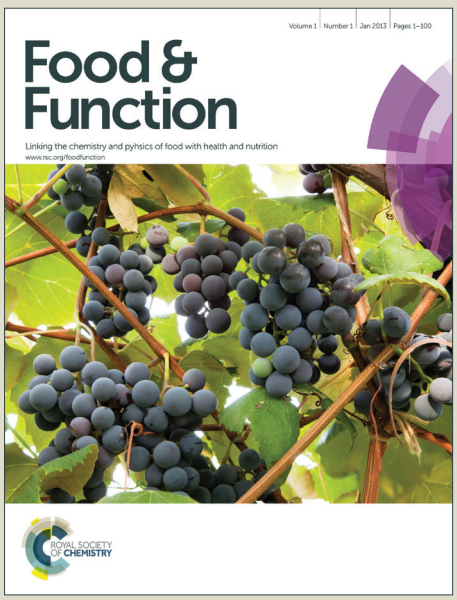
Food & Function

Accepted Manuscript



This is an *Accepted Manuscript*, which has been through the Royal Society of Chemistry peer review process and has been accepted for publication.

Accepted Manuscripts are published online shortly after acceptance, before technical editing, formatting and proof reading. Using this free service, authors can make their results available to the community, in citable form, before we publish the edited article. We will replace this *Accepted Manuscript* with the edited and formatted *Advance Article* as soon as it is available.

You can find more information about *Accepted Manuscripts* in the Information for Authors.

Please note that technical editing may introduce minor changes to the text and/or graphics, which may alter content. The journal's standard Terms & Conditions and the Ethical guidelines still apply. In no event shall the Royal Society of Chemistry be held responsible for any errors or omissions in this *Accepted Manuscript* or any consequences arising from the use of any information it contains.

ROYAL SOCIETY OF CHEMISTRY

www.rsc.org/foodfunction

Research article

Ethanolic extract of Rhizome of *Ligusticum Chuanxiong* Hort. (Chuanxiong) enhances endothelium-dependent vascular reactivity in ovariectomized rats fed with high-fat diet

Chun-Mei Li[1,2], Yu-Qing Guo[2,3], Xiao-Li Dong[4], He Li[1], Bo Wang[2], Jian-Hong Wu[2], Man-Sau Wong[2,4,*], Shun-Wan Chan[2,4,*]

[1] Department of Biochemistry & Molecular Biology, Guangdong Pharmaceutical College, Guangzhou, China

[2] State Key Laboratory of Chinese Medicine and Molecular Pharmacology, Shenzhen, China

[3] Institute of Medicinal Plant Development, Chinese Academy of Medical Sciences & Peking Union Medical College, Beijing, China

[4] Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong, China

[*]Author for correspondence; Dr. Shun-Wan Chan, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR, P. R. of China. *E-mail address:* bcswchan@polyu.edu.hk (S.W. Chan).

Dr. Man-Sau Wong, Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University, Hong Kong SAR, P. R. of China. *E-mail address:* bcmswong@polyu.edu.hk (M.S. Wong).

Short Title: Chuanxiong' vasoprotective effect on HFD-fed OVX rats

Abstract

Rhizome of *Ligusticum chuanxiong* Hort. (LC), also known as Chuanxiong, is a very common herb widely used to treat cardiovascular and cerebrovascular diseases. It is also used as a major ingredient in soups for regular consumption to promote good health. To study the protective effect of LC ethanolic extract (LCEE, 600 mg/kg/day, *p.o.*) on the integrity of the vascular system, ovariectomized (OVX) rats were fed with a high-fat diet (HFD) plus LCEE for 12 weeks. The animal model mimics the dyslipidemic condition seen in postmenopausal women. LCEE was found to significantly reduce the body weight gain, improve serum lipid profiles (lowering total cholesterol and low density lipoprotein cholesterol but raising high density lipoprotein cholesterol) and protect vascular endothelium in the HFD-fed OVX rats. It is postulated that LCEE could provide its vascular protective effect through multiple targets by (1) improving serum lipid profiles to reduce the detrimental effects given by cholesterol; (2) reducing ROS level inside the body via enhancing hepatic anti-oxidative activity or antioxidant level to scavenge the reactive oxygen species generated in the postmenopausal hypercholestemic condition; (3) stimulating eNOS-derived nitric oxide production; and (4) counteracting the up-regulation of inflammatory cytokine (TNF-α, VCAM-1 and ICAM-1) expressions so as to reduce endothelium damage.

Keywords: *Ligusticum chuanxiong* Hort.; vascular reactivity; antioxidant; high-fat diet; ovariectomized rat

1. Introduction

It is well-known that the vascular endothelium plays a pivotal role in the local regulation of vascular tone.[1] Endothelium injury characterized by abnormal endothelium-dependent vasorelaxation can result from the disruption of normal vascular homeostatic balance and clinically significant cardiovascular diseases such as atherosclerosis, thrombus and coronary heart disease.[1] Current research findings have suggested that oxidative stress alters many vascular functions including endothelium-dependent vasorelaxation tone.[2] Epidemiological studies have shown that postmenopausal women have a higher risk of developing cardiovascular diseases than premenopausal women partly because of estrogen deficiency-induced oxidative stress.[3,4] Hormone therapy in postmenopausal women not only relieves postmenopausal syndrome but also exerts beneficial effects on serum lipid profiles and vascular endothelial function.[5,6] However, recent findings have shown that exposure to estrogen is associated with increased risks of breast and ovarian cancers.[6] Hence, alternative approaches for prevention/treatment of postmenopausal cardiovascular diseases are needed.

Rhizome of *Ligusticum chuanxiong* Hort. (LC), also called Chuanxiong, is a very common herb widely used to treat cardiovascular and cerebrovascular diseases in China.[7] It is also used as a major ingredient in soups for regular consumption to promote good body health. LC ethanolic extract (LCEE) and its major constituents, such as ferulic acid and ligustrazine, have been found to improve serum lipid profiles, increase blood fluidity, inhibit platelet aggregation and exert antioxidative activity.[8,9,10,11] Ferulic acid was found to increase serum level of estrogen, improve menopausal symptoms in postmenopausal rat model or women, and display estrogen-like effect.[12,13] Some of the LC active compounds were found to exhibit vasorelaxation effects in rat isolated aorta.[14,15,16] In addition, LC has been used, alone or in combination with other herbs, for the improvement of menstrual disorders such as irregular menses, amenorrhoea and dysmenorrhoea.[17] However, no information is available regarding the vascular protective effect of LC under postmenopausal status.

Ovariectomized (OVX) animals are a well-known experimental model similar to human estrogen loss. Such an animal model is commonly used to investigate the mechanisms responsible for postmenopause-related complications in humans.[18] In order to study the role of LC supplement in protecting the integrity of vascular system, OVX (the same as estrogen-deficient) rats were fed a high-fat diet (HFD) to mimic dyslipidemia as seen in postmenopausal women. In the present study, changes in body weight, uterine weight index, serum lipid profiles [total cholesterol, triacylglycerols, high density lipoprotein cholesterol (HDL-cholesterol) and low density lipoprotein cholesterol (LDL-cholesterol)] and vasorelaxation of the HFD-fed OVX rats with or without treatment of LCEE for 12 weeks were investigated. Further analyses were made on the oxidant/antioxidant levels in the livers, *in vitro* aortic nitrate/nitrite production and mRNA expressions of endothelial nitric oxide synthase (eNOS), tumor necrosis factor α (TNF-α), intercellular cell adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1) in isolated aorta rings.

2. Materials and Methods

2.1. Chemicals

17β-estradiol, phenylephrine hydrochloride, acetylcholine hydrochloride, sodium nitroprusside, pentobarbital sodium, ferulic acid and tetramethylpyrazine were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). Kits for analyzing plasma lipid profile were obtained at Biosino Bio-technology and Science Inc. (Beijing, China). All other reagents are analytically pure and used without further purification.

2.2. Preparation of herbal extract

Dried LC herb was purchased from the Beijing Guancheng Pharmaceutical Co. Ltd. of China (Beijing, China). A voucher specimen was deposited in the Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University. After grinding into powder, the dried herb (1 kg) was extracted twice with 6 L of 80% ethanol (ethanol:distilled water = 8:2, v/v). The extracted solutions were filtered, combined, concentrated by a rotary evaporator and freeze-dried. The extract yield was equal to 13.03%. The dried extract (LCEE) was stored at -20°C and dissolved in distilled water before administrating to the animals.

