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Mulberry Leaf Polyphenols Possess Antiatherogenesis Effect via Inhibiting LDL Oxidation and Foam Cell Formation

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ABSTRACT: Oxidized low-density lipoprotein (ox-LDL) and its uptake by machrophage are the hallmark in atherogenesis. In the present study, we aimed to investigate the antiatherogenic effect of mulberry leaf extracts (MLE) and the polyphenolic extracts (MLPE), which contained polyphenols including quercetin (11.70%), naringenin (9.01%) and gallocatechin gallate (10.02%). Both MLE and MLPE inhibited the oxidation and lipid peroxidation of LDL, while MLPE was shown to be more potent. As 1.0 mg/mL MLE reduced 30% of ox-LDL-generated ROS, 0.5 mg/mL MLPE decreased 46% of the ROS and was shown to be more potent on elevating SOD-1 and GPx in macrophages. At the same dose of 0.5 mg/mL, MLPE exhibited 1.5-fold potency than MLE in decreasing the formation of foam cells. Both MLE and MLPE reduced the expression of PPARγ, CD36 and SR-A, implicating the molecular regulation on ox-LDL uptake. These results suggested that MLPE potentially could be developed as an antiatherogenic agent and deserve further investigation.

KEYWORDS: mulberry leaf, antiatherogenesis, oxidized LDL, foam cell

INTRODUCTION

Atherosclerosis is the major risk of coronary artery disease (CAD), which has become a leading cause of death in developed countries. The pathogenesis of atherosclerosis is a cascade of inflammatory processes involving soluble mediators, monocytes, endothelial cells and vascular smooth muscle cells.[1]

The formation of oxidized low density lipoprotein (ox-LDL) and oxidative stress are thought to play a critical role in early atherogenesis, eliciting endothelial dysfunction and macrophage activation.[2] Ox-LDL stimulates the macrophage to accumulate cholesterol and to form the foam cells, which compose fatty streak, the hallmark in early atherosclerotic lesions, followed by the development of fibrous and atheromatous plaques.[3] Recent data support the importance of macrophage surface proteins CD36 and class A scavenge receptors (SR-A), which can specially bind ox-LDL in the atherogenesis.[4,5] Meanwhile, the transcription factor peroxisome proliferator-activated receptor-γ (PPAR-γ) is highly expressed in activated monocytes and lipid-accumulating macrophages.[6] It has been demonstrated that SR-A and CD36 are the target genes for PPAR-γ.[7,8]

In addition to the activation by ox-LDL, the oxidized macrophages increase their ability to oxidize LDL, thus further promoting foam cell formation.[9] The oxidative stress could be eliminated by endogenous antioxidative enzymes such as superoxide dismutase (SOD), catalase, and glutathione peroxidase (GPx), or the exogenous application of antioxidants.

Mulberry leaf (the leaf of *Morus alba*), commonly used as the silkworm diet, has been used in the traditional medicine for antidiabetes, antihyperlipidemics, and the prevention of CAD.[10−12] In the literature, mulberry leaf possessed anticancer effects, restored arterial pressure, inhibited hyperglycemia, and attenuated atherosclerotic lesion development.[13−16] It contains a lot of nutritional components including flavonoid, which is known as a powerful polyphenol and antioxidant.[17] In the previous reports, dietary mulberry leaf showed antiatherogenic effects in certain animal models. Mulberry leaf and its butanol extracts inhibited the oxidative modification of rabbit and human LDL,[12] and attenuated atherosclerosis in the LDL receptor or apo E deficient mice.[18]

In the present study, we aimed to investigate the putative effect of mulberry leaf extracts (MLE) and mulberry leaf polyphenolic extracts (MLPE). Whether they could influence LDL oxidation, macrophage activation, foam cell formation, and the protein expression of related signals needed to be clarified.

MATERIALS AND METHODS

Materials. The mulberry leaves were collected in Dadu Township, located in central Taiwan. All used chemicals were purchased from Sigma (St. Louis, MO). The anti-CD36, GPx, PPARγ, SOD-1 and SR-A antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz,

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CA). Anti-β-actin, catalase and HRP-conjugated secondary antibodies were obtained from Sigma.

Preparation of MLE and MLPE. Fresh mulberry leaves (100 g) were harvested and immediately dried at 50 °C. The dried leaves were heated in 1500 mL of deionized water. After filtration, we removed the residue. The suspension was stored at −80 °C overnight and then evaporated with a freeze-dryer. The dried powder remaining was an aqueous fraction of mulberry leaves (MLE). Analysis of MLE revealed that it contained 16.94 ± 0.14% total phenolic acids (using gallic acid and quercetin as the standard), 3.90 ± 0.19% flavonoids (using quercetin and naringenin as the standard), 25.43 ± 3.56% carbohydrate, 2.43 ± 0.26% protein and 8.40 ± 1.63% lipid. For preparation of the polyphenol extract of mulberry leaves (MLPE), 100 g dried powder of mulberry leaves was merged in 300 mL of ethanol and heated at 50 °C for 3 h. The extract was filtered and thereafter lyophilized under reduced pressure at room temperature. The powder was then resuspended in 500 mL of 50 °C distilled water, followed by extraction with 180 mL of ethyl acetate three times, redissolved in 250 mL of distilled water, stored at −70 °C overnight, and lyophilized. The analysis of MLPE revealed that it contained 47.94 ± 0.20% total phenolic acids (using gallic acid and quercetin as the standard), 21.67 ± 0.26% flavonoids (using quercetin and naringenin as the standard). The presence and proportion of the main constituents of MLPE were then analyzed by HPLC. MLE and MLPE were filtrated by 0.22 μm filter before used in cell culture.

HPLC Analysis. HPLC was performed with a Hitachi HPLC system (Hitachi, Danbury, CT, USA) which consisted of a pump (L-6200A), an ultraviolet detector (L-4250) and the Hitachi D-7000 HPLC System Manager program. A reported procedure was used for analyzing the phenolic acids, which contained column, Mightysil RP-18 GP 250 (Kanto, Tokyo, Japan); mobile phase solvent A, acetic acid/water (2:98, v/v), and solvent B, 0.5% acetic acid in water/acetonitrile (50:50, v/v). The flow rate was 1 mL/min. The gradient for the separation was 100% solvent A at 0 min, 70% solvent A and 30% solvent B at 5 min, 65% solvent A and 35% solvent B at 50 min, 60% solvent A and 40% solvent B at 55 min, 0% solvent A and 100% solvent B at 60 min, followed by a 5 min postrun with HPLC grade water. Phenolic acids were detected at 260 nm.

