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Atherosclerosis

Gentiana scabra Reduces SR-A Expression and Oxidized-LDL Uptake in Human Macrophages

Chin-Sheng Lin,[1] Pang-Yen Liu,[1] Chen-Hao Lian,[2] Ching-Heng Lin,[3] Jenn-Haung Lai,[4] Ling-Jun Ho,[5] Shih-Ping Yang[1] and Shu-Meng Cheng[1]

Background: Macrophages can imbibe low-density lipoprotein (LDL) through scavenger receptors to become foam cells, which is critical in the initiation and progression of atherosclerosis. Mounting evidence suggests that the anti-inflammatory nature of Chinese herbs have the capacity to halt the complex mechanisms underlying atherosclerosis. This study examined the effects of Chinese herbs on foam cell formation.

Methods: Chinese herbs were obtained from the Sun Ten pharmaceutic company. Using oxidized LDL (OxLDL) uptake and a cell toxicity assay, we screened more than 30 types of Chinese herbs. Western blotting was used to determine expressions of scavenger receptors (SRs) and extracellular-signal-regulated kinase (ERK) activities.

Results: We found that *Gentiana scabra* reduced oxidized LDL uptake effectively in THP-1 macrophages ($p < 0.05$ vs. OxLDL treated control). Moreover, treatment with *Gentiana scabra* in THP-1 macrophages resulted in decreased expression of scavenger receptor- A (SR-A) ($p < 0.05$ vs. control). Molecular investigation revealed that *Gentiana scabra* inhibited SR-A protein expression, possibly by regulating ERK signaling pathways ($p < 0.05$ vs. control).

Conclusions: By regulating SR-A expression, *Gentiana scabra* reduced oxidized LDL uptake in human macrophages. These results support the potential use of *Gentiana scabra* in treating atherosclerosis.

Key Words: Atherosclerosis • Foam cells • *Gentiana scabra* • Macrophage • Oxidized LDL

INTRODUCTION

Atherosclerosis is a chronic inflammatory disease that originates in the walls of large- and medium-sized arteries, and causes luminal narrowing.[1-5] One of the critical steps during the process of atherosclerosis is macrophages ingesting lipids through scavenger receptors (SRs) and becoming foam cells or lipid-laden macrophages in the arterial intimae. These foam cells can release pro-inflammatory cytokines and chemokines, which further activate vascular endothelial cells and cause progress of atherosclerosis.[6] Despite the comprehensive understanding of the inflammatory events leading to atherogenesis, no specific and effective anti-inflammatory therapy has been described in the literature, and the disease remains the leading cause of death worldwide.

Herbal medicines are a common form of alternative therapy because of their supposed low toxicity and natural origins.[7,8] Recently, advanced Western medical techniques have been used to study herbal medicine, particularly to determine their therapeutic efficacy and underlying mechanisms. Several herbal medicines have been reported to have cardioprotective effects and may facilitate preventing cardiovascular disease. Recently, Tsai et al. suggested that treatment with *ginkgo biloba* extract reduced foam cell formation and suppressed

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[1]Division of Cardiology, Department of Medicine, Tri-Service General Hospital, National Defense Medical Center, Taipei, Taiwan; [2]Division of General Laboratory, Ministry of Health and Welfare, KinMen Hospital, Kinmen; [3]Best Health Chinese Medicine Clinic, Taipei; [4]Division of Allergy, Immunology and Rheumatology, Department of Internal Medicine, Chang Gung Memorial Hospital, Tao-Yuan; [5]Institute of Cellular and System Medicine, National Health Research Institute, Zhunan, Taiwan.
Address correspondence and reprint requests to: Dr. Shu-Meng Cheng, Division of Cardiology, Tri-Service General Hospital, No. 325, Sec. 2, Cheng-Kung Rd., Neihu 114, Taipei, Taiwan. Tel: 886-2-8792-7160; Fax: 886-2-6601-2656; E-mail: dmscmsc@yahoo.com.tw

atherosclerosis in atherogenesis-prone mice.[9] A study by Ling et al. demonstrated that *Salvia miltiorrhiza* and *Panax notoginseng* effectively inhibited tumor necrosis factor-α-induced adhesion molecule expressions in human umbilical vein endothelial cells.[8] Moreover, Chinese herbs have been reported to have antioxidative effects, inhibit low density lipoprotein (LDL) oxidation, and improve endothelial dysfunction.[10-12] Such evidence indicates the importance of Chinese herbs in treating cardiovascular disease, including atherosclerosis.

