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Analysis of the synergistic effect of glycyrrhizin and other constituents in licorice extract on lipopolysaccharide-induced nitric oxide production using knock-out extract

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ABSTRACT

The pharmacological evidence for synergism between natural compounds is not fully elucidated. In this study, we investigated the synergistic function of one target compound in medicinal plant extract by using knock-out (KO) extract, which is one target compound-eliminated extract from whole crude extract. Licorice is the most important ingredient used in the traditional Chinese medicine (TCM) and the Japanese Kampo medicine, and one of the major active components of licorice is glycyrrhizin (GC). To identify the potential role of GC, we prepared GC-removed extract (GC–KO extract) from licorice extract (LE) using immunoaffinity column conjugated with anti-GC monoclonal antibody (MAb), which could eliminate 99.5% of GC from LE. LE inhibited nitric oxide (NO) production and inducible NO synthase (iNOS) expression in lipopolysaccharide (LPS)-stimulated RAW264 murine macrophage cells. However, treatment of GC alone could not show the suppression of NO production and iNOS expression. Interestingly, the inhibitory effect of GC–KO extract was significantly attenuated compared with LE. Furthermore, the combined treatment with GC–KO extract and GC could improve the attenuated inhibition. Taken together, our results indicate that GC may exert synergistic suppression of iNOS expression when coexisting with the other constituents contained in LE, and KO extract is a useful approach for determination of real pharmacological functions of natural compound in the phytochemical mixture.

1. Introduction

Interactions between natural compounds contained in medicinal plants or functional foods have been investigated in a number of studies and have demonstrated the possibility of synergism. For instance, the single-compound treatment of (−)-epigallocatechin gallate (EGCG) from green tea, genistein from soybean, and benzylisothiocyanate (BITC) from Cruciferae plants were inactive for inhibition of nitric oxide (NO) production in lipopolysaccharide (LPS)- and interferon-γ (INF-γ)-treated RAW264.7 cells, while the combined treatment of EGCG/genistein, EGCG/BITC, and BITC/genistein significantly blocked NO generation [1]. EGCG together with luteolin contained in the green vegetables such as celery, broccoli, and cauliflower could exert anti-tumor activity than either agent alone in several cancer cell lines and xenograft mouse tumor models [2]. Moreover, resveratrol from grapes or wine and catechin from green tea have synergistic protection from β-amyloid-induced PC12 cell death [3]. Although many such studies examined the synergistic pharmacological effects by the co-treatment with individual natural compound, there is no report investigating the synergistic roles of single compound contained in the component mixture such as crude extract.

Licorice (*Glycyrrhiza* spp.) is the most important ingredient for the traditional Chinese medicine (TCM) and the Japanese Kampo medicine and has been reported to show various pharmaceutical functions, including anti-inflammation, antiulcer, antivirus, anti-allergy, and liver function improvement [4]. Accumulated evidence indicated that glycyrrhizin (GC), a main saponin component of licorice, is one of the biologically active compounds, and it has been reported that GC exhibited anti-inflammation, anti-ulcer, anti-tumor, anti-allergy, and hepatoprotective activities [4]. On the other hand, a number of studies by HPLC profiles suggested that licorice has many other components, including flavonoids, isoflavonoids and chalcones [4,5]. Biological studies showed that various flavonoid glycosides and their aglycones of licorice exhibit anti-inflammatory, anti-oxidative, anti-microbial, superoxide scavenging, and anticarcinogenic activities [4,5]. However, the interaction between GC and other components in licorice extract (LE) have not been studied yet since it is believed that the preparation of GC-free extract from LE is impossible.