Determination of Free Radical Quenching Capacity. Free radical quenching capacities of MLE and MLPE were tested by the method of bleaching stable DPPH.[19] A reaction mixture containing methanol (3 mL), DPPH (1 mM), and MLE (0.005 to 10 mg/mL) or MLPE (0.005 to 10 mg/mL) was allowed to stand at room temperature for 30 min before mixing with redistilled water (1 mL) and toluene (3 mL). The solution was then centrifuged, and absorbance of the upper phase was measured at 517 nm. DPPH without sample solution was used as the control. Decrease of DPPH solution absorbance indicated the increase of DPPH radical-scavenging activity. The capability of scavenging was calculated using the following equation:

$$\text{bleaching effect}\ (\%) = [1 - (\text{absorbance of sample at 517 nm} / \text{absorbance of control at 517 nm})] \times 100$$

LDL Isolation and Oxidization. Blood was obtained from healthy volunteers and collected in the presence of 0.01% EDTA. LDL (1.019 to 1.063 g/mL) was isolated by sequential density ultracentrifugation at 4 °C in an Optima TL Beckman ultracentrifuge (Beckman Instruments, U.S.A.) as described previously.[20] After the isolation, EDTA existing in LDL was removed by a Sephadex G-25 column (Pharmacia PD-10) equilibrated with phosphate-buffered saline (PBS). Protein concentration was measured using a Bradford protein assay kit (Bio-Rad, USA). LDL was diluted in PBS (100 μg/mL) and incubated at 37 °C in the presence of 10 μM $CuSO_4$ for 24 h to prepare the oxidized LDL (ox-LDL), with or without the treatment of MLE or MLPE. After the incubation, the formation of ox-LDL was confirmed by the electrophoretic mobility. Samples were sterilized by filtration (pore size 0.45 μm), stored at 4 °C, and used within two weeks.

Electrophoretic Mobility of Ox-LDL. The LDL (100 μg/mL) was incubated with 10 μM $CuSO_4$ at 37 °C for 24 h in the presence or absence of different concentrations of MLE or MLPE. The electrophoretic mobility of native or ox-LDL was detected by agarose gel electrophoresis,[21] which was performed using a Beckman paragon lipo gel electrophoresis system (Beckman Analytic, Milan, Italy). Using the buffer containing 10 mM 5,5-diethylbarbituric acid and 50 mM 5,5-diethylbarbituric acid sodium salt, samples were electrophoresed on the agarose gel (0.5% agarose and 1.0% barbital buffer) under 100 V for 30 min. After electrophoresis, the lipoproteins in the gel were immobilized in a fixative solution (containing 60% absolute alcohol, 30% deionized water, and 10% glacial acetic acid), and the gel was dried to a film. The lipoprotein pattern was visualized by staining with a lipid-specific stain.

Determination of Lipid Peroxidation of Ox-LDL. The LDL peroxidation was measured by thiobarbituric acid-reacting substances (TBARS) analysis. LDL (100 μg/mL) was incubated with 10 μM $CuSO_4$ at 37 °C for 24 h in the presence or absence of different concentrations of MLE or MLPE. TBARS assay was performed according to the previous report.[22] To each tube containing 0.55 mL of the incubated LDL, 0.5 mL of 25% (w/v) trichloroacetic acid (TCA) was added to denature the protein. Then the samples were centrifuged (10000 rpm) at 10 °C for 30 min to remove the pellets. Thiobarbituric acid (TBA; 1%, 0.5 mL) in 0.3% NaOH was added to the supernatant, and the mixed reagents reacted at 90 to 95 °C for 40 min in darkness. After completing the reactions, samples were detected with excitation at 532 nm and emission at 600 nm in a Hitachi F2000 spectrophotofluorimeter. The concentration of TBARS expressed as equivalents of 1,1,3,3-tetraethoxypropane was used as the standard. Quantification of TBARS was performed by comparison with a standard curve of malondialdehyde (MDA) equivalents generated by acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane.

Cell Culture and Differentiation. Murine macrophage cell line J774A.1 (BCRC 60140) and the human monocytic leukemia cell line THP-1 (BCRC 60430) were purchased from the Bioresource Collection and Research Center (BCRC, Food Industry Research and Development Institute, Hsinchu, Taiwan, ROC). J774A.1 cells were maintained in Dulbecco modified Eagle's medium (DMEM, GIBCO-BRL), while THP-1 cells were maintained in RPMI-1640 medium (GIBCO-BRL). Both cell line cultures were supplemented with heat-inactivated 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, and 2 mM glutamine at 37 °C under 5% CO_2. Before each experiment, THP-1 cells were pretreated with TPA (200 nM) for 48 h to induce cell differentiation.

Cell Viability. Cells were seeded on 24-well plates at a density of 4×10^4/mL and treated with indicated concentrations of MLE or MLPE for 24 h. After incubation, the cytotoxicity was determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, 5 mg/mL MTT solution (Sigma, St. Louis, MO, USA) was added to each well and incubated for 4 h at 37 °C. After washing with PBS, the purple blue formazan was dissolved in 1 mL of isopropanol, and the absorbance was determined at 563 nm. Cell viability was proportional to the amounts of formazan.

Detection of Intracellular Oxidative Stress. J774A.1 macrophage cells (1×10^6) were incubated with 10 μM dichlorofluorescin-diacetate (DCFH-DA) for 30 min at 37 °C. Under oxidative stress, DCFH was oxidized to DCF, a fluorescent compound. Cellular fluorescence was determined with a FACScan (Becton Dickinson).

Foam Cell Formation and Oil Red O Stain. J774A.1 cell (2×10^5) was seeded on a 6-well culture plate and cultured with ox-LDL (50 μg/mL), with or without MLE or MLPE for 20 h. After the medium was removed, cells were washed twice with PBS and then fixed with 4% paraformaldehyde in PBS (pH 7.3) for 30 min. The fixed cells were

washed with distillated water and then stained with Oil Red O for 1 h. Cells were observed under a microscope, and their pictures were taken at 200×.

Cellular Lipid Analysis. Cellular layers were extracted in hexane: isopropanol (3:2, v/v) as described.[23] After centrifugation (1500*g* for 10 min), the lower clear organic phase solution was transferred into a new glass tube and lyophilized. The lyophilized powder was dissolved in isopropanol as the cellular lipid extract and stored at −20 °C for use within 3 days. The cellular cholesterol and TG were measured by enzymatic colorimetric methods using commercial kits (Human, Wiesbaden, Germany).

Immunoblotting Assay. Total protein extracts were prepared in a lysis buffer (1% NP-40, 50 mM Tris-base, 0.1% SDS, 0.5% deoxycholic acid, and 150 mM NaCl) (pH 7.5) containing leupeptin (17 μg/mL) and sodium orthovanadate (10 μg/mL). Protein concentration was measured using a Bradford protein assay kit (Bio-Rad, USA). Equal amounts of protein samples (50 μg) were subjected to SDS−polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Millipore, Bedford, MA, USA). Membranes were blocked with 5% nonfat milk powder with 0.1% Tween-20 in Tris-buffered saline (TBS) and then incubated with the primary antibody at 4 °C overnight. Afterward, membranes were washed three times with TBS Tween-20 (TBST) and incubated with the secondary antibody conjugated to horseradish peroxidase (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.). Membrane was extensively washed with TBST, and the reactive signal was detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, U.K.). The β-actin expression was used as the internal control. Band detection was thereafter revealed by enhanced chemiluminescence using ECL Western blotting detection reagents and exposed to FUJFILM LAS-3000 (Tokyo, Japan). Protein quantitative was determined by densitometry using FUJFILM-Multi Gauge V2.2 software.

