Journal of Ethnopharmacology 128 (2010) 213–220

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Chrysanthemum morifolium Ramat. reduces the oxidized LDL-induced expression of intercellular adhesion molecule-1 and E-selectin in human umbilical vein endothelial cells

Chong-Kuei Lii[a], Yen-Ping Lei[b], Hsien-Tsung Yao[a], Yun-Sheng Hsieh[b], Chia-Wen Tsai[a], Kai-Li Liu[b,c,**], Haw-Wen Chen[a,*]

[a] Department of Nutrition, China Medical University, No.91 Hsueh-Shih Road, Taichung 404, Taiwan
[b] Department of Nutrition, Chung Shan Medical University, No.110, Sec.1, Jianguo N. Road, Taichung 402, Taiwan
[c] Department of Dietitian, Chung Shan Medical University Hospital, Taichung 402, Taiwan

ARTICLE INFO

Article history:
Received 17 October 2009
Received in revised form
24 December 2009
Accepted 5 January 2010
Available online 15 January 2010

Keywords:
Chrysanthemum morifolium Ramat.
E-selectin
Human umbilical vein endothelial cells (HUVECs)
Intercellular adhesion molecule-1 (ICAM-1)
Oxidized LDL (oxLDL)

ABSTRACT

Ethnopharmacological relevance: The flower of *Chrysanthemum morifolium* Ramat. (CM) with antioxidant, cardiovascular protective and anti-inflammatory functions, has been widely used in China for hundreds of years as a healthy beverage and medicine.
Aims of the study: The purpose of the present study is to investigate the effects of HCM (a hot water extract of the flower of *Chrysanthemum morifolium* Ramat. [CM]), ECM (an ethanol extract of CM), and the abundant flavonoids apigenin and luteolin in CM on the oxidized LDL (oxLDL)-induced expression of ICAM-1 and E-selectin in human umbilical vein endothelial cells (HUVECs). The possible mechanism of these effects was also determined.
Materials and methods: MTT assay was for cell viability. Western blot was used for ICAM-1 and E-selection protein expression, and for activation of protein kinase B (PKB) and cAMP responsive element binding protein (CREB) proteins. Fluorescence flow cytometry was for ICAM-1 and E-selectin expression on cell surface. DCF-DA flow cytometric assay was used for reactive oxygen species (ROS) production.
Results: HCM, ECM, apigenin, and luteolin dose-dependently inhibited ICAM-1 and E-selectin expression and adhesion of HL-60 by oxLDL. HCM, ECM, apigenin, and luteolin reversed the inhibition of phosphorylation of Akt and CREB by oxLDL; however, this reversion was abolished by wortmannin. In addition, wortmannin abrogated the inhibitory effects of CM extracts, apigenin and luteolin on adhesion molecule expression. The ROS scavenging capability of HCM, ECM, apigenin, and luteolin proceeded dose-dependently in the presence of oxLDL.
Conclusion: CM is a plant with cardiovascular-protective potential and the inhibitory effects of CM on ICAM-1 and E-selectin expression are, at least partially, attributed to its antioxidant activity and modulation of the PI3K/Akt signaling pathway.

1. Introduction

Increased LDL and especially oxidized LDL (oxLDL) are recognized as risk factors for cardiovascular disease (CVD) (Middleton et al., 2000). CVD is the leading cause of death throughout the Western world and is the second most common cause of death worldwide (Braunwald, 1997). Atherosclerosis is an inflammatory disease, and the early stages of atherosclerosis are initiated by accumulation of oxLDL and activation of endothelial cells with subsequent expression of adhesion molecules and increased recruitment of leukocytes to the vascular endothelium (Aviram, 1993; Sigurdardottir et al., 2002). oxLDL has been shown to increase the expression of intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), and E-selectin and the adherence of monocytes to human umbilical vein endothelial cells (HUVECs) (Ou et al., 2006). In addition, oxLDL is involved in the production of various proinflammatory cytokines and growth factors (Ito and Ikeda, 2003), the proliferation and migration of vascular smooth muscle cells (Taguchi et al., 2000), and the retardation of endothelial regeneration (Boissonneault et al., 1995), which are critical steps in atherosclerosis.

* Corresponding author. Tel.: +886 4 2205 3366x7520; fax: +886 4 2206 2891.
** Corresponding author at: Department of Nutrition, Chung Shan Medical University, No. 110, Sec. 1, Jianguo N. Road, Taichung 402, Taiwan.
Tel.: +886 4 2205 3366x7520; fax: +886 4 2206 2891.
E-mail addresses: kaililiu@csmu.edu.tw (K.-L. Liu), chenhw@mail.cmu.edu.tw (H.-W. Chen).

0378-8741/$ – see front matter © 2010 Elsevier Ireland Ltd. All rights reserved.
doi:10.1016/j.jep.2010.01.018

The flower of *Chrysanthemum morifolium* Ramat. (CM) has been widely used in China for hundreds of years as a healthy beverage and medicine. CM is reported to have various biological features including antioxidation (Kim and Lee, 2005), cardiovascular protection (Jiang et al., 2004), anti-tumorigenesis (Miyazawa and Hisama, 2003), and anti-inflammation (Ukiya et al., 2001). *Chrysanthemum* species have been shown to contain a wide variety of flavonoids, phenols, and phenolic acids (Lee et al., 2003; Hu et al., 1994; Matsuda et al., 2002), and the health benefits of CM are confirmed to be associated with the flavonoids (Hertog et al., 1993). Apigenin and luteolin are the 2 major bioactive flavonoids in vivo, as shown when CM extract is orally administered to male Sprague–Dawley rats (Chen et al., 2007).

Flavonoids or related compounds are involved in the prevention of atherosclerosis by inhibiting LDL oxidation and by increasing cellular resistance to the deleterious effects of oxLDL. In a study in elderly Dutch men, an inverse correlation was found between dietary flavonoid intake and the incidence of coronary artery disease (Hertog et al., 1993). The flavones apigenin and luteolin were shown to inhibit THP-1 cell adhesion to oxLDL-activated HUVECs and to abolish the oxLDL-induced expression of VCAM-1 and E-selectin in HUVECs (Jeong et al., 2007). Therefore, apigenin and luteolin are qualified as anti-atherogenic agents in LDL systems and may be implicated in the attenuation of endothelial dysfunction-related atherosclerosis (Jeong et al., 2007). Jeong et al. (2007) reported that the ability of apigenin and luteolin to prevent the oxLDL-induced expression of adhesion molecules may be attributed to their antioxidant capacity. Previous studies showed that antioxidant systems inhibit the oxidative stress-induced expression of adhesion molecules (Seguí et al., 2005; Kokura et al., 2001). Hence, it is reasonable to infer that the cardiovascular benefits of flavonoids come from their local antioxidant effect on endothelial cells and subsequent modulation of the intracellular redox environment, cell signaling, and gene expression (Lotito and Frei, 2006).