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NO, synthesized by NO synthase (NOS) from L-arginine, is an important regulatory/modulatory mediator for several physiological processes [6]. However, excess NO production by inducible NOS (iNOS) stimulated by bacterial LPS and inflammatory cytokines participates in the pathogenesis of inflammatory diseases [7]. Chronic exposure to excess NO frequently causes a variety of inflammatory diseases including sepsis, psoriasis, arthritis, multiple sclerosis, and systemic lupus erythematosus [8]. Thus, NO production by iNOS may reflect the degree of inflammation resulting that the inhibition of NO production by blocking the iNOS expression may be useful strategy for treating various inflammatory diseases.

Our previous study demonstrated the preparation of monoclonal antibody (MAb) against GC [9]. Moreover, we established competitive enzyme-linked immunosorbent assay (ELISA) and Eastern blotting method using anti-GC MAb [10,11]. In the present study, we prepared GC-removed LE (GC–knock-out (KO) extract) by one-step isolation using the immunoaffinity column conjugated with anti-GC MAb in order to investigate the combinational effects of GC and the other components in LE on NO production in LPS-stimulated murine macrophage RAW264 cells. By using this approach, we found the potential function of GC on LE-derived suppression of NO production and iNOS expression.

2. Material and methods

2.1. Materials and cell culture

Licorice root powder (Japanese pharmacopeia grade) was purchased from Uchida Wakanyaku Corporation (Tokyo, Japan). GC was purchased from Wako Pure Chemical Industries (Osaka, Japan). LPS (*Escherichia coli* Serotype 055:B5) was purchased from Sigma (St. Louis, MO). Antibodies against iNOS and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA) and Cell Signaling Technology (Beverly, MA), respectively. Fetal bovine serum (FBS) was from GIBCO (Gaithersburg, MD). The murine macrophage cell line, RAW264 was obtained from RIKEN BioResource Center Cell Bank, and cultured at 37 °C under 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS.

2.2. Preparation of LE

The dried powder of licorice root (100 mg) was extracted with methanol (1.2 ml) under sonication five times, filtered and then evaporated with N_2 gas at 60 °C. The dried extract was dissolved in 100% of DMSO to a concentration of 100 mg/ml and diluted with media (DMSO 0.2%, final concentration) for the cell experiments.

2.3. Preparation of anti-GC MAb and determination of GC concentration by competitive ELISA

The preparation of the anti-GC MAb has been reported in our previous work [9]. The cross-reactivities of the anti-GC MAb against glycyrrhetic acid-3-*O*-glucuronide and glycyrrhetic acid were 0.585% and 1.865%, respectively. The other related compounds (deoxycholic acid, ursolic acid, and oleanolic acid) were all less than 0.005%. The quantitative measurement of GC in LE and GC–KO extract was measured with competitive ELISA using anti-GC MAb as described previously [9,10].

2.4. Preparation of immunoaffinity column using anti-GC MAb and one-step isolation of GC from LE

The anti-GC MAb Affi-Gel Hz gel column was prepared and optimized according to our previous study [12]. In brief, 60 mg of purified anti-GC MAb in coupling buffer (Bio-Rad Affi-gel Hz coupling buffer) was dialyzed and oxidized with $NaIO_4$ for 1 h. After the reaction, glycerol was added to the reaction mixture and then dialyzed. The oxidized and desalted anti-GC MAb was coupled to 25 ml of an Affi-Gel Hz gel (Bio-Rad) for 24 h with gentle stirring at room temperature, which was packed into a plastic column (300 mm × 28 mm i.d.). Twelve milligrams of LE was dissolved in loading buffer (5% MeOH), and then applied on the anti-GC-MAb Affi-Gel Hz gel column. The loading buffer was continuously circulated by a peristaltic pump (Perista pump, ATTO) overnight at 4 °C to enhance the binding efficiency. The column was washed with 5% MeOH and then eluted with elution buffer (20 mM phosphate buffer-30% MeOH). After separation, each fraction was deionized and the solvent was lyophilized.