Quantification of DiI-acLDL Uptake. To analyze acLDL uptake, cells were washed twice in PBS and then incubated in basal culture medium supplemented with MLE (0.5 or 1 mg/mL) or MLPE (0.25 or 0.5 mg/mL) in the presence or absence of 5 μg/mL DiI (1,19-dioctadecyl-1-3,3,39,39-tetramethylindocarbocyanine perchlorate)-labeled acLDL (DiI-acLDL; Molecular Probes, Inc.) for 4 h at 37 °C. Cells pretreated with rosiglitazone (1 μM) and GW9662 (1 μM) for 48 h were used as the positive control. Cells were washed and harvested with PBS, and then fixed in a 4% solution of paraformaldehyde and analyzed on a FACScan (Becton Dickinson).

Statistics. Data were analyzed using an unpaired *t* test and represented as means ± standard deviation (SD). Statistical differences were evaluated using the unpaired *t* test and considered significant at the level $p < 0.05$. All data collected was analyzed using an unpaired *t* test after one-way ANOVA testing showed a significant difference among all the groups ($P < 0.001$).

RESULTS

Mulberry Extract Is Rich in Polyphenols. Figure 1 and Table 1 showed that PCA (3.72%), gallocatechin (1.11%), gallocatechin gallate (10.02%), caffeic acid gallocatechin (2.12%), rutin (5.43%), quercetin (11.70%) and naringenin (9.01%) are contained in the composition of MLPE.

MLE and MLE Inhibit the Oxidation and Lipid Peroxidation of LDL. MLE and MLPE exhibited their antioxidative ability on the DPPH blocking (Figure 2**A**), whereas MLPE was shown to be more potent. The scavenging effect of 0.5 mg/mL MLPE was 80% greater than the same dose of MLE. At the dose of 1 mg/mL, MLPE exhibited 2-fold scavenging ability compared to MLE.

Figure 2**B** and Figure 2**C** showed that both MLE and MLPE inhibited Cu^{2+}-induced oxidation dose-dependently. Noticeably, MLPE significantly reduced the oxidation at the dose of 0.01 mg/mL. At the same dose of 0.05 mg/mL, MLE slightly reduced the LDL oxidation, while MLPE reduced more than 55% of the oxidation, indicating that MLPE was more potent than MLE.

TBARS analysis showed similar results (Figure 2**D**). MLE at the doses of 0.1 mg/mL and 0.2 mg/mL reduced lipid peroxidation about 30% and 70% respectively. MLPE 0.05, 0.1, 0.2 mg/mL respectively reduced 30%, 60%, and 80% of the peroxidation. These data suggested that MLPE could exert the antioxidative effect at lower doses.

MLE and MLPE Attenuate the Ox-LDL-Induced ROS Production in Macrophages. Ox-LDLs induce the ROS generation of macrophages, thus mediating the cascades of atherosclerotic formation. We observed if MLE and MLPE could inhibit the ox-LDL-induced ROS production in macrophages.

We first tested the cell viability for evaluating the doses used in the following experiments. Figure 3**A** showed that MLE was nontoxic for cultured cells, while MLPE inhibited the cell viability at dose higher than 0.5 mg/mL. Hence 0.5 mg/mL was used as the maximum dose in the following experiments.

Incubation of ox-LDL increased 4-fold of the intracellular ROS compared with the control (Figure 3**B**). MLE 1.0 mg/mL reduced 30% of the ROS generation. MLPE 0.25 mg/mL and 0.5 mg/mL significantly decreased 40% and 46% of the intracellular ROS, respectively. While at the lower doses, MLPE still exhibited its superior effect compared to MLE.

MLE and MLPE Stimulate the Expressions of Antioxidative Enzymes. Figure 4**A** and 4**B** showed that MLE and MLPE stimulated the expressions of SOD-1, catalase and GPx.

Although both MLE and MLPE increased the expression of catalase dose-dependently, MLPE was shown to be more potent on elevating SOD-1 and GPx expressions.

MLE and MLPE Inhibit Ox-LDL-Induced Foam Cell Formation and Lipid Deposition. Ox-LDL increased the amount of foam cells about 3-fold. Figure 5**A** and Figure 5**B** showed that treatment of MLE and MLPE dose-dependently inhibited the foam cell formation. At the same dose of 0.5 mg/mL, MLPE exhibited 1.5-fold potency in decreasing the formation of foam cells.

Intracellular lipid analysis showed that both MLE and MLPE decreased the TG and cholesterol dose-dependently. Compared with the same dose of MLE, MLPE exhibited more than 2-fold of the lipid-lowering effect (Figure 5**C** and Figure 5**D**).

MLE and MLPE Inhibit the Ox-LDL Uptake of Macrophages. To investigate if the foam cell formation and lipid deposition associated with the uptake ability of macrophages, we used flow cytometry to analyze the uptake of acLDL, which could be viewed as ox-LDL as reported in the literature. Although both MLE and MLPE inhibited the acLDL uptake, MLPE still exerted the superior effect (Figure 6**A**). Similar results were found in TPA-activated monocytes (Figure 6**B**).

MLE and MLPE Decrease the Expressions of Scavenger Receptors and PPAR-γ. Figure 7**A** showed that ox-LDL increased CD36 and SR-A, which are recognized as the macrophage scavenger receptors mediating the ox-LDL uptake. MLE decreased the expression of CD36 and SR-A above the dose of 0.5 mg/mL, whereas MLPE reduced 30% of CD36 and 20% of SR-A at 0.1 mg/mL.

Figure 7**B** showed that ox-LDL also increased the amount of PPAR-γ. Both MLE and MLPE decreased the ox-LDL-induced

