Short communication

Dual inhibitory effect of *Glycyrrhiza glabra* (GutGard™) on COX and LOX products

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ABSTRACT

Glycyrrhiza glabra and its phytoconstituents have been known to possess widespread pharmacological properties as an anti-inflammatory, anti-viral, antitumour and hepatoprotective drug. In this study, we examined the inhibitory potential of extract of *G. glabra* (GutGard™) root and its phytoconstituents (glabridin, glycyrrhizin, and isoliquiritigenin) on both cyclooxygenase (COX) and lipoxygenase (LOX) products in order to understand the mechanism of its anti-inflammatory action. Inhibitory effect of GutGard™ and its phytoconstituents on lipopolysaccharide (LPS) induced prostaglandin E$_2$ (PGE$_2$), calcimycin (A23187) induced thromboxane (TXB$_2$), and leukotriene (LTB$_4$) release was studied using murine macrophages (J774A.1) and human neutrophil (HL-60) cells. Results revealed that, *G. glabra* and glabridin significantly inhibited PGE$_2$, TXB$_2$ (COX) and LTB$_4$ (LOX), while, isoliquiritigenin exerted inhibitory effect only against COX products but failed to suppress LOX product. However, glycyrrhizin at the tested concentrations failed to exhibit inhibitory effect on both COX and LOX products. Here, we report for the first time that *G. glabra* (almost devoid of glycyrrhizin) exhibits anti-inflammatory property likely through the inhibition of PGE$_2$, TXB$_2$ and LTB$_4$ in mammalian cell assay system, which could be influenced in part by glabridin and isoliquiritigenin.

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Introduction

Glycyrrhiza glabra (*G. glabra* L.; Family: Papilionaceae/Fabaceae) is a well-known medicinal herb that has long been used in the traditional medicinal system for multiple pharmacological activities (Khattak and Simpson 2010). It is a highly reputed ayurvedic plant used in the herbal preparations as a tonic, expectorant and demulcent (Ambawade et al. 2002). It has been reported to have antioxidant (Toshio et al. 2003), immunostimulant, and anti-allergenic (Zore et al. 2008) activities. Recently, Mukherjee et al. (2010) reported the anti-ulcer and anti-oxidant activity of standardized extract of *G. glabra* (GutGard™).

Phytochemical investigation has demonstrated that major bioactive components of licorice root are flavonoids and pentacyclic triterpene saponin, including isoliquiritigenin, glycyrrhizin, glabridin and glycyrrhizic acid (Kamei et al. 2003). Isoliquiritigenin is a flavonoid from licorice having diverse biological activities

such as antiallergic (Kakegawa et al. 1992), antioxidant (Vaya et al. 1997), etc. Apparently, the major pharmacological action of the plant is mainly attributed to glabridin, a chief active flavonoid, which exhibits wide range of biological activities (Kang et al. 2005). However, little is known about the pharmacological mechanisms underlying these actions, although some of its anti-inflammatory effects have been investigated.

Cyclooxygenase-2 (COX-2) and 5-lipoxygenase (5-LOX) are both key enzymes involved in the arachidonic acid (AA) cascade (Funk 2001). The prostaglandins (PGs) and thromboxanes (TXs) produced by COX plays a major role in inflammatory reactions and are responsible for the characteristic inflammatory symptoms (Smith et al. 1996; Gaddi et al. 2004). Considering the potent pro-inflammatory properties of LTB$_4$, the modulation of this pathway should be interesting in the treatment of numerous diseases such as allergic and inflammatory disorders (Brain and Williams 1990). Previous reports have suggested that *G. glabra* inhibits both COX-2 and 5-LOX enzymes in cell free system (Herold et al. 2003). In order to elucidate the mechanism of its anti-inflammatory activity, we investigated the inhibitory effect of extract of *G. glabra* root and its active principles (glabridin, glycyrrhizin, and isoliquiritigenin) on COX and LOX products in cell based assays. We examined LPS induced PGE$_2$ release in murine macrophages (J774A.1) and A23187 stimulated LTB$_4$ and TXB$_2$ levels in human promyelocytic leukemic (HL-60) cells.

