

## Original Article

# Vascular relaxation induced by *Eucommiae Ulmoides Oliv.* and its compounds Oroxylin A and wogonin: implications on their cytoprotection action

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**Abstract:** The vascular relaxation action of *Eucommiae Ulmoides Oliv.* also known as Duzhong has been seen on arteries of the heart such as the aorta and the coronary artery which are elastic in nature. Duzhong is historically an active ingredient commonly used in hypertensive herbal prescriptions in China. This work investigated the vasodilating effect of Duzhong and its compounds (wogonin 10  $\mu$ M and oroxylin-A) in the isolated intact rat heart, perfused retrograde according the method of Langendorff and the cytoprotective effect in EA.hy926 cell lines. Coronary perfusion pressure was monitored with a pressure transducer connected to a side-arm of the aortic perfusion cannula. Duzhong induced vasorelaxation in a dose dependent manner, on precontracting the vessels with endothelin-1, Duzhong 10 mg/ml, wogonin 10  $\mu$ M and oroxylin-A 10  $\mu$ M could significantly lower the perfusion pressure in reference to positive control SNP, Duzhong induced vasodilation was not inhibited by L-NAME (nitric oxide inhibitor), but was significantly inhibited by Tetraethyl ammonium (TEA, a K<sup>+</sup> channel blocker and almost abolished by potassium chloride. The underlying mechanism was carried out in EA.hy926 cell lines. When these cells were treated with H<sub>2</sub>O<sub>2</sub>, there was higher expression of NOX-4, TNF- $\alpha$  and COX-2 mRNA. However, wogonin treatment attenuated the mRNA of NOX-4, TNF- $\alpha$  and COX-2. Wogonin also upregulated the mRNA expression of CAT, SOD-1 and GSR in oxidative stress induced by H<sub>2</sub>O<sub>2</sub> EA.hy926 cells. Duzhong and compounds can exert an in vitro relaxation effect of the coronary artery and improve the heart function in Langendorff apparatus. This action appears to be endothelium dependent but not NO mediated. Cell culture findings indicated that wogonin can exert vascular and cellular protection by scavenging Reactive Oxygen Species.

**Keywords:** *Eucommiae Ulmoides Oliv.*, endothelium, vasorelaxation, hypertension

## Introduction

Cardiovascular disease is the major cause of morbidity and mortality in human population and the damage induced by oxidative stress has been purported as a major sign for cardiovascular health hazard. In hypertension and atherosclerosis, increased vessel wall oxidative stress is the major pathogenic feature (Rajagopalan et al., 1996). It's also a major cause of endothelial dysfunction mainly by attenuating NO production, promoting inflammation and through its involvement in the activation of intracellular signal transduction pathways, therefore influencing intracellular com-

munication, ion channel activation and gene expression preventing the cessation of cell division and premature senescence. *Eucommiae Ulmoides Oliv.* of *Eucommiaceae* family has been used as traditional Chinese medicine in Asia, in countries like Japan, China and Korea. Traditionally, it has various pharmacological properties including strengthening tendons and bones, reinforcing muscle, benefit to the liver and kidney, prevention of miscarriage, increasing longevity and lowering blood fat and pressure (Deyama et al., 2001). The leaf and bark of this plant have been widely used in traditional Chinese medicine for the treatment of hypertension (Chiu-Yin Kwan, 2004; Deyama et al.,

**Table 1.** Duzhong compounds

Flavonoids	β-sitosterol
	wogonin
	oroxylin-A
	Baicalein
	α-O-β-D-glucopyranosyl-4, 2', 4'-trihydroxydihydrochalcone
Iridoids	Genipin
	Geniposide
	Geniposidic acid
	Aucubin
Lignanans	pinoresinol 4'-O-β-D-glucopyranoside
	pinoresinol di-O-β-D-glucopyranoside
	syringaresinol 4'-O-β-D-glucopyranoside
	syringaresinol di-O-β-D-glucopyranoside
Triterpenoids	betulinic acid
	betulin

**Table 2.** The primer sequences for human TNF-α, Nox4, COX-2, SOD1, CAT and GSR

Gene	Sequence
GAPDH	S 5': GAAGGTGAAGGTCTGGAGTCAAC3'
	As 5': CTGGAAGATGGTGATGGGATTC3'
Cu/Zn-SOD	S 5': GAGTTTGGAGATAATACAGCAGGCTGT3'
	As 5': TTTCATGGACCACCACTGTGC3'
GSR	S 5': ATCCCCGGTGCCAGCTTAGG3'
	As 5': 5AGCAATGTAACCTGCACCAACAA3'
CAT	S 5': 5GTTACTCAGGTGCGGGCATTCTAT3'
	As 5': 5GAAGTTCTTGACCGCTTTCTCTG3'
NOX-4	S: 5'CAGAAGGTTCCAAGCAGGAG3'
	As: 5'GTTGAGGGCATTACCCAGAT3'
TNF-α	S 5'-AACATCCAACCTTCCCAAACG-3'
	As 3'-GACCCTAAGCCCCAATTCTC-5'
COX-2	S 5'-CACCATGTCAAAACCGAGG-3'
	As 5'-CCGGTGTTGAGCAGTTTCTC-3'

2001; Pharmacopoeia, 2010.). Many in vivo and in vitro studies have proved that some extracts and components from EU have antihypertensive action. The identified antihypertensive components can be classified into four categories: lignans, iridoids, flavonoids and triterpenoids as shown in **Table 1**. These components have their own mechanisms (Chan et al., 2000; Nishida and Satoh, 2004; Taubert et al., 2002). Pinoresinol di-β-d-glucoside (PDG, 30 mg/kg) and geniposide acid (GPA, 30 mg/kg) elicit strong blood pressure lowering activity (Deyama et al., 2001). However, they have a very low content in EU (< 0.3%) (Qi, 2000; Xu, 2000) which cannot explain why 6 g of *Eucommia* bark can

produce a great decrease in blood pressure of on (SHRs) (Pharmacopoeia, 2010.).

Recent studies indicated that aqueous extracts from EU have antihypertensive effect via releasing vascular relaxing molecules including nitric oxide (NO) and endothelium-derived hyperpolarizing factor (EDHF) from the endothelium (Jin et al., 2008; Kwan et al., 2003). Pharmacological studies has proved that *Eucommia ulmoides* cortex exhibit components like antioxidants (Chen, 2003; Lee et al., 2005; Qu, 2006; Xu et al., 2010; Zhang et al., 2005). But phenolics and flavonoids make up a group of nonessential dietary components possessing the bioactivity of an antioxidant, antimutagenic, anti-inflammatory and anticarcinogenic, therefore they can suppress atherosclerosis and cancer (M.T. HUANG, 1992) as prophylactic agents (Laranjinha et al., 1994). Wogonin is one of the flavonoids found in EU that pocess anti-respiratory syncytial virus activity (Ma et al., 2002), anti-hepatitis B virus (R.L. Huang, 2000), antioxidant and free radical scavenging effects and Oroxylin A pocess an anti-respiratory syncytial virus activity just like wogonin (Ma et al., 2002) and a tocolytic effect (Shih et al., 2009).

In the present study, we evaluated the vasodilatory effects of *Eucommiae Ulmoides* and its two compounds Wogonin and Oroxylin A in isolated intact heart, perfused retrograde according to the method of Langendorff as a measure of endothelial function. In this model, the intact coronary bed was studied, and changes in coronary perfusion pressure was used to monitor changes in coronary vascular resistance. Subsequently the antioxidant action of wogonin and Oroxylin A in EA.hy926 cells was further investigated. To further study the endothelial function and dysfunction, cells were treated with H<sub>2</sub>O<sub>2</sub> to induce Reactive Oxygen Species. Subsequent addition of the compounds wogonin and Oroxylin A was done to check their cytoprotective activity against cell damage by ROS.

## Materials and methods

### Chemicals and reagents

Reagents used in Langendorff and cell culture were from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. L-NAME N (o-nitro-L-

arginine) and endothelin-1 (human, porcine) were purchased from Sigma-Aldrich. The other compounds were dissolved in dimethyl sulfoxide (DMSO) and further diluted. DMSO concentration used in cell experiment was not more than 0.1% (Hong Wang, 2011). In this concentration, DMSO showed no pharmacological effect (data not shown). DMEM/with high glucose was purchased from Thermo scientific, Streptomycin from Invitrogen, Trypsin and FBS from Biological industries (Kibbutz Beit Haeme of Israel)  $H_2O_2$  was from Sigma Aldrich, USA, Duzhong, Duzhong Extract, Wogonin and Oroxylin A were got from Tianjin Key Laboratory of modern Chinese Medicine, Tianjin University of Traditional Chinese Medicine.