2.3. High performance liquid chromatography (HPLC) analysis on LCEE

HPLC analysis was performed using a Shimadzu 10advp HPLC system (Shimadzu, Japan) coupled to a photodiode array detector. All samples were filtered through a 0.45 μm Millipore filter unit (Millipore, Bedford, MA, USA) and separated on a C18 column (250 mm × 4.6 mm; 5 μm) (Elite, Dalian, China) using linear gradient elution with a mobile phase containing acetonitrile (solvent A) and 0.05% trifluoroacetic acid (solvent B). Linear gradient profile (A:B, v/v) started at 4:96 for 25 min, then increased to 25:75 for the last 3 min. The flow rate was set at 1.0 mL/min (25°C). An autosampler was utilized for sample injection with an injection volume of 20 μL. Detection was performed with a UV wavelength detector at 280 nm. The level of

ferulic acid and tetramethylpyrazine in LCEE was calculated from the relevant peak area with external standard method. Since ferulic acid is used as a marker to evaluate the quality of LC in Chinese medicine pharmacopoeia[19] and tetramethylpyrazineisone is one of the important active ingredients in LC, ferulic acid and tetramethylpyrazine were used to express the quality of the LC 80% ethanolic extract used in the current study.

2.4. Animals and experimental settings

Twenty-eight female Sprague-Dawley rats (3-month-old) were supplied by Guangdong Provincial Medical Laboratory Animal Center (Guangdong, China). All rats were housed under standard conditions (temperature 20 ± 1°C, humidity 60 ± 10%, light–dark cycle 12h:12 h) with free access to water and rat chow until they were 6 months old. After rats were randomly divided into four different groups (each with 7 animals), they underwent bilateral ovariectomy (OVX, n = 21) or laparotomy (Sham, n = 7) under anaesthetization with pentobarbital sodium. The rats were allowed to recover for 4 weeks and received different treatments according to their assigned groups: (1) Control: Sham rats with low fat control diet (LFD) (Harlan Teklad, TD.10592) + vehicle; (2) Model: OVX rats fed with high fat diet (HFD) (Harlan Teklad, TD.10586) + vehicle; (3) E2: OVX rats with HFD + 17β-estradiol (1 mg/kg/day, *p.o.*) and (4) CX: OVX rats with HFD + LCEE (600 mg/kg/day, *p.o.*). Since Formulation of the testing dosage of LCEE in the present study was based on its standard clinical dosage (6 – 10 g crude herb per patient).[20]

The rats were pair-fed with their corresponding commercial diets and received different treatments by oral gavage (10 mL/kg) once every morning for 12 weeks. During the whole experimental period, the body weight of each animal was monitored daily. At the end of the experimental period, the rats were fasted overnight and killed by cervical dislocation. Uteruses were weighed and blood samples, livers and aortas were collected for further analyses. The experimental protocol was conducted under the animal license issued by the Shenzhen Research Institute of The Hong Kong Polytechnic University. All procedures were consistent with the Guide for the Care

and Use of Laboratory Animals published by the US National Institutes of Health and the principles outlined in the Declaration of Helsinki. Every effort was made to limit animal suffering and the number of animals used in this study.

2.5. Analysis of lipid levels in blood samples

After cervical dislocation, blood was collected immediately in chilled centrifuge tubes through cardiac puncture and allowed to clot for 2 hr. The clotted blood was then centrifuged (2000 ×g) at 4°C for 20 min to get serum. Total serum cholesterol (TC), triacylglycerol (TG), LDL-cholesterol and high density lipoprotein (HDL)-cholesterol were measured on biochemical autoanalyzer (Alcyon abbot-300, USA) by using their commercially available kits (Biosino Bio-technology and Science Inc., Beijing, China).

2.6. Determination of oxidant/antioxidant levels in the livers

Liver tissue was kept at -80°C before measurement of the oxidant/antioxidant levels. Each frozen sample was homogenized in ice-cold saline (1:9, w/v) and centrifuged at 2000 ×g for 15 min (4°C). The supernatant was collected and stored at -20°C until further analysis. The levels of malondialdehyde (MDA) as well as the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) were measured in duplicate with spectrophotometry-based commercial kits (Keygen Biotech. Co. Ltd., Nanjing, PR China). The protein content was measured by Bradford method (Bio-Rad, Hercules, CA, USA). Absorbance for various assays was determined using a POLARstar Galaxy Plate Reader (BMG, Offenburg, Germany). MDA levels were expressed as nmol/mg protein; while SOD, CAT and GPx activities were expressed as U/mg protein.

2.7. Isolation of thoracic aorta

At the end of the experiment period, all rats were sacrificed and their thoracic aortas were immediately isolated and placed in 4°C Tyrode's solution (118 mM NaCl, 4.7

mM KCl, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 11 mM glucose, 2.5 mM $CaCl_2$, and 1.2 mM $MgSO_4$). After the careful removal of fat and connective tissue, each aorta was cut into three ring segments for vascular reactivity study (4 mm in length), *in vitro* nitrate/nitrite production (15 mm in length) and real-time PCR analysis (15 mm in length).

2.8. Study of vascular reactivity

Vascular reactivity was investigated in the isolated thoracic aortic ring as described previously.[21,22,23] The 4 mm-long aortic rings were placed in 5 mL-organ baths containing Tyrode's solution (37°C) bubbled with a mixture of 95% O_2 and 5% CO_2 gases. A resting tension of 1.2 g was applied to each aortic ring and Tyrode's solution was changed every 15 min until the end of the 60 min equilibrium period. Then the aortic rings were sensitized with 60 mM KCl for three times. The aortic rings were contracted with phenylephrine (1 μM) for about 20 min, and then cumulative concentrations of acetylcholine (ACh) (an endothelium-dependent vasodilator, 10 nM – 10 μM) or sodium nitroprusside (SNP, a nitric oxide donor, 0.1 nM – 0.3 μM) were added to measure the endothelium-dependent and endothelium-independent responses (isometric tension, in g), respectively. Throughout the experimental period, neostigmine (1 μM) and indomethacin (1 μM) were present in the Tyrode's solution. Changes in tension of the isolated aorta rings were recorded by isometric force-displacement transducers connected to the PowerLab Data Acquisition system (AD Instruments, Sydney, NSW, Australia). Concentration-response curves were plotted as percentage vasorelaxation against the logarithmic cumulative concentration of either ACh or SNP in the organ baths.

2.10. *In vitro* nitrate/nitrite production in isolated thoracic aorta

Nitric oxide (NO) production was evaluated by *in vitro* nitrate/nitrite production in the thoracic aorta. After incubation with 2 mL Tyrode's solution with ACh (1 μM) and neostigmine (1 μM) at 37°C for 2 hr, each 15 mm-long segment was blotted dry and weighed. The incubated culture solution of each segment was collected and dried by

vacuum freeze-drying, and subsequently re-dissolved with 300 μL of distilled water. Nitrate/nitrite levels were detected with a commercial kit (Promega, USA, Cat.: G2930). Absorbance (at 520 nm) was determined using a POLARstar Galaxy Plate Reader (BMG, Offenburg, Germany). The concentrations of nitrate/nitrite per mg of aorta were calculated by following the instructions of the kit.

2.11. Real-time polymerase chain analysis

For determination of the molecular mechanism of the vasoprotection of LCEE, we analyzed mRNA levels of eNOS, TNF-α, ICAM-1 and VCAM-1 in the artery. Total RNA was isolated from the other 15 mm-long arteries using Trizol Reagent (Invitrogen, Carlsbad, CA, USA, Cat. No. 15596-018), and then reverse-transcribed into cDNA with random hexamers using RevertAid™ first strand cDNA synthesis kit (Fermentas, Cat. No. K1622). PCR amplifications using cDNA as template were run in 20 μL reaction volumes containing 0.4 μM specific primers and IQ™ SYBR®Green SuperMix (Cat. No.170-8884) with a CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). All the primers used were synthesized by TaKaRa Biotechnology Co., Ltd. (Dalian, China) (Table 1). The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the invariant control to correct for differences in quantity and quality between RNA samples, and the expression levels of various mRNA of interest were expressed as a ratio to GAPDH mRNA.

2.12. Statistical analysis

All data were expressed as the mean ± S.E.M. Relaxations (%) were expressed as the percentage of phenylephrine (1 μM)-induced contraction. GraphPad Prism 5.02 (San Diego, California, USA) was used for curve fitting and estimating the magnitude of maximum relaxation (R_{max}). Statistical analysis was carried out by one-way analysis of variance (ANOVA) and Tukey's test as post-test after ANOVA for multiple comparisons by the statistical software, GraphPad Prism 5.02 (San Diego, California, USA) for Windows. A significant level of $p < 0.05$ was used for all comparisons.

3. Results

3.1. HPLC analysis on LCEE

The chemical characteristic of LCEE was determined using the HPLC-UV method in order to ensure the consistency of its quality and standardization of the sample tested. Representative HPLC chromatograms of standards (tetramethylpyzine and ferulic acid) and LCEE were shown in Fig. 1. It was found that LCEE (1 g) contained 3.00 ± 0.16 mg of tetramethylpyzine and 4.22 ± 0.23 mg of ferulic acid.