On the basis of the evidence in the literature that CM has anti-inflammatory and anti-CVD effects, the present study was designed to explore the effects of a hot water extract of CM (HCM), an ethanol extract of CM (ECM), and the abundant flavonoids apigenin and luteolin in CM on the oxLDL-induced expression of ICAM-1 and E-selectin in HUVECs and the adherence of HL-60 cells to oxLDL-activated HUVECs. We also studied the possible mechanisms of these effects.

2. Materials and methods

2.1. Materials

HUVECs and monocytic HL-60 cells were obtained from Clonetics Co. (San Diego, CA) and Bioresources Collection and Research Center (BCRC, Taiwan), respectively. Apigenin and luteolin were purchased from Sigma (St. Louis, MO). Medium 199 and RPMI 1640 were from Gibco-BRL (Grand Island, NY). Monoclonal antibodies to ICAM-1 and E-selectin were purchased from Cell Signaling Technology (Beverly, MA) and Santa Cruz Biotechnology (Santa Cruz, CA), respectively. Polyclonal antibodies to Akt, phospho-Akt (Ser473), cAMP responsive element binding protein (CREB), and phospho-CREB (Ser133) were obtained from Cell Signaling Technology (Beverly, MA). HEPES, heparin, $CuSO_4$, and 2′,7′-dichlorodihydrofluorescein diacetate (DCF-DA) were obtained from Sigma (St. Louis, MO). *bis*-Carboxyethyl-carboxyfluorescein acetoxymethyl ester (BCECF-AM) was ordered from Molecular Probes (Eugene, OR). Dry *Chrysanthemum morifolium* Ramat. was procured from Taipei, Taiwan, and identified by Dr. R. S. Lin (Department of Horticulture, National Chung-Hsing University, Taichung, Taiwan). Voucher specimens (No. 257) were deposited in our laboratory, Department of Nutrition, and in the Institute of Chinese Pharmaceutical Sciences, China Medical University, Taichung, Taiwan, for further reference.

2.2. Preparation of CM extracts

Powdered flower (1 g) was extracted with 25 ml of boiling water for 30 min (HCM) or with 25 ml of 95% ethanol by stirring overnight at room temperature (ECM). After cooling, the HCM and ECM extracts were collected by centrifugation at $3200 \times g$ at 4 °C for 10 min. HCM was freeze-dried and ECM was concentrated by using a rotary evaporator, stored at −20 °C, and dissolved in dimethylsulfoxide when used.

2.3. Cell cultures

HUVECs with passages between 7 and 9 were used in this study. Cells were grown in 10 ml of medium 199 supplemented with 20 mmol/l HEPES (pH 7.4), 30 mg/l endothelial cell growth supplement (Upstate Biotechnology, Lake Placid, NY), 100 mg/l heparin, 20% fetal bovine serum (Biological Industries, Canada), 100,000 U/l penicillin, and 100 mg/l streptomycin at 37 °C under 5% CO_2. The HL-60 cells were cultured in T-75 tissue culture flasks in a RPMI 1640 medium supplemented with 10% fetal bovine serum, 100,000 units/l penicillin, and 100 mg/l streptomycin.

2.4. LDL isolation and oxLDL preparation

Blood was collected from healthy volunteers to isolate LDL. Written informed consent as approved by the Review Board for Human Research of the Chung Shan Medical University was signed by all participants. Plasma in the presence of EDTA was used to isolate LDL by sequential ultracentrifugation ($1.019 < d < 1.063$ kg/l) (Claise et al., 1999). Afterward, native LDL was dialyzed at 4 °C for 48 h against 500 volumes of PBS to remove EDTA. To initiate oxidation, LDL in an amount of 0.5 g/l protein was exposed to 5 μmol/l $CuSO_4$ for 18 h. The generation of thiobarbituric acid-reactive substances was monitored by the fluorometric method as described by Fraga et al. (1988), and the values of malondialdehyde equivalents increased from 0.83 ± 0.17 nmol/mg protein of native LDL to 18.9 ± 1.2 nmol/mg protein of $CuSO_4$-treated LDL. The freshly prepared oxLDL was dialyzed at 4 °C for 48 h against 500 volumes of PBS to remove Cu^{2+} and was sterilized by passage through a 0.45-μm filter. The protein contents of native LDL and the oxLDL preparations were measured by the Lowry assay (Lowry et al., 1951).

2.5. Cell treatments

For each experiment, HUVECs at 80% confluence were incubated with HCM, ECM, apigenin, or luteolin at the indicated concentrations for 16 h and were then stimulated with 40 mg/l of oxLDL for an additional 24 h. Cells were lysed in a lysis buffer (10 mmol/l Tris–HCl, pH 8.0, 320 mmol/l sucrose, 5 mmol/l EDTA, 0.1% Triton X-100, 1 mmol/l phenylmethylsulfonyl fluoride, 1 mg/l leupeptin, 1 mg/l aprotinin, and 1 mmol/l dithiothreitol). The cell lysates were then sonicated at 20 W for 15 s. All of the cell extracts were centrifuged at $20{,}000 \times g$ at 4 °C for 15 min. The supernatants were recovered and the total protein was analyzed by use of the Coomassie Plus protein assay reagent kit (Pierce Biotechnology Inc., Rockford, IL). The cytotoxicity of HCM, ECM, apigenin, and luteolin was determined by MTT (3-(4,5)-dimethylthiahiazo (-z-y1)-3,5-di-phenytetrazoliumromide) assay (Denizot and Lang, 1986).

2.6. Immunoblotting

Equal amounts of cellular proteins were electrophoresed in a SDS-polyacrylamide gel, and proteins were then transferred to polyvinylidene fluoride membranes (Amersham Biosciences Co., Piscataway, NJ). Nonspecific binding sites on the membranes were blocked with 5% nonfat milk at 4 °C overnight. Membranes were probed with mouse anti-human ICAM-1 and E-selectin or rabbit anti-Akt, phospho-Akt, CREB, phospho-CREB, and β-actin antibodies. The membranes were then probed with the secondary antibody labeled with horseradish peroxidase. The bands were visualized by using an enhanced chemiluminescence kit (PerkinElmer Life Science, Boston, MA) and were quantitated with an AlphaImager 2000 (Alpha Innotech).