#### *Isolation and standardization of Duzhong part 6*

Standards of geniposidic acid, chlorogenic acid, geniposide, genipin, pinorensin di-O- $\beta$ -D-glucopyranoside, licoagroside F (( $\alpha$ R)- $\alpha$ -O- $\beta$ -D-glucopyranosyl-4, 2', 4' trihydroxydihydrochalcone), syringaresin di-O- $\beta$ -D-glucopyranoside, baicalein, wogonin and oroxylin A were isolated from *E. ulmoides* which were identified by direct comparison of their spectra data (MS,  $^1H$  NMR and  $^{13}C$  NMR) with those reported in the literatures. The purities were above 98% using LC analysis. LC-grade water was produced by Milli-Q water purification system (Millipore, Bedford, MA, USA). Formic acid, methanol, and acetonitrile of LC grade were purchased from Fisher Scientific (Pittsburg, PA, USA).

#### *Sample preparation*

The sample (20 mg) was dissolved separately in 50% methanol aqueous solution (5 mL) in an ultrasonic water bath at room temperature. The solution was centrifuged at 14,000 rpm for 10 min. Aliquot (3  $\mu$ L) of the supernatant solution was injected into UPLC for analysis.

#### *Standard solutions*

Accurately weighed ten compounds were dissolved in DMSO to prepare stock solutions. A certain amount of each stock solution was placed in a 5 mL volumetric flask and diluted to volume with 50% methanol aqueous solution at the concentration of 103.3  $\mu$ g/mL geniposidic acid, 26.17  $\mu$ g/mL chlorogenic acid, 23.09  $\mu$ g/mL geniposide, 33.96  $\mu$ g/mL genipin, 60.36

$\mu$ g/mL pinorensin di-O- $\beta$ -D-glucopyranoside, 2.190  $\mu$ g/mL licoagroside F, 18.47  $\mu$ g/mL syringaresin di-O- $\beta$ -D-glucopyranoside, 0.126  $\mu$ g/mL baicalein, 0.0159  $\mu$ g/mL wogonin and 0.0319  $\mu$ g/mL oroxylin A.

#### *UPLC analysis*

UPLC analyses were carried out using a Waters Acquity UPLC system (Waters, Milford, MA, USA), composed of a column heater, a sample manager, a binary solvent manager and a TUV detector. The chromatographic separation was performed on an Acquity UPLC<sup>TM</sup> BEH  $C_{18}$  column (100 mm  $\times$  3.0 mm, 1.7  $\mu$ m particle size; Waters) by fixing the column heater at 50°C. The mobile phase consisted of acetonitrile (A) and water containing 0.1% formic acid (B) with the flow rate at 0.5 mL/min. A gradient elution program was employed as follows: 5-16% A at 0-12 min, 16-50% A at 12-13 min, 50-58% A at 13-16 min, and decreasing from 58 to 5% A at 16-16.1 min. The composition was held at 5% A for a further 3 min for re equilibration. The detection wavelength was set at 237 nm in 0-3 min, 277 nm in 3-6 min, 237 nm in 6-9.2 min, 227 nm in 9.2-12 min, and 275 nm in 12-16 min.

#### *Isolated rat hearts*

Both female and male Wistar rats with a body-weight of 240-300 g were purchased from Beijing Hua FuKang Bio-technology Co. Ltd. The rats were sacrificed by a sharp blow on the neck within one minute (until the insertion of the aortic cannula) to avoid ischemia and loss of high energy phosphates (they were connected to an artificial respirator by a PE-tube inserted into the trachea (respiration frequency 40/min, respiration volume 160-180 ml/min). A polyethylene (PE) catheter was inserted into the thoracic aorta via the right carotid artery to measure heart rate (HR), diastolic blood pressure (DBP), systolic blood pressure (SBP) and mean arterial pressure (MAP) via a Powerlab data acquisition system (A.D. Instruments, Castle Hill, Australia). Subsequently, the hearts and lungs were rapidly removed and immersed in a Krebs-Henseleit solution of the following composition (mM): NaCl 118; KCl 4.7;  $CaCl_2$  1.25;  $MgSO_4$  1.2;  $KH_2PO_4$  1.2; glucose 11.0;  $NaHCO_3$  25.0. The solution was gassed with a mixture of 95%  $O_2$  and 5%  $CO_2$  (carbogen). The aorta was mounted on a cannula (ID 2.0 mm) attached to a perfusion device. The hearts

were perfused at a constant flow according to the method of Langendorff with non-recirculated Krebs-Henseleit solution (pH 7.4 gassed with carbogen). Coronary flow (CF) was set to 10 ml/min by means of a peristaltic pump (Ismatech SA, LaborTechnik, Zurich, Switzerland). The perfusion solution was pumped through a bubble trap which formed a “wind-kessel” with a volume of 1 ml. The hearts were paced throughout at a frequency of 6 Hzz (pulse width 5 ms, voltage 10% above threshold, 2-4 V) via platinum electrodes, placed at the level of the atria. The left ventricular end-diastolic pressure (LVEDP) and systolic pressure (SP) was measured by means of a water-filled balloon inserted into the left ventricle and connected to a Baxter Uniflow pressure transducer and recorded via the Powerlab data acquisition system. By subtracting the LVEDP from the SP, the left ventricular pressure (LVP) could be calculated. The balloon filling pressure, corresponding to the diastolic perfusion pressure, was kept at 10 mmHg. Coronary perfusion pressure (CPP) was measured with a second transducer connected to a side arm of the perfusion line. Changes in CPP were used to monitor.

Changes in coronary vascular resistance, and these were continuously recorded via the Powerlab data acquisition system. Drug infusions, Sodium Nitro pruside (SNP) and all other drugs to be studied were infused via a side arm just above the aortic cannula.

#### Cell culture

EA.hy926 cells were obtained from ATCC, USA. The cells were maintained in an incubator at 37°C with a humidified atmosphere of 5% CO<sub>2</sub> and cultured in Dulbecco's modified Eagle's medium (DMEM) with high glucose containing 10% (v/v) fetal calf serum and 100 U/ml penicillin or 100 µg/ml streptomycin. This study was divided into three groups: H<sub>2</sub>O<sub>2</sub> treatment, H<sub>2</sub>O<sub>2</sub> + EuO treatment, and control (H<sub>2</sub>O<sub>2</sub> and wogonin free). In the H<sub>2</sub>O<sub>2</sub> and H<sub>2</sub>O<sub>2</sub> + wogonin groups, 50 µmol/L of H<sub>2</sub>O<sub>2</sub> was applied, and in various concentrations of wogonin (10<sup>-6</sup>, 10<sup>-5</sup>, 10<sup>-4</sup>) were applied.

#### Measurement of cell viability

The viability OF EA.hy926 cells was measured by a colorimetric MTT assay. Briefly, after the different concentrations of wogonin and H<sub>2</sub>O<sub>2</sub> were added to the wells to incubate for 24 h,

following addition of MTT solution (5 mg/mL) to each well, the plates were incubated for 4 h at 37°C. After the medium had been removed, the dye crystal was dissolved in 150 µL DMSO. Finally, the optical density (OD) of each well was immediately measured on 96 well plate (Axygen) at 540 nm to represent cellular viability. The OD of formazan formed in control group of cells was taken as 100% viability.

#### Total RNA extraction and qPCR

After treatment for 24 hours, cells were harvested by centrifugation and washed twice in PBS. Total RNA was isolated from the cells using High Pure RNA Isolation Kit, according to manufacturer's instructions. The primer sequences for TNF-α, Nox4, COX-2, SOD1, CAT and GSR are as listed in **Table 2**. The qPCR reaction was performed with 0.5 µl of cDNA, 10 µM of 0.5 µl each forward and reverse primer and 12.5 µl of IQ SYBR Green Super mix (Bio-Rad, USA) in Bio-Rad Cyclor (Bio-Rad, USA) with reaction profile of: 40 cycles of 95°C (10 seconds) and 61°C (30 seconds). For each sample, PCRs were performed in triplicate for each gene of interest, and reference gene to normalize for input cDNA. The threshold cycle (C<sub>T</sub>) value was determine d and the relative mRNA expression of the genes was calculated as follows:  $2^{-\Delta\Delta C_T}$  with  $\Delta\Delta C_T = C_{T\_GAPDH} - C_{T\_gene}$  of interest.

