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Polyphenols from *Ilex latifolia* Thunb. (a Chinese bitter tea) exert anti-atherosclerotic activity through suppressing NF- κ B activation and phosphorylation of ERK1/2 in macrophages

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Ilex latifolia Thunb is a kind of herbal tea and widely consumed as a functional tea beverage in Asian countries. In this study, polyphenols were extracted from *I. latifolia* and the major compounds were identified by liquid chromatography-mass spectrometry (LC-MS), then the effect on oxidized low-density lipoprotein (ox-LDL)-induced macrophage foam cell formation was investigated. Results showed that the polyphenols could significantly inhibit ox-LDL-induced macrophage foam cell formation and suppress lipid droplet accumulation and cholesterol uptake in RAW 264.7 cells. Additionally, the secretion of pro-inflammatory cytokines, such as tumor necrosis factor (TNF- α), interleukin (IL)-1 β , IL-6 and inducible nitric oxide synthase (iNOS), was significantly inhibited. Moreover, the polyphenols could suppress the expression of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) and clusters of differentiation 36 (CD 36), which were receptors for ox-LDL. Mechanistically, *I. latifolia* polyphenols could inhibit macrophage foam cell formation by suppressing NF- κ B activation and phosphorylation of ERK1/2.

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1. Introduction

Atherosclerosis is a chronic inflammatory disease, which is characterized by the accumulation of lipids and fibrous elements that result from interactions between vascular cells and inflammatory cells.¹ Inflammation has been suggested to be a key mediator of many events during atherosclerosis development, from initiation through to progression, and ultimately drives the thrombotic complications of atherosclerosis.² In the pathogenesis of atherosclerosis, macrophages and oxidized low-density lipoproteins (ox-LDL) are important for intracellular lipid accumulation and foam cell formation.³ Additionally, ox-LDL stimulate macrophages to release pro-inflammatory cytokines such as interleukin (IL)-1 β and tumor necrosis factor (TNF)- α .⁴

Pharmacological optimization as well as limited revascularization and reconstructive or replacement options are the primary therapies currently, but they are accompanied by a high rate of recidivism. Despite advances in the interventional and pharmacological therapy of atherosclerotic disease, it is still the leading cause of morbidity and mortality in many countries. Due to security issues on the use of the above treatments, dietary supplements are becoming attractive alternatives as atherosclerosis-preventive agents.

Ilex latifolia Thunb, called ku-ding-cha in Chinese, is a kind of herbal tea and widely consumed in Asian countries, such as China, Singapore, Malaysia, Vietnam and so on.⁵ *I. latifolia* has become more popular in recent years due to its public acceptance as a functional tea beverage with highly advantageous nutrients and many beneficial functions including antioxidant, anti-obesity, anti-diabetic, hepatoprotective and neuroprotective effects.⁶ Recently, an increasing number of researchers have become interested in the extraction and identification of the main active substances in *I. latifolia* and their potential health benefits.⁵ We have successfully separated five compounds from the ethyl acetate fraction of *I. latifolia* in our previous study, and the major compounds are chlorogenic acid and its derivatives, such as 3,4-di-O-caffeoylquinic acid methyl ester and 3,5-di-O-caffeoylquinic acid methyl ester.⁶ Therefore, searching for alternative natural active ingredients such as polyphenols in *I. latifolia* is

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necessary for its application in food additives and health products.

However, little attention has been paid to the effects of polyphenols from *I. latifolia* on ox-LDL-induced foam cell formation in RAW 264.7 macrophages. In this context, the aim of this work was to investigate the effects of polyphenols from *I. latifolia* on ox-LDL-induced foam cell formation in RAW 264.7 macrophages and to explore the possible molecular mechanism involved.

2. Results and discussion

2.1. Tentative identification of the major compounds in polyphenols from *I. latifolia*

As shown in Fig. 1 and Table 1, LC-MS analysis of polyphenols from *I. latifolia* revealed the presence of (1) quinic acid, (2) 3-caffeoylquinic acid, (3) 5-caffeoylquinic acid, (4) shikimic acid, (5) 4-caffeoylquinic acid, (6) rutin, (7) hyperoside, (8) 3,4-di-caffeoylquinic acid, (9) 3,5-di-caffeoylquinic acid and (10) 4,5-di-caffeoylquinic acid by comparison with the previously reported spectral data, and their chemical structures are shown in Fig. 2.^{7–10} The major compounds in polyphenols from *I. latifolia* are chlorogenic acid and its derivatives.

