.5(Glc-4), 80.0(Glc-5), 68.3(Glc-6), 109.0(Apiose-1), 75.6(Api-2), 78.7(Api-3), 73.2(Api-4), 63.0(Api-5). The NMR spectral data were in agreement with those of daidzein 8-*C*-apiosyl (1-6) glucoside reported (Kingo et al., 1987) and the structure

was also supported by ^{1}H-^{1}H COSY, TOCSY, HSQC and HMBC experiments, so Compound 5 was elucidated as daidzein 8-*C*-apiosyl (1-6) glucoside.

DISCUSSION

Hypoxia injury to the endothelial cells is known to change the redox state of cell and result in pathological changes in the tissue (Wang et al., 2011; Castillo et al., 2002; Ma et al., 2011). Previous studies reported that hypoxia-induced apoptosis and necrosis of endothelial cells was related to the formation of free radicals (Sun et al., 2011; Mattson et al., 1995). In this study, we investigated the protective effects of *n*-butanol extract of *P. anserina* in the endothelial cells during the hypoxia compared with composite salviae extract (as positive control) for the first time. At the same time, the bioactive constituents of *n*-butanol extract of *P. anserina* were first analyse.

In the trypan blue coloration experiment, it can be seen from the images that the number of cells is greatly reduced after subjecting the cells to hypoxia. Pretreatment for 3 mg/ml *n*-butanol extract of *P. anserina* 30 min before induction of hypoxic conditions resulted in cell morphology more like that seen with the normoxic cells, with a more normal cell shape, which showed that *n*-butanol extract of *P. anserina* was more effective at preventing cell death caused by hypoxia.

It is possible that the trypan blue exclusion assay is overestimating the number of surviving cells since the dead cells could have been washed away (despite care being taken to avoid this), and that cells in the early stages of dying are still able to exclude trypan blue. Therefore, to examine the extent of damage to EAhy926 cells caused by hypoxia, the LDH release assay was preformed (James and Tewin 2011). The vitality of LDH can be used as an important indicator of the extent of endothelial cells damaged. When endothelial cells were hypoxia damaged, the release of LDH was increased (Xu et al., 2002). SOD play key role in detoxification of reactive oxygen metabolites (peroxides) and reactive electrophilic compounds and are commonly considered as antiperoxidative biomarkers (Kalpakcioglu and Senel, 2008; Nakamura, 2003). Therefore, we detected the concentrations of LDH and SOD to evaluate the cell injury induced by hypoxia and the ability in scavenging free radicals in hypoxia.

Hypoxia result in endothelial cells injury and even in death, which lead to ET-1 release. ET-1, a 21-amino acid peptide produced primarily in endothelial cells, is the most powerful endogenous vasoconstrictor agent and has been identified as a key player in endothelial dysfunction resulting from endothelial cell activation and injury (Abraham and Dashwood, 2008). NO is an unstable substance produced in endothelial cells and a sensitive biomarker of endothelial cell injury, which is involved in the regulation of blood pressure, inhibition of the adhesion of leukocytes to endothelium, decreased platelet and blood vessel wall interaction, inhibition of apoptosis induced by various apoptotic stimuli and proliferation and migration of vascular smooth muscle cells. Due to the very unstable chemical nature of NO, endothelium-derived NO can be rapidly inactivated by free radicals due to the formation of a complex between NO and $O^{2\cdot-}$, and thus, an enhanced production of oxygen-free radical species appears to be involved in the accelerated breakdown of NO. Excessive vascular oxidative stress leads to the impairment of NO production, resulting in reduced NO in endothelial cells (Wang et al., 2005). Therefore, we detected the concentrations of ET-1 and NO to assess EAhy926's function in regulating vasoconstriction and vasodilatation and injury of EAhy926 cell.