2.5. Profiling of GC–KO extract by TLC and Eastern blotting

TLC was carried out by using n-BuOH/H_2O/CH_3COOH (7:2:1) as the developing solvent on pre-coated Merck Silica gel 60 F_{254} plate, and then the samples on the TLC were visualized with a UV lamp (254 nm) or 50% H_2SO_4 spray reagent with heating. Eastern blotting was performed as described previously [10]. Briefly, LE, GC–KO extract, and GC were applied to a polyethersulfone (PES) membrane, and then this membrane was developed by acetonitrile/H_2O/formic acid (45:55:2). The developed PES membrane was dried and dipped into $NaIO_4$ (10 mg/ml). After incubation for 1 h at room temperature, the membrane was washed with water, and then incubated with 50 mM carbonate buffer solution (pH 9.6) containing 1% BSA for another 3 h. The PES membrane was washed with PBS and treated with PBS containing 5% skimmed milk for 2 h to reduce nonspecific adsorption. The membrane was immersed in anti-GC MAb and stirred at room temperature for 3 h. After washing twice with PBST, a 1:1000 dilution of peroxide-labeled goat anti-mouse IgG in PBS containing 0.2% gelatin was added, and the mixture was stirred at room temperature for 1 h. The membrane was washed twice with PBST and then exposed to dimethylaminoazobenzene/4-chloro-1-naphthol solution for 15 min at room temperature. The reaction stopped by washing with water, and the immunostained membrane was allowed to dry.

2.6. Nitrite assay

RAW264 cells were plated at 4×10^5/well in 24-well plates and then treated with or without LE or GC before exposure to 50 ng/ml LPS. After 12 h of incubation, the cell culture supernatant was collected. For determining NO concentration, the level of nitrite, a stable oxidized product of NO, was measured using the Griess reagent as described previously [13].

2.7. Western blotting analysis

RAW264 cells (1×10^6 cells) were plated in 6-cm dishes for 24 h, and then treated with LE or GC for 30 min before exposure to 50 ng/ml LPS. The harvested cells were lysed and the supernatants were boiled for 5 min. Protein concentration was determined by using dye-binding protein assay kit (Bio-Rad) according to the manufacturer's manual. Equal amounts of lysate protein were subjected to SDS–PAGE, followed by Western blotting as described previously [13].

2.8. RNA isolation and RT-PCR analysis

RAW264 (1×10^6 cells) was pre-cultured in 6-cm dishes for 24 h, and then treated with or without LE for 30 min before exposure to 50 ng/ml LPS. After 6 h incubation, total RNA was extracted

using Trizol Reagent (Invitrogen) according to the manufacturer's instructions. The RT-PCR for iNOS and GAPDH was performed as previously described [14].

2.9. Statistical analyses

Difference between the treated and the control was analyzed by Student's *t*-test. *P* value less than 0.05 were considered to be significant.

3. Results

3.1. Preparation of GC–KO extract using anti-GC MAb immunoaffinity column

To investigate the potential function of GC, we prepared the GC-free extract from crude LE using immunoaffinity column conjugated with anti-GC MAb. To eliminate GC from LE, 12 mg of LE (GC content: 1275.0 μg) in loading buffer was applied on the anti-GC MAb immunoaffinity column, and then the loading buffer was continuously circulated through the column to enhance the binding efficiency. After overnight circulation at 4 °C, the un-bound fraction was separated. After washing the column completely, the bound fraction was eluted by the elution buffer. After deionization and lyophilization of the each fraction, the recovery ratio of GC was checked by ELISA (Table 1). In the un-bound fraction, 3.50 μg of GC (0.27% of the applied GC) was detected. On the other hand, 1269.26 μg of GC (99.55% of the applied GC) was obtained in the bound fraction. To further characterize the two fractions, the TLC analysis and Eastern blotting were performed. As shown in Fig. 1A, many spots including GC were detected in LE (lane 2). However, the un-bound fraction contained all of the components in the LE except GC (lane 3). Eastern blotting using anti-GC MAb (Fig. 1B) clearly detected GC in LE (lane 2), but the spot of GC was completely disappeared in the un-bound fraction (lane 3). These data indicated that the immunoaffinity column using anti-GC MAb could eliminate only GC from LE with high efficiency, so we named this un-bound fraction GC–KO extract.