2.7. ICAM-1 and E-selectin expression on cell surfaces

The suppression of HCM, ECM, apigenin, and luteolin on ICAM-1 and E-selectin expression on plasma membranes was measured by fluorescence flow cytometry. After treatment, cells were detached with trypsin and centrifuged at $400 \times g$ for 5 min. Cells were reacted with FITC-conjugated goat anti-mouse ICAM-1 and E-selectin antibodies (Serotec, Oxford, UK) at 4 °C for 45 min in the dark. After incubation, cells were washed three times with cold PBS, re-suspended in 500 μl of PBS and immediately subjected to cytometric analysis performed with Becton Dickinson FACSCalibur (BD Biosciences, San Jose, CA).

2.8. ROS determination

The state of ROS production in HUVECs was measured by using a DCF-DA fluorescence flow cytometric assay. At the end of HCM, ECM, apigenin, luteolin, and oxLDL treatment, the fluorogenic substrate solution was added to the M199 medium and was incubated for an additional 45 min at 37 °C under 5% CO_2. Cells were washed twice with cold PBS. The cells were kept cool and were then quickly analyzed by Becton Dickinson FACSCalibur.

2.9. LDL peroxidizability

LDL used for the peroxidizability assay was prepared by equilibrium density-gradient ultracentrifugation from freshly withdrawn blood. LDL was co-treated with various concentrations of HCM and ECM and 5 μmol/l of copper sulfate. Conjugated diene formation was monitored at 37 °C every 5 min for 2.5 h by use of a U-2000 spectrophotometer (Hitachi, Tokyo, Japan) and with the results expressed as absolute absorbance at 234 nm. The LDL oxidation lag time was obtained from curve analysis according to the description in a previous study (Yoshida et al., 2005).

2.10. Monocyte adhesion assay

One milliliter of 1×10^8 HUVECs/l was plated in 24-well plates and was allowed to grow to 80% confluence. At the end of HCM, ECM, apigenin, luteolin, and oxLDL treatment, a total of 4×10^5 BCECF-AM-labeled HL-60 cells was added to each well and the cells were co-incubated with HUVECs at 37 °C for 30 min. The wells were washed and filled with cell culture medium, and the plates were sealed, inverted, and spun at $100 \times g$ for 5 min to remove nonadherent HL-60 cells. Bound HL-60 cells were lysed in a 1% SDS solution, and the fluorescence intensity was determined in a PerkinElmer HTS 7000 plate reader (PerkinElmer Instruments, Norwalk, CT) with an excitation wavelength of 490 nm and an emission wavelength of 520 nm. A control study showed that fluorescence is a linear function of HL-60 cells in the range of 3000–60,000 cells/well. On the basis of the standard curve obtained, the results are reported as the number of adherent HL-60 cells per well (Yoshida et al., 2000).

2.11. Determination of apigenin and luteolin in CM extracts by LC/MS

The identity and content of apigenin and luteolin in CM extracts were determined by LC/MS according to the method of Lai et al. (2007) with some modification. Because of the high amount of glycosides in CM extracts, hydrochloric acid (2.5 M final concentration) was added to hydrolyze glycosides prior to determination of apigenin and luteolin. HCM contains 309 and 1149 μg/g dry weight of apigenin and luteolin, and ECM contains 2110 μg/g and 1689 μg/g dry weight of apigenin and luteolin.

2.12. Statistical analysis

Data were analyzed by using analysis of variance (SAS Institute Inc., Cary, NC). Values are expressed as means ± SD ($n = 3$). The significance of the difference between mean values was determined by one-way analysis of variance followed by Tukey's test; P values <0.05 were taken to be statistically significant.

3. Results

3.1. Cell viability

By use of the MTT assay, we found no adverse effects of HCM, ECM, apigenin, or luteolin on the growth of HUVEC at concentrations up to 2 mg/ml of HCM, 0.5 mg/ml of ECM, and 50 μM of apigenin and luteolin for 24 h, respectively. On the basis of these results, the maximum concentrations of HCM or ECM, apigenin, and luteolin used in the following experiments were 1000 μg/ml, 250 μg/ml, and 50 μM, respectively.

3.2. Effects of HCM, ECM, apigenin, and luteolin on the oxLDL-induced expression of ICAM-1 and E-selectin and HL-60 cell adhesion

In our previous study, the optimal concentration of oxLDL for the induction of adhesion molecule has been determined and it was 40 μg/ml (Lei et al., 2008). In the following experiments, we used 40 mg/l of oxLDL to induce the expression of ICAM-1 and E-selectin. oxLDL induced the expression of ICAM-1 (Fig. 1A) ($F = 26.5$, $P < 0.05$) and E-selectin (Fig. 1B) ($F = 25$, $P < 0.05$) on the cell surface, and this increase in expression was inhibited by HCM, ECM, apigenin, and luteolin in a dose-dependent manner. Apigenin and luteolin showed similar potency at inhibiting the oxLDL-induced expression of ICAM-1 and E-selectin.

Immunoblots indicated that oxLDL significantly induced total cellular ICAM-1 and E-selectin expression in HUVEC, and this induction was inhibited by the pretreatment of cells with HCM, ECM, apigenin, and luteolin (Fig. 1C). ECM was more potent than HCM at inhibiting the oxLDL-induced expression of both ICAM-1 and E-selectin.

Consistent with the induction of expression of ICAM-1 and E-selectin on the cell surface, oxLDL significantly increased the adhesion of HL-60 cells to HUVEC compared with that of control cells (4-fold, $F = 59.2$, $P < 0.05$; Fig. 1D). Pretreatment of HUVEC with HCM, ECM, apigenin, or luteolin inhibited the adhesion of the HL-60 cells to oxLDL-induced HUVEC in a dose-dependent manner. A 25%, 37%, 29%, and 33% decrease in the adhesion of HL-60 cells was noted in HUVEC treated with 250 μg/ml of HCM and ECM and 50 μM of apigenin and luteolin, respectively.