Our results demonstrate a clear divergence in the responsiveness of LDH, SOD, NO and ET-1 in EAhy926 cells following changes to *n*-butanol extract of *P. anserina* absence and presence during hypoxia injury. Treatment of *n*-butanol extract of *P. anserina* during hypoxia incubation for 2 h increased the activity of SOD and reduced the release of LDH in the culture medium in a concentration-dependent manner, which indicated that these *n*-butanol extract could improve the process of cell metabolism, the activity of scavenging free radical and ameliorate the permeability of cell membrane further to protect the cell against hypoxia injury. At the same time, the *n*-butanol extract of *P. anserina* also could reverse the changes of ET-1 and NO in a concentration-dependent manner to regulate the endothelial function that occurred during hypoxia. These indicated that the *n*-butanol extract of *P. anserina* obviously reduced the degree of hypoxia injury to EAhy926 cells and ultimately improved endothelial cell survival.

In order to determine the active ingredients of *n*-butanol extract of *P. anserina*, the active components were isolated and identified by using a variety of modern spectroscopic techniques, and five compounds were determined as: adenosine (1), daidzin (2), puerarin (3), 3'-methoxypuerarin (4) and daidzein 8-*C*-apiosyl glucoside (5). Among them, compound 2 to 5 were isolated from genus *Potentilla* for the first time. Compound 1 was first isolated from the title plant. Previously, chemical studies were reported that triterpenoids, flavonoids, phenolic acids and polysaccharide were isolated and determined from this genus (Michal and Klaus 2009). But few chemical studies were reported from this plant except some triterpenoids and sterols. It is very exciting that adenosine and four isoflavones were indentified from this bioactive extract. Many research confirmed that adenosine decreased hypoxic damage as was detected by LDH release, MTT absorbance, reactive oxygen species measurement or desmin immunostaining when cardiomyocytes were subjected to hypoxia (Dalia et al., 2007). Recently, a study showed that the effects of a group of 20 structurally

related flavonoids, including flavones, flavonols and isoflavones, on the production of vascular endothelial growth factor (VEGF) induced by hypoxia in NCI-H157 cells were analyzed. VEGF is the main regulator of physiological and pathological angiogenesis and is highly stimulated by hypoxia-inducible factor 1 (HIF-1) (Sun et al., 2011). Structure-activity relationships demonstrated that flavones derivatives were the most active compounds and that hydroxylation of the A ring at positions 5 and 7 and of the B ring at position 4 were important for this activity (Elena et al., 2010). As shown in Figure 2, four isoflavones all have two OH group at position 7 of ring A and position 4 of ring B, but the OH group at position 7 of ring A in compound 2 were glycoside with glucose. So it was supposed that adenosine (1), daidzin (2), puerarin (3), 3'-methoxypuerarin (4) and daidzein 8-*C*-apiosyl glucoside (5) are likely to be anti-hypoxia active ingredients of *n*-butanol extract of *P. anserina* for the first time. Therefore, further studies regarding the underlying mechanisms of four isoflavones protecting EAhy926 cells against hypoxia-induced injury need to be conducted.

Conclusion

This study showed that *n*-butanol extract of *P. anserina* could improve the process of cell metabolism, the activity of scavenging free radical further to protect the EAhy926 cell under hypoxia injury. To investigate the ingredients of *n*-butanol extract, five compounds were isolated and determined as adenosine (1), daidzin (2), puerarin (3), 3'-methoxypuerarin (4) and daidzein 8-*C*-apiosyl glucoside (5), which are likely to be anti-hypoxia active ingredients of *n*-buthanol extract of *P. anserina.*

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REFERENCES

Abraham D, Dashwood M (2008). Endothelin—role in vascular disease. Rheumatology (Oxford), 47(Suppl. 5): 23-24.

Castillo M, Bellot JL, Garcis-Cabanes C, Miquel J, Orts A, Palmero M (2002). Effects of Hypoxia on Retinal Pigmented Epithelium Cells: Protection by Antioxidants. Ophthalmic. Res., 34(6): 338-342.

Cogo A, Fischer R, Schoene R (2004). Respiratory Diseases and High Altitude. High Alititude Med. Biol., 5: 435-444.

Dalia EA, Reuven Z, Yaakov M, Asher S (2007). Adenosine and TNF-α exert similar inotropic effect on heart cultures, suggesting a cardioprotective mechanism against hypoxia. Life Sci., 81(10): 803-813.

Elena A, Alicia Z, Marta I, Maria CU, Ana R, Juan MI (2010). Flavonoids inhibit hypoxia-induced vascular endothelial growth factor expression by a HIF-1 independent mechanism. Biochem. Pharmacol., 79(11): 1600-1609.