2.2. The effect of polyphenols from *I. latifolia* on ox-LDL-induced foam cell formation in macrophages

Foam cells, pathogenic cells in atherosclerosis, are derived from monocytes/macrophages and vascular smooth muscle

cells.¹¹ RAW264.7 cells display many features in common with macrophages, which can be induced to form foam cells by ox-LDL *in vitro*.³ To determine whether polyphenols extracted from *I. latifolia* can affect foam cell formation, RAW264.7 cells were treated with ox-LDL in the absence or presence of different doses of polyphenols for 24 h and were then stained with oil-red O solution. First, the cytotoxicity of polyphenols on RAW 264.7 cells was detected by an MTT assay and the results were shown as relative cell viability compared to a control (equal to 100%). As shown in Fig. 3A, the viability of RAW 264.7 cells upon treatment with polyphenols was high (>97%), indicating that polyphenols from *I. latifolia* did not cause cytotoxicity within RAW 264.7 cells in a concentration range between 100 and 300 $\mu\text{g mL}^{-1}$; therefore, these concentrations of polyphenols were used in the subsequent *in vitro* experiments. RAW264.7 cells exhibited foamy characteristics with oil red staining of lipid droplets after incubation with ox-LDL for 24 h, but treatment with polyphenols significantly reduced the foam cell formation in a concentration-dependent manner (Fig. 3B). Moreover, the cellular cholesterol level in RAW 264.7 cells was evaluated by ELISA. Results showed that ox-LDL stimulation for 24 h resulted in a significant increase in cellular cholesterol (Fig. 3C). Furthermore, treatment with different concentrations of polyphenols could dramatically inhibit the cellular cholesterol levels in ox-LDL-induced RAW 264.7 cells in a dose-dependent manner, which was consistent with the results in the oil red staining

Fig. 1 LC-MS chromatogram of polyphenols from *I. latifolia* (peak assignments are listed in Table 1).

Table 1 Identification of the chemical constituents in polyphenols from *I. latifolia*

No.	t_R (min)	$[M - H]^- m/z$	Formula	MS ⁿ	Proposed compound
1	1.07	191.0558	C ₇ H ₁₁ O ₆	MS ² : 173, 127	Quinic acid
2	2.94	353.0874	C ₁₆ H ₂₁ O ₁₄	MS ² : 191.0559, 179.0347 MS ³ : 173.0449, 127.0396, 85.0293, 93.0343	3-Caffeoylquinic acid
3	4.59	353.0874	C ₁₆ H ₂₁ O ₁₄	MS ² : 191.0559, 179.0347 MS ³ : 173.0449, 127.0396, 85.0293, 93.0343	5-Caffeoylquinic acid
4	5.14	173.0453	C ₇ H ₉ O ₅	MS ² : 155.0343, 111.0446, 93.0342	Shikimic acid
5	6.75	353.0874	C ₁₆ H ₂₁ O ₁₄	MS ² : 191.0559, 179.0347 MS ³ : 173.0449, 127.0396, 85.0293, 93.0343	4-Caffeoylquinic acid
6	12.1	609.1450	C ₂₇ H ₂₉ O ₁₆	MS ² : 301.0341 MS ³ : 178.9981, 151.0033, 271.0237, 255.0289	Rutin
7	12.9	463.0873	C ₂₁ H ₁₉ O ₁₂	MS ² : 301.0342 MS ³ : 178.9981, 151.0033, 271.0237, 255.0289	Hyperoside
8	14.92	515.1186	C ₂₅ H ₂₃ O ₁₂	MS ² : 353.0867, 434.1022 MS ³ : 191.0555, 179.0344	3,4-Di-caffeoylquinic acid
9	15.47	515.1186	C ₂₅ H ₂₃ O ₁₂	MS ² : 353.0867, 434.1022 MS ³ : 191.0555, 179.0344	3,5-Di-caffeoylquinic acid
10	18.04	515.1186	C ₂₅ H ₂₃ O ₁₂	MS ² : 353.0867, 434.1022	4,5-Di-caffeoylquinic acid

experiment. All of these results suggested that polyphenols from *I. latifolia* could significantly reduce foam cell formation, which was mainly due to inhibiting lipid droplet accumulation and cholesterol uptake in macrophages. The anti-atherosclerotic activity of polyphenols from *I. latifolia* may be related to its phenolic substrates, especially chlorogenic acid and its derivatives.