3.2. Body weight and uterine relative weight

Table 2 summarized the body weight, body weight gain, uterine dry weight and uterine weight index (a ratio of uterine weight to body weight) of animals in the Control, Model, E2 and CX groups. Ovariectomy plus HFD (Model) could significantly increase the body weight gain ($p < 0.001$) and decrease the uterine index of rats ($p < 0.001$) as compared with those parameters of the rats in the Control group. Consumption of 17β-estradiol could prevent body weight gain ($p < 0.001$) and stimulate the growth of the atrophic uterus ($p < 0.05$) in HFD-fed OVX rats as compared with those parameters in the Control animals. Interestingly, treatment of LCEE markedly decreased the increased body weight gain induced by ovariectomy plus HFD ($p < 0.05$), but did not significantly alter the uterine dry weight and uterine index (CX vs Model: both with $p > 0.05$).

3.3. Serum lipid profiles

The serum lipid profiles: total cholesterol, triacylglycerol, HDL-cholesterol and LDL-cholesterol levels of rats in different treatment groups by the end of the experiment were summarized in Table 3. In the Model group, there were significant elevations in the contents of serum total cholesterol ($p < 0.01$) and LDL-cholesterol ($p < 0.05$) but no significant differences in the contents of triacylglycerol and HDL-cholesterol as compared with the Control group. Treating the animals with 17β-estradiol could suppress the increased serum levels of total cholesterol ($p < 0.001$)

and LDL-lipoprotein ($p < 0.001$) induced by ovariectomy plus HFD. Interestingly, administration of LCEE not only could markedly suppress the increased serum total cholesterol and LDL-cholesterol contents (both with $p < 0.05$) induced by ovariectomy plus HFD but also enhance serum HDL-cholesterol level in the HFD-fed OVX rats (CX vs Model: $p < 0.001$)**.**

3.4. Oxidant/antioxidant levels in the liver

Oxidative stress was evaluated by the production of MDA (a biomarker of lipid peroxidation) and antioxidant level was estimated by the activities of the anti-oxidative enzymes SOD, CAT and GSH-Px in the liver (Fig. 2). MDA levels were raised in the HFD-fed OVX rats compared with the Control rats (~87% increase, $p < 0.01$) and the increase decreased markedly after treatment of 17β-estradiol ($p < 0.05$) or LCEE ($p < 0.001$) for 12 weeks. Compared to the Control group, the activities of SOD, CAT and GPx reduced significantly in the HFD-fed OVX rats [with ~30% ($p < 0.01$), ~30% ($p < 0.05$) and ~26% ($p < 0.05$) reduction, respectively). Administration of LCEE, similar to administration of 17β-estradiol, could significantly ($p < 0.05$) elevate the activities of these enzymes back to the levels as observed in the Control group.

3.5. Vascular reactivity

The contractions induced by phenylephrine (1 μM) on isolated aortic rings from different groups were not significantly different ($p > 0.05$, Table 4). The ACh-induced endothelium-dependent relaxation was significantly suppressed under the condition of ovariectomy and HFD (Control vs Model: $p < 0.001$) (Table 4 and Fig. 3A). The maximum ACh-induced vasorelaxation observed in the isolated aortic rings from the CX group (R_{max}: 57.39 ± 13.87%) was slightly higher than that from the E2 group (R_{max}: 55.51 ± 3.63%). The isolated aortic rings of both the CX and E2 groups could provide significantly enhanced ACh-induced vasorelaxation as compared with those of the Model group (both, $p < 0.001$). Interestingly, there were no significant differences in the vasorelaxation elicited by SNP, a NO donor, among all the

experimental groups ($p > 0.05$, Table 4 and Fig. 3B). All these findings indicated that the vascular dysfunction induced by the condition of ovariectomy plus HFD could be prevented by either LCEE or 17β-estradiol treatment.

3.6. *In vitro* NO production in the isolated thoracic aorta

Nitrate/nitrite production in the isolated thoracic aorta from various groups of animals was tested to estimate NO production and the result was presented in Fig. 4. In the Control rats, the average of nitrate/nitrite production in the isolated thoracic aortic ring was 1.07 nmol/mg aortic tissue. It was found that nitrate/nitrite production was markedly attenuated in the aortic rings from the Model group (0.40 nmol/mg aortic tissue). With 17β-estradiol or LCEE treatment, the extent of decrease in nitrite/nitrate production induced by ovariectomy plus HFD was obviously antagonized as compared with that of the Model group (E2 *vs* Model, $p < 0.05$; CX *vs* Model, $p < 0.05$).

3.7. Real-time polymerase chain reaction analysis in the isolated thoracic aorta

In order to understand the molecular mechanism of the effect of LCEE on preventing vascular dysfunction induced by ovariectomy plus HFD, the mRNA levels of four genes related to vascular functions were studied by real-time polymerase chain reaction analysis (Fig. 5). It was found that the combined actions of ovariectomy and HFD (as indicated in Model) markedly down-regulated the mRNA expression of eNOS ($p < 0.01$) and up-regulated the mRNA expressions of TNF-α, VCAM-1 and ICAM-1 (for all genes, $p < 0.001$) as compared with those in the Control rats (Fig. 5). It was found that treating the HFD-fed OVX rats with either 17β-estradiol or LCEE significantly enhanced the eNOS expression (E2 *vs* Model: $p < 0.05$; CX *vs* Model: $p < 0.01$) and reduced the up-regulation of TNF-α, VCAM-1 and ICAM-1 mRNA expressions (E2 *vs* Model: for all genes, $p < 0.001$; CX *vs* Model: for all genes, $p < 0.001$).

4. Discussion

There is increasing evidence suggesting that postmenopausal women with estrogen deficiency are much more prone to suffering from abnormal serum lipid profiles, increased oxidative stress and impaired vascular endothelium than their premenopausal counterparts.[5,24] Thus, postmenopausal women have higher incidence rates for cardiovascular diseases. In the present study, we successfully combined HFD-feeding with OVX in female SD rats to simulate the cardiovascular condition of postmenopausal women with dyslipdemia, high oxidative stress and endothelial dysfunction. To evaluate the applicability of the current animal model, 17β-estradiol was used as a positive control in the present study. The consumption of 17β-estradiol was shown to reverse the deterioration induced by ovariectomy plus HFD. The current study demonstrated for the first time that treatment of LCEE for 12 weeks could provide an overall health benefit by counteracting the detrimental effects, such as body weight gain, serum lipid profile abnormality, decreased hepatic anti-oxidative activity and vascular dysfunction, induced by ovariectomy plus HFD.

In order to ensure its quality consistency and standardization of the LCEE used in the present study, the chemical characteristics of it were determined using the HPLC-UV method. Two markers, ferulic acid and tetramethylpyrazine, were selected to express the quality of LCEE since ferulic acid is used as a marker to evaluate the quality of LC in Chinese medicine pharmacopoeia[19] and tetramethylpyrazineisone is one of the important active ingredients in LC. Although the chemical composition of LCEE is mostly known, the current HPLC fingerprint provided is to help compare the quality of LCEE produced from other team so as to ensure the reproducibility of the current experimental data.

LC has been reported to have phytoestrogenic-like effect *in vitro*[25], the total amount of phytoestrogen in LCEE and phytoestrogenic-like effect provided by LCEE were relatively low. Therefore, the beneficial effects of LCEE on the HFD-fed OVX rats observed in the current study didn't appear fully consistent with those of 17β-estradiol, especially the beneficial effects on uterine index. It is believed that the

beneficial effects of LCEE may be through a non-estrogenic pathway or a synergistic interaction with multiple active components (both phytoestrogenic and non-phytoestrogenic compounds). It is interesting to note that the beneficial effects given by LCEE were mainly on cardiovascular system. The effects are similar to those of statin, a potent hypocholesterolemic and hypolipidemic drug.[22,23] Further studies with simvastatin as another positive control could definitely help comparing and dissecting the pharmacological mechanisms of LCEE.

Cholesterol is the underlying agent responsible for causing atherosclerosis and other cardiovascular diseases. If serum cholesterol levels get too high, it can form plaques inside the blood vessels and make blood vessels more fragile and easier to break.[26] Clinical and epidemiological evidence has demonstrated that total and/or LDL-cholesterol levels are significantly elevated in postmenopausal women as compared with premenopausal women.[27,28] In the present study, we found LCEE treatment for 12 weeks on the HFD-fed OVX rats not only significantly decreased the elevated serum total cholesterol and LDL-cholesterol, but also markedly increased serum HDL-cholesterol. HDL-cholesterol was shown to reverse cholesterol transport, prevent endothelial dysfunction and maintain the critical balance needed for normal vascular function.[29] Many natural products, such as *Bacopa monniera* Linn. and *Cynanchum wilfordii*, were found to exerted their vasculoprotection mainly through increasing HDL-cholesterol and improving antioxidant status.[30,31] Our results suggested that consumption of LCEE could modulate serum lipid profiles (including decrease in serum total cholesterol and LDL-cholesterol and increase in HDL-cholesterol) and subsequently reduce the risk of atherosclerosis and other cardiovascular diseases in postmenopausal women, but the molecular mechanism is still unclear and awaits further studies.