According to the current understanding of the chronic inflammatory nature of atherosclerosis, it is evident that Chinese herbs are beneficial in treating atherosclerosis. Berberine, the main alkaloid of *Coptis chinensis*, has been reported to exhibit its anti-atherosclerotic effects by regulating nuclear factor-κB and cyclooxygenase-2 (COX-2) signaling.[13-15] Tanshinone IIA, a pharmacologically active component of *Salvia miltiorrhiza* Bunge (Danshen), reduces atherosclerosis by inhibiting macrophage cholesterol accumulation and proinflammatory cytokine expression.[16] In addition to berberine and tanshinone IIA, other anti-inflammatory herbs may produce anti-atherosclerotic effects.

In this study, more than 30 types of anti-inflammatory Chinese herbs were screened. By using an oxidized LDL (OxLDL) uptake assay and cell cytotoxicity assay, we observed that *Gentiana scabra* effectively reduced OxLDL uptake in nontoxic therapeutic concentrations, possibly through the suppression of scavenger receptor-A (SR-A) expression. Moreover, *Gentiana scabra* reduced SR-A expression by regulating extracellular signal-regulated kinase (ERK) signaling. Although further *in vivo* and human studies are required, these results indicate the therapeutic potential of *Gentiana scabra* in treating atherosclerosis and related cardiovascular disorders.

MATERIALS AND METHODS

Reagents

The ERK inhibitor, PD98059, was purchased from Calbiochem (Darmstadt, Germany) and Dii-OxLDL was purchased from Intracel Resources (Frederick, MD, USA). Unless otherwise specified, all other reagents were purchased from Sigma-Aldrich Chemical Company.

Preparation of Chinese herb extracts

For this study, Chinese herbs were obtained from SunTen pharmaceutic company. To prepare the Chinese herb extracts, 1000 mL of distilled water was added to 20 grams of Chinese herbs and the solution was boiled for about 40 minutes until the total volume was 200 mL. The solutions were then filtered using a 0.22 μm filter (Millipore) and used for subsequent experiments. The extract was stored at 20 °C until use.

Cell culture

Human monocytic cell line THP-1 cells (Bioresource Collection and Research Center; Hsinchu, Taiwan) were cultured in a RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), penicillin (100 U/mL), and streptomycin (100 μg/mL). THP-1 cells at a density of 1 × 10^6/mL were incubated with 100 ng/mL PMA for 3 days to enable them to differentiate into macrophages.[9]

DiI-OxLDL uptake assays

THP-1 macrophages were incubated with 1,19-dioctadecyl-3,3,39,39-tetramethylindocarbocyane perchlorate (DiI)-labeled oxidized LDL (DiI-OxLDL) in RPMI 1640 containing 10% FBS at 37 °C for 24 hrs. Thereafter, DiI-OxLDL uptake was analyzed by flow cytometry and fluorescence microscopy.[17,18]

Measurement of nonspecific cytotoxicity of drugs

The release of lactate dehydrogenase (LDH), as an indicator of damage to the plasma membrane and cell death, was measured according to the manufacturer's instructions (Roche, Indianapolis, IN, USA). The percentage of cytotoxicity was calculated as: ([sample value – medium control]/[high control – medium control]) × 100, where the sample values were the averages of the absorbance values from triplicates of indicated dose of various Chinese herb-treated THP-1 macrophage supernatants, after subtraction of the absorbance values from the background control. The average absorbance values of untreated cell culture supernatants, which were used as the medium control, were similarly calculated. Equal amounts of cells treated with 1% Triton X-100 were used as the high control.

Western blotting

Enhanced chemiluminescence Western blotting

(Amersham) was performed as previously described.[19] After extensive washing, the treated cells were pelleted and re-suspended in a lysis buffer. Protein concentrations were measured and equal amounts of whole cellular extracts were then analyzed on an 8-10% SDS-PAGE and transferred to a nitrocellulose filter. For immunoblotting, the nitrocellulose filter was incubated with TBS-T containing 5% nonfat milk (milk buffer) for 1 hour, and then blotted with primary antibodies against SR-A, CD36, total ERK (Santa Cruz Biotechnology, Dallas, TX, USA), actin (Chemicon, Temecula, CA, USA), or phosphorylated ERK (Cell Signaling, Danvers, MA, USA) at 4 °C overnight. After being washed with a TBS-T buffer, the filter was incubated with secondary antibodies conjugated to horseradish peroxidase at a concentration of 1:5000 for 1 hour. The filter was then incubated with the substrate and exposed to X-ray film.