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Materials and methods

Source of materials

Lipopolysaccharide (LPS), calcimycin (A23187), acetylsalicylic acid, MTT [1-(4, 5-Dimethylthiazol-2-yl)-3, 5-diphenylformazan], dexamethasone (D-2915) and captopril were purchased from Sigma–Aldrich (St. Louis, MO, USA). Iscove's modified Dulbecco's media (IMDM), and Dulbecco's modified Eagle's medium (DMEM) were supplied by Gibco Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) was purchased from Hyclone (Logan, US).

Investigational substance

The investigational substance GutGard™ (Batch No: RD1972) is an extract derived from roots of *Glycyrrhiza glabra* (Linn.) developed by Natural Remedies Pvt. Ltd, Bangalore. *G. glabra* used in this study was identified at NISCAIR (National Institute of Science Communication And Information Resources). A voucher specimen (No. 51) was deposited in our herbarium. The roots of *G. glabra* were refluxed with acetone in the proportion of 1:4 (thrice). The liquid extract obtained from each extraction wash was filtered and distilled under vacuum (at <55 °C) until a thick paste with a total solid content of 40–50% (w/w) was obtained, which was mixed and dried under vacuum (<65 °C, 500 mm Hg) to get the final extract.

HPLC analysis

HPLC was performed on GutGard™ using Shimadzu Model LC-2010A (Shimadzu Corporation, Tokyo, Japan). Briefly, the stationary phase used was an octadecylsilane column (C_{18}, 5 μ, 250 × 4.6 mm, Phenomenex Luna). The mobile phase consisted of gradient mixture of phosphate buffer (solvent A) and acetonitrile (solvent B) [HPLC grade, Qualigens, India], which were separately filtered through filter (0.45 μ) and degassed by sonicating for 3 min. The solvents were mixed in a linear gradient manner so that the concentration of solvent B was increased from 5% to 45% from 0 to 18 min. Then, the concentration was increased from 45% to 80% in 18–25 min and from 25 to 28 min it was maintained at a concentration of 80%. From 28 to 35 min the concentration of solvent B was decreased from 80% to 45% and from 35 to 45 min the concentration of solvent B was decreased from 45% to 5%. The flow rate of mobile phase was maintained at 1.5 ml/min throughout the analysis and detector wavelength was kept at 280 nm for glabridin and isoliquiritigenin (Fig. 1B); 254 nm for glycyrrhizin (Fig. 1A). Three reference standards (glabridin, isoliquiritigenin and glycyrrhizin) were used to confirm their presence in the extract. Glabridin and isoliquiritigenin were procured from ChromaDex (ChromaDex Inc., CA, USA) and glycyrrhizin from Sigma–Aldrich (Sigma–Aldrich, Inc., St. Louis, MO, USA). The reference compounds, glabridin and isoliquiritigenin were separately dissolved in methanol and glycyrrhizin in water.

The standards and GutGard™ solutions (20 μl each) were injected into HPLC and concentration (% w/w) of each compound in the sample was calculated using the formula: [(sample area/standard area) × (standard concentration/sample concentration)] × standard purity. For total flavonoid estimation, the flavonoid peaks in the sample were identified based on the characteristic UV pattern exhibited by such class of compounds (Markham 1982) and their contents were calculated using glabridin peak area in the standard solution.