Oxidative stress, defined as the imbalance that is in favor of reactive oxygen species (ROS) generation over antioxidant defense, exerts several negative effects on the arterial wall, such as damage on endothelium and decrease in bioavailability of NO.[32] Previous studies have indicated that postmenopausal women have higher chance to have hypercholesterolemic atherogenesis.[33,34] In fact, abnormality in the

redox state is often observed in OVX or HFD-fed rat models.[4,35] In the present study, high MDA (a biomarker of lipid peroxidation) with low activities of the antioxidant enzymes (SOD, CAT and GPx) were recorded in the livers of the HFD-fed OVX rats. 17*β*-Estradiol consumption could markedly prevent the oxidative stress induced by ovariectomy plus HFD. A significant reduction of MDA content as well as significant increase in SOD, CAT and GPx activities were observed in the liver of the HFD-fed OVX animals treated with 17*β*-Estradiol. Similar results were obtained in the HFD-fed OVX rats treated with LCEE. Previous studies have shown that the extract,[36] essential oils[37,38] and some active ingredients[9,10] of LC herb could strongly alleviate oxidative stress. It could help to explain why LC is widely used to prevent and treat oxidative disorders, such as ischemic stroke and ischemia/reperfusion injury.[9,10]

Endothelium plays a pivotal role in modulating vascular tone by producing and releasing vasoactive substances such as NO.[39] Endothelial vasodilator dysfunction contributes to the progression of vascular disease and vascular reactivity abnormality.[40,41,42] Oxidative stress induced by menopause could also result in endothelial dysfunction.[43,44] Hormone therapy in postmenopausal women (or OVX animal) could limit the uptake of cholesterol ester into the arterial wall, reduce oxidative stress, improve NO-mediated endothelium-dependent vasodilation and exert cardiovascular protection.[6,45,46] In the present study, ovariectomy plus HFD reduced the vasorelaxation induced by ACh (endothelium-dependent relaxation) without affecting that induced by SNP (endothelium-independent relaxation), suggesting LCEE could protect the endothelium from the detrimental effect given by ovariectomy plus HFD. It was found that there was a partial loss of endothelial function in aorta from the control group rats since the maximum relaxation with ACh is around a 60% of the contraction induced by phenylephrine (1 μM) (Fig. 3). It may be resulted from the age (8-month old) of the rats at the end of the experiment. In our previous studies, end point measurement on aortic endothelium-dependent relaxation from younger rats (3-month old) gave a higher ACh-induced maximum relaxation (>80%).[47,48] It further confirmed that age is inversely related to the endothelium function.

Oral administration of LCEE to the HFD-fed OVX rats increased the vascular response to ACh via up-regulating eNOS mRNA expression and inducing the NO release from the isolated aortic rings. Additionally, LC has been report to have direct *in vitro* vasorelaxation effect.[49] However, whether the LCEE-induced improvement on endothelium-dependent relaxation is due to a direct pathway via up-regulation of eNOS mRNA expression in the endothelium or as a result of an indirect mechanism through improving lipid profile/antioxidant level or both is presently unclear and awaits further studies.

Inflammation plays a crucial role in the pathogenesis of various vascular diseases, including atherosclerosis. Estrogen (or 17β-estradiol) could antagonize the acute vascular injury response and the development of other forms of vascular pathology, in part, by altering the vessel wall's ability to express various growth factors, adhesion molecules and chemokines, such as TNF-α, ICAM-1 and VCAM-1.[6,50] TNF-α, a pro-inflammatory cytokine, is mainly derived from mononuclear phagocytes and primarily targeted some cells including vascular endothelial cells.[51] Animal under OVX and/or HFD-fed condition was found to with elevated TNF-α expression.[52,53,54,55] The increase in TNF-α expression could induce the gene expressions of various inflammatory cytokines, stimulate the production of ROS, impair NO bioavailability and result in endothelial dysfunction.[51,56] In the present study, up-regulations of the mRNA expressions of TNF-α, VCAM-1and ICAM-1 were observed in the isolated aortic tissues from the HFD-fed OVX rats. LCEE treatment for 12 weeks could significantly counteract the up-regulations of inflammatory cytokine (TNF-α, VCAM-1 and ICAM-1) expressions induced by ovariectomy plus HFD. The current results are in agreement with pervious *in vitro* studies which showed that LC and its main ingredients had protective effects against endothelial dysfunction in vitro by down-regulating TNF-α, VCAM-1 and ICAM-1 expressions.[57,58,59] In order to confirm the mechanism of LCEE's vascular protective effect through the anti-inflammatory pathway, further studies with monitoring on the protein expressions are warranted.

5. Conclusions

The current findings demonstrated for the first time that oral administration of LCEE (600 mg/kg/day) to HFD-fed OVX rats for 12 weeks had significant beneficial effect in terms of reducing body weight gain, enhancing hepatic anti-oxidative activity improving serum lipid profiles and preventing vascular endothelial dysfunction. Based on the outcome of the current study, the mechanisms by which LCEE exerted vascular protective effect are summarized in Fig. 6. It is postulated that LCEE could provide its vascular protective effect via multiple targets, (1) improving serum lipid profiles to reduce the detrimental effects given by cholesterol; (2) reducing ROS level inside the body via enhancing hepatic anti-oxidative activity or antioxidant level to scavenge the reactive oxygen species generated in the postmenopausal dyslipidemic condition; (3) stimulating eNOS-derived nitric oxide production; and (4) counteracting the up-regulations of inflammatory cytokine (TNF-α, VCAM-1 and ICAM-1) expressions so as to reduce endothelium damage. Although the precise molecular mechanism(s) exerted by LCEE to protect the vascular system is still unknown, the present study clearly demonstrated that LCEE treatment could provide an overall beneficial effect to the body under postmenopausal dyslipidemic condition.

Acknowledgements:

This research was financially supported by the Department of Applied Biology and Chemical Technology, The Hong Kong Polytechnic University and Shenzhen Key Laboratory Advancement Program (project code: CXB201104220020A). Special thanks go to Ms. Josephine Hong-Man Leung for proofreading and providing critical comments to the manuscript.

Conflict of interest statement

There is no conflict of interest associated with the authors of this paper.

References

1. H. Drexler and B. Hornig, *J. Mol. Cell Cardiol.*, 1999, **31**, 51-60.
2. H. Cai and D. G. Harrison, *Circ. Res.*, 2000, **87**, 840-844.
3. H. Suzuki and K. Kondo, *Hypertens. Res.*, 2012, **35**, 142-147.
4. W. B. Kannel, M. C. Hjortland, P. M. McNamara and T. Gordon, *Ann. Intern. Med.*, 1976, **85**, 447-452.
5. I. R. Pereira, A. A. Faludi, J. M. Aldrighi, M. C. Bertolami, M. H. Saleh, R. A. Silva, Y. Nakamura, M. F. Campos, N. Novaes and D. S. Abdalla, *Menopause*, 2006, **13**, 942-950.
6. D. Xing, S. Nozell, Y. F. Chen, F. Hage and S. Oparil, *Arterioscler. Thromb. Vasc. Biol.*, 2009, **29**, 289-295.
7. J. C. Tang, J. N. Zhang, Y. T. Wu and Z. X. Li, *Phytother. Res.*, 2006, **20**, 1046-1051.
8. L. Fan, K. Wang, Z. Shi, J. Die, C. Wang and X. Dang, *J. Vasc. Surg.*, 2011, **54**, 192-200.
9. F. Jiang, J. Qian, S. Chen, W. Zhang and C. Liu, *Pharm. Biol.*, 2011, **49**, 856-863.
10. H. Qi, S. O. Siu, Y. Chen, Y. Han, I. K. Chu, Y. Tong, A. S. Lau and J. Rong, *Chem. Biol. Interact.*, 2010, **183**, 380-389.
11. H. M. Chao, D. E. Lin, Y. Chang, W. M. Hsu, S. M. Lee, F. L. Lee, C. W. Chi, W. H. Pan, T. Y. Liu, W. Y. Lui, L. T. Ho, C. D. Kuo, C. C. Chan and F. P. Chao, *J. Ocul. Pharmacol. Ther.*, 2008, **24**, 461-472.
12. M. Ishihara, *Asia Oceania J. Obstet. Gynaecol.*, 1984, **10**, 317-323.
13. S. Sassa, T. Kikuchi, H. Shinoda, S. Suzuki, H. Kudo and S. Sakamoto, *In vivo*, 2003, **17**, 277-280.
14. S. S. Chan, T. Y. Cheng and G. Lin, *J. Ethnopharmacol.*, 2007, **111**, 677-680.
15. W. F. Bi, H. Y. Yang, J. C. Liu, T. H. Cheng, C. H. Chen, C. M. Shih, H. Lin, T. C. Wang, W. S. Lian, J. J. Chen, H. C. Chiu and N. C. Chang, *Clin. Exp. Pharmacol. Physiol.*, 2005, **32**, 536-540.
16. S. S. Chan, A. O. Choi, R. L. Jones and G. Lin, *Eur. J. Pharmacol.*, 2006, **537**, 111-117.
17. L. S. Lim, P. Shen, Y. H. Gong and E. L. Yong, *Phytochem.*, 2006, **67**, 728-734.
18. E. A. Thorndike and A. S. Turner, *Front. Biosci.*, 1998, **3**, c17-26.
19. Pharmacopoeia Commission of People's Republic of China. China. Pharmacopoeia of the People's Republic of China. Beijing: China Chemistry Industry Press; 2010.
20. Pharmacopoeia Commission of the Ministry of Public Health. Chinese Pharmacopoeia. Beijing, China: Chemical Industrial Press; 2005.
21. C. M. Li, X. L. Dong, X. D. Fan, J. H. Wu, Q. H. Wang, X. L. Tian, D. J. Guo, M. S. Wong, T. Q. Qiu and S. W. Chan, *Menopause*, 2012.
22. J. H. Wu, G. P. Leung, Y. W. Kwan, T. T. Sham, J. Y. Tang, Y. H. Wang, J. B. Wan, S. M. Lee and S. W. Chan, *J. Funct. Foods*, 2013, **5**, 1159-1169.
23. J. H. Wu, Q. H. Wang, F. Li, Y. L. Shu, C. O. Chan, D. K. W. Mok and S. W.