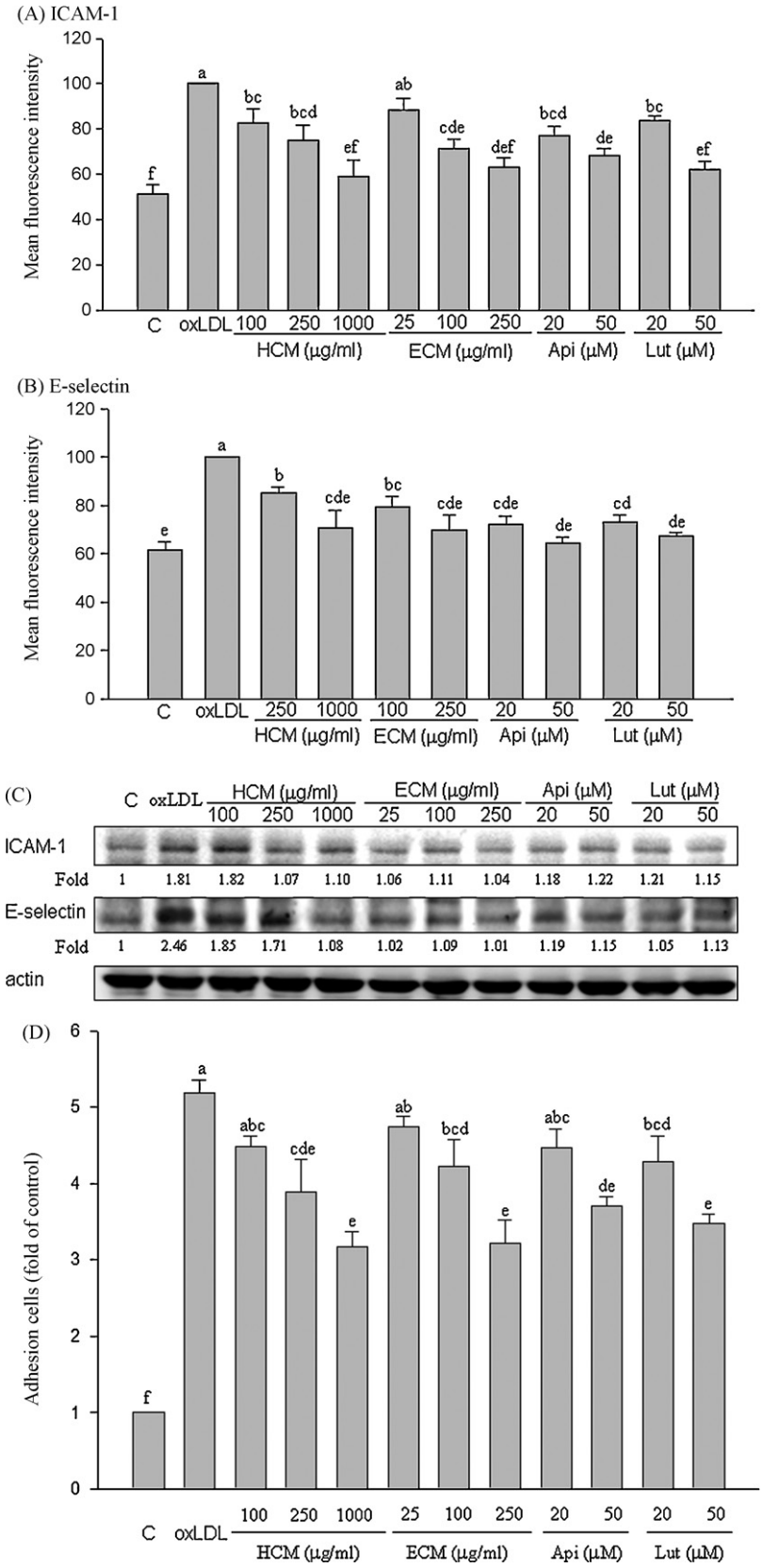

Fig. 1. Effects of HCM, ECM, apigenin, and luteolin on the oxLDL-induced expression of ICAM-1 (A) and E-selectin (B) on cell surfaces, on the total cellular expression of ICAM-1 and E-selectin (C), and on the oxLDL-induced HL-60 cell adhesion (D). Cells were pretreated with HCM, ECM, apigenin, or luteolin for 16 h before being challenged with 40 μg/ml oxLDL for another 24 h. Control cells were maintained in the vehicle before challenge with oxLDL. Values are means ± SD, $n = 3$. Bars with different letters are significantly different within the group ($P < 0.05$). One representative immunoblot out of three independent experiments is shown.

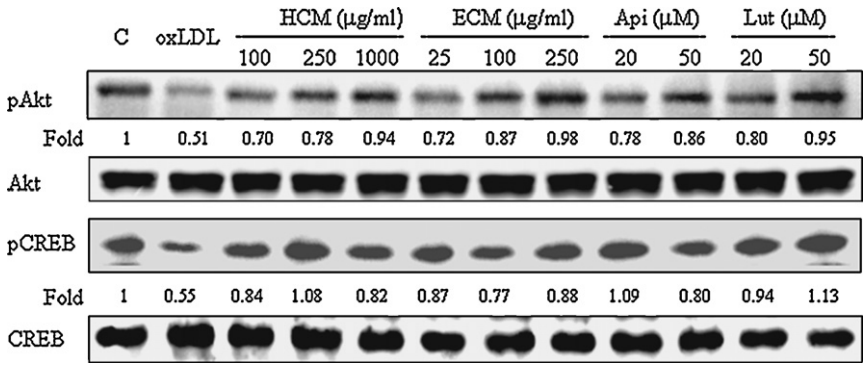

Fig. 2. Effects of HCM, ECM, apigenin, and luteolin on oxLDL-induced dephosphorylation of Akt and CREB. Cells were pretreated with 100, 250, or 1000 μg/ml HCM; 25, 100, or 250 μg/ml ECM; or 20 or 50 μM apigenin or luteolin for 16 h before being challenged with 40 μg/ml oxLDL for another 24 h. Control cells were maintained in the vehicle. One representative immunoblot out of three independent experiments is shown.

3.3. *Effects of HCM, ECM, apigenin, and luteolin on oxLDL-induced Akt and CREB activation*

To test whether the phosphorylation or dephosphorylation states of Akt and CREB were involved in the amelioration of adhesion molecule expression by HCM, ECM, apigenin, and luteolin, we performed an immunoblot assay (Fig. 2). As noted, oxLDL caused a significant dephosphorylation of Akt in HUVEC. With pretreatment with HCM, ECM, apigenin, and luteolin, however, the dephosphorylation of Akt induced by oxLDL was dose-dependently reversed. Furthermore, restoration of the phosphorylation of Akt by HCM, ECM, apigenin, or luteolin paralleled their inhibition of oxLDL-induced ICAM-1 and E-selectin expression.

In addition to Akt, CREB was significantly dephosphorylated in response to oxLDL as well (Fig. 2). Also, the phospho-CREB level was significantly reversed by the pretreatment of cells with HCM, ECM, apigenin, and luteolin.