2.3. The effect of polyphenols from *I. latifolia* on the mRNA expression of pro-inflammatory cytokines and iNOS in ox-LDL-induced macrophages

Macrophages have been demonstrated to be abundant in plaque lesions and play a central role in atherosclerosis development.¹² In addition to inducing foam cell formation, ox-LDL can contribute to the chronic inflammatory process and are known to be a potent inducer of inflammatory cytokines such as TNF- α , IL-1 β and IL-6, thus promoting atherosclerosis.¹³ Inducible nitric oxide synthase (iNOS) is an enzyme expressed in atherosclerotic lesions, which could generate high concentrations of nitric oxide and superoxide.¹⁴ It is not found in healthy vessels, but in the microenvironment of inflammatory atherosclerotic lesions. To examine the effect of polyphenols from *I. latifolia* on the ox-LDL-induced production of TNF- α , IL-1 β , IL-6 and iNOS in macrophages, the production of these cytokines by ox-LDL-stimulated RAW264.7 cells was determined in the absence or presence of polyphenols. Ox-LDL stimulation for 24 h led to marked increases ($P < 0.01$) of TNF- α , IL-1 β , IL-6 and iNOS mRNA expression (Fig. 4). Furthermore, treatment with polyphenols (at the concentration of 100, 200 and 300 $\mu\text{g mL}^{-1}$) could significantly inhibit ox-LDL-induced production of TNF- α , IL-1 β , IL-6 and iNOS in a dose-dependent manner, which suggested that polyphenols might inhibit ox-LDL-induced pro-inflammatory cytokines and iNOS expression at the transcriptional level.

2.4. Polyphenols from *I. latifolia* inhibit ox-LDL-induced LOX-1 and CD36 expression in macrophages

LOX-1 is a lectin-like receptor for ox-LDL in endothelial cells, smooth muscle cells, and macrophages, and plays important roles in the development of atherosclerosis.¹⁵ To explore whether polyphenols from *I. latifolia* can restrain foam cell formation by blocking ox-LDL-induced LOX-1 expression, RAW 264.7 cells were treated with ox-LDL in the absence or presence of polyphenols for 24 h. Then the effect of polyphenols from *I. latifolia* on ox-LDL-induced LOX-1 expression was investigated by western blot analysis of RAW264.7 cell proteins. Results found that the incubation of RAW 264.7 cells with ox-LDL resulted in marked up-regulation of LOX-1 expression compared with an untreated control (Fig. 5A). However, co-treatment of the cells with polyphenols (100–300 $\mu\text{g mL}^{-1}$) could significantly inhibit the ox-LDL-mediated increase of LOX-1 protein levels in a dose-dependent manner (Fig. 5A).

CD36 (cluster of differentiation 36), a class B scavenger receptor, plays a quantitatively crucial role in ox-LDL uptake and cholesterol accumulation in macrophages, which are well-known risk factors for the development of atherosclerosis.¹⁶ Approximately 60–70% of macrophage-derived foam cell formation is caused by CD36-mediated ox-LDL uptake. Inhibition of CD36 expression could significantly decrease the ability of macrophages to accumulate ox-LDL and reduce the development of atherosclerosis, suggesting that CD36 plays a pro-atherogenic role in foam cell formation and it could be an important target for therapeutic treatment.¹⁷

To examine whether polyphenols from *I. latifolia* can induce CD36 expression in macrophages, the CD36 protein levels were evaluated by western blotting. The CD36 expression of ox-LDL treated cells was markedly up-regulated compared to unstimulated cells, but was significantly reduced in a concentration-dependent manner when cells were treated with polyphenols (100–300 $\mu\text{g mL}^{-1}$) (Fig. 5B). To

