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Inflammopharmacology

SHORT COMMUNICATION

Modulation of lipopolysaccharide-induced pro-inflammatory mediators by an extract of *Glycyrrhiza glabra* and its phytoconstituents

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Abstract
Objective To evaluate the inhibitory property of de-glycyrrhizinated extract of *Glycyrrhiza glabra* root and its phytoconstituents (glabridin, isoliquiritigenin and glycyrrhizin) on LPS-induced production of pro-inflammatory mediators.
Materials and methods Inhibitory effect of *G. glabra* extract and its phytoconstituents were studied on lipopolysaccharide (LPS)-induced nitric oxide (NO), interleukin-1 beta (IL-1 beta) and interleukin-6 (IL-6) levels in J774A.1 murine macrophages.
Results *G. glabra* and isoliquiritigenin significantly inhibited LPS stimulated NO, IL-1 beta and IL-6 production. Glabridin showed significant inhibition of NO and IL-1 beta release, but failed to attenuate IL-6 levels at the tested concentrations. In addition, glycyrrhizin did not exhibit inhibitory response towards any of the LPS-induced pro-inflammatory mediators at the tested concentrations.
Conclusion From the results we speculate that the inhibitory effect of *G. glabra* extract on LPS-induced pro-inflammatory mediators is influenced by glabridin and isoliquiritigenin and is not contributed by glycyrrhizin.

Keywords *Glycyrrhiza glabra* · Phytoconstituents (glabridin, isoliquiritigenin and glycyrrhizin) · Inflammation · Pro-inflammatory mediators (NO, IL-1 beta and IL-6)

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Introduction

Glycyrrhiza glabra (Papilionaceae/Fabaceae) also known as licorice/sweetwood is indigenous to Mediterranean and certain areas of Asia. Many components such as triterpene saponins, flavonoids, isoflavonoids and chalcones have been isolated from licorice, with glycyrrhizic acid being the major biologically active component (Asl and Hosseinzadeh 2008). The licorice root is one of the most popular herbal medicines in the world due to its pronounced pharmacological properties (Fiore et al. 2005). It has been reported to have antioxidant (Fukai et al. 2003), hepatoprotective (Asl and Hosseinzadeh 2008), immunostimulant and anti-inflammatory (Olukoga and Donaldson 2000) activities. Active components isolated from the root are mainly flavonoids and saponins, which include isoliquiritigenin and glycyrrhizin (Kamei et al. 2003). Moreover, many studies have revealed that several licorice-derived compounds, i.e., glycyrrhizin, isoliquiritigenin, licochalcone, and glabridin, have a variety of pharmaceutical effects (Fukai et al. 2003; Yokota et al. 1998; Inoue et al. 1986; Zhou et al. 2004). Glabridin is one of the major active flavonoids in licorice which exhibits inhibitory effects on inducible nitric oxide synthase (iNOS) protein expression (Kang et al. 2005).

Isoliquiritigenin is a flavonoid known to have many pharmacological activities such as antiallergic (Kakegawa et al. 1992), antioxidant (Vaya et al. 1997), etc. Apparently, the major pharmacological action of the plant is mainly due to glabridin (Kang et al. 2005). Although the underlying mechanism of anti-inflammatory action of *G. glabra* remains unresolved, some of its anti-inflammatory effects have been already investigated. Many studies have reported that the anti-inflammatory activity of licorice is majorly influenced by glycyrrhizin (Inoue et al. 1986; Ohuchi et al.

1981). In our recent publication we demonstrated the dual inhibitory (COX and LOX products) property of de-glycyrrhizinated extract of *G. glabra* root. We had demonstrated that inhibitory property of the *G. glabra* extract towards PGE_2, TXB_2 and LTB_4 production is attributable to its phytoconstituents (Chandrasekaran et al. 2010b). In addition, in another study, we had shown the anti-ulcer activity of de-glycyrrhizinated extract of *G. glabra* root (Mukherjee et al. 2010). These studies apparently reflect that the anti-ulcer activity of the *G. glabra* extract might be related to its dual inhibitory property. In this view, the present study was conducted to analyze the effect of the extract and its phytoconstituents on LPS-induced pro-inflammatory mediators secreted from macrophages.