3.4. *The role of the PI3K/Akt signaling pathway in the inhibition of ICAM-1 and E-selection expression by HCM, ECM, apigenin, and luteolin*

Wortmannin, an inhibitor of the PI3K/Akt signaling pathway, reversed the inhibition of ICAM-1 and E-selectin expression by HCM, ECM, apigenin, and luteolin in the presence of oxLDL (Fig. 3A). Wortmannin also prevented the restoration of phosphorylation of both Akt and CREB by HCM, ECM, apigenin, and luteolin (Fig. 3B). These results suggest that the PI3K/Akt signaling pathway plays an important role in the inhibition of expression of ICAM-1 and E-selectin exerted by the CM extracts and flavonoids. The expression of ICAM-1 and E-selectin may be regulated by the phosphorylation

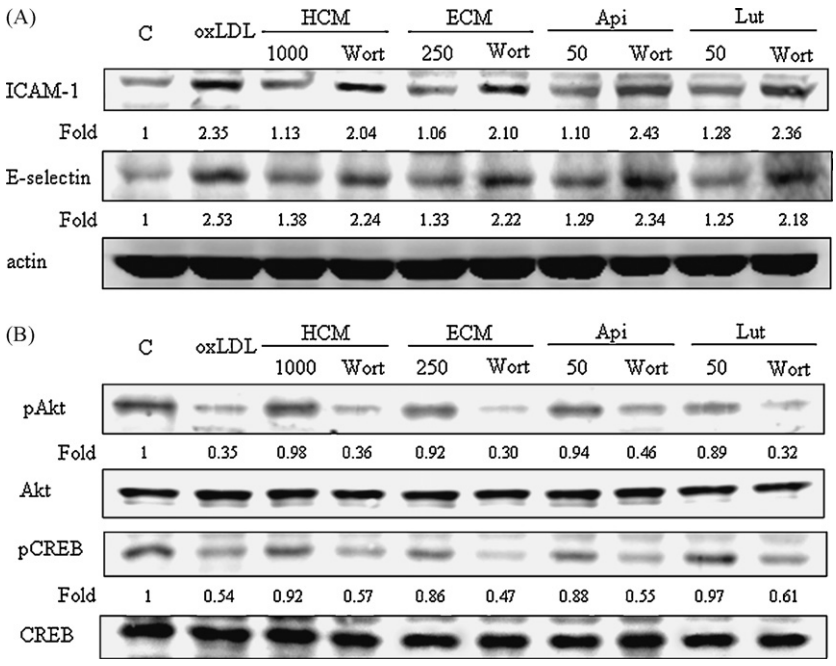

Fig. 3. Effect of wortmannin on the inhibition of the oxLDL-induced expression of ICAM-1 and E-selectin by HCM, ECM, apigenin, and luteolin (A) and on the reversal of phosphorylation of Akt and CREB by HCM, ECM, apigenin, and luteolin (B). Cells were pretreated with 100 nM wortmannin for 1 h; then HCM, ECM, apigenin, and luteolin for 16 h; and finally 40 μg/ml oxLDL for another 24 h. Control cells were maintained in the vehicle. One representative immunoblot out of three independent experiments is shown.

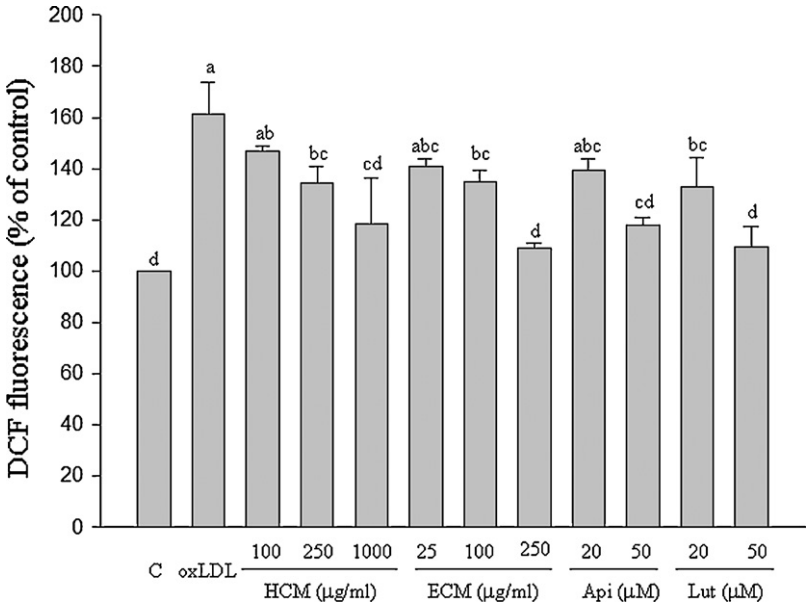

Fig. 4. Effects of HCM, ECM, apigenin, and luteolin on oxLDL-induced ROS production. Cells were pretreated with 100, 250, or 1000 μg/ml HCM; 25, 100, or 250 μg/ml ECM; or 20 or 50 μM apigenin or luteolin for 16 h before being challenged with 40 μg/ml oxLDL for another 24 h. Control cells were maintained in the vehicle without oxLDL challenge. The oxLDL group consisted of cells pretreated with nothing but challenged with 40 μg/ml oxLDL for 24 h. Values are means ± SD, $n = 3$. Bars with different letters are significantly different within the group ($P < 0.05$).

or dephosphorylation states of Akt, which affects the activation of transcription factors such as CREB.

3.5. Effects of HCM, ECM, apigenin, and luteolin on oxLDL-induced ROS production in HUVECs

In the DCF-DA assay, treatment of cells with oxLDL significantly increased intracellular fluorescence intensity, which represents the cellular levels of ROS ($F = 15.4$, $P < 0.05$; Fig. 4). Pretreatment of cells with HCM, ECM, apigenin, and luteolin inhibited the cellular production of ROS induced by oxLDL in a dose-dependent manner. Significant inhibitory effects were observed for 250 and 1000 μg/ml HCM, 100 and 250 μg/ml ECM, 50 μmol/l apigenin, and 20 and 50 μmol/l luteolin ($P < 0.05$).

3.6. Inhibition of LDL oxidation by HCM and ECM

The antioxidant activity of HCM and ECM was also measured by using an in vitro $CuSO_4$-induced LDL oxidation assay. As shown in Table 1, the LDL oxidation lag time was 43.9 min; however, the LDL oxidation lag time was dose-dependently increased by both HCM and ECM. ECM showed better antioxidant potency than did HCM.

Table 1
HCM and ECM inhibit LDL oxidation.

Treatment	CM concentration (μg/ml)	Lag time (min)
oxLDL		43.9 ± 8.0[e]
oxLDL + HCM	0.5	66.1 ± 1.8[d]
	2.0	87.4 ± 12.5[c]
	5.0	124.0 ± 9.0[b]
oxLDL + ECM	0.5	67.1 ± 6.4[d]
	2.0	143.8 ± 28.1[b]
	5.0	204.0 ± 7.5[a]

Values are the mean ± SD ($n = 3$–6). Values were analyzed by one-way ANOVA followed by Tukey's test. Values not sharing the same letter are significantly different ($P < 0.05$).