Chan, *Evid. Based Compl. Alt.*, 2012.
24. V. Pialoux, A. D. Brown, R. Leigh, C. M. Friedenreich and M. J. Poulin, *Hypertension*, 2009, **54**, 1014-1020.
25. Q. Hao, J. Wang, J. Niu, P. Zhao, Y. Cui, L. Sun and X. Xue, *Zhongguo Zhong Yao Za Zhi*, 2009, **34**, 620-624.
26. T. J. Verbeuren, F. H. Jordaens, L. L. Zonnekeyn, C. E. Van Hove, M. C. Coene and A. G. Herman, *Circ. Res.*, 1986, **58**, 552-564.
27. K. K. Koh, C. Cardillo, M. N. Bui, L. Hathaway, G. Csako, M. A. Waclawiw, J. A. Panza and R. O. Cannon, *Circulation*, 1999, **99**, 354-360.
28. L. W. Lissin, R. Oka, S. Lakshmi and J. P. Cooke, *Vasc. Med.*, 2004, **9**, 26-30.
29. S. Madahian, K. D. Navab, N. Pourtabatabaei, S. Seyedali, S. Safar, S. Vazirian and G. Hough, *Curr. Med. Chem.*, 2014.
30. D. H. Choi, Y. J. Lee, J. S. Kim, D. G. Kang and H. S. Lee, *Immunopharmacol. Immunotoxicol.*, 2012, **34**, 4-11.
31. V. Kamesh and T. Sumathi, *Asian Pac. J. Trop. Med.*, 2012, **5**, 949-955.
32. A. J. Wadley, J. J. Veldhuijzen van Zanten and S. Aldred, *Age*, 2012.
33. G. A. Behr, C. E. Schnorr, A. Simoes-Pires, L. L. da Motta, B. N. Frey and J. C. Moreira, *Cell. Biol. Toxicol.*, 2012, **28**, 317-330.
34. U. Forstermann, *Pflugers Arch.*, 2010, **459**, 923-939.
35. C. Y. Kwok, C. N. Y. Wong, M. Y. C. Yau, P. H. F. Yu, A. L. Au, C. C. Poon, S. W. Seto, T. Y. Lam, Y. W. Kwan and S. W. Chan, *J. Funct. Foods*, 2010, **2**, 179-186.
36. M. Ramalingam and P. Yong-Ki, *Pharmacogn. Mag.*, 2010, **6**, 323-330.
37. J. B. Jeong, S. Y. Ju, J. H. Park, J. R. Lee, K. W. Yun, S. T. Kwon, J. H. Lim, G. Y. Chung and H. J. Jeong, *Cancer Epidemiol.*, 2009, **33**, 41-46.
38. J. G. Wu, L. Ma, S. Y. Zhang, Z. Z. Zhu, H. Zhang, L. P. Qin and Y. J. Wei, *Pharm. Biol.*, 2011, **49**, 86-93.
39. D. Behrendt and P. Ganz, *Am. J. Cardiol.*, 2002, **90**, 40L-48L.
40. A. Virdis, L. Ghiadoni, S. Pinto, M. Lombardo, F. Petraglia, A. Gennazzani, S. Buralli, S. Taddei and A. Salvetti, *Circulation*, 2000, **101**, 2258-2263.
41. J. P. Camporez, E. H. Akamine, A. P. Davel, C. R. Franci, L. V. Rossoni and C. R. Carvalho, *J. Physiol.*, 2011, **589**, 2585-2596.
42. L. M. Yung, W. T. Wong, X. Y. Tian, F. P. Leung, L. H. Yung, Z. Y. Chen, X. Yao, C. W. Lau and Y. Huang, *PLoS One*, 2011, **6**, e17437.
43. D. E. Bush, C. E. Jones, K. M. Bass, G. K. Walters, J. M. Bruza and P. Ouyang, *Am. J. Med.*, 1998, **104**, 552-558.
44. J. Wagner, H. Tennen, G. Mansoor and G. Abbott, *J. Diabetes Complications*, 2009, **23**, 18-24.
45. K. K. Koh, A. Blum, L. Hathaway, R. Mincemoyer, G. Csako, M. A. Waclawiw, J. A. Panza and R. O. Cannon, *Circulation*, 1999, **100**, 1851-1857.
46. Q. Liao, X. Li, S. Zhou, L. Liu, S. Zhao, Y. Lian and H. Dong, *J. Cardiovasc. Pharmacol.*, 2011, **57**, 174-182.
47. C. Y. Kwok, C. Li, H. L. Cheng, Y. F. Ng, T. Y. Chan, Y. W. Kwan, G. P. H. Leung, S. M. Y. Lee, D. K. W. Mok, P. H. F. Yu and S. W. Chan, *J. Funct. Foods*,

2013, **5**, 1326-1335.
48. Q. Li, J. H. Wu, D. J. Guo, H. L. Cheng, S. L. Chen and S. W. Chan, *Planta Med.*, 2009, **75**, 1203-1208.
49. M. J. Liang, L. C. He and G. D. Yang, *Life Sci.*, 2005, **78**, 128-133.
50. A. J. Jakimiuk, M. Bogusiewicz, P. Skorupski, A. Adamiak, P. Miotla, J. Haczynski and T. Rechberger, *Gynecol. Endocrinol.*, 2007, **23**, 584-589.
51. L. A. Madge and J. S. Pober, *Exp. Mol. Pathol.*, 2001, **70**, 317-325.
52. Y. H. Lai, L. J. Chen and J. T. Cheng, *Horm. Metab. Res.*, 2012.
53. T. Long, G. Liu, Y. Wang, Y. Chen, Y. Zhang and D. Qin, *J. Sex. Med.*, 2012, **9**, 1801-1814.
54. X. L. Ma, F. Gao, C. L. Yao, J. Chen, B. L. Lopez, T. A. Christopher, J. Disa, J. L. Gu, E. H. Ohlstein and T. L. Yue, *J. Pharmacol. Exp. Ther.*, 2000, **295**, 786-792.
55. A. Albayrak, M. H. Uyanik, F. Odabasoglu, Z. Halici, A. Uyanik, Y. Bayir, F. Albayrak, Y. Albayrak, B. Polat and H. Suleyman, *J. Surg. Res.*, 2011, **169**, 67-75.
56. H. Zhang, Y. Park, J. Wu, X. Chen, S. Lee, J. Yang, K. C. Dellsperger and C. Zhang, *Clin. Sci. (Lond)*, 2009, **116**, 219-230.
57. H. J. Wu, J. Hao, S. Q. Wang, B. L. Jin and X. B. Chen, *Eur. J. Pharmacol.*, 2012, **674**, 365-369.
58. L. Q. Zhu, S. R. Wang and Y. Qin, *Zhongguo Zhong Xi Yi Jie He Za Zhi*, 2009, **29**, 238-241.
59. Y. Huang, H. J. Yin, X. J. Ma, J. S. Wang, Q. Liu, C. F. Wu and K. J. Chen, *Chin. J. Integr. Med.*, 2011, **17**, 355-360.

Figure legends

Fig. 1. Representative high performance liquid chromatography chromatograms of (A) standards and (B) LCEE. 1 = tetramethylpyzine; 2 = ferulic acid.

Fig. 2. Oxidant/antioxidant levels in the liver obtained from the Control, Model, E2 and CX groups. Data are expressed as means ± S. E. M., n = 7. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$ represent significant differences when compared with the Control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ represent significant differences when compared with the Model group.