Inflammation is a beneficial host response to a foreign challenge or tissue injury that ultimately leads to the restoration of normal tissue structure and function. However, prolonged inflammation contributes to the pathogenesis of many inflammatory diseases. Macrophage plays an essential role in the inflammatory response and serves as a crucial interface between innate and adaptive immunity (Kim et al. 2006). Activation of macrophages with LPS induces the expression of various inflammatory mediators, including NO, inflammatory cytokines, etc. (Kim et al. 2006; Kwon et al. 2005). In the present study, we report the inhibitory effects of de-glycyrrhizinated extract of *G. glabra* root and its phytoconstituents (glabridin, isoliquiritigenin and glycyrrhizin) on production of pro-inflammatory mediators (NO, IL-1 beta and IL-6) in LPS stimulated J774A.1 murine macrophages.

Materials and methods

Lipopolysaccharide (LPS), MTT [1-(4, 5-dimethylthiazol-2-yl)-3,5-diphenylformazan], 1400W (*N*-[[3-(aminomethyl) phenyl]methyl]-ethanimidamide) dihydrochloride, sulphanilamide, naphthyl ethylene diamine dihydrochloride (NEDD) and dexamethasone were purchased from Sigma-Aldrich, St. Louis, USA. Dulbecco's modified Eagle's medium (DMEM) was supplied by Gibco Life Technologies, Grand Island, NY. Fetal bovine serum (FBS) was purchased from Hyclone, Logan, USA.

Preparation of extract and isolation of phytoconstituents

G. glabra used in this study was identified at NISCAIR (National Institute of Science Communication And Information Resources) and voucher specimen (No. 51) has been deposited in our herbarium. Preparation of the extract and isolation of the phytoconstituents was performed as described in our previous publication (Chandrasekaran et al. 2010b). Briefly, the dried roots were extracted thrice using acetone in 1:4 proportions at room temperature. The resultant liquid extract obtained after every extraction was mixed and filtered under vacuum at temperature <55°C until a thick paste was obtained. This fraction amassed a total solid content of 40-50% (w/w). The extract was further filtered and dried under vacuum (<65°C, 500 mmHg) to get de-glycyrrhizinated extract of *G. glabra* (56 g). The phytoconstituents present in the extract were isolated and identified by HPLC analysis.

The extract was found to contain 4.5%w/w of glabridin, 0.1% w/w of isoliquiritigenin, 16%w/w of total flavonoids and 0.019%w/w of glycyrrhizin. The extract had moisture content of 2.4%w/w and ash content of 0.02%w/w (USP 2009). Glycyrrhizin was dissolved in plain media. *G. glabra* extract, glabridin and isoliquiritigenin were dissolved in DMSO and filter sterilized through 0.2 µm positively charged nylon DMSO compatible filter. The final concentration of DMSO was maintained at 0.2%, at which it did not show cytotoxicity or influence the stimulant-induced release of any of the inflammatory mediators. For all the assays respective reference standards were used.

Cell culture and cell viability assay

J774A.1 murine macrophage cell line (TIB-67^{TM}) was procured from American Type Culture Collection (ATCC) (Rockville, MD, USA). The cells were maintained in DMEM supplemented with 10% FBS at 37°C under 5% CO_2 humidified air. The effect of *G. glabra* extract and the phytoconstituents on cell viability was studied using MTT method (Mosmann 1983). Briefly, J774A.1 cells were seeded at a density of 1×10^5 cells/well in a 96-well plate and treated with different concentrations of extract/phytoconstituents. Following treatment, 10 µl of MTT solution (5 mg/ml) was added to each well and further incubated for 1 h at 37°C. Subsequently, the MTT solution was removed and added with 200 µl of dimethyl sulfoxide (DMSO) to solubilize formazan crystals. The optical density (OD) was measured at 570 nm. Based on the cell viability results, different non-cytotoxic concentrations were selected for each study.