Fig. 2 The chemical structures of main polyphenols in *I. latifolia*: (1) quinic acid; (2) 3-caffeoylquinic acid; (3) 5-caffeoylquinic acid; (4) shikimic acid; (5) 4-caffeoylquinic acid; (6) rutin; (7) hyperoside; (8) 3,4-di-caffeoylquinic acid; (9) 3,5-di-caffeoylquinic acid; and (10) 4,5-di-caffeoylquinic acid.

determine whether the reduced expression levels of CD36 could be attributed to the reduced mRNA levels of CD36, real-time PCR was performed. As shown in Fig. 5C, polyphenols from *I. latifolia* significantly suppressed the mRNA levels of CD36, which indicated that polyphenols may inhibit ox-LDL-induced CD36 expression at the transcriptional level.

2.5. The effect of polyphenols from *I. latifolia* on NF- κ B activation and phosphorylation of ERK1/2 in ox-LDL-induced macrophages

It has been found that ox-LDL could induce the LOX-1 expression and related inflammatory cytokines, such as TNF- α and IL-6, through nuclear factor κ B (NF- κ B) activation.¹⁸ NF- κ B activation in endothelial cells and macrophages plays a

Fig. 3 Polyphenols extracted from *I. latifolia* inhibit ox-LDL-induced foam cell formation. (A) RAW264.7 cells were treated with various doses of polyphenols for 24 h, and the MTT assay was performed. Polyphenols showed no cytotoxicity in RAW264.7 cells. (B) RAW264.7 cells were treated with various doses of polyphenols along with ox-LDL (50 $\mu\text{g mL}^{-1}$) for 24 h, and the oil red staining experiment was performed. Representative images are shown. (C) Cellular cholesterol content was determined by an enzymatic method in RAW264.7 cells. The values indicate the means $\pm$ SD from three independent experiments. Different letters represent significant differences ($P < 0.05$).

central role in modulating gene expression by multiple atherogenic factors.¹⁹ In order to explore whether the anti-atherosclerotic reactions by polyphenols from *I. latifolia* were mediated through the NF- κ B pathway, we assessed the effects of polyphenols on ox-LDL-induced NF- κ B activation in ox-LDL-stimulated RAW 264.7 macrophages. As shown in Fig. 6A, the expression of NF- κ B increased (compared with the control group) after treatment with ox-LDL (3 h) in RAW 264.7 macrophages. The co-treatment with polyphenols from *I. latifolia* (100–300 $\mu\text{g mL}^{-1}$) significantly inhibited ox-LDL-induced NF- κ B activation in a concentration-dependent pattern. The experimental results are well consistent with those of published reports. It has been previously reported that NF- κ B activation was significantly suppressed by hydrogen sulfide in ox-LDL-stimulated RAW 264.7 macrophages.²⁰ These data suggest that the NF- κ B pathway might be related to the anti-atherosclerotic activity of polyphenols from *I. latifolia*.

The activation of MAPK (mitogen-activated protein kinases)-signaling pathways has been associated with the stimulatory effects of ox-LDL, relevant to vascular pathology, in cultured cells implicated in macrovascular disease.²¹ Extracellular signal regulated kinase (ERK), c-jun N-terminal kinase (JNK) and P38 are three important parallel pathway factors in the MAPK pathway. Some previous reports have indicated that ox-LDL could activate the phosphorylation of ERK1/2, which is considered to be involved in PPAR γ expression induced by several stimuli in macrophages.^{22,23,28} To further explore whether polyphenols from *I. latifolia* could inhibit the ERK1/2 signaling pathway, the effects of polyphenols on ox-

LDL-induced ERK1/2 phosphorylation in RAW 264.7 cells were examined. As shown in Fig. 6B, incubation with ox-LDL for 3 h led to a high level of ERK1/2 phosphorylation compared with un-stimulated cells. When given the co-treatment with polyphenols from *I. latifolia* at the concentration of 300 $\mu\text{g mL}^{-1}$, the ox-LDL-induced ERK1/2 phosphorylation activation was decreased to some extent, suggesting that polyphenols from *I. latifolia* could inhibit the activation of ERK1/2 signaling. These above results suggested that polyphenols from *I. latifolia* could inhibit macrophage foam cell formation by suppressing NF- κ B activation and the phosphorylation of ERK1/2 (Fig. 7).