Statistical analysis

Statistical analysis was performed using one-way analysis of variance with Bonferroni post-hoc tests for multiple comparisons and Student's t-test for comparison between two groups. $p < 0.05$ was considered significant.

RESULTS

Screening effects of Chinese herbs on Dii-OxLDL uptake in THP-1 macrophages

To examine the effects of Chinese herbs on Dii-OxLDL uptake in PMA-treated human THP-1 macrophages, THP-1 macrophages were pretreated with Chinese herbs for 2 hours, and then incubated with Dii-OxLDL for 24 hours. *Gentiana scabra* and some Chinese herbs inhibited Dii-OxLDL uptake significantly in THP-1 macrophages (data not shown). We then performed LDH assays to evaluate the cytotoxic effects of these potential therapeutic Chinese herbs on THP-1 macrophages. Only the concentrations of *Gentiana scabra* used in the experiments were not cytotoxic (Figure 1A).

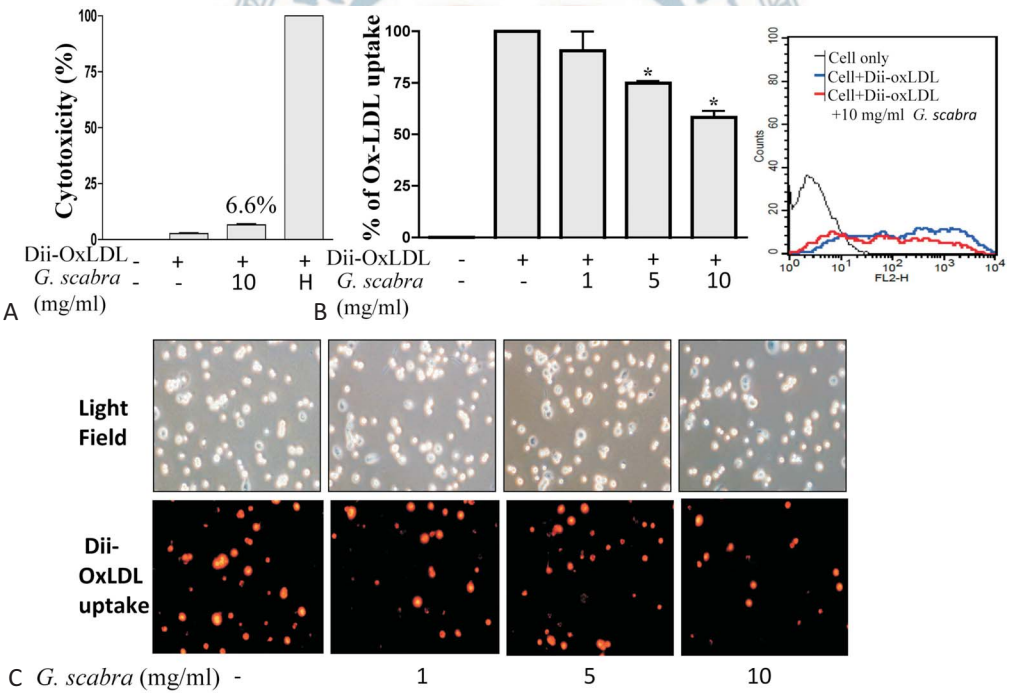

Figure 1. *Gentiana scabra inhibited OxLDL uptake in THP-1 macrophages. (A) THP-1 macrophages were pretreated with Gentiana scabra at a concentration of 10 mg/ml for 2 h and were then incubated with 10 μg/ml Dii-OxLDL for another 24 h. The culture supernatants were collected to determine the possible cytotoxic effects of Gentiana scabra by using LDH release assays. Cells treated with 1% Triton X-100 were taken as the positive control (indicated as H). (B) THP-1 macrophages were pretreated with various doses of Gentiana scabra for 2 h, incubated with 10 μg/ml Dii-OxLDL for another 24 h, and then analyzed using flow cytometry. Representative and average percentages of Dii-OxLDL uptake from at least three independent experiments are presented. * $p < 0.05$ vs. OxLDL treated control. (B) After treatment, cells were observed using fluorescence microscopy. LDH, lactate dehydrogenase; OxLDL, oxidized low-density lipoprotein.*

Gentiana scabra dose-dependently suppressed Dii-OxLDL uptake in THP-1 macrophages

To further confirm the effects of *Gentiana scabra* on Dii-OxLDL uptake, we treated THP-1 macrophages with various doses of *Gentiana scabra* and evaluated the effects on Dii-OxLDL uptake. By using flow cytometric analysis and microscopic examination, we determined that *Gentiana scabra* dose-dependently inhibited Dii-OxLDL uptake in THP-1 macrophages (Figure 1B and 1C).