Quantification of NO and IL-6 production in cultured LPS-induced J774A.1 cells

J774A.1 murine macrophages were seeded at a density of 1×10^5 cells/well and incubated overnight. The cells were replaced with new growth media and then incubated for 1 h with the extract/phytoconstituents at different non-cytotoxic concentrations, followed by addition of LPS (0.1 µg/ml) and further incubated for 24 h (Chandrasekaran et al. 2010a). The cell supernatant was collected and analyzed for nitrite

and IL-6 estimation. NO concentration in the cultured medium was determined using the Griess reagent (Weissman and Gross 2001). Specifically, 50 µl of supernatant from each well was mixed with 50 µl of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% NEDD in water). After an incubation of 5 min at room temperature, the optical density was determined at 540 nm. IL-6 levels in the cell supernatants from the respective treatment were quantified using enzyme-linked immunosorbent assay (ELISA) kit OptEIA^{TM} from BD biosciences (USA) according to the manufacturer's instructions. The reference standards used for NO scavenging and IL-6 inhibition assays were 1400W dihydrochloride (0.11–108 µM) and dexamethasone (0.0013–100 µM), respectively.

Measurement of IL-1 beta accumulation in cultured LPS-induced J774A.1 cells

J774A.1 cells were seeded at a density of 1×10^5 cells/well and incubated overnight. Post-incubation of the cells was treated with the extract/phytoconstituents for 1 h and then stimulated with LPS (0.1 µg/ml) for 6 h. The treated cells were lysed using cell lysis buffer [0.1% Triton X-100 and protease cocktail inhibitor (1X)] in combination with repeated freeze thaw cycles (Chandrasekaran et al. 2010a). The level of IL-1 beta in the treated lysates was quantified using ELISA kit from R&D Systems, Minneapolis, USA according to the manufacturer's instructions. Dexamethasone (0.006–6.25 µM) was used as a reference standard.

Statistical analysis

The data are presented as mean ± standard error mean (SEM) of triplicates. The results were analyzed by one-way ANOVA, followed by Dunnett's test for multiple comparisons using GraphPad Prism 5·0 software (GraphPad Software Inc., San Diego, CA, USA). $P < 0.05$–0.01 was considered significant. The half maximal inhibitory concentration (IC_{50}) was calculated using median effect plot (Wolfe and Liu 2007).

Results and discussion

The roots and rhizomes of *G. glabra* are one of the most popular herbal medicines and have been used as antidotes, expectorants and for allergic inflammations (Belinky et al. 1998). Previous studies have demonstrated that ethanol extracts of roasted licorice inhibits release of inflammatory mediators in LPS-activated RAW264.7 cells (Kim et al. 2006). Active constituents isolated from *G. glabra* are mainly flavonoids and saponins, e.g., glycyrrhizin, isoliquiritigenin, licochalcone, and glabridin which are reported to have variety of pharmaceutical effects (Zhou et al. 2004; Fukai et al. 2003; Yokota et al. 1998; Inoue et al. 1986). Despite the pharmacological activities of glycyrrhizin, high doses and chronic consumption causes serious side effects that emulate a hypermineralcorticoid state. This condition is clinically characterized by electrolyte imbalances, hypertension, and possible cardiac abnormalities. Though the side effects are not common and the risk gets further minimized with proper monitoring of the patient and knowledge of dosage, removal of the glycyrrhizin compound completely eliminates the risk (Kaczor 2009). The de-glycyrrhizinated extract has been extensively studied for its anti-ulcer property (Mukherjee et al. 2010) nevertheless the anti-inflammatory property still remains unresolved. The purpose of this study was to investigate the inhibitory potential of de-glycyrrhizinated extract of *G. glabra* and its active phytoconstituents on release of pro-inflammatory mediators—NO, IL-1 beta and IL-6 in murine macrophages.