Cell lines and culture conditions

HL-60 human promyelocytic leukemia cell line (CCL-240™) and J774A.1 murine macrophage cell line (TIB-67™) were procured from American Type Culture Collection (ATCC) (Rockville,
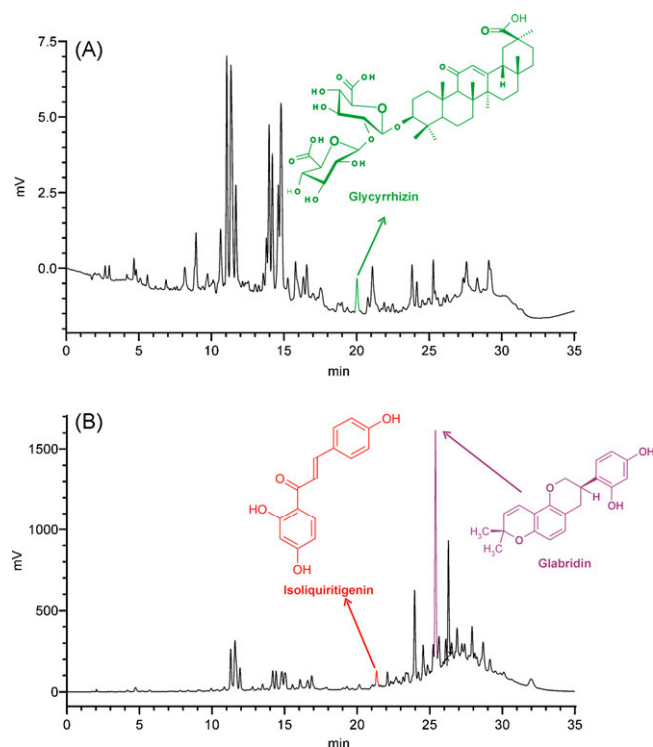

Fig. 1. (A) HPLC chromatogram of GutGard™ (with glycyrrhizin peak identified). (B) HPLC chromatogram of GutGard™ (with isoliquiritigenin and glabridin peak identified).

MD, USA). The cells were cultured in appropriate ATCC recommended medium and maintained at 37 °C under 5% CO_2 humidified air.

Preparation of Ringer's buffer

Ringer's buffer was prepared using the following components: 118 mM NaCl, 4.6 mM KCl, 1.0 mM $CaCl_2$, 1.0 mM KH_2PO_4, 1.10 mM $MgSO_4$, 24.9 mM $NaHCO_3$, 5.0 mM HEPES, 0.1% BSA and 11.1 mM D-glucose. The pH was maintained at 7.4.

Cytotoxicity assay

Cytotoxic effect of extract of *G. glabra* and its phytoconstituents was studied in the respective cell lines by using MTT. Based on the cell viability results, different non-cytotoxic concentrations were selected for each study. All the experiments were conducted in quadruplicates per treatment using 96 well tissue culture plates.

PGE_2 inhibition assay

J774A.1 murine macrophages (passage number between 10 and 25) were plated at a density of 1×10^5 cells/well. After overnight incubation, the macrophages were pretreated with the extract, glabridin, glycyrrhizin and isoliquiritigenin at the non-cytotoxic concentrations followed by addition of LPS (0.1 μg/ml) and incubated for 24 h. Dexamethasone was used as reference standard. PGE_2 levels in macrophage supernatants were quantified as per the method described by the kit manufacturer [Homogenous Time Resolved Fluorescence (HTRF) kit, CisBio, France].

TXB_2 and LTB_4 inhibition assay

HL-60 promyelocytic leukemia cells (passage number between 2 and 10) were differentiated into metamyelocytes using DMSO to

Table 1
Summary of activity profile of *G. glabra* and its phytoconstituents.

S. No.	Test substance	COX products ~ IC_{50}		LOX product ~ IC_{50}
		PGE_2	TXB_2	LTB_4
1.	*Glycyrrhiza glabra* (μg/ml)	5.50	6.30	5.20
2.	Glabridin (μg/ml)	3.61 (11 μM)	3.70 (11.3 μM)	1.73 (5.3 μM)
3.	Isoliquiritigenin (μg/ml)	2.00 (7.8 μM)	>5 (>19.5 μM)	NA
4.	Dexamethasone (nM)	120	–	–
5.	Captopril (μM)	–	–	48
6.	Acetyl salicylic acid (μM)	–	5.5	–

NA – not active at the tested concentrations.