#### Statistical analysis

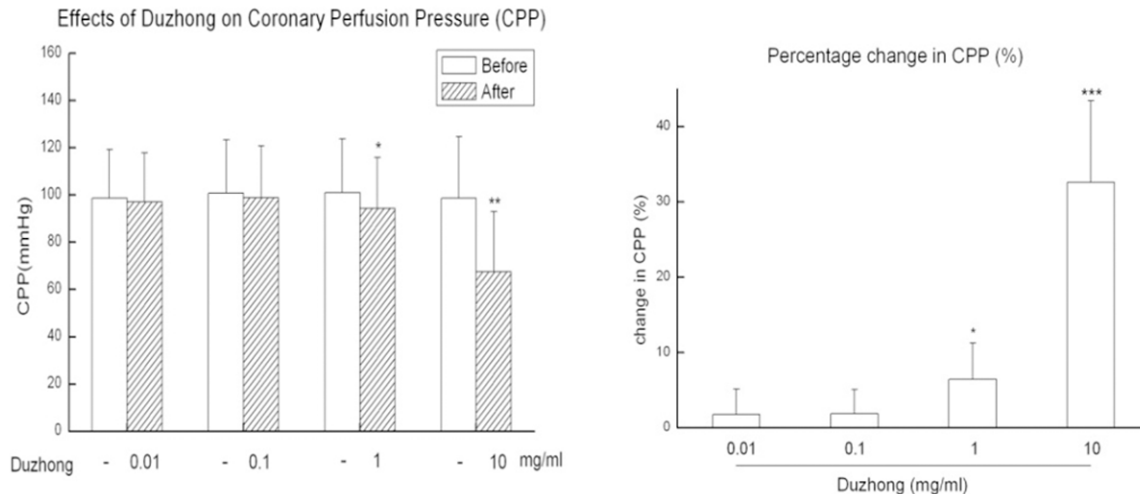
The results were expressed as means ± SEM. If appropriate, Student's t-test or one-way analysis of variance (ANOVA), followed by the Dennett post test for comparison versus values before adding medicine, control and hydrogen treated groups were performed. *P* values < 0.05 or 0.01 were considered to be statistically significant values < 0.001 were considered very statistically significant. Statistical analyses were performed using SPSS 16.0 software.

#### Results

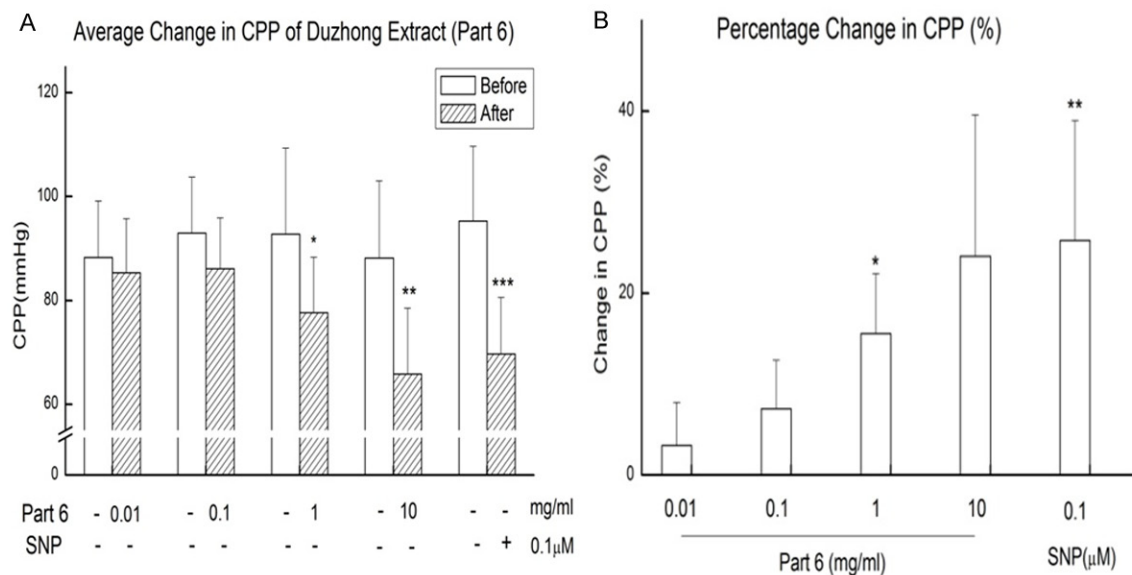
##### *Effects of Duzhong, compound and Sodium nitropruside on coronary perfusion pressure in an isolated rat heart, a langendorff preparation*

Crude Duzhong (0.01 mg/ml, 0.1 mg/ml, 1 mg/ml and 10 mg/ml were previously prepared. Bolus injections of 0.2 µl Duzhong significantly





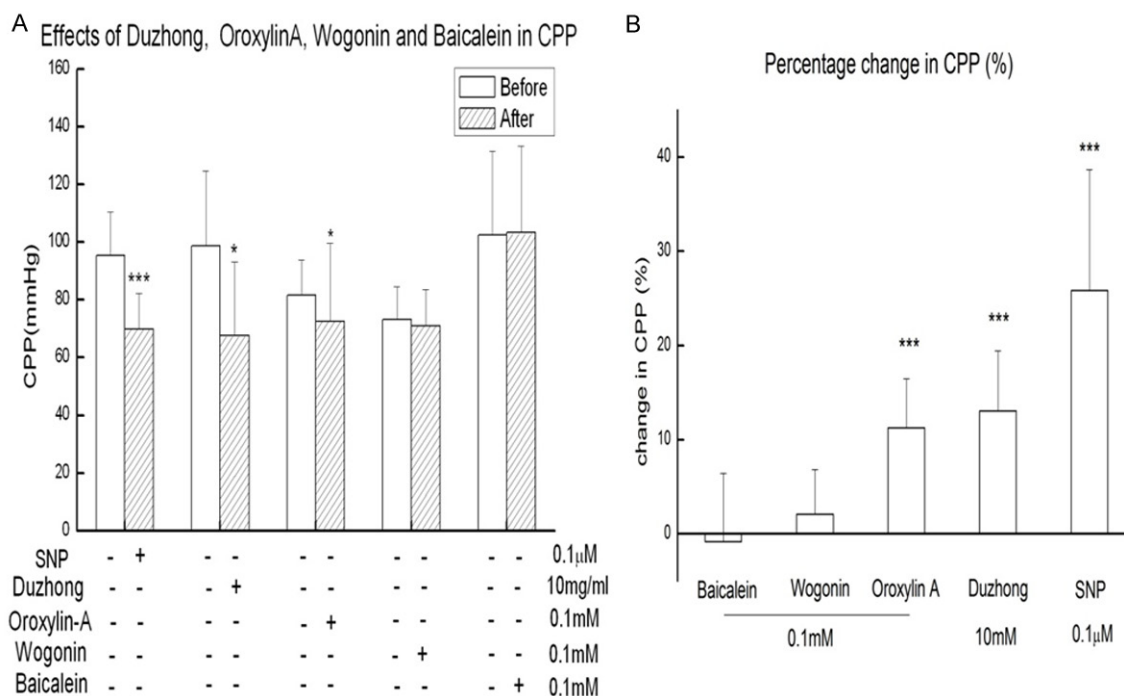
**Figure 1.** Effects of Duzhong on CPP in an isolated rat heart, a langendorff preparation. A. Reduction in coronary perfusion pressure (CPP) caused by different concentrations of ethanol extract of *Eucommiae Ulmoides Oliv*. (Duzhong) in langendorff preparation of isolated hearts. Open columns show values before adding medicine and Shaded columns show values after adding medicine. Data is expressed as the mean  $\pm$  SEM  $n = 10$ . \* $P < 0.05$ , \*\* $P < 0.01$  vs. values before adding medicine. B. Percentage change in CPP calculated as (Before medicine-After medicine)/Before Medicine  $\times 100\%$ . Data is expressed as a percentage change in CPP. \* $P < 0.05$ , \*\*\* $P < 0.001$  vs. 0.01 mg/ml, values are presented as the mean  $\pm$  SEM from 10 different experiments.