3. Materials and methods

3.1. Plant materials and reagents

I. latifolia, cultivated in Hainan Province, China, was purchased from Forest Drugstore Chain Co. (Guangzhou, China) and was authenticated by the South China Botanical Garden, Chinese Academy of Sciences, where voucher specimens were kept. Then they were air-dried under shade for one week and ground into a fine powder with a cutting mill, which was stored at room temperature (25 ± 2 °C) in a well-closed container until use.

Dulbecco's modified Eagle medium (DMEM), foetal bovine serum (FBS) and penicillin/streptomycin were purchased from GIBCO (Grand Island, NY, USA). (3-4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), oil red O and a bicinchoninic acid (BCA) kit were obtained from Sigma

Fig. 4 Polyphenols extracted from *I. latifolia* significantly inhibit ox-LDL-induced production of TNF- α (A), IL-1 β (B), IL-6 (C) and iNOS (D). RAW264.7 cells were stimulated with ox-LDL in the presence of different concentrations of polyphenols for 24 h. The mRNA levels of TNF- α , IL-1 β , IL-6 and iNOS were measured by real-time PCR. The values indicate the means $\pm$ SD from three independent experiments. ** $P < 0.01$ as compared with the control group. Different letters represent significant differences ($P < 0.05$).

Aldrich (St. Louis, MO, USA). Ox-LDL were obtained from Yiyuan Biotechnologies (Guangzhou, China). TRIzol Reagent was purchased from Invitrogen (Carlsbad, CA, USA). The antibodies against phospho-ERK, NF- κ B and GAPDH were obtained from Cell Signaling Technology (Beverly, MA, USA). The antibody against LOX-1 was obtained from R&D systems (Minneapolis, MN, USA). The antibody against CD36 was obtained from NOVUS biologicals (Littleton, CO, USA). The water used in all assays was ultrapure due to use of a Milli-Q water purification system from Millipore. All other chemicals and solvents used in this study were of analytical grade.

3.2. Extraction of polyphenols

Ultrasound-assisted extraction was conducted following the previously reported method with some modifications.²⁴ Briefly, ten grams of the dried powder was mixed with 200 mL of 50% (v/v) aqueous ethanol, and then the mixture was subjected to continuous ultrasonic treatment in a 60 W (ultrasonic power) ultrasonic bath for 100 min at 30 °C. After centrifuging at 1500 $\times$ g for 10 min, the supernatant was recov-

ered and then evaporated to near dryness under a vacuum at 40 °C. The obtained polyphenols were stored at 4 °C in a refrigerator for further analysis.

The Folin-Ciocalteu method was used to determine the total phenolic content (TPC) with some modifications.²⁵ The total polyphenolic content was calculated on the basis of the standard curve for gallic acid and expressed as mg of gallic acid equivalents (GAE) per g dry material plant (mg GAE per g plant material). The calibration curves (five data points) were linear with $R^2 = 0.999$.

3.3. LC-MS analysis

LC analysis was carried out on a Thermo Accela ultra-high performance liquid chromatography (UHPLC) system (Thermo Fisher Scientific, San Jose, CA, USA) equipped with a quaternary pump, a diode-array detector (DAD), an auto-sampler, and a thermostatically controlled column compartment.²⁶ The chromatographic separation of analytes was carried out by an AcQuity UPLC™ BEH C18 column (2.1 mm $\times$ 50 mm, 1.7 μm). The final mobile phase was composed of

Fig. 5 Polyphenols extracted from *I. latifolia* attenuate ox-LDL-induced LOX-1 and CD36 expression. RAW264.7 cells were treated with ox-LDL ($50 \mu\text{g mL}^{-1}$) in the absence or presence of polyphenols for 24 h. LOX-1 (A) and CD36 (B) protein levels were measured by western blot analysis. (C) CD36 mRNA expression was measured using real-time PCR. The values indicate the means $\pm$ SD from three independent experiments. $**P < 0.01$ as compared with the control group. Different letters represent significant differences ($P < 0.05$).

acetonitrile (A) and water containing 0.1% formic acid (B) using the following gradient program: 12% A (0 min), 18% A (6 min), 26% A (20 min) and 95% A (24 min). A pre-equilibration period of 4 min was used between individual

runs. The mobile phase flow rate was $400 \mu\text{L min}^{-1}$, and the injection volume was $2 \mu\text{L}$. The online UV spectra were recorded in the range of 200–400 nm.