Gentiana scabra inhibited expression of SR-A, but not CD36, in THP-1 macrophages

Because the majority of modified lipoproteins are ingested in macrophages through SR-A and CD36,[20] we evaluated the effects of *Gentiana scabra* on the expression of these two SRs. We found that *Gentiana scabra* significantly inhibited the expression of SR-A, but not CD36, in THP-1 macrophages (Figure 2).

Gentiana scabra inhibited SR-A expression through ERK signaling

We previously demonstrated that ERK signaling is involved in PKCδ-regulated SR-A expression.[21] Li et al. reported that ERK is crucial to the interferon (IFN)-γ-induced uptake of modified lipoproteins in human macrophages.[22] We examined the effects of *Gentiana scabra* on ERK activation and demonstrated that treatment with *Gentiana scabra* reduced ERK activation in a dose-dependent manner (Figure 3A). Moreover, treatment with an ERK inhibitor, PD98059, reduced the expression of SR-A, but not CD36 (Figure 3B).[21]

Collectively, these results suggested that *Gentiana scabra* effectively reduced OxLDL uptake and SR-A expression, supporting its potential use as a therapeutic agent in treating atherosclerosis. Figure 4 summarizes the proposed mechanisms of *Gentiana scabra* suppressing OxLDL uptake and foam cell formation.

DISCUSSION

In the present study, by screening the effects of potential anti-inflammatory Chinese herbs on foam cell formation, we demonstrated that *Gentiana scabra* effectively suppressed OxLDL uptake in THP-1 macrophages. Moreover, *Gentiana scabra* inhibited SR-A expression by regulating ERK activity. These results indicate the therapeutic potential of *Gentiana scabra* in treating atherosclerosis.

Gentiana, an important genus of the Gentianaceae family, comprises more than 400 species that are extensively distributed worldwide. *Gentiana scabra*, also called "longdan", has been used as a crude drug to treat inflammation and to stimulate appetite and gastric infections.[23] Recent pharmacological research indicated that polysaccharides isolated from *Gentiana scabra*

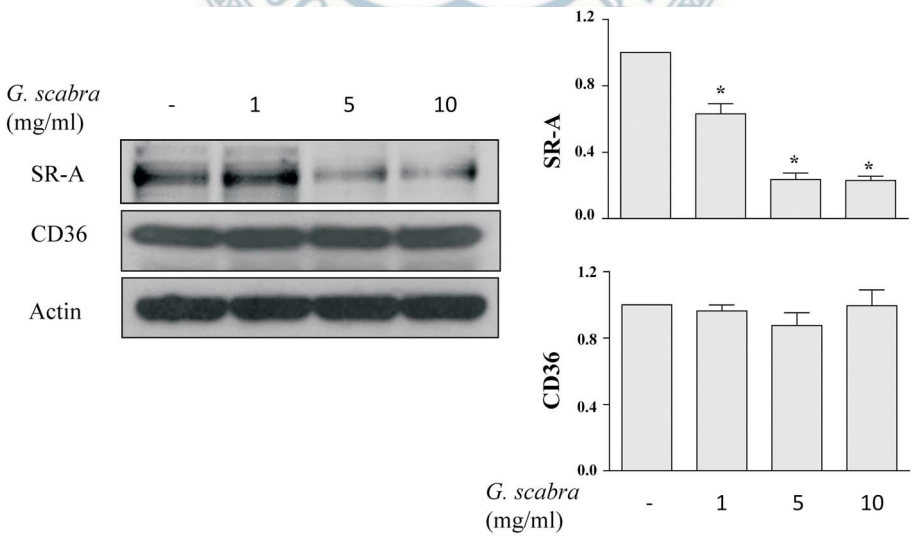

Figure 2. *Gentiana scabra suppressed SR-A expression in THP-1 macrophages. THP-1 macrophages were treated with Gentiana scabra at indicated concentrations for 24 h. The expressions of SR-A, CD36, and actin were determined. Values representative and relative protein expressions of SR-A and CD36 from at least three independent experiments were presented after densitometric analysis. * p < 0.05 vs. control. SR-A, scavenger receptor-A.*