**Figure 2.** Effects of Sodium Nitropruside and Duzhong extract (named as part 6) in decreasing coronary perfusion pressure. A. Decrease in coronary perfusion pressure (CPP) caused by different concentrations of water extract of *Eucommiae Ulmoides Oliv*. (Duzhong) in langendorff preparation of isolated hearts and  $10^{-7}$  mol/L sodium nitropruside used as the control. Open columns show values before adding medicine and Shaded column show values after adding medicine. Data is expressed as mean  $\pm$  SEM on different values of  $n$  whereby 0.01 mg/ml ( $n = 6$ ), 0.1 mg/ml ( $n = 8$ ), 1.0 mg/ml ( $n = 8$ ), 10 mg/ml ( $n = 8$ ) SNP ( $n = 10$ ). \* $P < 0.05$ , \*\* $p < 0.01$ , \*\*\* $P < 0.001$  vs. values before adding medicine. B. Percentage change in CPP calculated as (Before medicine-After medicine)/Before Medicine  $\times 100\%$ . Data is expressed as a percentage change in CPP. \* $P < 0.05$ , \*\* $P < 0.01$  vs. 0.01 mg/ml, values are presented as the mean  $\pm$  SEM from 10 different experiments.

decreased the coronary perfusion pressure in a dose dependent manner. With changes in CPP observed in 1 mg/ml and 10 mg/ml, the

average values showed maximum effect with 10 mg/ml (**Figure 1A and 1B**). 10  $\mu$ g/ml SNP was added as a positive control with Duzhong



**Figure 3.** Effects of crude Duzhong, SNP, baicalein, Oroxylin A and wogonin. A. Reduction in coronary perfusion pressure (CPP) caused by Duzhong compounds in langendorff preparation of isolated hearts. Open columns show values before adding medicine and Shaded columns show values after adding medicine. Data is expressed as an average change in CPP and are presented as the mean  $\pm$  SEM from different experiments where wogonin n = 8, Oroxylin A n = 10 and SNP n = 11. \* $P < 0.05$ , \*\*\* $P < 0.001$  vs. values before adding medicine. B. Percentage change in CPP as (Before medicine-After medicine)/Before Medicine  $\times 100\%$ . Data is expressed as a percentage change in CPP with SNP, Duzhong and Oroxylin A having a significant decrease. Values are presented as the mean  $\pm$  SEM from different experiments where Duzhong n = 10, wogonin n = 8, Oroxylin A n = 10 and SNP n = 11 showing a significant decrease. \*\*\* $P < 0.001$  vs. wogonin.

extract (part 6) at different concentrations tested. The results as in **Figure 2A** and **2B**. On incorporation of single Duzhong compounds, Baicalein, Oroxylin A and wogonin was tested (**Figure 3A** and **3B**). In another experiment, Genipin, Baicalein and Geniposide tested at the same time had no effect on the coronary pressure but betulinic acid significantly raised the coronary perfusion pressure 106.07 mmHg-119.82 mmHg (data not shown).

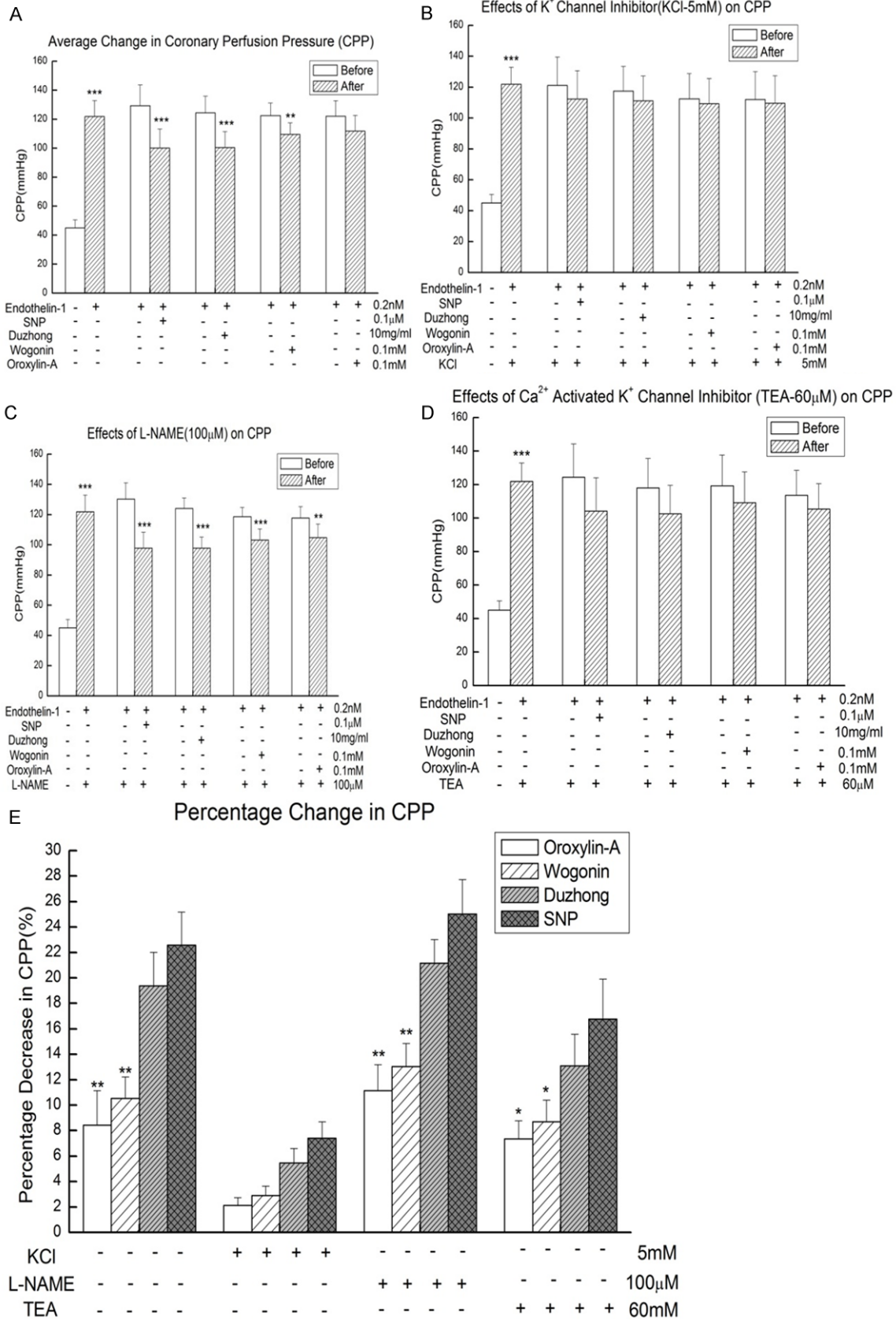
#### Effects Duzhong, wogonin and Oroxylin A on addition of a potent vasoconstrictor (Endothelin-1)

Hearts were then treated with endothelin-1 (0.2 nM), and significant increase in CPP was observed. Endothelin-1 was used to sensitize the heart and to constrict the vessels thereby inducing hypertension. Subsequent administration of Duzhong, SNP, wogonin and Oroxylin A was done, after which different parameters such L-NAME, KCl and TEA were administered as treatments after pre contrac-

tion with endothelin-1 and CPP studied. Endothelin-1 significantly increased the CPP, thereby inducing hypertension as shown in (**Figure 4A**). Administration of SNP ( $10^{-7}$ ), Duzhong (10 mg/ml), wogonin ( $10^{-4}$  M) and Oroxylin A ( $10^{-4}$  M) had an effect on CPP with SNP ( $10^{-7}$  M), Duzhong (10 mg/ml) and wogonin ( $10^{-4}$  M) showing a significant decrease in CPP.

To determine the mechanisms of the vasodilator effects of Duzhong 10 mg/ml, wogonin( $10^{-4}$  M) and Oroxylin A ( $10^{-4}$  M) on the coronary bed, the effects of inhibitor of Nitric Oxide Synthase (L-Nitroarginine Methyl Ester-L-NAME) 100  $\mu$ M, 60 mM KCL (non-selective potassium inhibitor channel, 5 mM Tetraethylammonium (TEA; a nonselective  $Ca^{2+}$  activated  $K^{+}$  channel inhibitor) on the dilator response to SNP ( $10^{-7}$  M) Duzhong 10 mg/ml, wogonin ( $10^{-4}$  M) and Oroxylin A ( $10^{-4}$  M) were examined. The endothelium remained intact and pre contraction of the vessels with endothelin-1 (0.2 nM) was maintained in the whole experiment.

# Cytoprotection of *Eucommiae Ulmoides Oliv*



**Figure 4.** Effects of SNP, Duzhong, wogonin and Oroxylin A on addition of a potent Vasoconstrictor (Endothelin-1). A: Reduction in coronary perfusion pressure (CPP) caused by SNP, Duzhong, wogonin and Oroxylin A on preconstruction of the vessels with endothelin-1 in langendorff preparation of isolated hearts. Open columns show values before adding medicine and Shaded columns show values after adding medicine. Data is expressed as an average change in CPP and are presented as the mean  $\pm$  SEM from different experiments where  $n = 8$ . \*\*\* $P < 0.001$ , \*\* $p < 0.01$  compared to values before adding medicine. B: Effects of KCl on CPP.  $n = 8$ . \*\*\* $p < 0.001$  compared to values before adding medicine. C: Effects of L-NAME on CPP.  $N = 8$ . \*\*\* $P < 0.001$ , \*\* $P < 0.01$  compared to values before adding medicine. D: Effects of TEA on CPP.  $N = 8$ . \*\*\* $P < 0.001$  compared to values before adding medicine. E percentage change in coronary perfusion pressure. E: Percentage change in CPP calculated by getting the difference between values before medicine and values after medicine, the difference divided by the original value then multiplied by 100% (Before medicine-After medicine)/Before Medicine  $\times$  100%. Data is expressed as a percentage change in CPP with L-NAME parameter showing a significant decrease. \* $P < 0.05$ , \*\* $p < 0.01$  vs. SNP, values are presented as the mean  $\pm$  SEM from different experiments where  $n = 8$ .