Macrophages are the initiator cells activated during the early stages of inflammatory response and are also responsible for the production of pro-inflammatory cytokines. The chemical substances derived from plants are of potential interest for therapeutic intervention in inflammatory diseases. They act either by inhibiting the pro-inflammatory enzymes or by inhibition of cytokine release, etc., that are known contributors to chronic inflammatory disorders (Dharmappa et al. 2010). Hence, inhibition of pro-inflammatory mediators is legitimate in the neutralization of inflammation.

Nitric oxide is essential for host's innate immune responses to pathogens and mediates a variety of biological functions (Barbato and Tzeng 2004). However, overproduction of NO leads to cytotoxicity in host tissue thereby, triggering DNA injury and development of several inflammatory diseases (Yoon et al. 2009). Thus, inhibition of NO production is a major target for anti-inflammatory agent development.

G. glabra extract (10–40 µg/ml) significantly inhibited LPS-induced NO production at the indicated concentrations with an IC_{50} value of 24 µg/ml (Fig. 1a). Except glycyrrhizin both glabridin and isoliquiritigenin dose-dependently inhibited NO release. Glabridin (5–10 µg/ml) showed moderate inhibition in NO levels with maximum inhibition of 33% at the highest tested concentration (Fig. 2a). Further, isoliquiritigenin (2.5–10 µg/ml) displayed dose-dependent inhibition at the indicated concentrations with 50% inhibition attained at 7.5 µg/ml (29 µM). The results from the cell viability study showed that the inhibitory activity of *G. glabra* and its phytoconstituents was not due to the cytotoxic effect. These results are similar to previous study which reported inhibition of LPS-induced NO release in RAW264.7 cells by glabridin