3.2. Effects of LE and GC on NO production and iNOS expression in LPS-stimulated RAW264 cells

To examine the inhibitory effect of the LE on the NO production, we used murine macrophage RAW264 cells, which can produce NO upon stimulation of LPS. Cells were pre-incubated with the different concentrations of LE for 30 min and stimulated with LPS for 12 h. As shown in Fig. 2A, LPS caused a dramatic increase of NO from the basal level of 0.4 ± 0.1 μM to 16.4 ± 0.6 μM, and this induction was inhibited in a dose-dependent manner by treatment of LE. To investigate whether the inhibitory effect of LE on NO production was related to iNOS expression, we analyzed the iNOS expression at protein and mRNA levels using Western blotting analysis and RT-PCR, respectively (Fig. 2B and C). The iNOS expression of protein and mRNA were markedly upregulated upon exposure to LPS, and the pre-treatment with LE showed the dose-dependent inhibition of these upregulations although the expressions of β-actin and GAPDH as internal control remained unchanged. These results suggest that LE suppressed NO production

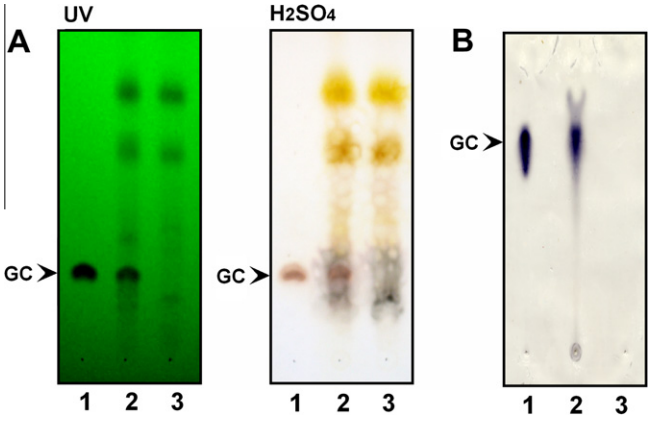

Fig. 1. Characterization of GC–KO extract by TLC (A) and Eastern blotting by anti-GC MAb (B). Lane 1, GC standard; Lane 2, LE; and Lane 3, Un-bound fraction (GC–KO extract).

by blocking iNOS expression at the transcriptional level. In addition, no cytotoxity was observed at any concentrations of LE by MTT assay (Supplementary Fig. 1), clearly indicating that these inhibitory effects were not attributable to cytotoxic effects.

As shown in Fig. 2B and C, 100 μg/ml of LE led to complete inhibition of iNOS protein and mRNA. ELISA using anti-GC MAb demonstrated that 100 μg of LE contains 10.6 ± 0.618 μg of GC. To further define the role of GC in the suppression of iNOS expression, we treated the cells with GC at around 10.6 μg/ml (the concentration range from 5.3 to 21.2 μg/ml) before exposure to LPS. However, GC did not show any significant inhibition of NO production (Fig. 2D) and iNOS protein (Fig. 2E). Thus, the treatment of GC alone contained in 100 μg/ml of LE could not directly block the NO production and iNOS expression.