To assess the possible mechanisms under-lying the vasodilatation induced by the bolus injection of SNP ( $10^{-7}$  M), Duzhong 10 mg/ml, wogonin ( $10^{-4}$  M) and Oroxylin A ( $10^{-4}$  M), with or without 60 mM KCl, L-NAME (100  $\mu$ M) and 60 mM TEA were studied, separately. The vasodilator effects of SNP ( $10^{-7}$  M), Duzhong 10 mg/ml, wogonin ( $10^{-4}$  M) and Oroxylin A ( $10^{-4}$  M) were shown in **Figure 4E**.

Percentage change in CPP calculated by getting the difference between values before medicine and values after medicine, the difference divided by the original value then multiplied by 100% (Before medicine-After medicine)/Before Medicine  $\times$  100% **Figure 4E**.

All the studies were carried out prior to contraction of the coronary artery with endothelin-1.

*Effects of Duzhong compounds ( $10^{-4}$  M) on hydrogen peroxide (50 M/L) induced cell apoptosis*

A morphological observation of EA.hy926 cells was performed to confirm the apoptotic process. Cells grew normally, adhering to the bottom of the plate. The shapes of endochylema were fusiform, polygonal, and irregular with round cell nuclei. On treatment with  $H_2O_2$  (50  $\mu$ mol/L), a high proportion of cells showed apoptosis like changes such as being detaching from plate bottom and cytoplasmic condensation, leading to cellular swelling, rounding, microvillus disappearing and vacuoles appearing. In contrast, wogonin and wogonin + hydrogen peroxide treatment groups, showed apoptosis decrease in a dose-dependent manner (**Figure 5A**). At the same time, the definitive quantification of the effect of wogonin and Oroxylin A at various concentrations of  $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$  M) on  $H_2O_2$ -induced EA.hy926 cell apoptosis, the absorbance of cells was detect-

ed by MTT method. The result is as shown in **Figure 5B** and **5C** respectively.

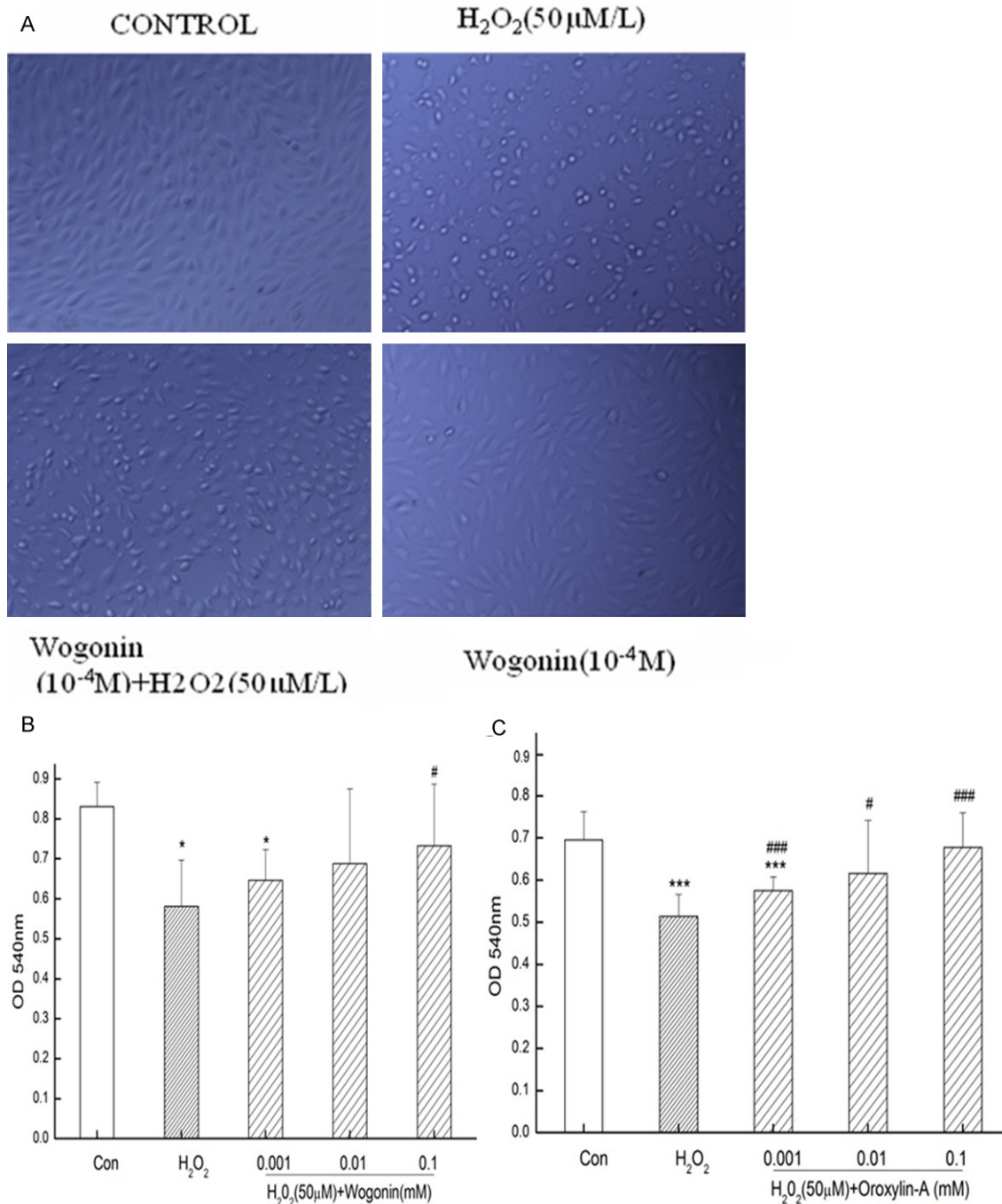
#### PCR results

In qPCR wogonin treatment with the same concentrations was done to evaluate its effects on gene expression. Treatment with wogonin ( $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$  M) and  $H_2O_2$  (50  $\mu$ M/L) significantly downregulated Nox-4 mRNA expression in EA.hy926 cells compared to the control and  $H_2O_2$  groups, with wogonin at a concentration of ( $10^{-4}$  M) +  $H_2O_2$  (50  $\mu$ M/L) producing very significant level compared to control (**Figure 6**). When stimulated with  $H_2O_2$  (50  $\mu$ M/L), EA.hy926 cells expressed higher level of Nox-4 mRNA expression. On the other hand, Wogonin treatment upregulated the expression of ROS inactivating enzymes; SOD1, CAT and GPx (**Figure 7**) in EA.hy926 cells. The result showed that SOD-1 mRNA expression was significantly expressed.