Fig. 3. Cumulative concentration-response curves of (A) acetylcholine-induced or (B) sodium nitroprusside-induced vasorelaxation on the phenylephrine pre-constructed thoracic aorta isolated from the Control, Model, E2 and CX groups. Data [a decrease in steady-state tension elicited by phenylephrine (1 μM)] are expressed as means ± S. E. M., n = 7. ***$p < 0.001$ represents significant differences when compared with the Control group. $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ represent significant differences when compared with the Model group.

Fig. 4. *In vitro* nitrate/nitrite production in isolated thoracic aorta from the Control, Model, E2 and CX groups. Data are expressed as means ± S. E. M., n = 7. ***$p < 0.001$ represents significant differences when compared with the Control group. $^{\#}p < 0.05$ represents significant differences when compared with the Model group.

Fig. 5. Real-time PCR analysis of the gene expressions of (A) eNOS, (B) TNF-α, (C) VCAM-1 and (D) ICAM-1 in isolated aortas from the Control, Model, E2 and CX groups. The expression level of each gene was normalized to that of the GAPDH gene. Data are expressed as means ± S. E. M., n = 7. **$p < 0.01$ and ***$p < 0.001$ represent significant differences when compared with the Control group. $^{\#}p < 0.05$, $^{\#\#}p < 0.01$ and $^{\#\#\#}p < 0.001$ represent significant differences when compared with the Model

group.

Fig. 6. Outline of the proposed mechanistic pathways for the vascular protective effect of LCEE in HFD-fed OVX rats. They are: (1) improving lipid profiles; (2) enhancing hepatic anti-oxidative activity; (3) stimulating eNOS mRNA expression; (4) counteracting the up-regulations of inflammatory cytokine expressions. EC, endothelial cell; HFD, high fat diet; LC, liver cells; VSMC, vascular smooth muscle cell; ROS, reactive oxygen species; OVX, ovariectomized. Dashed arrow represents stimulatory effect. Broken arrow represents inhibitory effect.

Table 1: The primer sets used for real-time PCR analysis.

Genes	Forward primer (5' to 3')	Reverse primer (5' to 3')	Product's Size (bp)	Accession number
eNOS	GGATTCTGGCAAGACCGATTAC	GGTGAGGACTTGTCCAAACACT	159	NM_021838.2
ICAM-1	AGACACAAGCAAGAGAAGAA	GAGAAGCCCAAACCCGTATG	105	NM_012967.1
VCAM-1	GGAGCCTGTCAGTTTTGAGAATG	TTGGGGAAAGAGTAGATGTCCAC	234	NM_012889.1
TNF-α	TGAGGCTACAATCTGTTTTCTCAG	AGCAGCAGAAGTTATGAATGTCAG	131	NM_182950.4
GAPDH	TGCACCACCAACTGCTTAG	AGTGGATGCAGGGATGATGT	180	NM_017008

Table 2: Body weight and uterine dry weight of the Control, Model, E2 and CX groups at the end of the 12-week treatment.

Groups	Body weight (g)	Body weight gain (g)	Uterine dry weight (mg)	Uterine index (mg/g)
Control	358.0 ± 8.59	18.96 ± 5.21	1.09 ± 0.29	0.75 ± 0.08
Model	446.2 ± 11.65***	87.38 ± 6.01***	0.12 ± 0.01**	0.12 ± 0.01***
E2	380.5 ± 10.06$^{\#\#\#}$	20.76 ± 6.86$^{\#\#\#}$	0.38 ± 0.02$^{\#\#\#}$	0.40 ± 0.02$^{\#\#\#}$
CX	442.7 ± 6.20	61.41±7.57$^{\#}$	0.12 ± 0.01	0.13 ± 0.01

Results correspond to the means ± S. E. M., n = 7. $^{**}P < 0.01$ and $^{***}P < 0.001$ represents significant differences when compared with the Control group. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ represent significant differences when compared with the Model group.

Table 3: Serum lipid profiles of the Control, Model, E2 and CX groups at the end of the 12-week treatment.

Groups	Total cholesterol (mmol/L)	Triacylglycerol (mmol/L)	LDL-cholesterol (mmol/L)	HDL-cholesterol (mmol/L)
Control	2.47 ± 0.13	1.92 ± .19	0.26 ± 0.04	1.82 ± 0.09
Model	3.50 ± 0.13**	1.61 ± 0.20	0.42 ± 0.04*	2.06 ± 0.14
E2	2.03 ± 0.13$^{\#\#\#}$	1.74 ± 0.15	0.14 ± 0.06$^{\#\#\#}$	1.68 ± 0.06
CX	2.84 ± 0.23$^{\#}$	1.08 ± 0.21	0.33 ± 0.01$^{\#}$	2.77 ±0.18$^{\#\#\#}$

Results correspond to the means ± S. E. M., n = 7. $^{*}P < 0.05$ and $^{**}P < 0.01$represents significant differences when compared with the Control group. $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.001$ represent significant differences when compared with the Model group.

Table 4: Vascular contraction induced by 1 μM phenylephrine and maximum vasorelaxation to acetylcholine and sodium nitroprusside in the isolated thoracic aorta from different groups of rats.

Groups	Contraction (to phenylephrine, g)	Maximum relaxation (induced by acetylcholine, %)	Maximum relaxation (induced by sodium nitroprusside, %)
Control	0.42 ± 0.11	54.60 ± 0.358	99.79 ± 3.05
Model	0.48 ± 0.18	31.12 ± 5.84***	109.40 ± 2.37
E2	0.45 ± 0.13	55.51 ± 3.63$^{###}$	111.80 ± 3.69
CX	0.54 ± 0.19	57.39 ± 13.87$^{###}$	99.99 ± 2.62

Results correspond to the means ± S. E. M., n = 7. $^{***}P < 0.001$ represents significant differences when compared with the Control group. $^{###}P < 0.001$ represents significant differences when compared with the Model group.