Eltzschig HK, Collard CD (2004). Vascular ischaemia and reperfusion injury. Br. Med. Bull., 70(1): 71-86.

Kingo JE, Furusawa JI, Baba J, Takashi T, Masaki Y, Toshihiro N (1987). Studies on the Constituents of Pueraria lobata. III. Isoflavonoids and Related Compounds in the Roots and the Voluble Stems. Chem. Pharm. Bull., 35(12): 4846-4850.

James MB, Tewin T (2011). Rhinacanthus nasutus Protects Cultured Neuronal Cells against Hypoxia Induced Cell Death. Molecules, 16: 6322-6338.

Laura MS, Seeber, Nicole H, Marc A.G.G. Vooijs A, Peter M, Heintz, Elsken van der Wall, René H.M. V, Paul JD (2011). The role of hypoxia inducible factor-1alpha in gynecological cancer. Critical Rev. Oncol. Hematol., 78(3): 173-184.

Ma HP, Fan PC, Jing LL, Yao J, He XR, Yang Y, Chen KM, Jia ZP (2011). Anti-hypoxic activity at simulated high altitude was isolated in petroleum ether extract of Saussurea involucrata. J. Ethnopharm., 137(3): 1510-5.

Mattson MP, Barger SW, Begley IG, Mark RJ (1995). Chapter 10 Calcium, Free Radicals, and Excitotoxic Neuronal Death in Primary Cell Culture. Methods Cell Biol., 46: 187.

Mcloughtin P, Hyvelin JM, Howell K (2005). Pulmonary Hypertension. New Engl. J. Med., 352(4): 418-419.

Michal T, Klaus PL (2009). Potentilla—A review of its phytochemical and pharmacological profile . J. Ethnopharmacol., 122(2): 184-204.

Rochat I, Sayegh Y, Gervaix A, Rimensberger PC, Argiroffo CB (2004). Acute hypoxic respiratory failure as the first manifestation of systemic-onset juvenile rheumatoid arthritis in a child. Pediatric Pulmonol., 38(6): 483-487.

Seema P, Ramesh CK, Bharath N (2007). The alternative reading frame tumor suppressor antagonizes hypoxia-induced cancer cell migration via interaction with the COOH-terminal binding protein corepressor. Cancer Res., 67(19): 9322-9329.

Sun GL, Su G, Zu Y, Zhang MCH, Shi QQ (2011). Effect of tetrandrine on the expressions of hypoxia-inducible factor-1α and vascular endothelial. Afr. J. Pharm. Pharm., 5(9): 1223-1227.

Tansuwangwong S, Hiroyuki Y, Kohzoh I, Vinitketkumnuen U (2006). Induction of apoptosis in RKO colon cancer cell line by an aqueous extract of Millingtonia hortensis. Asian Pac. J. Cancer Prev., 7: 641-644.

Wang C, Li YZ, Wang XR, Lv ZR, Shi DZ, Liu XH (2011). Panax Quinquefolium Saponins Reduce Myocardial Hypoxia-Reoxygenation Injury by Inhibiting Excessive Endoplasmic Reticulum Stress, p. 15.

Wang S, Wei W, Li LZ, Li Y, Li JY, Zhang L (2009). Protective effect of the silverweed cinquefoil root n-butanol fraction on the cardiomyocyte injured by hypoxia. Tianjin J. Tradit. Chin. Med., 26(3): 250-252.

Wang YK, Hong YJ, Huang ZQ (2005). Protective effects of silybin on human umbilical vein endothelial cell injury induced by H_2O_2 *in vitro*. Vascul. Pharm., 43: 198-206.

Xu LP, Wang HX, Li JL (2002). Protective effect of Xionggui capsule on the myocardial in mice. Chinese Mater. Med. Pharm. Clin., 18(6): 39-40.

Xue PF, Liang H, Wang B, Zhao YY (2005). Chemical constituents from Potentilla multifida L. J. Chin. Pharm. Sci., 14(2): 86-88.

Yuan SQ, Yu NJ, Zhao YM, Xu B (2008). Chemical constituents of Glycine max (Linn.) Merr. J. Chinese Med. Mater. 31(8): 1172-1174.