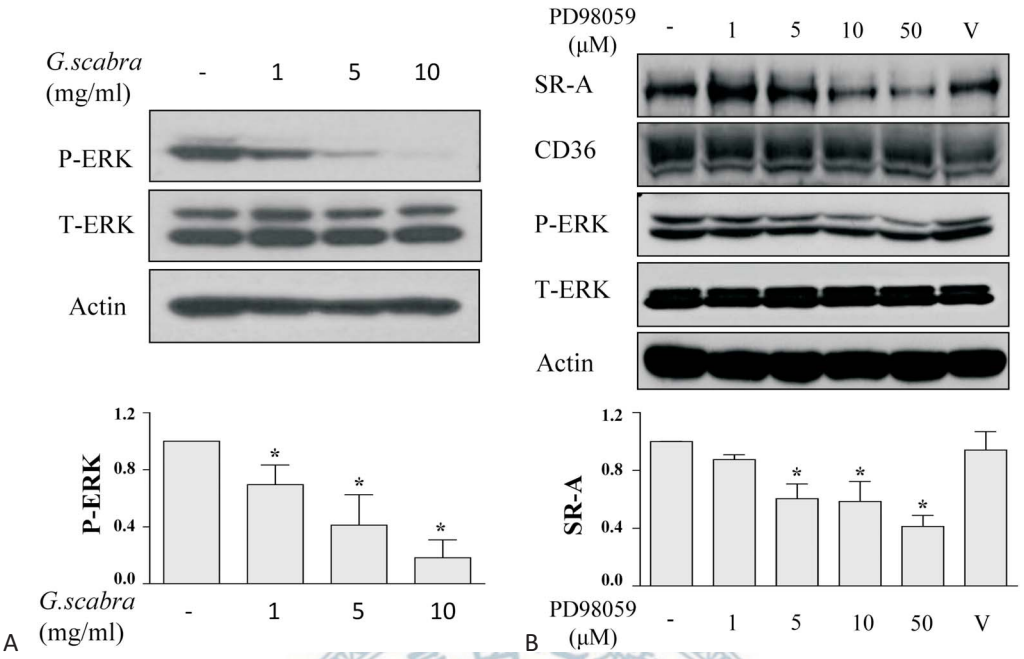

Figure 3. *ERK pathway is involved in Gentiana scabra regulating SR-A expression. (A) THP-1 macrophages were treated with Gentiana scabra at indicated concentrations for 24 h. The expressions of p-ERK, T-ERK, and actin were determined. Values representative and relative protein expressions of p-ERK from at least three independent experiments were presented after densitometric analysis. * p < 0.05 vs. control. (B) THP-1 macrophages were treated with various doses of an ERK inhibitor, PD98059, for 16 h. The cell lysates were collected to determine SR-A, CD36, p-ERK, T-ERK and actin expressions. "v." indicates vehicle control. Relative protein expressions of SR-A from at least three independent experiments were presented. * p < 0.05 vs. control.*

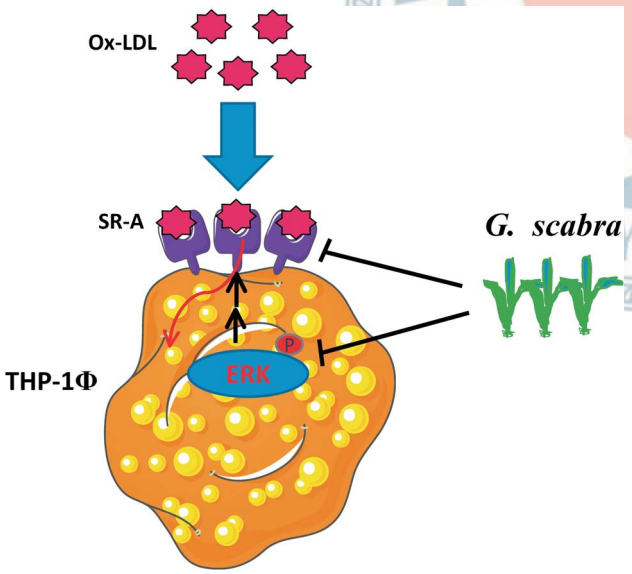

Figure 4. *Proposed mechanisms of Gentiana scabra suppressing SR-A expression and OxLDL uptake. The results of this study suggested that Gentiana scabra inhibits OxLDL uptake by regulating SR-A expression. A possible underlying mechanism may be through ERK signaling pathways.*