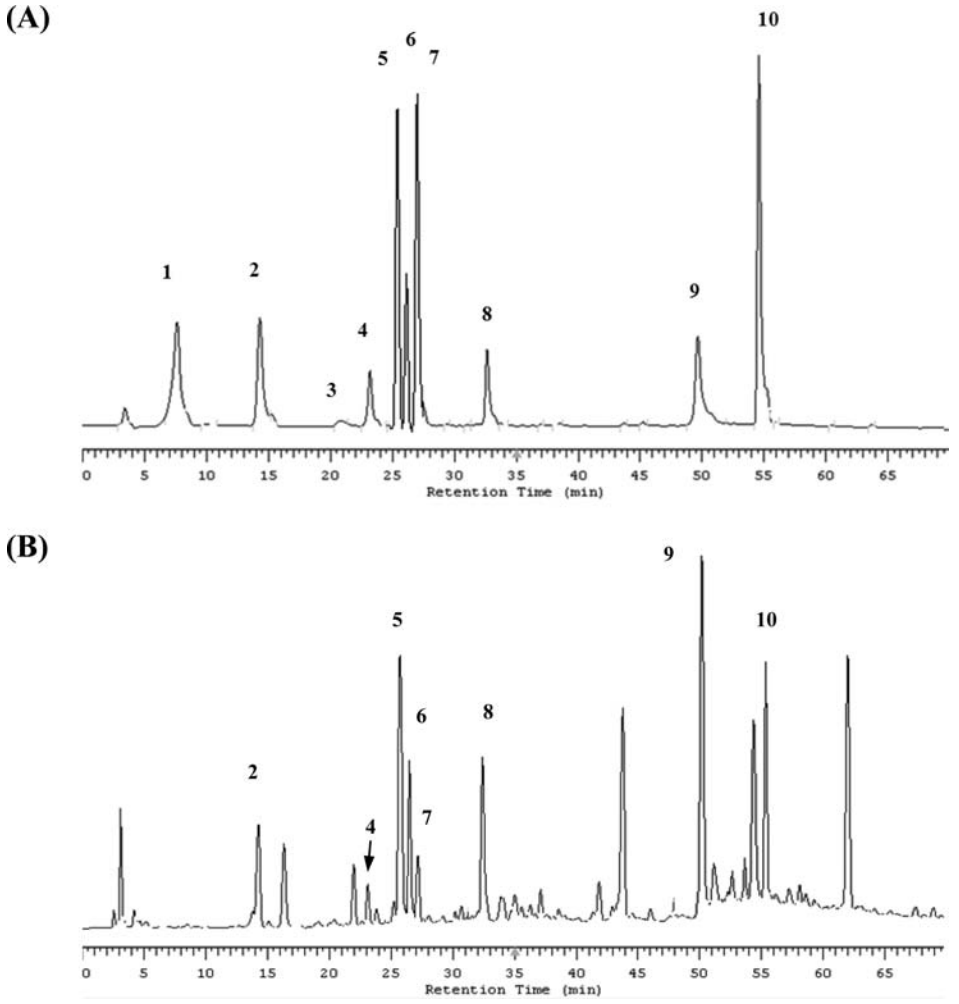

Figure 1. The HPLC chromatogram of MLPE. (**A**) HPLC chromatogram of nine kinds of standard polyphenols (1 mg/mL; 10 μL). Peaks: 1, gallic acid; 2, protocatechuic acid; 3, catechin; 4, gallocatechin; 5, gallocatechin gallate; 6, caffeic acid; 7, rutin; 8, quercetin; 9, naringenin. (**B**) HPLC chromatogram of free polyphenols from MLPE (10 mg/mL, 10 μL).

Table 1. Characterization of Phenolic Compounds of MLPE

peak no.	retention time (min)	assigned identity[a]	recovery (%)
2	14.43	PCA	3.72 ± 1.13
4	23.03	GC	1.11 ± 1.21
5	25.32	GCG	10.07 ± 2.48
6	26.21	Q3G	4.12 ± 0.66
7	27.18	CA	2.12 ± 0.88
8	32.49	R	5.43 ± 1.36
9	50.27	Q	11.70 ± 1.19
10	54.87	N	9.01 ± 1.72

[a] PCA, protocatechuic acid; GC, gallocatechin; GCG, gallocatechin gallate; Q3G, quercetin 3-glucoside; CA, caffeic acid; R, rutin; Q, quercetin; N, naringenin.

PPAR-γ expression. As GW9662 was used as the PPAR-γ antagonist, Figure 7**C** shows that PPAR-γ agonist rosiglitazone increased the acLDL uptake, while MLE and MLPE reduced the rosiglitazone-induced uptake. These results indicate that ox-LDL stimulated the expression of PPAR-γ, thus promoting the uptake of ox-LDL.

DISCUSSION

In the present investigation, we observed that both MLE and MLPE inhibited the oxidation of LDL and reduced the intracellular ROS generation of macrophages. MLE and MLPE possessed good abilities to quench free radicals and lipid peroxides. Furthermore, both of them enhanced the expression of antioxidant enzymes. MLE and MLPE decreased the expression of scavenger receptors via downregulating the transcription factor PPAR-γ, thus inhibiting the ox-LDL uptake, foam cell formation, and the intracellular lipid accumulation. However, compared with MLE, MLPE exhibited the superior effect.

The HPLC analysis showed that at least eight kinds of polyphenols were contained in the mulberry leaves, among which quercetin, gallocatechin gallate and naringenin are the main constitutes. Quercetin and naringenin, two kinds of flavonoids, inhibited lipopolysaccharide (LPS)-induced activation of macrophages, as they reduced the levels of NO, IL-1 and TNF-2.[24,25] Quercetin and its glycosides inhibited lipoxygenase-induced LDL oxidation.[26] Quercetin-3-glucuronide, a major quercetin metabolite in human plasma, suppressed inflammatory mediator production via regulating the MAPK and NF-κB pathways.[27] Enkhmaa et al. reported that mulberry leaves attenuated the