#### Discussion

The present study shows that both Duzhong, Duzhong extract (part 6), and the compounds Oroxylin A and wogonin produced concentration-dependent relaxation of CPP of the rat artery with functionally intact endothelium (**Figures 1-3**). Betulinic acid exhibited a significant vasoconstriction effect explaining that not all compounds in cortex *Eucommiae Ulmoides Oliv*. process a vasorelaxation activity, some counteracts the effect of other compounds. This is why Duzhong has been used as a combination of many compounds in the market since individual compounds are not potent anti-hypertensive. They work synergistically. The Pre treatment of arteries with endothelin-1 was done to temporarily induce hypertension and constrict the vessels (**Figure 4**). This finding





**Figure 5.** Effects of Duzhong compounds ( $10^{-4}$  M) on hydrogen peroxide (50 M/L) induced cell apoptosis. A: Cell morphology showing different groups and medicine treatments. B: Effect of wogonin on cell viability in  $H_2O_2$  treated EA.hy926 cells. The cells were incubated with  $H_2O_2$  (50  $\mu M/L$ ) with subsequent addition of  $H_2O_2$  with different concentrations of wogonin ( $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$  for 24 h. Cell viability was measured by MTT assay ( $n = 8$ ). Results were presented as mean  $\pm$  SEM. Values of the data obtained from three independent experiments performed in triplicate. \* $P < 0.05$ , compared with control group, # $P < 0.05$ , compared with  $H_2O_2$  group. C: Effect of Oroxylin A on cell viability in  $H_2O_2$  treated EA.hy926 cells. The cells were incubated with  $H_2O_2$  (50  $\mu M/L$ ) with subsequent addition of  $H_2O_2$  with different concentrations of Oroxylin A ( $10^{-6}$ ,  $10^{-5}$ ,  $10^{-4}$  for 24 h. Cell viability was measured by MTT assay ( $n = 8$ ). Results were presented as mean  $\pm$  SEM. values of the data obtained from three independent experiments performed in triplicate. # $P < 0.05$ , ### $P < 0.001$  compared to  $H_2O_2$  group, \*\*\* $P < 0.001$ , compared to control group 3.6. PCR Results.

indicates that the vasorelaxant effect of SNP, Duzhong, Oroxylin A and wogonin involved mainly endothelium independent mechanisms. To evaluate the involvement of endothelium-derived factors in vasorelaxation induced by SNP, Duzhong, oroxylin A and wogonin, the effects of L-NAME, an NO synthase inhibitor, KCl (non-selective K<sup>+</sup> channel inhibitor) and TEA (of Ca<sup>2+</sup> activated K<sup>+</sup> channel inhibitor) were investigated. Pretreatment with L-NAME significantly increased vasorelaxations in the rat aorta and coronary arteries, indicating the role of NO synthase in such endothelium dependent relaxation but in the presence of the inhibitor did not diminish the actions of SNP, Duzhong Oroxylin A and wogonin. Once released from endothelium, NO diffuses into the arterial smooth muscle to activate guanylate cyclase and thus increases cGMP formation, leading to vasorelaxation. The major finding of this study is that the vasodilation was markedly inhibited by the blockade of Ca<sup>2+</sup> activated K<sup>+</sup> channels by TEA and almost abolished by KCl medium. These findings suggested that the action is involved in activation of K<sup>+</sup> channels, which are associated with the release of EDHF.

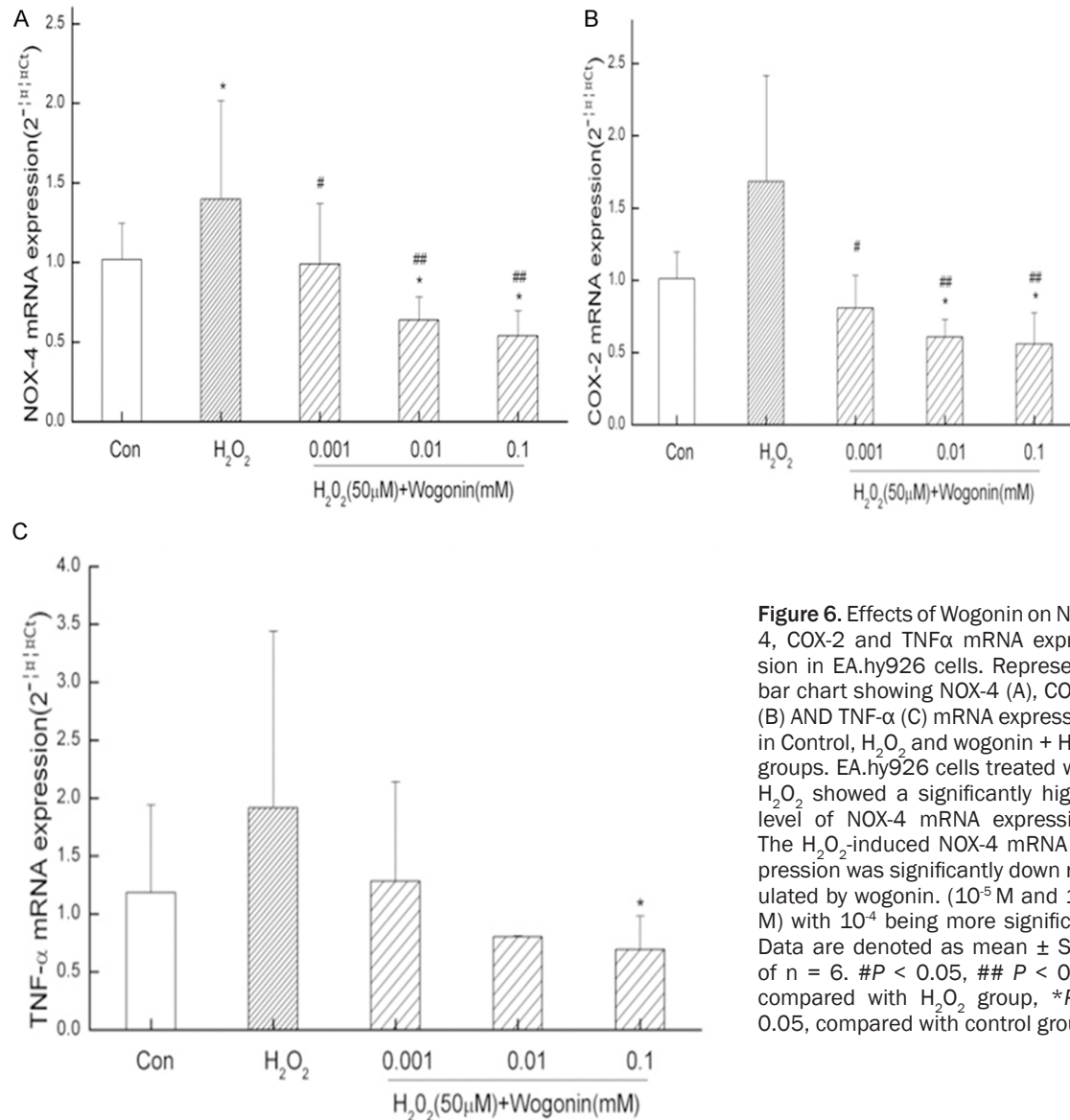
Vascular endothelial cells (ECs) are in direct contact with circulating leukocytes. During acute inflammation, they are exposed to activated leukocytes and their products. Studies has shown that some products of activated leukocytes, such as tumor necrosis factor (TNF) or interleukin-1 (IL-1), cause endothelial cells to undergo alterations in their constitution that enhance the capacity to promote inflammatory reactions (Poher and Cotran, 1990). These reversible changes, can be termed as “endothelial activation” (Poher, 1988; Sacks et al., 1978). Other products of activated leukocytes products like hydrogen peroxide have also been studied and identified as mediators of endothelial injury (Sacks et al., 1978; Varani et al., 1985; Varani et al., 1989; Weiss SJ, 1984). However, research suggests that hydrogen peroxide may also directly upregulate the pro inflammatory activity of vascular endothelium. For example, it has been reported to increase trans endothelial permeability (Shasby et al., 1985; Wilson et al., 1990) as well as neutrophil adhesion to endothelium in vitro (Lewis et al., 1988) and in vivo (Gasic et al., 1991). It is possible that hydrogen peroxide shares with cytokines the ability to activate endothelial gene expression. Hydrogen peroxide treated JURKAT cell line has been shown to activate the transcription factor

Nuclear Factor  $\kappa$ B (NF $\kappa$ B), (Schreck et al., 1991) a major mediator of TNF-induced gene expression, (Hohmann H-P, 1990; Hohmann et al., 1990; Israel A, 1989; Meichle et al., 1990) suggesting that they could have a shared mechanism of action.

NADPH oxidase generates superoxide by transferring electrons from NADPH inside the cell across the membrane thereby coupling them to molecular oxygen to produce superoxide anion, which is a reactive free-radical.

*Eucommiae ulmoides Oliv.* and its compounds exhibits a strong antioxidant activity (**Figure 5A-C**). The leaf extract has been reported to rather have a significant inhibitory effect on DNA damage induced by H<sub>2</sub>O<sub>2</sub> in lymphocytes (Yen, 2003). It was also found that the inhibitory effects of EuO on 1, 1-diphenyl-2-picrylhydrazyl (DPPH) radicals and low-density lipoprotein (LDL) oxidation has been realized in Duzhong tea in vitro (Ohmori, 2005). EuO's antioxidant effects may is in its inhibition on the cascade reaction of caspases. when treated with EuO, the initiator caspase 9 and effectors caspases 3, 6, and 7 were drastically downregulated both at mRNA and protein levels in H<sub>2</sub>O<sub>2</sub>-induced apoptotic model (Jun LIN1, 2011).