4. Discussion

CVD receives extensive attention owing to the large population worldwide affected by it; CVD is among the 10 leading causes of death worldwide (Braunwald, 1997). There is increasing interest in the utilization of traditional medicine to reduce the risk of diseases, including CVD. Atherosclerosis, one type of CVD, is an inflammatory disease and substances with anti-inflammatory properties may have a cardioprotective effect. oxLDL is recognized to be one of the risk factors for CVD, and the increased expression of adhesion molecules by oxLDL contributes to their CVD-promoting effect. In fact, the accumulation of oxLDL and activation of endothelial cells with subsequent expression of adhesion molecules and increased recruitment of leukocytes to the vascular endothelium are the early stages of atherosclerosis (Aviram, 1993; Sigurdardottir et al., 2002). Inhibition of the expression of endothelial adhesion molecules induced by inflammatory stimuli is regarded to be protective against CVD (Stangl et al., 2005). CM is reported to offer cardiovascular protection in isolated rat heart (Jiang et al., 2004) and to have anti-inflammatory properties in 12-*O*-tetradecanoylphorbol-13-acetate-induced mice (Ukiya et al., 2001). Although in vitro cardioprotective effects and in vivo anti-inflammatory effects of CM have been shown, the mechanism by which CM acts to prevent CVD is not clear. In the present study, we used HUVECs treated with oxLDL to simulate the in vivo cardiovascular condition and determined the effects of HCM and ECM and the major bioactive flavonoids in CM, apigenin and luteolin, on the oxLDL-induced expression of adhesion molecules.

Inflammatory cytokines, including TNF-α, have been implicated in atherosclerosis, and oxLDL enhances TNF-α secretion in macrophages (Jovinge et al., 1996). TNF-α upregulates the expression of ICAM-1, VCAM-1, and monocyte chemoattractant protein-1 (MCP-1) in the vasculature and also increases the expression of scavenger receptor class A (SRA), leading to oxLDL uptake into macrophages (Ohta et al., 2005). Mice in which the TNF-α gene is disrupted develop significantly fewer atherosclerotic lesions in the proximal aorta than do their normal counterparts (Ohta et al., 2005).

In recent years, interest has increased in the anti-inflammatory and anti-atherogenic effects of health foods and their active phytochemicals. Their protective effects are believed, in part, to inhibit the abnormal induction of adhesion molecules. For instance, apigenin and luteolin significantly inhibit the TNF-α-induced protein and mRNA expression of ICAM-1, VCAM-1, and E-selectin in HUVECs and THP-1 monocyte adherence to HUVECs in a dose-dependent fashion (Choi et al., 2004). The mechanism by which apigenin and luteolin inhibit the expression of adhesion molecules is via suppression of nuclear translocation and transactivation of NF-κB. Cinnamaldehyde found in the stem bark of *Cinnamomum cassia*, inhibits the adhesion of TNF-α-induced monocytes to the human endothelial cell line EA.hy926 and the expression of ICAM-1 and VCAM-1 (Liao et al., 2008). Cinnamaldehyde abolishes TNF-α-induced nuclear translocation of NF-κB in both time-dependent and dose-dependent manners. Cinnamaldehyde exerts its anti-inflammatory effects by two distinct mechanisms dependent on exposure period. In short-term pretreatments, cinnamaldehyde blocks the degradation of the inhibitory protein IκB-α, whereas over long-term pretreatments, it induces the expression of the Nrf2-mediated gene heme oxygenase-1 (HO-1). Elevated HO-1 protein levels are associated with suppression of TNF-α-induced ICAM-1 expression. Treatment of the EA.hy926 cell line with zinc protoporphyrin, an HO-1 inhibitor, partially reverses the anti-inflammatory effects of cinnamaldehyde.

In addition to the NF-κB/IκB pathway, the regulation of the expression of adhesion molecules is related to mitogen-activated protein kinases (MAPKs), protein kinase A (PKA), and PI3K/Akt signaling dependent on different stimuli. In ischemia-reperfusion-induced myocardial infarct, p38 MAPK activation plays an important role in the upregulation of ICAM-1 and P-selectin and the subsequent polymorphonuclear leukocyte accumulation in ischemic-reperfused myocardial tissue (Gao et al., 2002). PKA was shown to be involved in the suppression of oxLDL-induced VCAM-1 expression by the garlic organosulfur compounds diallyl disulfide and diallyl trisulfide in HUVECs (Lei et al., 2008). It is also reported that HDL-associated sphingosylphosphorylcholine inhibits the expression of adhesion molecules in TNF-α-induced HUVECs (Schmidt et al., 2006). Interaction of the HDL-associated sphingosylphosphorylcholine and sphingosylgalactosyl-3-sulfate with the G-protein-coupled endothelial differentiation gene receptor initiates a signaling cascade that activates the protein kinase Akt as the underlying mechanism. In human coronary artery endothelial cells, oxLDL causes dephosphorylation of Akt, increased expression of ICAM-1 and P-selectin, and enhanced adhesion of monocytes to the cells (Chen et al., 2003). In the present study, HCM, ECM, apigenin, and luteolin inhibited the oxLDL-induced expression of both total cellular and cell surface ICAM-1 and E-selectin (Fig. 1A, B, and C) and the dephosphorylation of Akt (Fig. 2). Pretreatment of cells with wortmannin, an inhibitor of the PI3K/Akt pathway, abolished the inhibitory effects of HCM, ECM, apigenin, and luteolin on the oxLDL-induced expression of ICAM-1 and E-selectin and the dephosphorylation of Akt (Fig. 3A and B). These results suggest that the Akt signaling pathway is likely to play a critical role in the suppression of the oxLDL-induced expression of ICAM-1 and E-selectin by HCM, ECM, apigenin, and luteolin.

In addition to Akt, oxLDL dephosphorylated CREB (Fig. 3B), and this effect was suppressed by pretreatment with HCM, ECM, apigenin, and luteolin. The transcriptional activities of CREB are induced by phosphorylation at serine 133, and CREB is phosphorylated at the same serine residue by both PKA and Akt (Du and Montminy, 1998). The reversal of the phosphorylation of CREB by pretreatment with HCM, ECM, apigenin, and luteolin in the presence of oxLDL was abolished by wortmannin (Fig. 3B). This suggests that the PI3K/Akt signaling pathway plays an important role in CREB phosphorylation and is consistent with the notion proposed by a previous study (Du and Montminy, 1998).