enable production of higher levels of LTB_4 and TXB_2. Cell differentiation was attained by incubating the cells in 1.3% DMSO for 5 days (Collins 1987). The differentiated HL-60 cells were Giemsa stained and examined under microscope. After confirmation, the differentiated HL-60 cells were seeded at a density of 1×10^5 cells/well in Ringer's buffer and pre-treated with the extract and phytoconstituents at different non-cytotoxic concentrations and incubated for 1 h, followed by stimulation with A23187 (5 μM) for 15 min. Captopril and acetyl salicylic acid were used as reference standards for LTB_4 and TXB_2 inhibition assays respectively. The aliquots were removed from the conditioned medium and used for the quantification of LTB_4 (HTRF kit, CisBio, France) and TXB_2 (ELISA kit, Sapphire Bioscience, Australia) levels according to the instructions by the kit manufacturer.

Statistical analysis

Results are expressed as mean percentage ± standard error mean (S.E.M.). One-way ANOVA was performed followed by a Dunnett's test for multiple comparisons using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA) statistical software package. The significance level was chosen at $P < 0.05$–0.01 for all statistical analyses. The half maximal inhibitory concentration (IC_{50}) was calculated using median effect plot (Wolfe and Liu 2007).

Results

Investigational substance

GutGard™ used in this study was found to contain 4.5% w/w of glabridin, 0.1% w/w of isoliquiritigenin, 16% w/w of total flavonoids (calculated as glabridin) and 0.019% w/w glycyrrhizin. The extract had moisture content of 2.4% w/w and ash content of 0.02% w/w (USP 2009).

Cytotoxicity of test substances

Cytotoxic effect of *G. glabra*, glabridin, glycyrrhizin, and isoliquiritigenin were assessed at different concentrations by MTT assay in J774A.1 and differentiated HL-60 cells. The extract did not show any significant toxicity up to a concentration of 40 μg/ml, while glabridin, glycyrrhizin, and isoliquiritigenin were non-cytotoxic up to a concentration of 10 μg/ml (30.5 μM), 50 μg/ml (59.5 μM) and 5 μg/ml (19.5 μM) respectively under our experimental conditions (data not shown). Hereafter, the non-cytotoxic concentrations were chosen for the further experiments.

Suppression of LPS induced PGE2 production in cultured macrophages

Inhibitory effect of *G. glabra* extract and its phytoconstituents on LPS (0.1 μg/ml) induced PGE_2 production in cultured macrophages was examined at various concentrations. The extract demonstrated significant dose-dependent inhibition at concentration ranging from 2.5 to 40 μg/ml (Fig. 2A) with an IC_{50} value of ~5.50 μg/ml. Glabridin and isoliquiritigenin significantly inhibited PGE_2 levels at concentrations ranging between 1.25–10 μg/ml (3.8–30.5 μM) and 1.25–5 μg/ml (4.9–19.5 μM) respectively (Fig. 2B, C). Glabridin and isoliquiritigenin produced 50% inhibition at concentrations of ~3.61 μg/ml (11 μM) and ~2.00 μg/ml (7.8 μM) respectively (Table 1). However, glycyrrhizin failed to show inhibitory effect up to a concentration of 50 μg/ml (59.5 μM). Dexamethasone was used as a reference standard which exhibited 50% inhibition at a concentration of ~120 nM.

Inhibition of A23187 induced TXB2 release in HL-60 promyelocytic leukemia cells

HL-60 cells were stimulated with A23187 (5 μM) to augment TXB_2 production. As shown in Fig. 3A *G. glabra* extract exhibited significant and dose-dependent decrease in TXB_2 levels at concentrations ranging between 2.5 and 40 μg/ml with an IC_{50} value of ~6.30 μg/ml. Similar effects were displayed by glabridin, where significant dose-dependent inhibition was obtained at concentrations ranging from 0.62 to 10 μg/ml (1.9–30.5 μM) (Fig. 3B) with 50% inhibition attained at ~3.70 μg/ml (11.3 μM). However, isoliquiritigenin demonstrated moderate inhibition at 2.5 μg/ml (9.8 μM) and 5 μg/ml (19.5 μM), with maximum inhibition of ~37% obtained at the highest concentration (Fig. 3C) (Table 1). Acetylsalicylic acid was used as a reference standard and IC_{50} value was found to be ~5.5 μM.