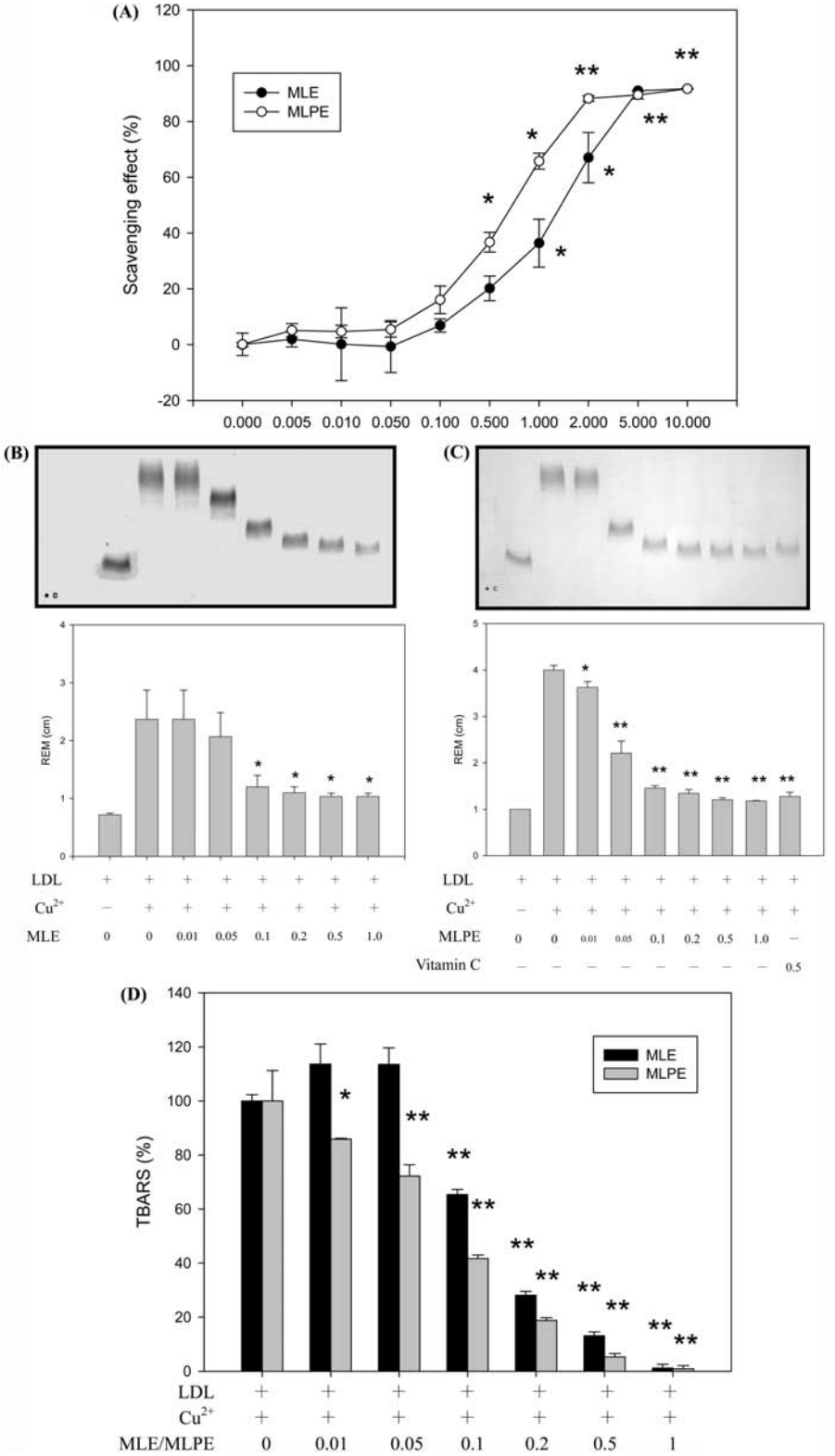

Figure 2. (**A**) The antioxidant effect of MLE and MLPE. DPPH (1 mM) was incubated with or without different concentrations of MLE or MLPE (mg/mL). Data are presented as mean ± SD (n = 3) from three independent experiments. *, $p < 0.05$; **, $p < 0.01$, as compared with the control. (**B**, **C**) Inhibitory effects of MLE and MLPE on the Cu^{2+}-induced oxidation of LDL. LDL (100 μg/mL) was incubated with 10 μM $CuSO_4$ at 37 °C for 24 h in the presence or absence of different concentrations of MLE (**B**) or MLPE (**C**). The change in relative electrophoretic mobility (REM) was determined. Vitamin C-treated group was used as a positive control. Data are presented as mean ± SD (n = 3) from three independent experiments. *, $p < 0.05$; **, $p < 0.01$, as compared with column 2. (**D**) Inhibitory effects of MLE and MLPE on the Cu^{2+}-induced lipid peroxidation of LDL. LDL (100 μg/mL) was incubated with 10 μM $CuSO_4$ at 37 °C for 24 h in the presence or absence of different concentrations of MLE or MLPE (0.1−1 mg/mL). Data are presented as mean ± SD (n = 3) from three independent experiments. *, $p < 0.05$; **, $p < 0.01$, as compared with the control.

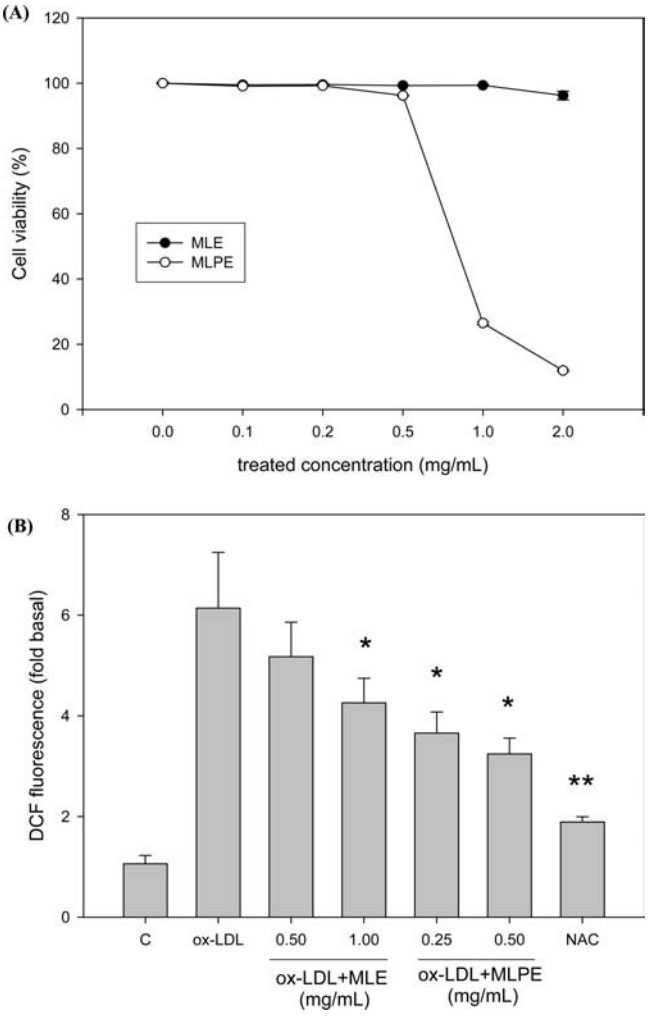

Figure 3. Effects of MLE and MLPE on ox-LDL-induced intracellular ROS production of macrophages. (**A**) J774A.1 cell was incubated with different concentrations of MLE or MLPE at 37 °C for 24 h. Cell viability was measured by MTT assay. (**B**) J774A.1 macrophages were preincubated with indicated concentrations of MLE or MLPE or 1 mM NAC for 1 h, and then incubated with 50 μg/mL ox-LDL for 30 min. The intracellular ROS level was determined by H_2DCF-DA. Data are presented as mean ± SD (n = 3) from three independent experiments. *, $p < 0.05$; **, $p < 0.01$, as compared with ox-LDL group.