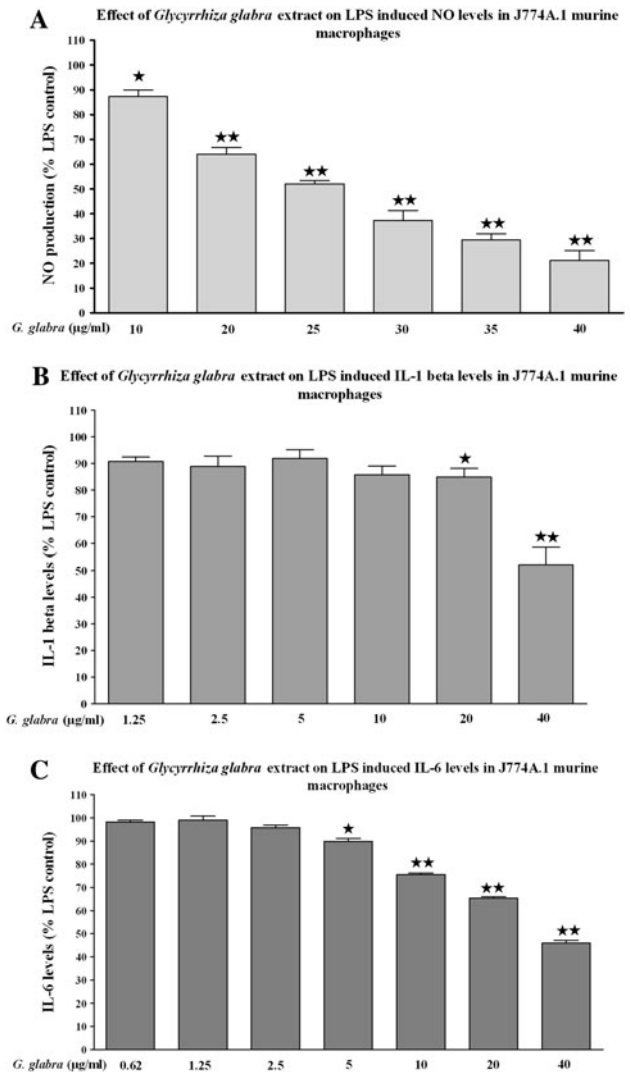

Fig. 1 Effect of *G. glabra* extract on LPS-induced NO, IL-1 beta and IL-6 levels. **a** J774A.1 cells were incubated in the presence/absence of *G. glabra* for 1 h, prior to LPS (0.1 µg/ml) stimulation for 24 h. After 24 h, the nitrite content in culture supernatant was analyzed by Griess reaction assay and expressed as a percentage of the control (LPS alone). **b** Cells were pretreated with the extract for 1 h. Then stimulated with LPS (0.1 µg/ml) for 6 h. The levels of IL-1 beta in the treated cell lysates were quantified using ELISA kit and the values are expressed as a percentage of the control (LPS alone). **c** Macrophage cells were incubated with extract for 24 h prior to stimulation with LPS (0.1 µg/ml) for 1 h. The cell supernatant was analyzed for IL-6 estimation, using ELISA technique. All the values are expressed as a percentage of the control (LPS alone). Data are represented as mean ± SEM. An *asterisk* indicates a significant ($*P < 0.05$ and $**P < 0.01$) difference from LPS control

(Kang et al. 2005). Prior study has reported that isoliquiritigenin (isolated from *G. uralensis*) elicits inhibitory effect on LPS-induced NO production in RAW264.7 cells by blocking the NF-kappa B signal pathway thereby inhibiting the expression of iNOS protein (Kim et al. 2008). On comparing with reference standard 1400W dihydrochloride,