3.3. Effects of GC–KO extract and the combination of GC–KO extract and GC on LPS-induced NO production and iNOS protein expression

We next examined the inhibitory effect of GC–KO extract and the combined treatment with GC–KO extract and GC on NO production. Since 100 μg/ml of LE contains 10.6 μg of GC and 89.4 μg of the other compounds, the cells were pre-treated with LE (100 μg/ml), GC–KO extract (89.4 μg/ml), or the combination of GC–KO extract (89.4 μg/ml) and GC (10.6 μg/ml). Fig. 3A indicated that the treatment of LE led to a marked suppression of NO production as compared to LPS treatment (inhibition ratio (IR) = 57.7%). Interestingly, the inhibitory effect of GC–KO extract was lower (IR = 17.8%) compared with LE although GC alone could not block NO production as indicated above. On the other hand, the combined treatment with GC–KO extract and GC significantly improved the inhibitory ability (IR = 33.5%). To determine whether the combinational effect of GC–KO extract and GC was related to iNOS expression, we performed Western blotting. As shown in Fig. 3B, the treatment of GC–KO extract diminished the inhibitory ability of LE on iNOS expression, and addition of GC to GC–KO extract could improve it. These results suggest that, although the single treatment of GC cannot suppress NO production and iNOS expression, the coexistence of GC and other constituents contained in LE can exert synergistic suppression.

Table 1
The GC contents of un-bound and bound fractions separated by the anti-GC MAb immunoaffinity column.

Applied GC (μg)	Fraction	GC (μg)	GC (% of loading GC)	Total GC (μg)	Recovery (%)
1275.00 (12 mg of LE)	Un-bound	3.50	0.27	1272.76	99.82
	Bound	1269.26	99.55		