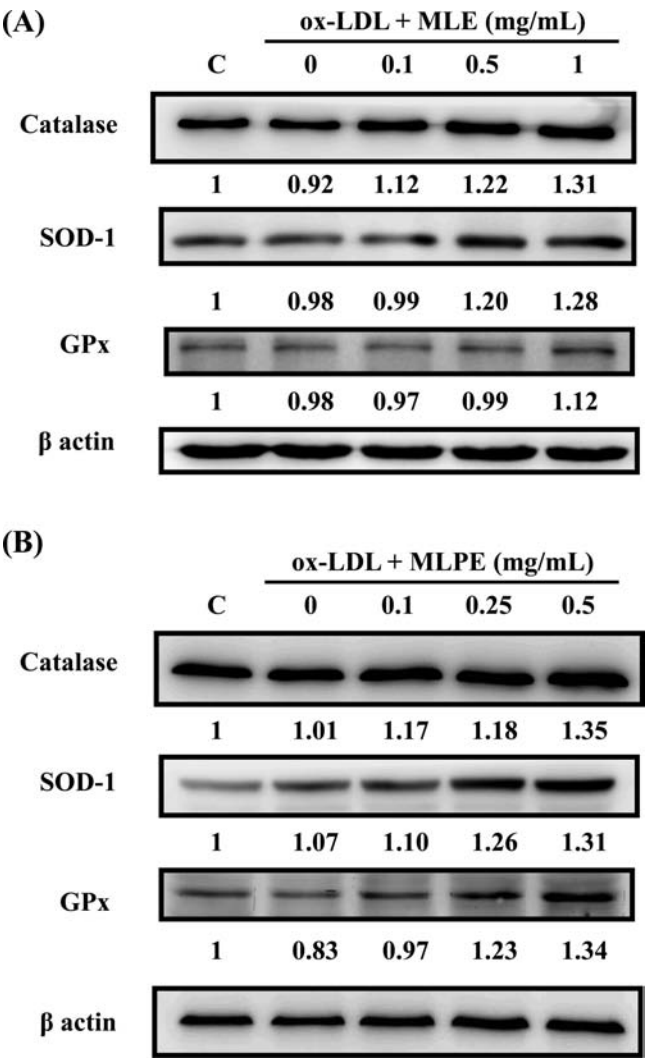

Figure 4. Effects of MLE and MLPE on the antioxidative enzyme protein expression in macrophages. J774A 1 cells were treated with or without 50 μg/mL ox-LDL for 20 h, in the presence of indicated concentrations of MLE or MLPE. The proteins isolated from cells were analyzed with Western blot and detected by anti-catalase, SOD-1 and GPx antibody. All data is presented as the fold relative to the control. Three independent experiments were conducted, all showing similar patterns of changes.

atherosclerotic lesion development attributing mainly to Q3MG, the quantitatively major flavonol glycoside.[28] In addition to quercetin derivatives, naringenin inhibited the AP-1 phosphorylation in LPS-stimulated macrophages.[25] Hence the antioxidative and macrophage-inhibitory effect of mulberry leaves could be attributed to the composition of flavonoids. On the other hand, about 10% of the MLPE were identified as gallocatechin gallate. It was shown that gallate-containing catechins inhibited IkappaBalpha degradation and NF-κB activation, therefore leading to the reduction of inflammatory cytokines.[29] Gallocatechin gallate also showed its potency in reducing LDL-oxidation.[30] Considering the association among inflammation, oxidation, and macrophage activation, the antiatherogenic effects of MLE or MLPE could be partly caused by gallocatechin gallate. Actually, the antiatherogenic effects could also be attributed to other identified components including rutin and protocatechuic acid, as well as the unidentified proportions contained in mulberry leaves.

In addition to acting as the antioxidants, MLE and MLPE also were shown to stimulate the expression of antioxidative enzymes like SOD-1, catalase and GPx, hence promoting ROS scavenging. Compared with MLE, MLPE was obviously more effective in elevating SOD-1 and GPx dose-dependently, indicating the superior effect of MLPE in scavenging the superoxide anion and activating the glutathione system.

The oxidative modification of LDL plays a crucial role in atherogenesis. However, LDLs in human comprise various subtypes which differ in metabolic behavior and pathologic role. This heterogeneity could result from different VLDL or IDL precursors, metabolic remodeling and direct production. LDLs have been grouped from the largest, buoyant to the smallest and densest ones, based on their density. The small dense LDL was commonly designated as the main atherogenic lipoprotein phenotype, which was suggested to have greater transport ability and increased oxidative modification.[31] The oxidative modification