Mass spectra were obtained on a Thermo-Fisher LTQ-Orbitrap XL hybrid mass spectrometer, which was connected to the LC system via an electrospray ionization (ESI) source as an interface. Full-scan mass spectra over the m/z range 150–1200 were acquired in negative ion mode. The basic conditions of MS analysis were as follows: ion spray voltage at 3.7 KV, capillary voltage at 37 V, capillary temperature at 320°C , sheath gas flow rate at 42 psi and auxiliary gas flow rate at 6 psi. The ion trap collision induced dissociation (CID) mode was used for selected reaction monitoring fragmentation. The selected ion width was $m/z \pm 1$ and the normalized collision energy was set at 35%.

3.4. Cell culture

RAW 264.7 murine macrophages from the cell bank of the Chinese Academy of Sciences (CAS, Shanghai, China) were cultured in plastic dishes containing DMEM supplemented

Fig. 6 Polyphenols extracted from *I. latifolia* inhibit ox-LDL-induced activation of NF- κ B and p-ERK signaling. RAW264.7 cells were stimulated with ox-LDL in the absence or presence of polyphenols for 3 h, and then western blot analysis of nuclear p65 and p-ERK was performed. Representative images of three independent experiments are shown. (A) The effect of polyphenols on ox-LDL-induced nuclear NF- κ B expression. (B) The effect of polyphenols on ox-LDL-induced p-ERK expression.

Fig. 7 Possible molecular mechanisms of the anti-atherosclerosis activity of polyphenols from *I. latifolia* in ox-LDL-induced macrophages.

with 10% FBS in a 5% CO₂ humidified atmosphere at 37 °C.²⁷

3.5. Cytotoxicity

The cytotoxicity of polyphenols was analyzed using an MTT assay.^{28,29} The dried polyphenols were firstly dissolved in DMSO, and the different concentrations of polyphenol solution were obtained by dilution with culture medium to keep DMSO at less than 0.1% (v/v) to avoid solvent toxicity. A total of 6×10^3 cells per well were seeded into a 96-well plate. After incubation with polyphenols for 24 h, the media solution was removed and 100 µL media containing 0.1% MTT (0.5 mg mL⁻¹) was added to each well. Plates were incubated for an additional 4 h before adding 150 µL dimethylsulfoxide to dissolve the formazan crystals. Absorbance was measured at 570 nm using a microplate reader. The cell viability of macrophages was calculated by the following formula:

$$\text{Cell viability (\%)} = A_0/A \times 100$$

where *A* is the absorbance of macrophages without sample; *A*₀ is the absorbance of the sample.

3.6. Oil red O staining

To observe lipid droplets in RAW264.7 macrophages, cells were stained with Oil red O. RAW 264.7 macrophages were

seeded in a 6-well plate (3×10^5 cells per well; total 2 mL) overnight. After treatment, the treated cells were washed with PBS and then fixed with 4% paraformaldehyde for 20 min. The fixed cells were washed with PBS three times and stained with oil red O (0.5% w/v in 60% isopropanol) for 30 min. Finally, the stained cells were washed with PBS three times and viewed using a microscope. All experiments were repeated three times, and the representative photographs were shown.

3.7. Enzyme-linked immunosorbent assay (ELISA)

After ox-LDL and polyphenol treatments, the RAW 264.7 cells were washed twice with PBS. The cellular total cholesterol level was tested by ELISA following the manufacturer's instructions. The cellular total protein was measured by a BCA assay kit.