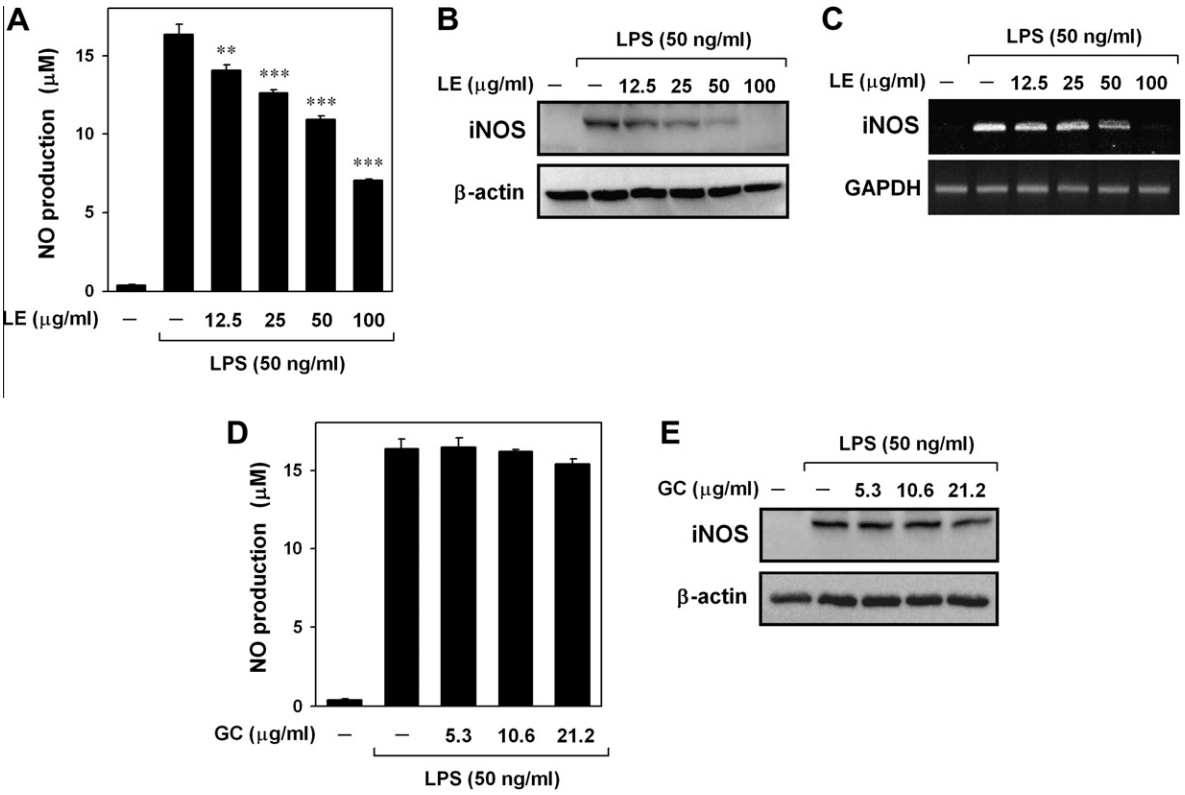

Fig. 2. Effects of LE and GC on NO production and iNOS expression in LPS-stimulated RAW264 cells. (A and D) Cells were pre-treated with the indicated concentrations of LE or GC for 30 min, followed by treatment of LPS (50 ng/ml) for 12 h. The amounts of NO in media were measured using the Griess reagent as described in Materials and methods. Each bar indicates the mean ± S.D. of four individual experiments. $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences from the LPS alone. (B and E) Cells were treated with LE or GC in presence of LPS as mentioned above. Cells were lysed, and iNOS and β-actin protein levels were determined by Western blotting. (C) Cells were incubated with the indicated concentrations of LE for 30 min, followed by treatment of LPS (50 ng/ml) for 6 h. Total mRNA were isolated, and iNOS and GAPDH mRNA levels were determined by RT-PCR.

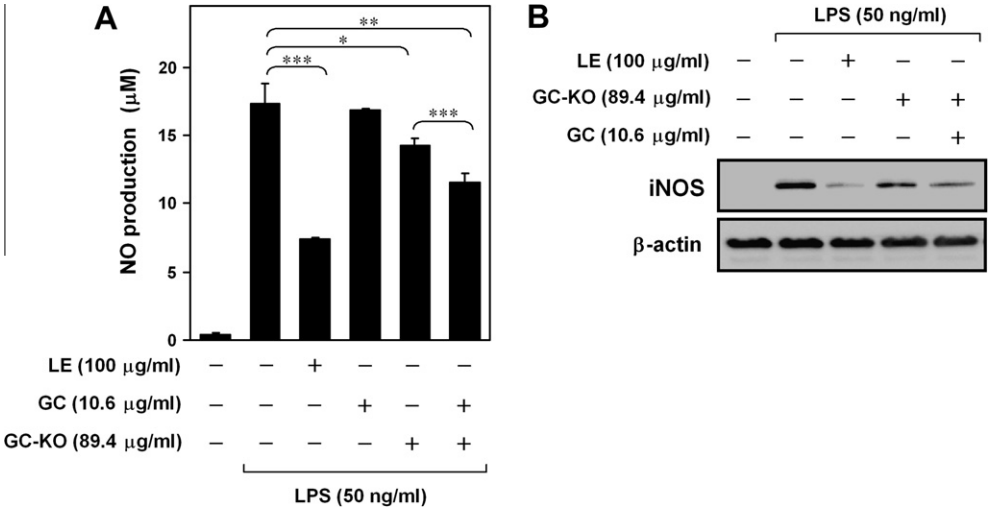

Fig. 3. Effects of GC–KO extract and the combination of GC–KO extract and GC on LPS-induced NO production and iNOS protein expression. (A) Cells were pre-treated with the indicated concentrations of LE, GC–KO extract, and GC for 30 min, followed by treatment of LPS (50 ng/ml) for 12 h. The amounts of NO in media were measured using the Griess reagent as described in Materials and methods. Each bar indicates the mean ± S.D. of four individual experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ and $^{***}P < 0.001$ indicate significant differences from the LPS alone. (B) Cells were treated with LE, GC–KO extract, and GC in presence of LPS. Cells were lysed, and iNOS and β-actin protein levels were determined by Western blotting.