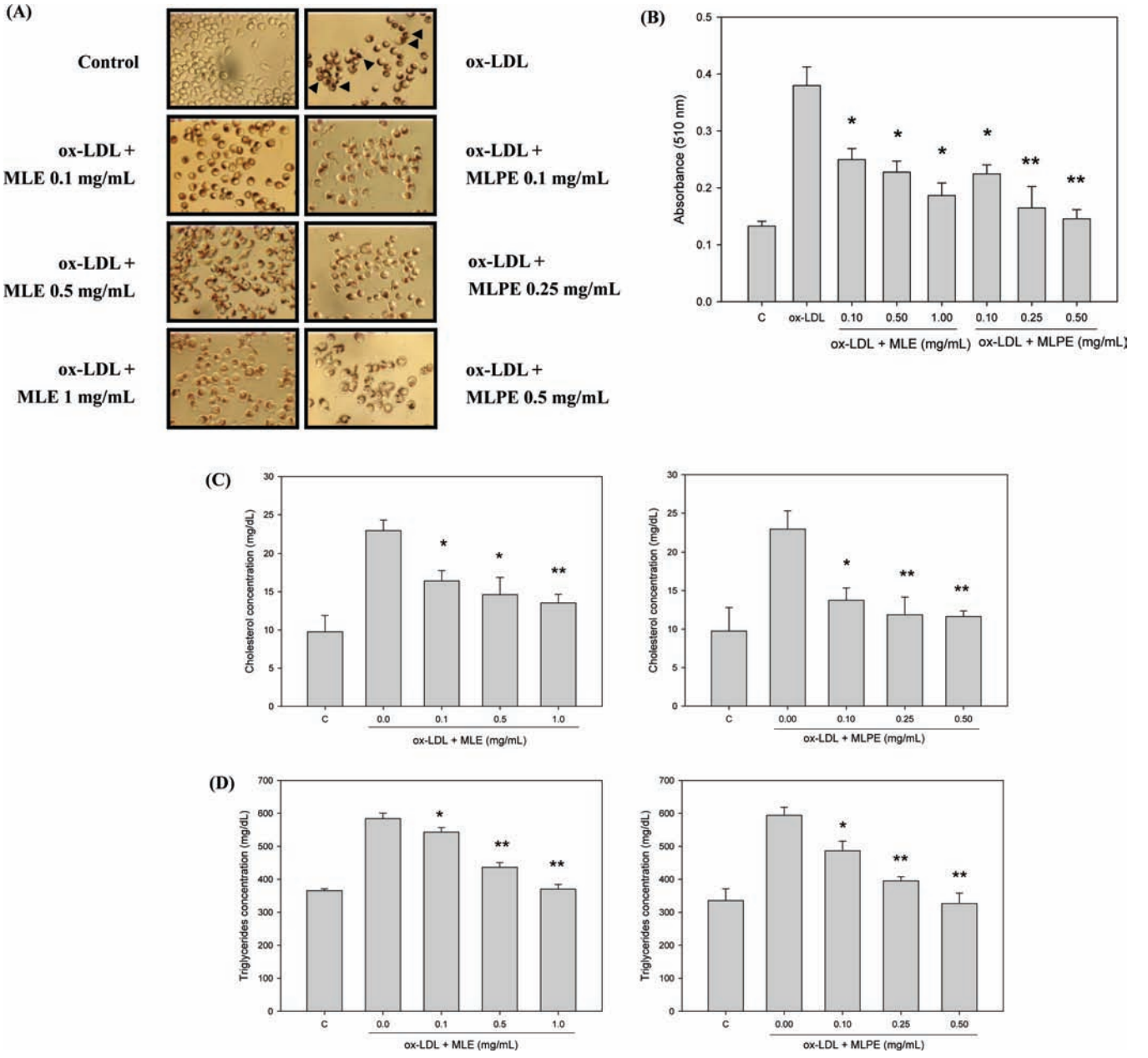

Figure 5. Inhibitory effects of MLE and MLPE on the ox-LDL-induced foam cell formation and intracellular lipid accumulation. (**A**) J774A.1 cells were cultured in DMEM medium with MLE or MLPE and then combined with 50 μg/mL of ox-LDL at 37 °C, 5% CO_2 for 24 h. These cells were stained with Oil Red O and then observed under the microscope (200×). The red droplets accumulated in the cells were indicated as the stained lipid (black arrows). (**B**) Adding 1 mL of isopropanol to the stained culture dish, the extracted dye was diluted 5× in ddH_2O, and then its absorbance was monitored at 510 nm. Total intracellular cholesterol (**C**) and triglyceride (**D**) were analyzed using enzymatic colorimetric method and expressed as mg/dL. Data were presented as mean ± SD (n = 3) from three independent experiments. *, $p < 0.05$; **, $p < 0.01$, as compared with ox-LDL group.

resulted in diminished affinity for LDL receptors and increased affinity for macrophage scavenger receptors.[32] Since MLE and MLPE inhibited LDL oxidation and macrophage scavenging, it deserved further investigation on whether MLE and MLPE could specially target the small dense LDL.

PPARγ is a member of a nuclear hormone receptor superfamily that heterodimerizes with the retinoid X receptor (RXR). These proteins are transcriptional regulators of genes that encode proteins involved in adipogenesis and lipid metabolism.[33] Two metabolites on ox-LDL, linoleic acid 9-hydroxyoctadecadienoic acid (9-HODE) and 13-HODE, were reported as potent activators of PPARγ.[34] Receptor-mediated endocytosis internalized PPARγ ligands and activators. Since CD36 was transcriptionally regulated by PPARγ,[8] macrophage expression of CD36 and foam cell formation might be perpetuated by a cycle in which ox-LDL drived its own uptake.

However, loss of SR-A or CD36-mediated lipid uptake did not ameliorate the atherosclerosis in hyperlipidemic mice.[35] Actually, ox-LDL can bind with high affinity to several receptors, including SR-A, CD36, CD68, LOX-1, and possibly others, which internalize ox-LDL and lead to its degradation.[36] On the other hand, since native LDL may provide greater amounts of lipid to generate

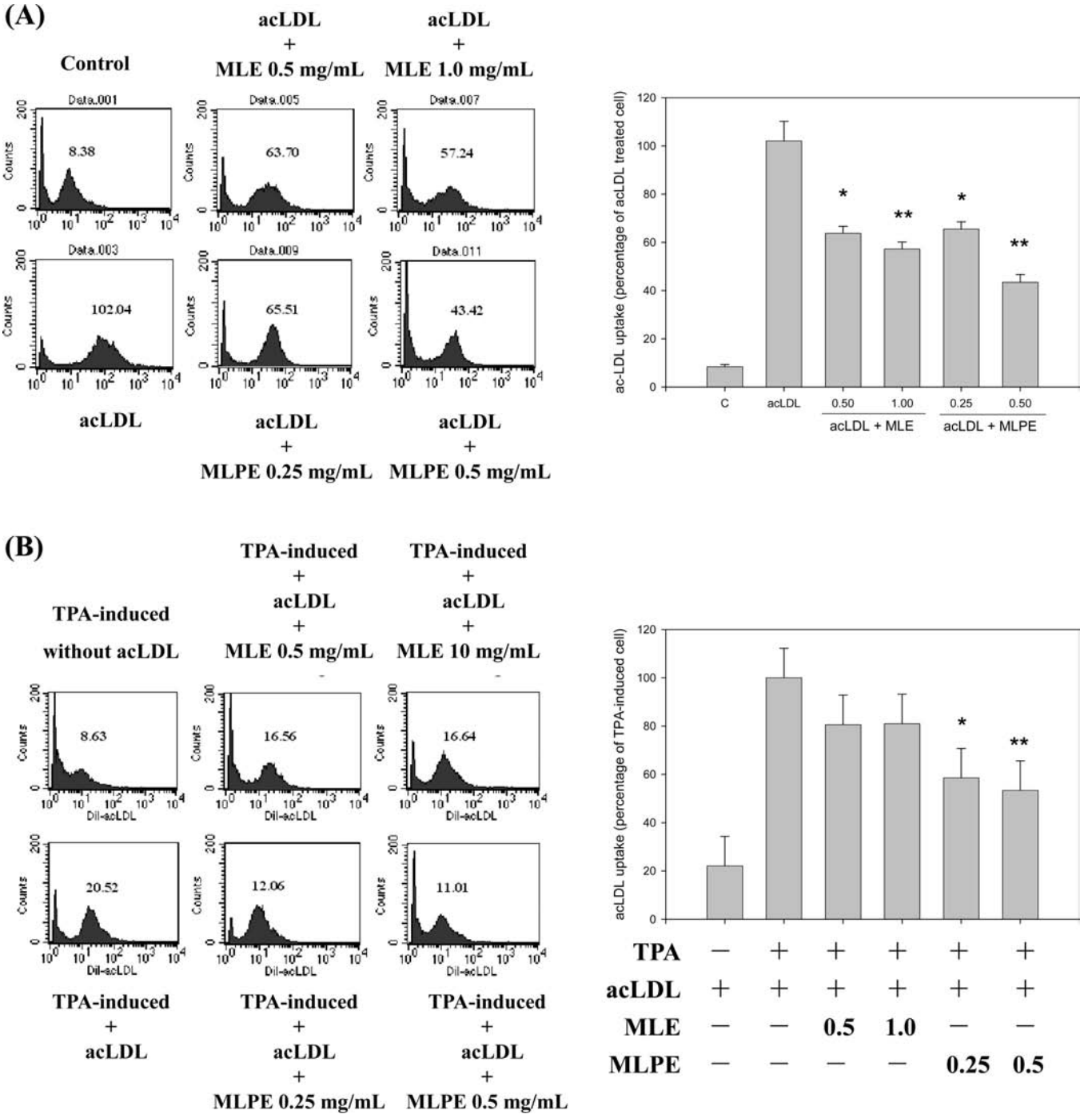

Figure 6. Dose-dependent inhibition of MLE and MLPE on DiI-acLDL uptake. (**A**) J774A.1 cells were treated with MLE (0.5 or 1 mg/mL) or MLPE (0.25 or 0.5 mg/mL) and incubated with 5 μg/mL of DiI-acLDL in DMEM medium for 4 h. (**B**) THP-1 cells were treated with or without TPA (200 nM) in the presence or absence of MLE (0.5 or 1 mg/mL) or MLPE (0.25 or 0.5 mg/mL), and then incubated with 5 μg/mL of DiI-acLDL in RPMI-1640 medium for 4 h. DiI fluorescence was analyzed by flow cytometry, and specific fluorescent intensity was determined by subtracting the mean fluorescent intensity of unlabeled cells (autofluorescence) from that of DiI-acLDL incubated cells. Data are presented as mean ± SD ($n = 3$) from three independent experiments. *, $p < 0.05$; **, $p < 0.01$, as compared with ox-LDL group.