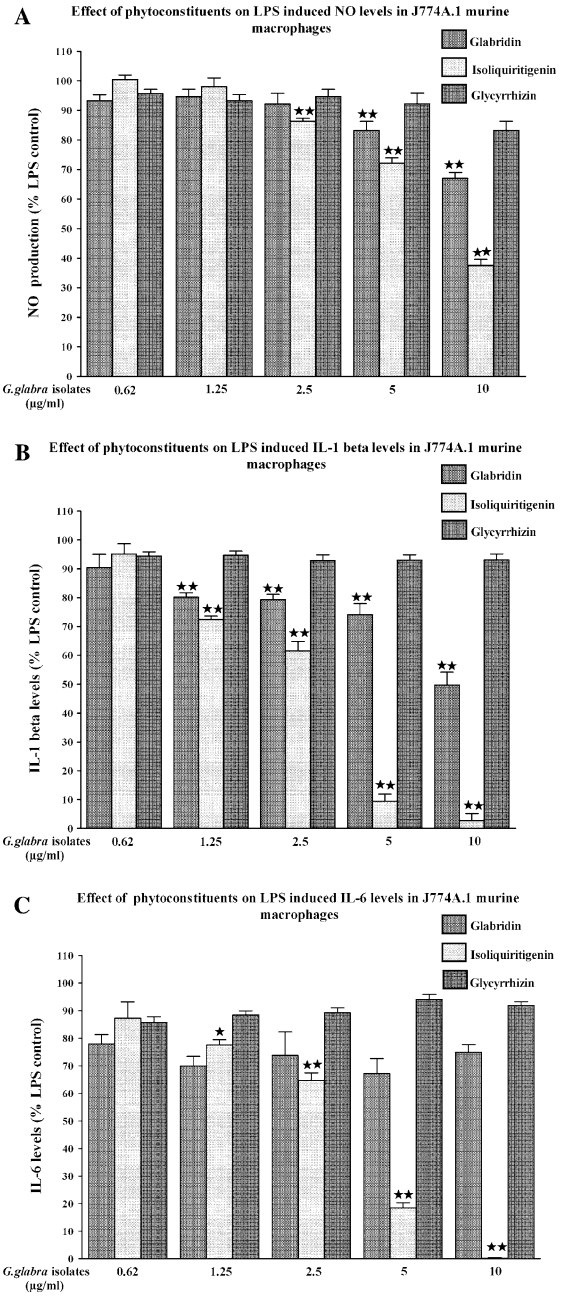

Fig. 2 **a** Effect of phytoconstituents on LPS-induced NO levels. J774A.1 cells were pre-incubated for 1 h with different concentration of phytoconstituents, followed by LPS (0.1 µg/ml) for 24 h. The nitrite content in culture supernatant was analyzed by Griess reaction assay and expressed as a percentage of the control (LPS alone). **b** Effect of phytoconstituents on LPS-induced IL-1 beta levels. Cells were pre-incubated for 1 h with different concentration of phytoconstituents, followed by LPS (0.1 µg/ml) for 6 h. The levels of IL-1 beta in the treated lysates were quantified using ELISA kit and the values are expressed as a percentage of the control (LPS alone). **c** Effect of phytoconstituents on LPS-induced IL-6 levels. The cells were pre-incubated for 1 h with different concentrations of phytoconstituents, followed by LPS (0.1 µg/ml) for 24 h. The cell supernatant was analyzed for IL-6 estimation using ELISA kit. All the values are expressed as a percentage of the control (LPS alone). Data are represented as mean ± SEM values. An *asterisk* indicates a significant ($*P < 0.05$ and $**P < 0.01$) difference from LPS control

isoliquiritigenin (IC_{50} = 29 μM) was found less potent than 1400W dihydrochloride (IC_{50} = 10.7 μM) in alleviating NO production. Studies report that over-expression of iNOS gene is implicated in increase of NO production (Kang et al. 2005), thus the inhibitory activity of *G. glabra* extract on NO levels might have resulted due to down-regulation of iNOS gene expression.

Surprisingly, glycyrrhizin failed to attenuate NO release at the tested concentrations. So, the anti-inflammatory activity of glycyrrhizin might be due to other factors such as inhibition of generation of ROS by neutrophils (Akamatsu et al. 1991), inhibiting the development of histamine-serotonin and bradykinin-induced edema and decreased vascular permeability (Nasyrov and Lazareva 1980). Consequently, the results indicate that the anti-inflammatory activity of *G. glabra* extract is either due to individual or due to synergistic effect of glabridin and isoliquiritigenin.

Bacterial LPS induces 12- to 16-fold increase in IL-1 beta, IL-6, and TNF-alpha mRNA levels (Omoigui 2007). LPS activates two distinct downstream signaling pathways: transcription factor nuclear factor-kappa B (NF-kappa B) and mitogen-activated protein kinase (MAPK). These two pathways induce the expression of various inflammatory mediators, including NO, inflammatory cytokines (IL-1 beta, IL-6, TNF-alpha), etc. (Kwon et al. 2005).

IL-1 beta is a multifunctional inflammatory cytokine that affects nearly all cell type often in concert with other cytokine or small mediators (Dinarello 1996). In this study, we scrutinized the inhibitory effect of *G. glabra* extract and its phytoconstituents on IL-1 beta production. We found that *G. glabra* extract (20–40 μg/ml) dose-dependently inhibited the LPS-induced IL-1 beta levels at the indicated concentrations (Fig. 1b). The maximum inhibition shown by the extract was 47.8% which was obtained at the highest tested concentration. We further tested the effect of the phytoconstituents on IL-1 beta production. Among the phytoconstituents only glabridin and isoliquiritigenin exhibited dose-dependent alleviation in IL-1 beta production at concentrations ranging from 1.25 to 10 μg/ml (Fig. 2b). Glabridin and isoliquiritigenin showed IC_{50} values of 10 μg/ml (30.8 μM) and 1.85 μg/ml (7.2 μM), respectively. The cell viability study showed that the inhibitory activity of the extract and phytoconstituents was not due to cytotoxic effect. However, surprisingly glycyrrhizin failed to demonstrate inhibitory effect towards LPS stimulated IL-1 beta release at the tested concentrations. Further, in order to evaluate the potency of these phytoconstituents, we compared their activity with the reference standard dexamethasone. Both glabridin (IC_{50} = 30.8 μM) and isoliquiritigenin (IC_{50} = 7.2 μM) showed lesser potency than dexamethasone (IC_{50} = 70 nM) in inhibiting IL-1 beta levels. Prior study reported that glabridin inhibits LPS-induced IL-1 beta levels in BV-2 microglial cells (Park et al. 2009).