Inhibition of A23187 induced LTB4 release in HL-60 promyelocytic leukemia cells

Inhibition of A23187 (5 μM) induced LTB_4 production in differentiated HL-60 cells was studied using various concentrations of extract of *G. glabra* and its phytoconstituents. *G. glabra* displayed significant inhibition at concentrations ranging from 5 to 40 μg/ml (Fig. 4A), with an IC_{50} value of ~5.2 μg/ml. Glabridin showed dose-dependent inhibition in LTB_4 levels with significance achieved between 1.25 and 10 μg/ml (3.8–30.5 μM) (Fig. 4B), 50% inhibition was obtained at ~1.73 μg/ml (5.3 μM) (Table 1). However, glycyrrhizin and isoliquiritigenin failed to inhibit LTB_4 release up to a concentration of 50 μg/ml (59.5 μM) and 5 μg/ml (19.5 μM) respectively. Captopril was used as a reference standard and IC_{50} value was found to be ~48 μM.

Discussion

COX-1 and COX-2 contribute substantially in the formation of PGs, TXs that are responsible for the characteristic inflammatory symptoms (Gaddi et al. 2004). In this study, we demonstrated that the GutGard™ and its constituent, glabridin clearly exhibit inhibitory effect on COX products (PGE_2 and TXB_2). This observation is in agreement with previous study that reported inhibitory
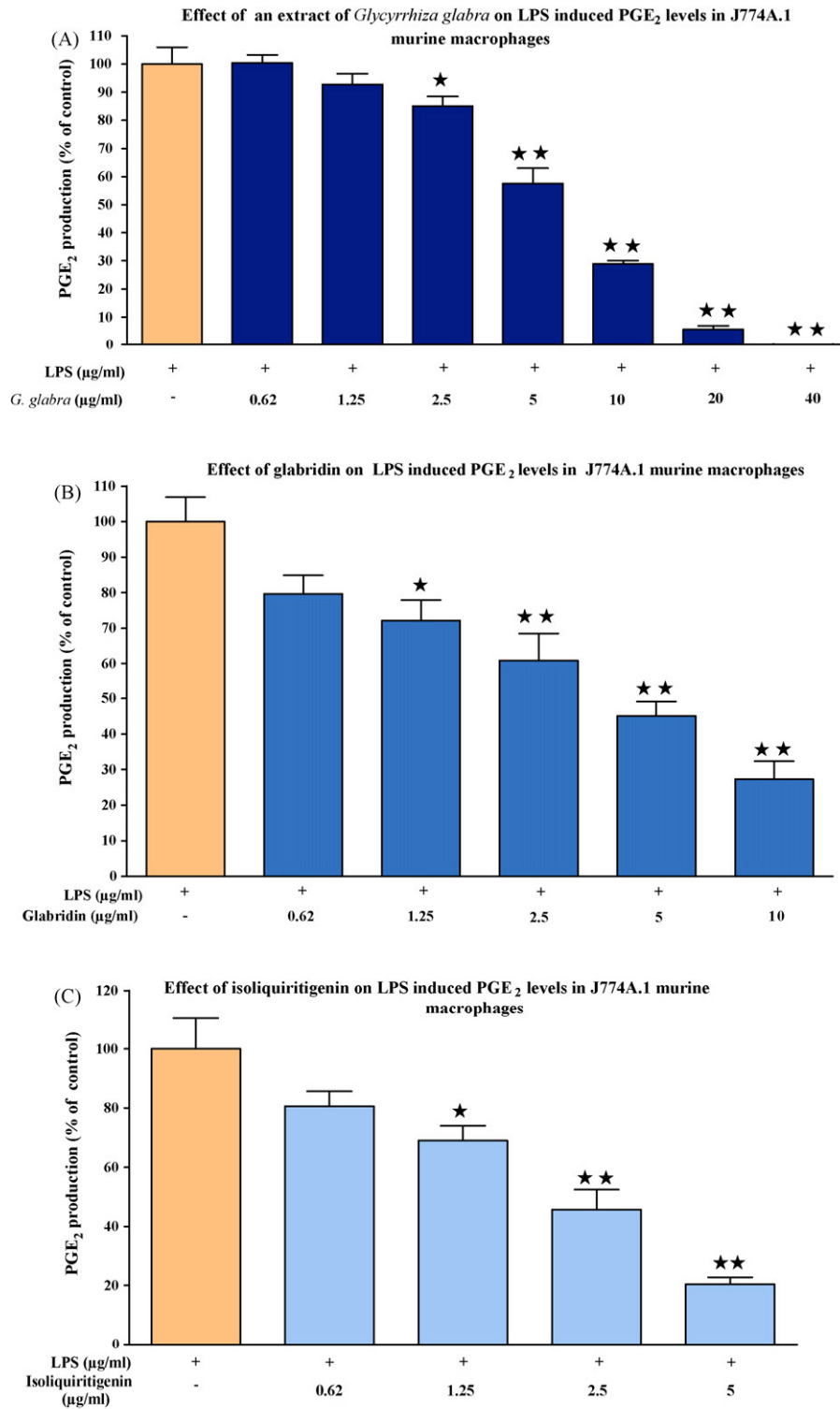