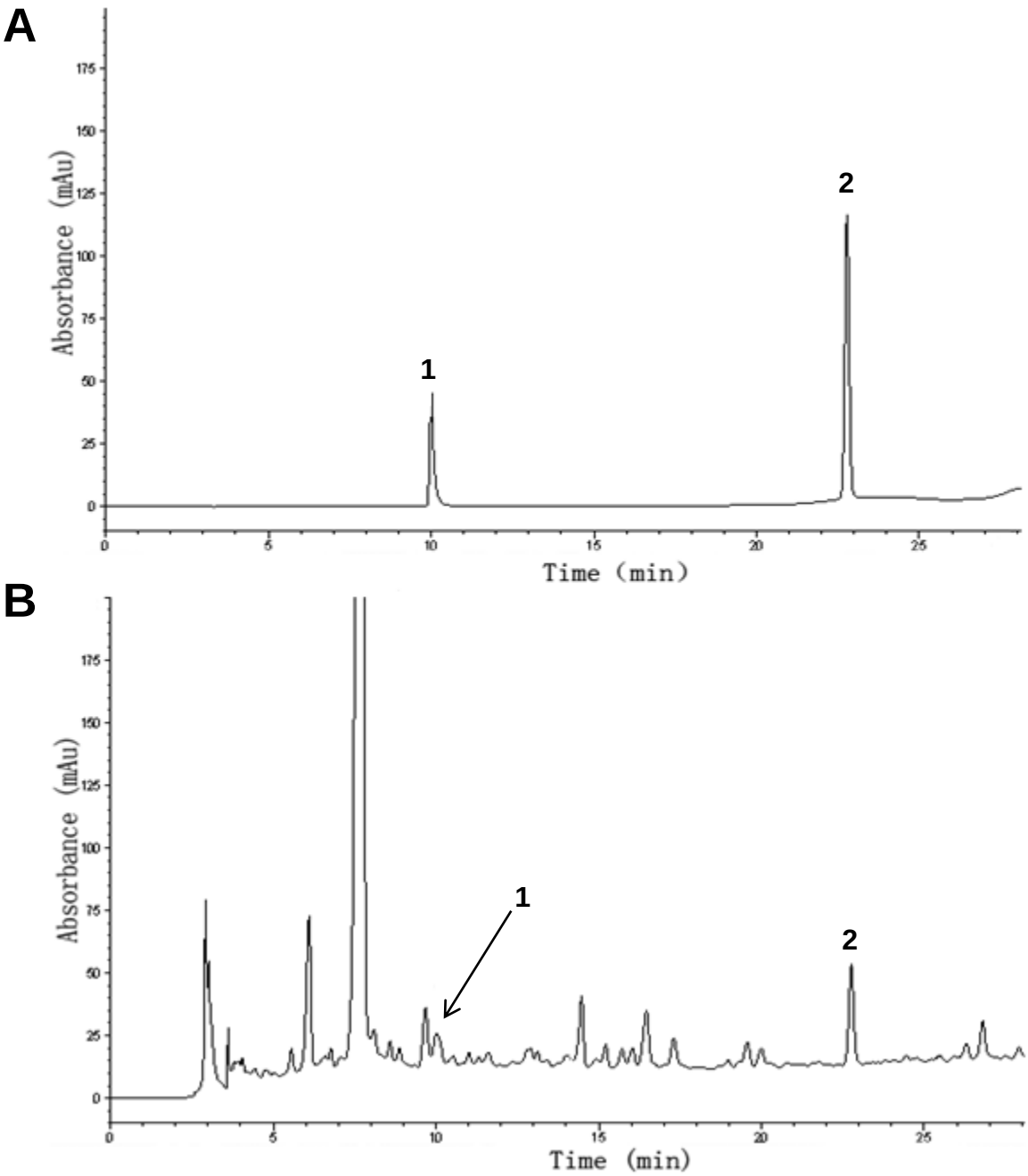


Figure 1

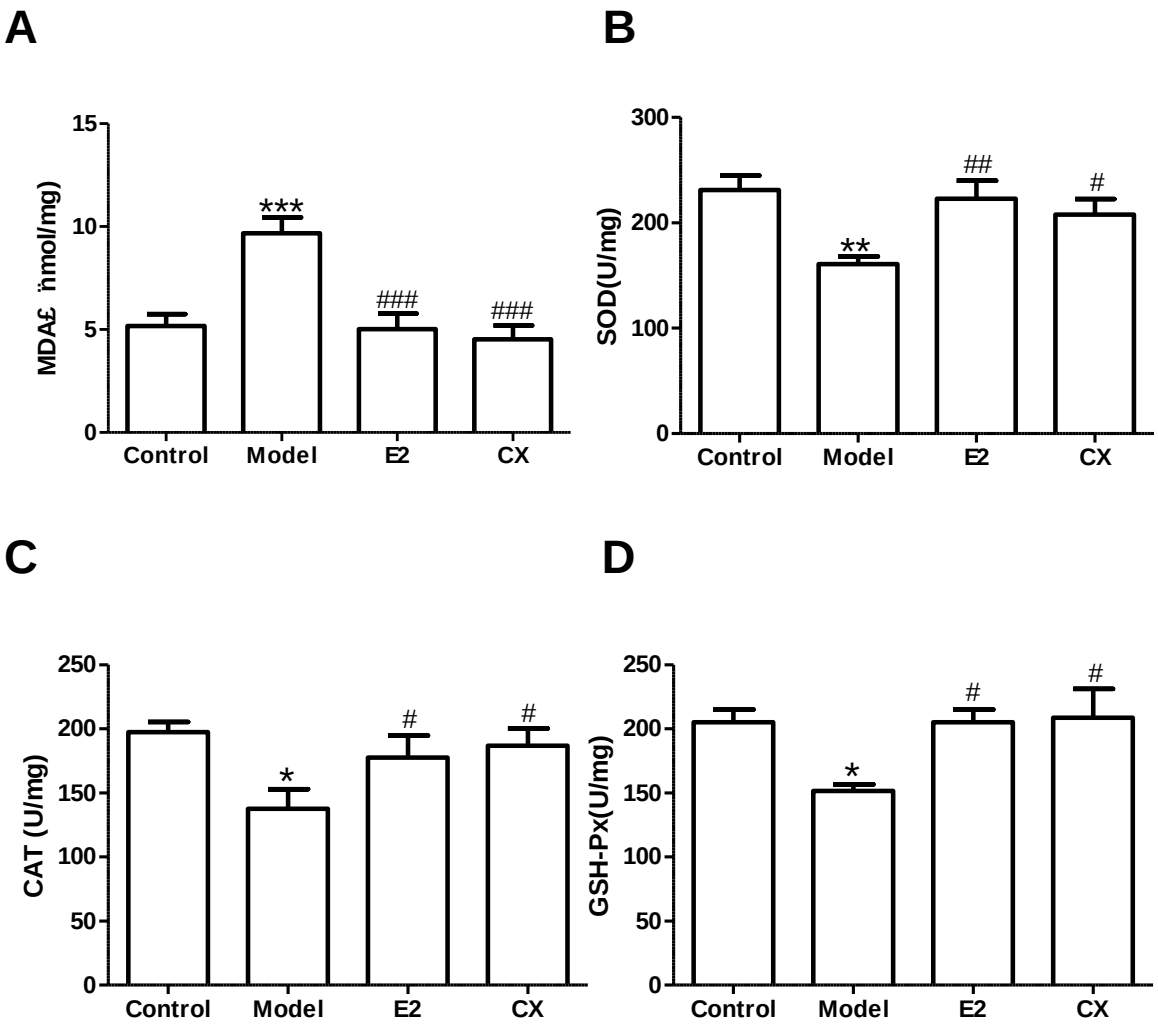


Figure 2

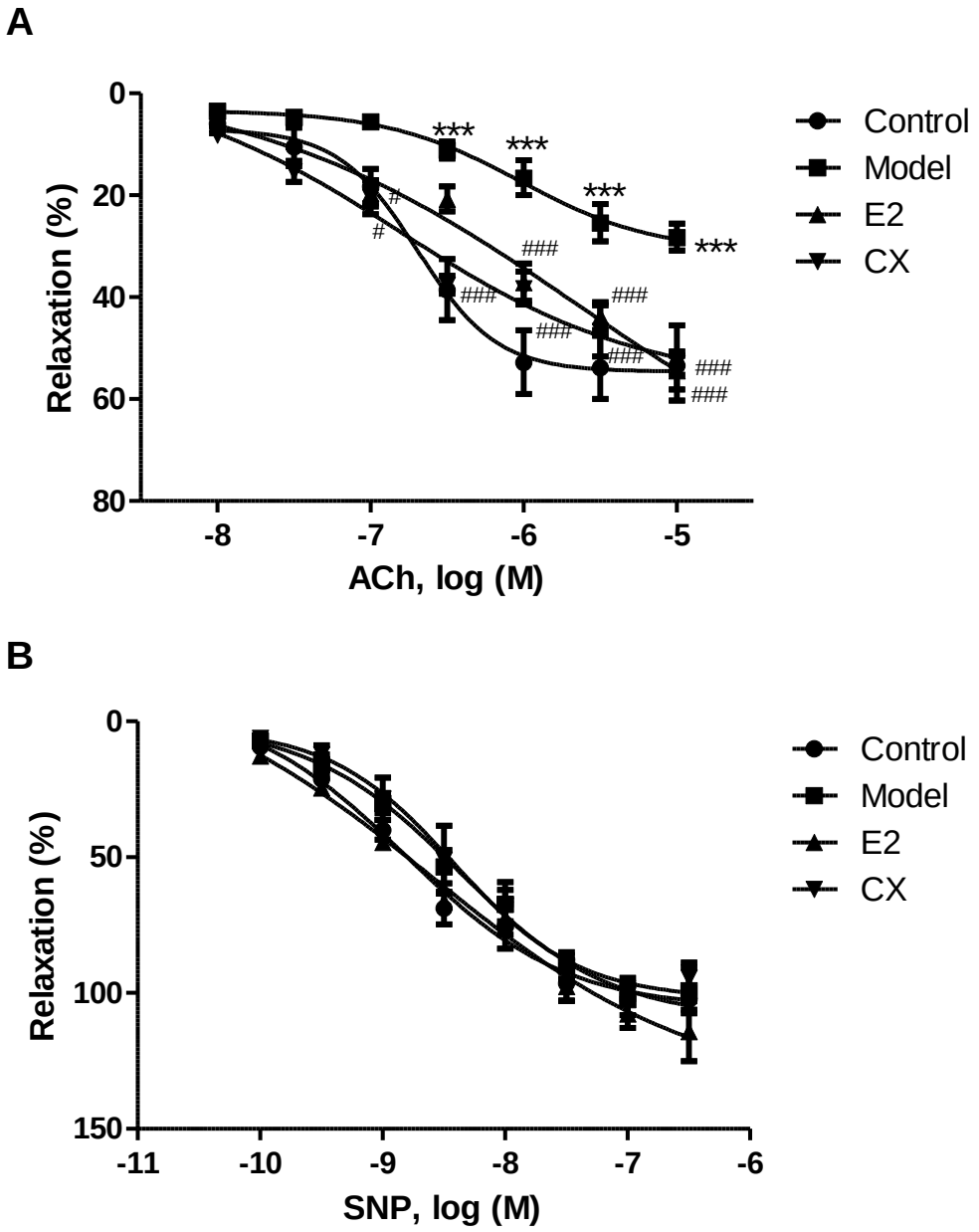


Figure 3