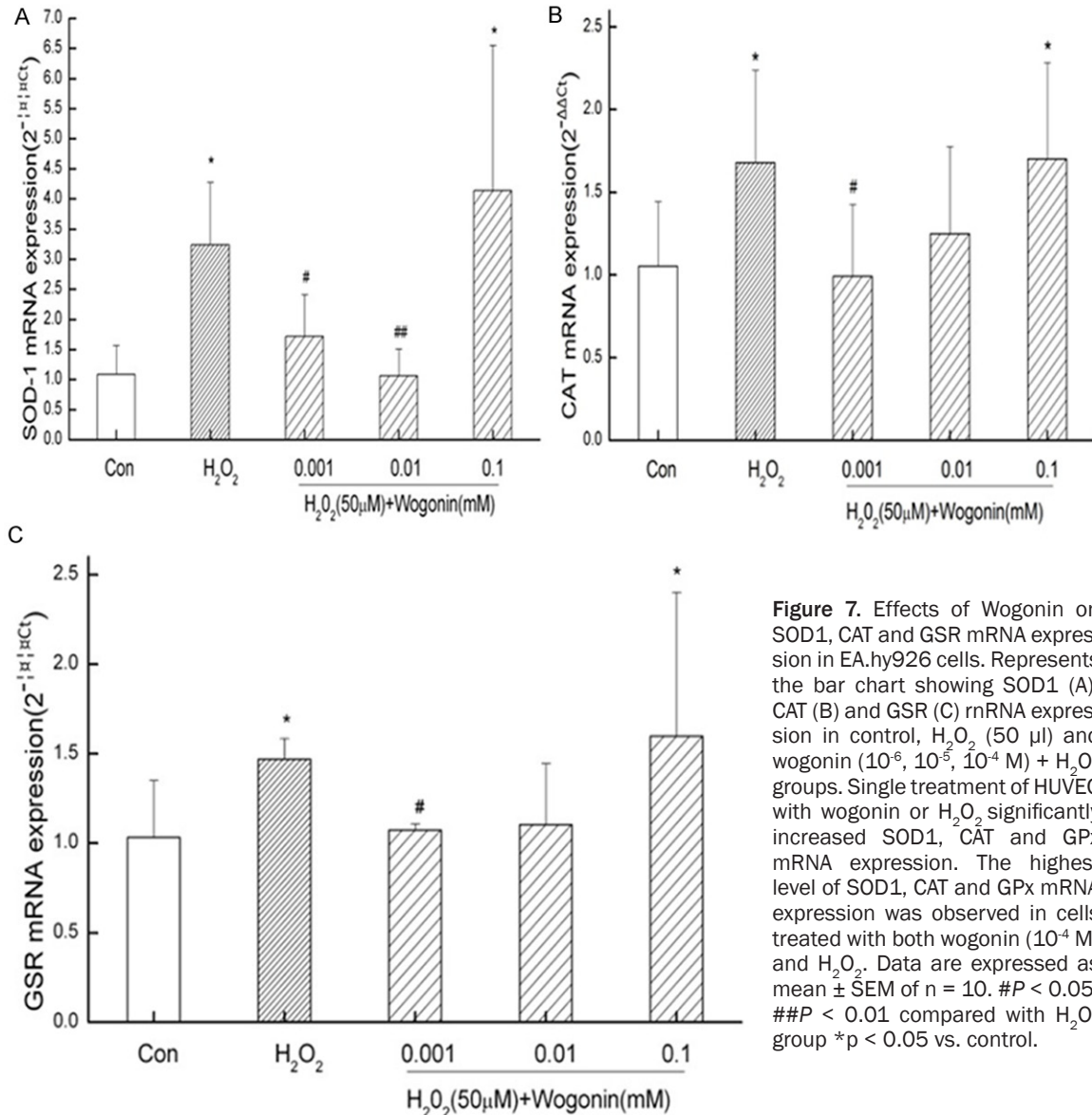
NF- $\kappa$ B regulates the immune response to infection as a major role (kappa light chains are critical components of immunoglobulins). Poor or improper regulation of NF- $\kappa$ B has been associated to cancer, inflammatory and autoimmune diseases, septic shock, viral infection, and improper immune development. It has also been implicated in processes of synaptic plasticity and memory. NF- $\kappa$ B's essential role of regulating the gene expression of cell adhesion molecules such as VCAM-1, ICAM-1 and E-selectin (Kang J, 2003) mediated by TNF- $\alpha$  is well known. Therefore, we hypothesized that wogonin has the ability to modulate the expression of TNF- $\alpha$  and cell adhesion molecules in H<sub>2</sub>O<sub>2</sub>-induced EA.hy926 cells. In this study, H<sub>2</sub>O<sub>2</sub> treatment group had a significant effect on the expression of TNF- $\alpha$  in EA.hy926 cells (**Figure 6C**). H<sub>2</sub>O<sub>2</sub> significantly induced NF- $\kappa$ B activated by TNF- $\alpha$  in porcine aortic endothelial cells but not in human aortic endothelial cells in another work, EA. Hy926 cells is a fused form of HUVECs therefore porcine endothelial cells might be more sensitive to H<sub>2</sub>O<sub>2</sub> compared to human endothelial cells (Lee S, 2007).



**Figure 6.** Effects of Wogonin on NOX-4, COX-2 and TNF $\alpha$  mRNA expression in EA.hy926 cells. Represents bar chart showing NOX-4 (A), COX-2 (B) AND TNF $\alpha$  (C) mRNA expression in Control, H<sub>2</sub>O<sub>2</sub> and wogonin + H<sub>2</sub>O<sub>2</sub> groups. EA.hy926 cells treated with H<sub>2</sub>O<sub>2</sub> showed a significantly higher level of NOX-4 mRNA expression. The H<sub>2</sub>O<sub>2</sub>-induced NOX-4 mRNA expression was significantly down regulated by wogonin. (10<sup>-5</sup> M and 10<sup>-4</sup> M) with 10<sup>-4</sup> being more significant. Data are denoted as mean  $\pm$  SEM of n = 6. #P < 0.05, ## P < 0.01 compared with H<sub>2</sub>O<sub>2</sub> group, \*P < 0.05, compared with control group.

Treatment with H<sub>2</sub>O<sub>2</sub> upregulated NOX-4 mRNA expression (**Figure 6A**). The H<sub>2</sub>O<sub>2</sub>-induced NOX-4 expression was significantly down regulated by both wogonin and H<sub>2</sub>O<sub>2</sub>. The effects of H<sub>2</sub>O<sub>2</sub> on the cellular adhesion molecules expression in this study, are in accordance with earlier research done (Bradley J, 1993). Treatment with 50  $\mu$ M/L H<sub>2</sub>O<sub>2</sub> upregulated COX-2 mRNA (**Figure 6B**). The pathophysiologic significance of our finding is that COX-2 as an oxidant stress-inducible gene may help to explain the enhanced expression of COX-2 during inflammation. ROS produced by inflammatory cells could lead to the deleterious amplification of prostanoids during inflammation. The most important physiological activators of COX are

Lipid hydroperoxides (Lands, 1984.). An important aspect of COX is that it exhibits auto accelerative reaction kinetics therefore time required to reach maximal velocity is decreased in the presence of lipid hydroperoxides (Hemler, 1978, 1979). In the observations made a small rise in the level of cellular peroxides can cause dramatic increases in COX activity thus increased production of ROS during inflammation could stimulate not only expression of COX-2, but also enzyme activity, resulting in an explosive production of prostanoids. The radical scavengers have been reported to block prostanoid formation by quenching the generation of hydro peroxides, which are the activators of COX.



**Figure 7.** Effects of Wogonin on SOD1, CAT and GSR mRNA expression in EA.hy926 cells. Represents the bar chart showing SOD1 (A), CAT (B) and GSR (C) mRNA expression in control, H<sub>2</sub>O<sub>2</sub> (50  $\mu$ l) and wogonin (10<sup>-6</sup>, 10<sup>-5</sup>, 10<sup>-4</sup> M) + H<sub>2</sub>O<sub>2</sub> groups. Single treatment of HUVEC with wogonin or H<sub>2</sub>O<sub>2</sub> significantly increased SOD1, CAT and GPx mRNA expression. The highest level of SOD1, CAT and GPx mRNA expression was observed in cells treated with both wogonin (10<sup>-4</sup> M) and H<sub>2</sub>O<sub>2</sub>. Data are expressed as mean  $\pm$  SEM of n = 10. #P < 0.05, ##P < 0.01 compared with H<sub>2</sub>O<sub>2</sub> group \*p < 0.05 vs. control.