Fig. 2. (A) Effect of extract of *G. glabra* on PGE_2 production in LPS stimulated J774A.1 murine macrophages. Cells were pretreated with indicated concentrations of *G. glabra* extract for 1 h, and then stimulated with LPS (0.1 μg/ml) for 24 h. The PGE_2 levels were dose-dependently decreased by *G. glabra* and the values are expressed as a percentage of the control (LPS alone). Data are represented as mean ± S.E.M. **$P < 0.01$ and *$P < 0.05$ compared with the LPS alone. (B) Effect of glabridin on PGE_2 production in LPS stimulated J774A.1 murine macrophages. Cells were pretreated with indicated concentrations of glabridin for 1 h, and then stimulated with LPS (0.1 μg/ml) for 24 h. The PGE_2 levels were dose-dependently decreased and the values are expressed as a percentage of the control (LPS alone). Data are represented as mean ± S.E.M. **$P < 0.01$ and *$P < 0.05$ compared with the LPS alone. (C) Effect of isoliquiritigenin on PGE_2 production in LPS stimulated J774A.1 murine macrophages. Cells were pretreated with indicated concentrations of isoliquiritigenin for 1 h, and then stimulated with LPS (0.1 μg/ml) for 24 h. The PGE_2 levels were markedly decreased dose-dependently and the values are expressed as a percentage of the control (LPS alone). Data are represented as mean ± S.E.M. **$P < 0.01$ and *$P < 0.05$ compared with the LPS alone.
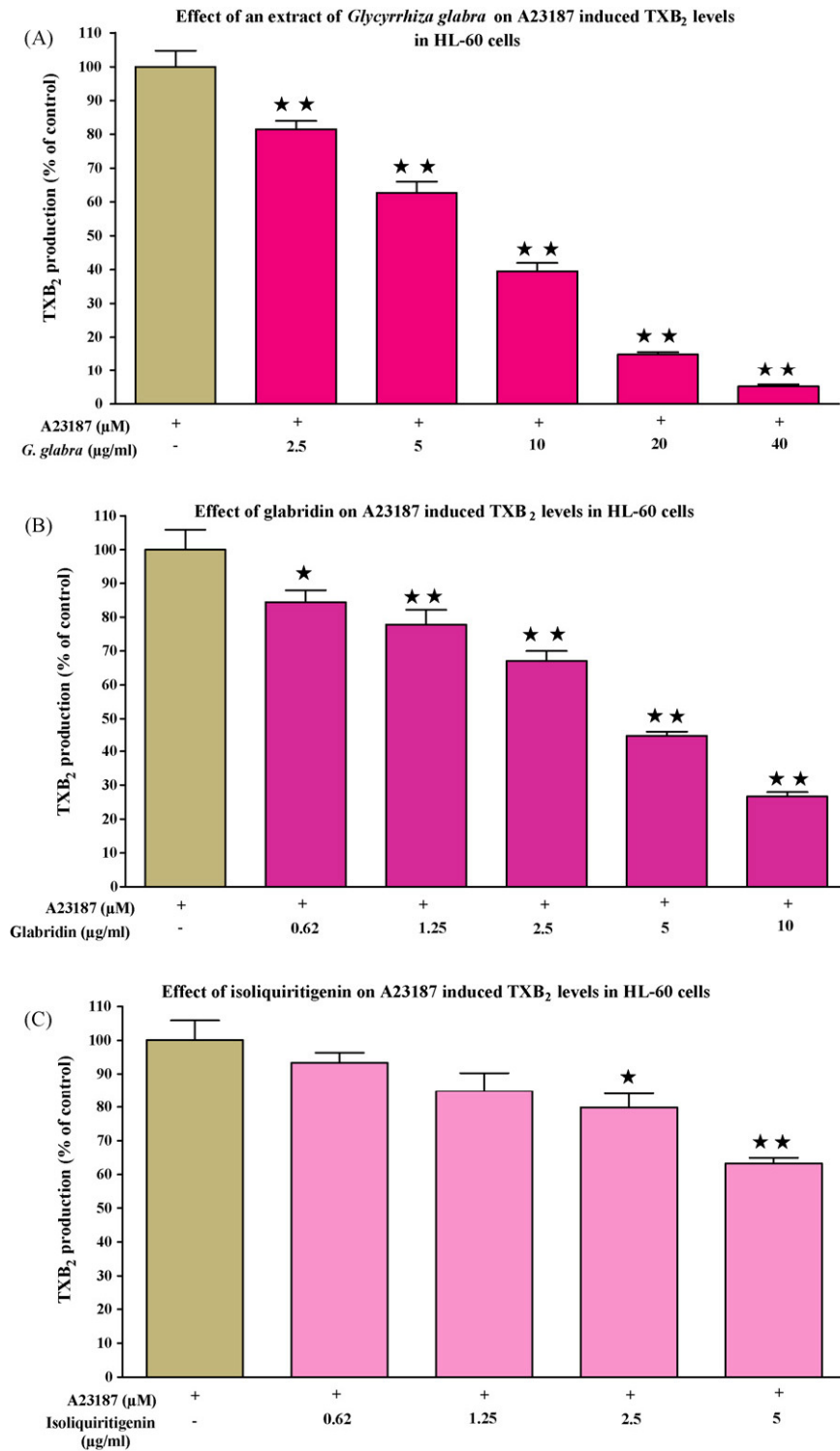