Several studies have demonstrated enhanced ROS production caused by TNF-α and that pretreatment with an antioxidant pyrrolidine dithiocarbamate inhibits TNF-α-induced ROS production, NF-κB activation, and expression of E-selectin, ICAM-1, and VCAM-1 in HUVECs and pulmonary artery endothelial cells (Weber et al., 1994; Rahman et al., 1998). In the present study, oxLDL treatment of HUVECs significantly increased ROS generation (Fig. 4); however, pretreatment with HCM, ECM, apigenin, and luteolin decreased the oxLDL-induced intracellular ROS levels in a dose-dependent manner. These results suggest that the inhibitory effects of HCM, ECM, apigenin, and luteolin on oxLDL-induced ICAM-1 and E-selectin expression may be related to their antioxidant activity, which is demonstrated to scavenge ROS. Water extracts of four CM varieties are shown to have antioxidant properties in vitro (Duh, 1999), and their antioxidant activity is much superior to that of butylated hydroxyanisole and tocopherol. In the present study as well, both HCM and ECM were shown to be effective in inhibiting $CuSO_4$ induction of LDL oxidation (Table 1).

In summary, HCM, ECM, apigenin, and luteolin could effectively inhibit the expression of ICAM-1 and E-selectin induced by oxLDL and subsequent HL-60 monocyte adherence to HUVECs. The inhibitory effects of HCM, ECM, apigenin, and luteolin are at least in part attributed to their antioxidant activity and modulation of the PI3K/Akt signaling pathway. According to the results of this study, we deduce that CM is a plant with cardiovascular-protective potential.

Acknowledgement

This work was supported by grant CMU97-209 from China Medical University, Taichung, Taiwan.

References

Aviram, M., 1993. Modified forms of low density lipoprotein and atherosclerosis. Atherosclerosis 98, 1–9.

Boissonneault, G.A., Wang, Y., Chung, B.H., 1995. Oxidized low-density lipoproteins delay endothelial wound healing: lack of effect of vitamin E. Annals of Nutrition and Metabolism 39, 1–8.

Braunwald, E., 1997. Shattuck lecture-cardiovascular medicine at the turn of millennium: triumphs, concerns, and opportunities. The New England Journal of Medicine 337, 1360–1369.

Chen, H., Li, D., Chen, J., Roberts, G.J., Saldeen, T., Mehta, J.L., 2003. EPA and DHA attenuate ox-LDL-induced expression of adhesion molecules in human coronary artery endothelial cells via protein kinase B pathway. Journal of Molecular and Cellular Cardiology 35, 769–775.

Chen, T., Li, L.P., Lu, X.Y., Jiang, H.D., Zeng, S., 2007. Absorption and excretion of luteolin and apigenin in rats after oral administration of *Chrysanthemum morifolium* extract. Journal of Agricultural and Food Chemistry 55, 273–277.

Choi, J.S., Choi, Y.J., Park, S.H., Kang, J.S., Kang, Y.H., 2004. Flavones mitigate tumor necrosis factor-alpha-induced adhesion molecule upregulation in cultured human endothelial cells: role of nuclear factor-kappa B. The Journal of Nutrition 134, 1013–1019.

Claise, C., Edeas, M., Chaouchi, N., Chalas, J., Capel, L., Kalimouttou, S., Vazquez, A., Lindenbaum, A., 1999. Oxidized LDL induce apoptosis in HUVEC but not in the endothelial cell line EA.hy 926. Atherosclerosis 147, 95–104.

Denizot, F., Lang, R., 1986. Rapid colorimetric assay for cell growth and survival. Modifications to the tetrazolium dye procedure giving improved sensitivity and reliability. Journal of Immunological Methods 89, 271–277.

Du, K., Montminy, M., 1998. CREB is a regulatory target for the protein kinase Akt/PKB. The Journal of Biological Chemistry 273, 32377–32379.

Duh, P.D., 1999. Antioxidant activity of water extract of four Harng Jyur (*Chrysanthemum morifolium* Ramat) varieties in soybean oil emulsion. Food Chemistry 66, 471–476.

Fraga, C.G., Leibovitz, B.E., Tappel, A.L., 1988. Lipid peroxidation measured as thiobarbituric acid-reactive substances in tissue slices: characterization and comparison with homogenates and microsomes. Free Radical Biology and Medicine 4, 155–161.

Gao, F., Yue, T.L., Shi, D.W., Christopher, T.A., Lopez, B.L., Ohlstein, E.H., Barone, F.C., Ma, X.L., 2002. p38 MAPK inhibition reduces myocardial reperfusion injury via inhibition of endothelial adhesion molecule expression and blockade of PMN accumulation. Cardiovascular Research 53, 414–422.

Hertog, M.G., Feskens, E.J., Hollman, P.C., Katan, M.B., Kromhout, D., 1993. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 342, 1007–1011.

Hu, C.Q., Chen, K., Shi, Q., Kilkuskie, R.E., Cheng, Y.C., Lee, K.H., 1994. Anti-AIDS agents, 10. Acacetin-7-*O*-beta-D-galactopyranoside, an anti-HIV principle from *Chrysanthemum morifolium* and a structure-activity correlation with some related flavonoids. Journal of Natural Products 57, 42–51.

Ito, T., Ikeda, U., 2003. Inflammatory cytokines and cardiovascular disease. Current Drug Targets-Inflammation and Allergy 2, 257–265.

Jeong, Y.J., Choi, Y.J., Choi, J.S., Kwon, H.M., Kang, S.W., Bae, J.Y., Lee, S.S., Kang, J.S., Han, S.J., Kang, Y.H., 2007. Attenuation of monocyte adhesion and oxidised LDL uptake in luteolin-treated human endothelial cells exposed to oxidised LDL. The British Journal of Nutrition 97, 447–457.

Jiang, H., Xia, Q., Xu, W., Zheng, M., 2004. *Chrysanthemum morifolium* attenuated the reduction of contraction of isolated rat heart and cardiomyocytes induced by ischemia/reperfusion. Die Pharmazie 59, 565–567.

Jovinge, S., Ares, M.P., Kallin, B., Nilsson, J., 1996. Human monocytes/macrophages release TNF-alpha in response to Ox-LDL. Arteriosclerosis. Thrombosis, and Vascular Biology 16, 1573–1579.

Kim, H.J., Lee, Y.S., 2005. Identification of new dicaffeoylquinic acids from *Chrysanthemum morifolium* and their antioxidant activities. Planta Medica 71, 871–876.