Bunge roots exhibit antioxidant and immunological effects.[24] *Gentiana scabra* extracts, probably secoiridoid and its glycosides, significantly inhibit severe acute respiratory syndrome (SARS) – associated coronavirus replication and could be a potential therapeutic agent for SARS.[25] Moreover, Ko et al. reported that the antioxidant effects of *Gentiana scabra* extracts produce hepatoprotective effects in the liver of carbon-tetrachloride-intoxicated mice.[26] These effects make *Gentiana scabra* a common ingredient in Chinese herbal medicine and health products.[23,24]

The formation of foam cells is principally regulated by the uptake of OxLDL or cholesterol efflux in macrophages, which leads to excessive lipid accumulation inside the cells.[27] The efflux of cholesterol is mediated by reverse cholesterol transporters including adenosine triphosphate (ATP)-binding cassette transporter A1 and G1 (ABCA1 and ABCG1), and it is generally accepted that SRs (SR-A and CD36) are responsible for the internalization of OxLDL.[20,28] Because the uptake of cholesterol-rich LDL by macrophages is believed to be critical in mediating the development of atherosclerotic plaques, understanding and targeting the pathways mediating LDL uptake by macrophages are crucial in treating athero-

sclerosis.[2,3,29,30] Moreover, Babaev et al. demonstrated that SR-A in macrophages contributes significantly to atherosclerotic lesion formation in an atherogenic mouse model.[31] By showing down-regulation of SR-A expression, our results provided further evidence demonstrating that *Gentiana scabra* reduces foam cell formation by affecting OxLDL uptake in THP-1 macrophages.

Regarding the underlying molecular mechanisms through which *Gentiana scabra* regulates SR-A expression, we observed that *Gentiana scabra* significantly reduced ERK activity in THP-1 macrophages. ERK signaling is critical in the IFN-γ-induced expression of several pro-inflammatory genes, such as the chemokines of monocyte chemoattractant protein-1, macrophage inflammatory protein 1β, and IFN-γ-induced protein 10, adhesion molecules of intercellular adhesion molecule 1, and suppressor of cytokine signaling 1, all of which have been implicated in atherosclerosis.[32] Our previous reports suggested that ERK is mediated in protein kinase Cδ-regulated foam cell formation and SR-A expression.[21] Additionally, previous studies have suggested that ERK signaling is critical in the IFN-γ mediated OxLDL uptake and the efflux of cholesterol from foam cells through the regulation of ABCA1 expression in macrophages.[22,33] Combined with the evidence that ERK is mediated in IFN-γ-induced STAT1 activation, the inhibitory effects of *Gentiana scabra* on ERK signaling in the current study indicate its anti-inflammatory and anti-atherogenic effects in treating atherosclerosis.[22]

Limitations

Although the current study revealed *Gentiana scabra* reduced OxLDL uptake significantly in THP-1 macrophages, some limitations should be noted before the findings are interpreted. First, atherosclerosis is a chronic inflammatory disease with a complex interplay of various cell types, such as endothelial cells (ECs), vascular smooth muscle cells (VSMCs), and macrophages in vessel walls. However, this study did not evaluate the effects of *Gentiana scabra* on ECs, VSMCs or in vivo animal models. Moreover, we used a mixture instead of pure compounds of *Gentiana scabra* extracts, which is a common limitation in studies of Chinese herbs. Nevertheless, the so-called "Junn-Chenn-Zuou-SS" theory illustrates a concept of coordinated effects from a combination of compounds or Chinese herbs, which might make Chinese herbs less toxic and more highly tolerated than Western drugs.[34]

CONCLUSIONS

In conclusion, we demonstrated for the first time that *Gentiana scabra* inhibited OxLDL uptake and the expression of SR-A in THP-1 macrophages, at least in part via ERK signaling. Further investigations, including research into other vascular cells and in vivo atherogenic-prone model and human studies, are necessary to elucidate the underlying mechanisms and evaluate the effects of *Gentiana scabra* on atherosclerosis.

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