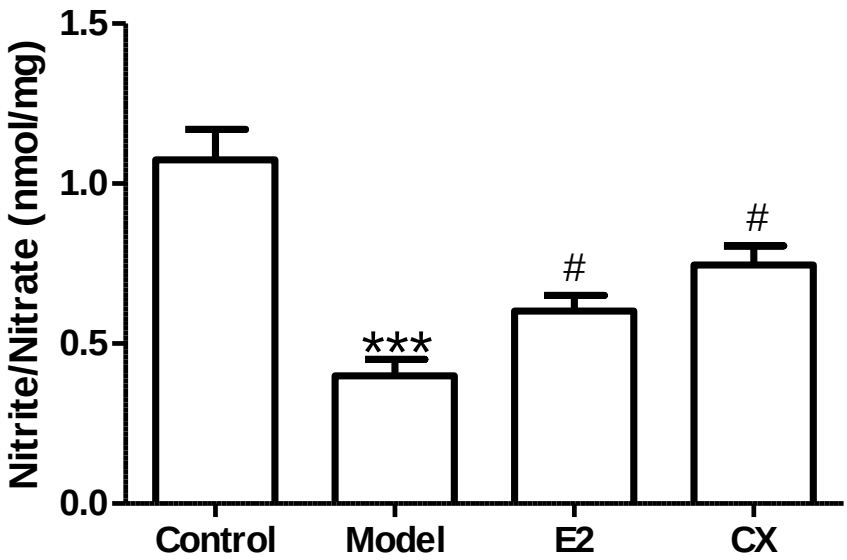


Figure 4

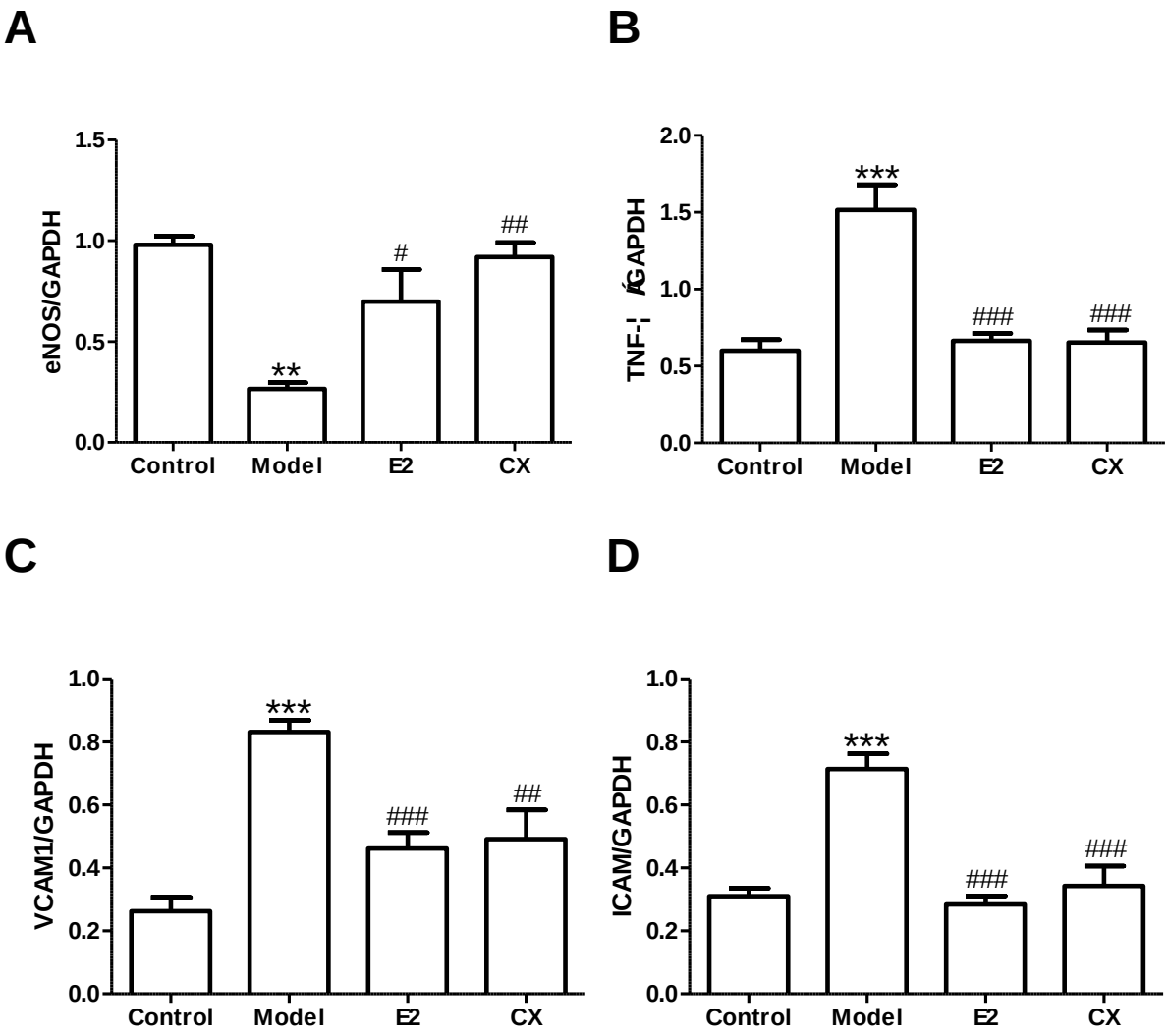


Figure 5

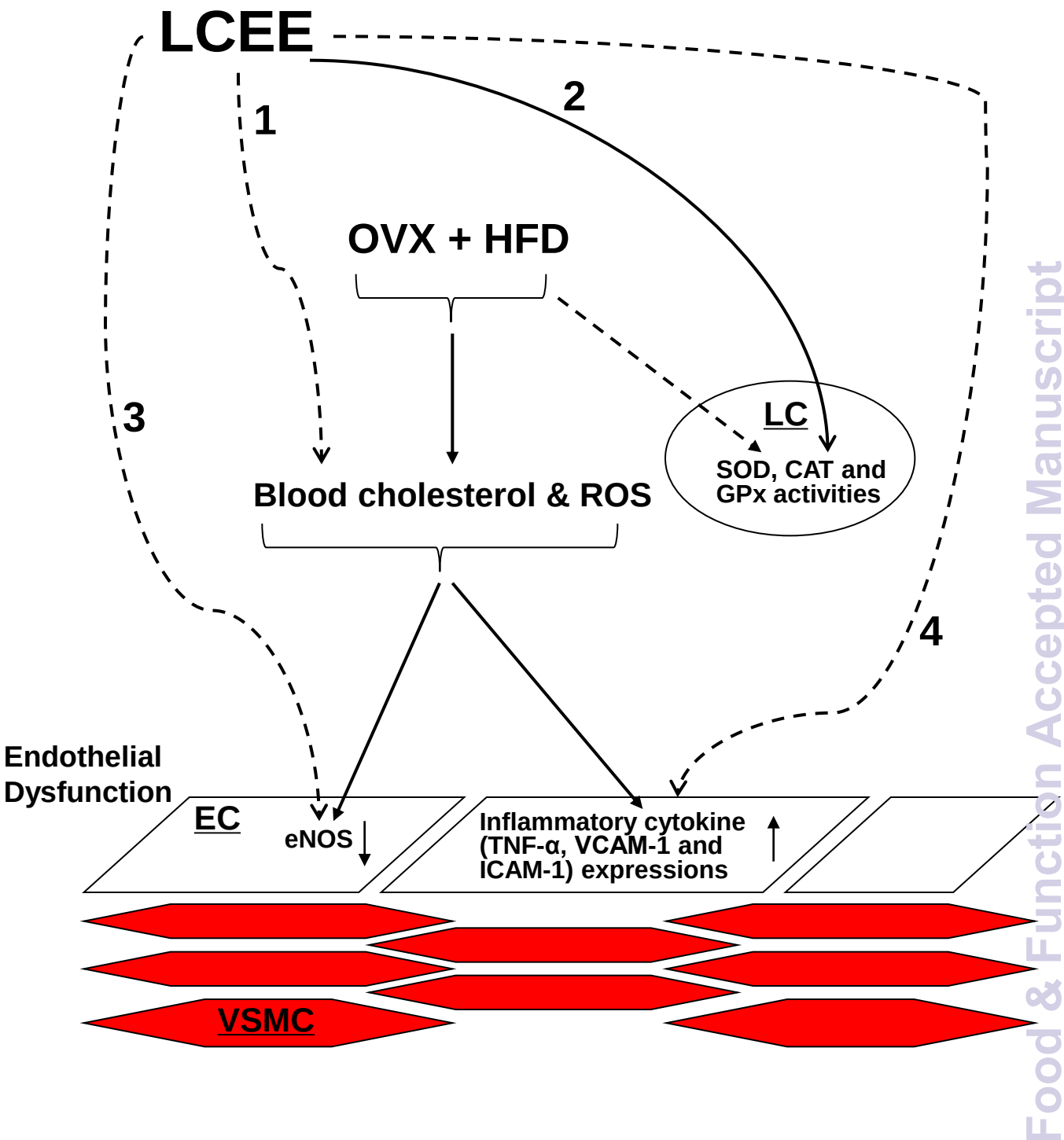


Figure 6