IL-6 is a multifunctional cytokine which regulates immune responses, inflammation, hematopoiesis, etc. Expression of IL-6 is regulated by variety of factors at both the transcriptional and post-transcriptional levels. Overproduction is associated with spectrum of age-related conditions including cardiovascular disease, osteoporosis, arthritis, etc. (Omoigui 2007). Our findings indicate that *G. glabra* extract at concentrations ranging from 5 to 40 μg/ml exhibited significant decrease in IL-6 release. The 50% inhibitory concentration was obtained at 37 μg/ml. Among the phytoconstituents glabridin did not show inhibitory effect on IL-6 production at the tested concentrations. Further, isoliquiritigenin at concentrations ranging from 1.25 to 10 μg/ml exhibited dose-dependent attenuation in IL-6 production (Fig. 2c), an IC_{50} value of 1.92 μg/ml (7.16 μM) was obtained. Surprisingly, glycyrrhizin did not alleviate LPS-induced IL-6 production up to a maximum tested concentration of 10 μg/ml. The cell viability study showed that the inhibitory activity of the extract and isoliquiritigenin was not due to cytotoxic effect. Further, in order to evaluate the potency of these phytoconstituents, we compared their activity with the reference standard dexamethasone. Isoliquiritigenin (IC_{50} = 7.16 μM) was found less potent than dexamethasone (IC_{50} = 58 nM) in inhibiting IL-6 levels. Former study reported that isoliquiritigenin (isolated from *G. uralensis*) alleviated LPS-induced IL-6 production by inhibiting the degradation and phosphorylation of IKB-alpha, and by blocking the activation of NF-kappa B (Kim et al. 2008).

In addition, various evidences suggest that production of eicosanoids and cytokines is interlinked viz. cytokines (IL-1 and TNF-alpha), induces PG synthesis in various cells and PG, in turn, modulates cytokine production. Similarly, dual COX/5-LOX inhibitors are known to preferentially inhibit IL-6 production (Rola-Pleszczynskic and Stankova 1992). Such kind of positive feedback loop is thought to potentially affect immune or inflammatory responses. In this view, based on the previous (Chandrasekaran et al. 2010b) and present findings we elucidate that the inhibitory response by *G. glabra* extract towards inflammatory and pro-inflammatory mediators is correlated. The inhibitory activity of the *G. glabra* extract on pro-inflammatory cytokines could be due to inhibition in PGE_2 production and vice versa. Besides, the dual inhibitory property of the extract can be attributed to alleviation of IL-6 production. Furthermore, increased expression of iNOS, COX-2 (Fu et al. 1999) and elevated levels of pro-inflammatory cytokines (Houghton and Wang 2005) have been observed in the gastric mucosa of patients suffering from *H. pylori* infection. Hence, our findings explain that the anti-ulcer

activity of *G. glabra* may be due to modulation of inflammatory and pro-inflammatory mediators.

In conclusion, although the precise mechanisms of regulating the anti-inflammatory activity of *G. glabra* is not yet known, the present study demonstrates that the de-glycyrrhizinated extract of *G. glabra*, glabridin and isoliquiritigenin effectively inhibits synthesis of pro-inflammatory mediators in LPS-activated murine macrophages. In addition, we propose that the inhibitory propensity of the extract is attributed to either individual or synergistic effect of isoliquiritigenin and glabridin and is not influenced by glycyrrhizin. Thus, the de-glycyrrhizinated extract of *G. glabra* and its phytoconstituents can be used as promising therapeutic agents for treating various inflammation-associated diseases.

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