Cellular studies demonstrated that wogonin downregulated the mRNA expression of ROS producing enzyme Nox-4 (**Figure 6A**) and at the same time, upregulated the expression of ROS inactivating enzymes; SOD1, CAT and GPx (**Figure 7A-C**) in EA.hy926 cells. Although several ROS generating systems have been described in endothelial and other vascular cells, NADPH oxidases (Nox) have now been recognized to be the major source of ROS in the vasculature (Guzik T, 2006). The Nox enzyme complex consists of two essential membrane bound subunits, gp91phox and p22phox, these are composed of cytochrome b558, and several cytosolic regulatory components. This

enzyme is dormant in resting cells, but on stimulation, the cytosolic subunits translocates to the cytochrome b558 at the membrane leading to activation of the enzyme and the release of large amounts of superoxides (Bradley J, 1993). NADPH oxidase

Governed by ROS production is regulated at two levels: gene expression of the Nox subunits and the enzyme activity (Bedard K, 2007). The study reported that wogonin decreased the expression of NOX-4, TNF- $\alpha$  and COX-2, mRNA expression in EA.hy926 cells (**Figure 6**). EA.hy926 cells treated with H<sub>2</sub>O<sub>2</sub> showed a significantly higher level of NOX-4 mRNA expres-



sion. The  $H_2O_2$ -induced NOX-4mRNA expression was significantly down regulated by wogonin and  $H_2O_2$ .

To the best of our knowledge, the present study reported that wogonin ( $10^{-4}$  M) +  $H_2O_2$  decreased the gene expression of Nox4, which is the predominant Nox isoform found in endothelial cells. The present study also showed that treatment of EA.hy926 with  $H_2O_2$  upregulated Nox4 mRNA expression. In another study,  $H_2O_2$  upregulated the Nox subunit p22phox mRNA and protein expression in endothelial cells (Djordjevic T, 2005).  $H_2O_2$  induced Nox4 mRNA expression was significantly down regulated by wogonin ( $10^{-4}$  M) +  $H_2O_2$ . This could be one of the mechanisms by which wogonin reduced endothelial oxidative stress. The antioxidant enzymes represent a first line of defense against ROS by metabolizing them to innocuous by products. The first enzymatic reaction in the reduction pathway of oxygen occurs during the dismutation of two molecules of superoxides when they are converted to  $H_2O_2$  and oxygen. The enzyme involved at this step is one of two isoforms of superoxide dismutase (SOD); CuZnSOD or SOD1, usually present in the cytosol while MnSOD or SOD-2 is located in the mitochondrial matrix. Although  $H_2O_2$  is not a free radical, it is reactive and is rapidly converted into the highly reactive hydroxyl anion in the presence of ferrous ion via the fenton reaction unless efficiently removed. The enzymes that participate in the removal of  $H_2O_2$  from the cellular environment are glutathione peroxidase (GPx) and catalase (CAT). Glutathione peroxidase is present in both the cytosol and mitochondria while catalase is present mainly in the peroxisomes. Both detoxify and breakdown  $H_2O_2$  into water and oxygen (J, 2000).

The antioxidant enzymes; SOD-1, CAT and GSR are thought to be effective for augmentation of antioxidant defenses in endothelial cells (V., 2001). Treatment with wogonin and  $H_2O_2$  upregulated the expression of SOD-1, CAT and GPx in EA. In hy926 cells as shown in **Figure 7**, in a dose dependent manner. These results suggested that the protective effects of wogonin against oxidative stress may be related to the increased ability to upregulate the antioxidant enzymes expression. The greatest inductions were in the levels of Cu/Zinc SOD mRNA; indicating that Cu/Zinc SOD plays an important role in scavenging  $H_2O_2$ . In another work, upregulation of antioxidant enzymes by resveratrol

protects aortic smooth muscle cells (Li Y, 2006,) and HUVECs (Spanier G, 2009) against oxidative stress. When EA.hy926 cells were exposed to  $H_2O_2$ , there was an increase in the mRNA expression of SOD1, CAT and GSR. This could be the possible defense mechanism of the cells to protect themselves better from the damage of  $H_2O_2$  (Rodriguez C, 2004). This study showed that wogonin significantly reduced Nox-4 expression and increased SOD1, CAT and GSR expression therefore it indicates an increase in cellular defense mechanism against oxidative stress and implies to the vasculature protective effects of wogonin. The work further improves the previous knowledge on the vasculature protective effects of wogonin as it explains that it can effectively increase cell defense mechanism against oxidative stress. *Eucommiae Ulmoides Oliv.* contained biologically active flavonoid compounds such as wogonin, oroxylin-A, baicalein and others which have been found to be potent antioxidants.

## Conclusion

In this work, we provided evidence that Duzhong and the two compounds induced endothelium dependent vasodilation of the isolated rat heart coronary artery mainly mediated by EDHF and involves the activation of  $K^+$  channel.

The study also described some novel effects of wogonin by decreasing the expression of Nox4, TNF- $\alpha$  and COX-2 and enhancing the expression of SOD1, CAT and GSR, it therefore represents a unique approach in reducing endothelial oxidative stress. wogonin has a protective effect against EA.hy926 cell damage induced by  $H_2O_2$  via anti-oxidative and anti-inflammatory pathways. The anti-inflammatory pathway is mediated by the decreased TNF- $\alpha$  and COX-2 mRNA expression induced by  $H_2O_2$  in EA.hy926 cells. Further, it inhibited the cell apoptosis induced by  $H_2O_2$  through promotion of NO release. The pharmacological studies done on *Eucommia ulmoides* cortex showed that possesses various activities such as antioxidant and antihypertensive (Chiu-Yin Kwan, 2004; Deyama et al., 2001; Pharmacopoeia, 2010).

The work has also given hopeful results that the flavonoid content from Duzhong have an antioxidant activity. It is therefore noteworthy that these findings may shed light on the pharmacological basis for the clinical application of traditional Chinese medicine in treatment of

atherosclerosis and hypertension relevant to endothelial cell damage. The limitation of this study was realized in Langendorff experiment whereby different concentrations of the compounds could not be prepared either by serial dilution or direct preparation. This was because Duzhong compounds are water insoluble. A concentration of  $10^{-4}$  M/L of Oroxylin A and wogonin was used after dissolving in DMSO and making  $10^{-3}$  M/L then using distilled water to dilute 10 fold but this still seemed to be high. Therefore more studies need to be carried out on the proper dilution method and administration into the isolated rat heart.

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### Abbreviations

ATCC, American Type Culture Collection;  $\text{Ca}^{2+}$ , Calcium; Carbogen, Carbon dioxide and Oxygen; CAT, Catalase; c DNA, Complementary Deoxyribonucleic Acid; COX-2, Cyclo Oxygenase-2; CPP, Coronary Perfusion Pressure; CT, Threshold Cycle; CVD, Cardiovascular Disease; CYP 450, Cytochrome P450;  $\Delta^{\text{CT}}$ , Delta Threshold Cycle; DMEM, Dulbecco's Modified Eagle Medium with high glucose; DMSO, Dimethylsulfoxide; DNA, Deoxyribonucleic Acid; EC, Endothelial Cell; ED, Endothelial Dysfunction; EDHF, Endothelial Derived Hyperpolarizing Factor; eNoS, Endothelial Nitric oxide Synthase; ET-1, Endothelin-1; FBS, Fetal Bovine Serum; GAPDH, Glyceraldehyde 3-Phosphate Dehydrogenase; GSH, glutathione; GPX, Glutathione Peroxidase; GSR, Glutathione Reductase; HLA, Human Leukocyte Antigen;  $\text{H}_2\text{O}_2$ , Hydrogen Peroxide; HUVECs, Human Umbilical Vein Endothelial Cells; IL-1K, Interleukin 1k; IL-6, Interleukin-6; IL-8, Interleukin-8; KCL, potassium Chloride; L-NAME- L, tro Arginine Methyl Ether; LVEDP, Left Ventricular End Diastolic Pressure; LVP, Left Ventricular Pressure; MCP-1, Monocyte Chemoattraction Protein-1; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NADPH (NOX-4), Nicotinamide Adenine Dinucleotide Phosphate Oxidase-4; NF-KB,

Nuclear Factor-Kappa light chain enhancer of activated B cells; NO, Nitric oxide; NOS, Nitric oxide Synthase; OD, Optical Density; q PCR, Quantitative Polymerase Chain Reaction; ROS, Reactive Oxygen Species; SHR, Spontaneously Hypertensive Rats; SNP, Sodium Nitro preside; SOD, Superoxide Dismutase; SP, systolic Pressure; TEA, Tetraethyl ammonium; TNF- $\alpha$ , Tissue Necrotic Factor- $\alpha$ ; VCAM-1, Vascular Cell Adhesion Molecule-1; XO, Xanthine Oxidase.

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