foam cells, the non-receptor-mediated pathways were also hypothesized. It was reported that macrophages can accumulate LDL via macropinocytosis, and enzyme modifications of LDL converted native LDL engendering foam cell formation. Furthermore, the LDL receptor-related protein (LRP) was implicated in the uptake of LDL.[35]

In our previous reports, MLE was demonstrated to inhibit vascular smooth muscle cell migration via blocking small GTPase and Akt/NF-κB signals.[37] MLE was rich in polyphenols and inhibited vascular smooth muscle cell proliferation by upregulation of p53 and inhibition of cyclin-dependent kinase.[38] In addition to the antiatherogenic effect, mulberry leaf was beneficial for

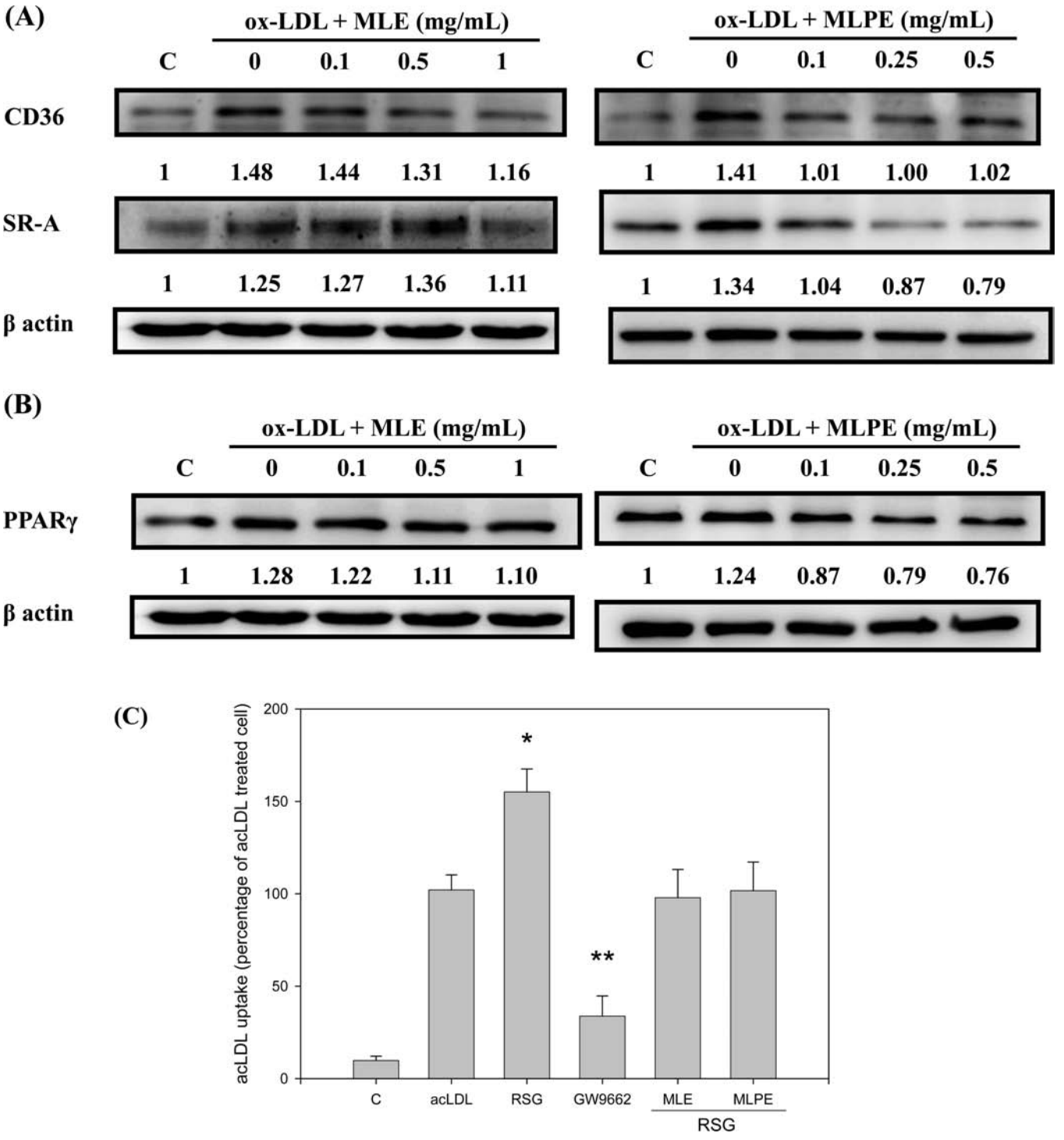

Figure 7. Effect of MLE and MLPE on the expressions of macrophage scavenger receptors. J774A.1 cells were treated with or without 50 μg/mL ox-LDL for 20 h, in the presence of indicated concentrations of MLE or MLPE. The proteins isolated from cells were analyzed with Western blot and detected by anti-CD36, SR-A (**A**) and PPAR-γ (**B**) antibody. All data was presented as the fold relative to the control. Three independent experiments were conducted, all showing similar patterns of changes. (**C**) J774A.1 macrophages were pretreated with rosiglitazone (1 μM), GW9662 (1 μM) for 48 h, and then incubated with MLE (1 mg/mL) or MLPE (0.5 mg/mL) in DMEM containing DiI-acLDL (5 μg/mL) for 4 h. The fluorescence was quantified by flow cytometry. Data are presented as mean ± SD (n = 3) from three independent experiments.

obesity and metabolic disorders. The ethanolic extract of *Morus alba* leaves were shown to lower hepatic lipid and body weight in diet-induced obese mice.[39] In the animal model of db/db mice, mulberry leaf decreased blood glucose and plasma triglyceride.[40] Treatment of mulberry leaf increased the expression of adiponectin but decreased the expression of TNF-alpha and MCP-1, thus ameliorating the adipocytokine dysregulation in white adipose tissue. As body weight increase was observed under the PPARγ agonist

pioglitazone treatment, combined treatment with mulberry leaf and pioglitazone showed additive effects, and reduced the adverse effects of pioglitazone used alone.[40]

In consideration of their synergistic effects on cellular changes of macrophages, VSMC, and the metabolic regulations, mulberry extract MLE and MLPE deserve to be further investigated and could be developed as an antiatherosclerotic agent in the future.

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