4. Discussion

The experimental evidences that the medicinal plant extracts are efficacious for the improvement of health, have recently increased much importance. Accumulating evidences from *in vivo* and *in vitro* studies demonstrated that the natural bioactive compounds derived from medicinal plant extracts have important roles for exerting the therapeutic or chemopreventive efficacies of the plant extract. But, the plant extracts are complex mixtures rich in effective phytochemicals which act synergistically or additively

on specific and/or multiple molecular and cellular targets, thus the interactions between these compounds are important to understand the real pharmacologically active compounds. Several reports defined the interactions of two or three different natural compounds, but these reports limited a fashion such as the co-treatment with each natural compound [1–3]. In this study, we prepared KO extract, which is one target compound-eliminated extract from whole crude extract by using immunoaffinity column conjugated with anti-natural compound MAb. This approach may make it possible to determine the real function of a single compound in a medicinal plant.

Our current data demonstrated that LE downregulated NO production and iNOS expression at protein and mRNA levels. NF-κB is one of critical factors for iNOS expression induced by LPS [15], and LE decreased LPS-induced DNA binding activity of p65 NF-κB subunit through the inhibition of IκB-α degradation and nuclear accumulation of p65 (Supplementary Fig. 2). Thus, the inhibitory effect of LE on iNOS expression occurs at the transcriptional level. Because a single treatment of GC had no effect on NO production and iNOS expression, we expected that GC–KO extract would have equal inhibitory potency compared to LE regarding the inhibition of NO and iNOS. However, interestingly, our results showed that the inhibitory effect of GC–KO extract was attenuated compared to LE. Furthermore, the attenuated inhibition by GC–KO extract could be recovered when combined with GC. These data imply that GC may exert synergistic suppression of iNOS expression with the other constituents contained in LE.

A number of phytochemical and biological investigations on licorice have revealed that GC is one of the biologically active compounds in licorice [4,16]. Furthermore, other species-specific compounds were isolated from licorice and show anti-inflammatory properties [4,16]. Glabridin has been reported to exert the suppression of melanogenesis and inflammation on B16 murine melanoma cells and guinea pig skins [17]. Additionally, glabridin attenuated LPS-induced NO production and iNOS gene expression by blocking NF-κB activation and reactive oxygen species generation in isolated mouse peritoneal macrophages and RAW 264.7 cells [18]. Licochalcone A also suppresses LPS-stimulated inflammatory mediators such as NO and prostaglandin E_2 in RAW264.7 cells and endotoxin shock in mice [19]. In recent study, licocalchone C also decreased the expression and activity of iNOS via NF-κB inhibition by influencing extracellular O_2^- production and modulating the antioxidant network activity such as superoxide dismutase (SOD), catalase, and glutathione peroxidase in THP-1 human myelomonocytic leukemia cells [20]. These reports suggest that multiple compounds contained in licorice exhibit potent anti-inflammatory activities. Our observation demonstrated that GC could not lead to direct inhibition of NO production and iNOS expression in the complex mixture of LE, thus GC may enhance the anti-inflammatory potencies together with the other bioactive compounds in licorice.

In this study, we demonstrated that anti-GC MAb immunoaffinity column could eliminate GC from LE with high efficiency resulting in one-step isolation of GC. Our laboratory has previously prepared MAbs against natural bioactive compounds such as ginsenosides [21–23], berberine [24], crocin [25], sennosides [26], and forskolin [27]. Previously the immunoaffinity column using anti-forskolin MAb succeeded the one-step isolation of forskolin [28]. Furthermore, we also reported the preparation of ginsenoside Rb1-KO ginseng extract by using the immunoaffinity column conjugated with anti-ginsenoside Rb1 MAb [29]. These MAbs and techniques are easily applicable to the preparation of series of KO extracts removing the target compounds from the crude extracts of medicinal plants and/or TCM.

This is the first report demonstrating the synergistic role of a single compound in the natural component mixture by using KO extract. The pharmacological analysis by KO extract may provide new insight of real and potential functions of natural compounds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.11.143.

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