Fig. 3. (A) Effect of extract of *G. glabra* on TXB_2 production in A23187 stimulated HL-60 cells. Cells were pretreated with the indicated concentration of *G. glabra* extract for 1 h. The cells were then stimulated with A23187 (5 μM) for 15 min, the levels of TXB_2 in the medium were quantified. *G. glabra* dose-dependently decreased the TXB_2 production and the values are expressed as a percentage of the control (A23187 alone). Data are represented as mean ± S.E.M. **$P < 0.01$ compared with the A23187 alone. (B) Effect of glabridin on TXB_2 production in A23187 stimulated HL-60 cells. Differentiated HL-60 cells were pretreated with indicated concentrations of glabridin for 1 h. After stimulation with A23187 (5 μM) for 15 min, the levels of TXB_2 in the medium were quantified. Glabridin dose-dependently decreased the TXB_2 production and the values are expressed as a percentage of the control (A23187 alone). Data are represented as mean ± S.E.M. **$P < 0.01$ and *$P < 0.05$ compared with the A23187 alone. (C) Effect of isoliquiritigenin on TXB_2 production in A23187 stimulated HL-60 cells. Differentiated HL-60 cells were pretreated with indicated concentrations of isoliquiritigenin for 1 h. After stimulation with A23187 (5 μM) for 15 min, the levels of TXB_2 in the medium were quantified. Isoliquiritigenin dose-dependently decreased the TXB_2 production and the values are expressed as a percentage of the control (A23187 alone). Data are represented as mean ± S.E.M. **$P < 0.01$ and *$P < 0.05$ compared with the A23187 alone.