3.8. Quantitative real-time PCR analysis

For the analysis of mRNA expression by quantitative real-time PCR, RAW264.7 cells were harvested after incubation with or without the indicated treatment. The total RNA was extracted using TRIzol reagent and transcribed into cDNA using an RT-PCR kit with an oligo-adaptor primer according to the manufacturer's protocol. A quantitative real-time PCR reaction was performed in a Light Cycler instrument with the FastStart DNA Master SYBR Green I kit. Each sample was run and analyzed in triplicate. Quantification was normalized to the

amount of endogenous GAPDH. The nucleotide sequences of the primers used were as follows: TNF- α (forward, 5'-GGG GAT TAT GGC TCA GGG TC-3', reverse, 5'-CGA GGC TCC AGT GAA TTC GG-3'), IL-1 β (forward, 5'-CCA TGG AAT CCG TGT CTT CCT-3', reverse, 5'-GTC TTG GCC GAG GAC TAA GG-3'), IL-6 (forward, 5'-GTA CTC CAG AAG ACC AGA GG-3', reverse, 5'-TGC TGG TGA CAA CCA CGG CC-3'), iNOS (forward, 5'-CGG CAA ACA TGA CTT CAG GC-3', reverse, 5'-GCA CAT CAA AGC GGC CAT AG-3'), CD36 (forward, 5'-GAA CCT ATT GAA GGC TTA CAT CC-3', reverse, 5'-CCC AGT CAC TTG TGT TTT GAA C-3') and GAPDH (forward, 5'-CAC TCA CGG CAA ATT CAA CGG CAC-3', reverse, 5'-GAC TCC ACG ACA TAC TCA GCA-3'). The expression levels relative to the control were estimated by calculating $\Delta\Delta C_t$ and subsequently analyzed using a $2^{-\Delta\Delta C_t}$ method.^{29,30}

3.9. Western blotting

Cells were seeded in 6-well plates 24 h prior to ox-LDL and polyphenol treatment, and the culture medium was discarded. The remaining cells were harvested at the indicated conditions for western blot analysis as described previously. The cells were washed twice using ice-cold PBS and the total proteins from the treated cells were extracted using lysis buffer. The protein concentration was determined using a BCA protein assay kit according to the manufacturer's instruction. A total of 30 μ g cellular protein lysates of each sample were subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto poly vinylidene fluoride (PVDF) membranes.³¹ The membranes were blocked using 5% skim milk for 1 h at room temperature and blots were incubated with appropriate specific primary antibodies overnight at 4 °C. After washing three times for 15 min each with TBST (TBS + 0.1% Tween 20), the blots were incubated with horseradish-peroxidase-conjugated secondary antibody for 2 h at room temperature, and then followed by ECL detection. Band densities were determined using densitometric analysis and were normalized to the GAPDH level.³²

3.10. Statistical analysis

Data were collected from several independent experiments, with three replicates per experiment. All analyses were carried out by one-way analysis of variance (ANOVA) with the Tukey *post hoc* test using SPSS 11.5 software. $p < 0.05$ and $p < 0.01$ were considered to indicate a statistical significance of differences between the mean values. Bars in the graphs represent standard deviation (S.D.).

4. Conclusion

In conclusion, polyphenols from *I. latifolia* showed strong inhibition of ox-LDL-induced macrophage foam cell formation, which is mainly due to suppressing lipid droplet accumulation and cholesterol uptake in RAW 264.7 cells. Additionally, treatment with polyphenols could significantly inhibit ox-

LDL-induced pro-inflammatory cytokine expression at the transcriptional level in a dose-dependent manner. Moreover, co-treatment of the cells with polyphenols from *I. latifolia* could significantly suppress the ox-LDL-mediated expression of LOX-1 and CD 36, which play important roles in the development of atherosclerosis. Mechanistically, polyphenols from *I. latifolia* could inhibit macrophage foam cell formation by suppressing NF- κ B activation and phosphorylation of ERK1/2. The anti-atherosclerotic activity of polyphenols from *I. latifolia* may be related to its phenolic substrates, especially chlorogenic acid and its derivatives including 5-caffeoylquinic acid, 4-caffeoylquinic acid, 3,4-di-caffeoylquinic acid, 3,5-di-caffeoylquinic acid and 4,5-di-caffeoylquinic acid. It is suggested that polyphenols from *I. latifolia* could be used as a functional factor applied in functional foods for the prevention and treatment of human atherosclerosis.

Conflicts of interest

The authors declare no competing interests.

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