Kokura, S., Rhoads, C.A., Wolf, R.E., Yoshikawa, T., Granger, D.N., Aw, T.Y., 2001. NF kappa b signaling in posthypoxic endothelial cells: relevance to E-selectin expression and neutrophil adhesion. Journal of Vascular Research 38, 47–58.

Lai, J.P., Lim, Y.H., Su, J., Shen, H.M., Ong, C.N., 2007. Identification and characterization of major flavonoids and caffeoylquinic acids in three compositae plants by LC/DAD–APCI/MS. Journal of Chromatography. B, Analytical Technologies in the Biomedical and Life Sciences 848, 215–225.

Lee, J.S., Kim, H.J., Lee, Y.S., 2003. A new anti-HIV flavonoid glucuronide from *Chrysanthemum morifolium*. Planta Medica 69, 859–861.

Lei, Y.P., Chen, H.W., Sheen, L.Y., Lii, C.K., 2008. Diallyl disulfide and diallyl trisulfide suppress oxidized LDL-induced vascular cell adhesion molecule and E-selectin expression through protein kinase A- and B-dependent signaling pathways. The Journal of Nutrition 138, 996–1003.

Liao, B.C., Hsieh, C.W., Liu, Y.C., Tzeng, T.T., Sun, Y.W., Wung, B.S., 2008. Cinnamaldehyde inhibits the tumor necrosis factor-alpha-induced expression of cell adhesion molecules in endothelial cells by suppressing NF-kappaB activation: effects upon IkappaB and Nrf2. Toxicology and Applied Pharmacology 229, 161–171.

Lotito, S.B., Frei, B., 2006. Dietary flavonoids attenuate tumor necrosis factor alpha-induced adhesion molecule expression in human aortic endothelial cells. Structure-function relationships and activity after first pass metabolism. The Journal of Biological Chemistry 281, 37102–37110.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J., 1951. Protein measurement with the Folin phenol reagent. The Journal of Biological Chemistry 193, 265–275.

Matsuda, H., Morikawa, T., Toguchida, I., Harima, S., Yoshikawa, M., 2002. Medicinal flowers. VI. Absolute stereostructures of two new flavanone glycosides and a phenylbutanoid glycoside from the flowers of Chrysanthemum indicum L. : their inhibitory activities for rat lens aldose reductase. Chemical and Pharmaceutical Bulletin 50, 972–975.

Middleton Jr., E., Kandaswami, C., Theoharides, T.C., 2000. The effects of plant flavonoids on mammalian cells: implications for inflammation, heart disease, and cancer. Pharmacological Reviews 52, 673–751.

Miyazawa, M., Hisama, M., 2003. Antimutagenic activity of flavonoids from *Chrysanthemum morifolium*. Bioscience, Biotechnology, and Biochemistry 67, 2091–2099.

Ohta, H., Wada, H., Niwa, T., Kirii, H., Iwamoto, N., Fujii, H., Saito, K., Sekikawa, K., Seishima, M., 2005. Disruption of tumor necrosis factor-alpha gene diminishes the development of atherosclerosis in ApoE-deficient mice. Atherosclerosis 180, 11–17.

Ou, H.C., Chou, F.P., Lin, T.M., Yang, C.H., Sheu, W.H., 2006. Protective effects of honokiol against oxidized LDL-induced cytotoxicity and adhesion molecule expression in endothelial cells. Chemico-Biological Interactions 161, 1–13.

Rahman, A., Kefer, J., Bando, M., Niles, W.D., Malik, A.B., 1998. E-selectin expression in human endothelial cells by TNF-alpha-induced oxidant generation and NF-kappaB activation. The American Journal of Physiology 275 (3 Pt 1), L533–544.

Schmidt, A., Geigenmüller, S., Völker, W., Buddecke, E., 2006. The antiatherogenic and antiinflammatory effect of HDL-associated lysosphingolipids operates via Akt → NF-kappaB signalling pathways in human vascular endothelial cells. Basic Research in Cardiology 101, 109–116.

Seguí, J., Gil, F., Gironella, M., Alvarez, M., Gimeno, M., Coronel, P., Closa, D., Piqué, J.M., Panés, J., 2005. Down-regulation of endothelial adhesion molecules and leukocyte adhesion by treatment with superoxide dismutase is beneficial in chronic immune experimental colitis. Inflammatory Bowel Diseases 11, 872–882.

Sigurdardottir, V., Fagerberg, B., Hulthe, J., 2002. Circulating oxidized low-density lipoprotein (LDL) is associated with risk factors of the metabolic syndrome and LDL size in clinically healthy 58-year-old men (AIR study). Journal of Internal Medicine 252, 440–447.

Stangl, V., Lorenz, M., Ludwig, A., Grimbo, N., Guether, C., Sanad, W., Ziemer, S., Martus, P., Baumann, G., Stangl, K., 2005. The flavonoid phloretin suppresses stimulated expression of endothelial adhesion molecules and reduces activation of human platelets. The Journal of Nutrition 135, 172–178.

Taguchi, S., Oinuma, T., Yamada, T., 2000. A comparative study of cultured smooth muscle cell proliferation and injury, utilizing glycated low density lipoproteins with slight oxidation, auto-oxidation, or extensive oxidation. Journal of Atherosclerosis and Thrombosis 7, 132–137.

Ukiya, M., Akihisa, T., Yasukawa, K., Kasahara, Y., Kimura, Y., Koike, K., Nikaido, T., Takido, M., 2001. Constituents of compositae plants. 2. Triterpene diols, triols, and their 3-o-fatty acid esters from edible chrysanthemum flower extract and their anti-inflammatory effects. Journal of Agricultural and Food Chemistry 49, 3187–3197.

Weber, C., Erl, W., Pietsch, A., Ströbel, M., Ziegler-Heitbrock, H.W., Weber, P.C., 1994. Antioxidants inhibit monocyte adhesion by suppressing nuclear factor-kappa B mobilization and induction of vascular cell adhesion molecule-1 in endothelial cells stimulated to generate radicals. Arteriosclerosis and Thrombosis 14, 1665–1673.

Yoshida, M., Chien, L.J., Yasukochi, Y., Numano, F., 2000. Differentiation-induced transmigration of HL60 cells across activated HUVEC monolayer involves E-selectin-dependent mechanism. Annals of the New York Academy of Sciences 902, 307–310.

Yoshida, H., Sasaki, K., Namiki, Y., Sato, N., Tada, N., 2005. Edaravone, a novel radical scavenger, inhibits oxidative modification of low-density lipoprotein (LDL) and reverses oxidized LDL-mediated reduction in the expression of endothelial nitric oxide synthase. Atherosclerosis 179, 97–102.