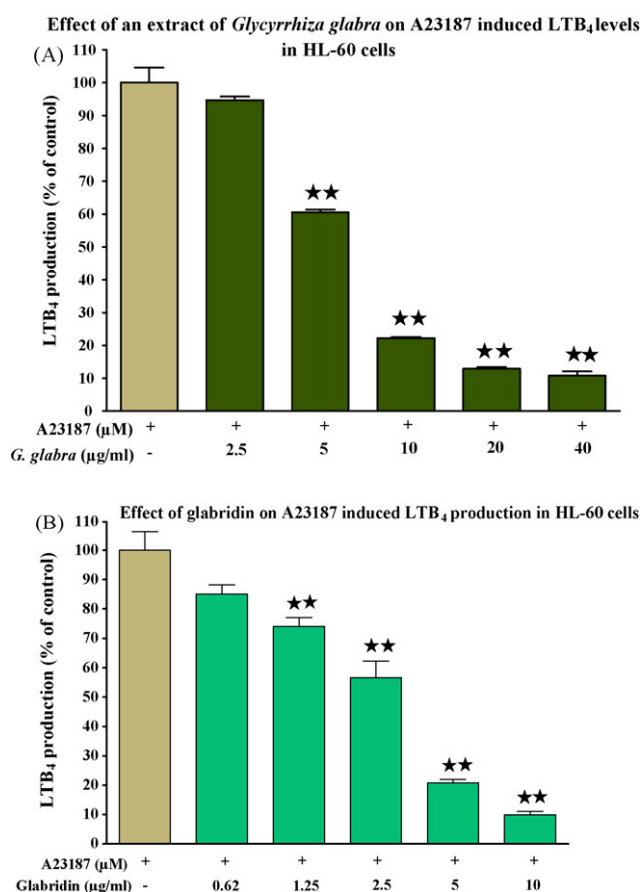
Effect of an extract of *Glycyrrhiza glabra* on A23187 induced LTB$_4$ levels in HL-60 cells

(A)

Effect of glabridin on A23187 induced LTB$_4$ production in HL-60 cells

(B)

Fig. 4. (A) Effect of *G. glabra* on LTB$_4$ production in A23187 stimulated HL-60 cells. Differentiated HL-60 cells were pretreated with indicated concentrations of *G. glabra* extract for 1 h. After stimulation with A23187 (5 μM) for 15 min, the levels of LTB$_4$ in the medium were quantified. *G. glabra* dose-dependently decreased the LTB$_4$ production and the values are expressed as a percentage of the control (A23187 alone). Data are represented as mean ± S.E.M. **$P < 0.01$ compared with the A23187 alone. (B) Effect of glabridin on LTB$_4$ production in A23187 stimulated HL-60 cells. Differentiated HL-60 cells were pretreated with indicated concentrations of glabridin for 1 h. After stimulation with A23187 (5 μM) for 15 min, the levels of LTB$_4$ in the medium were quantified. Glabridin dose-dependently decreased the LTB$_4$ production and the values are expressed as a percentage of the control (A23187 alone). Data are represented as mean ± S.E.M. **$P < 0.01$ compared with the A23187 alone.

effect of glabridin on cyclooxygenase activity (Yokota et al. 1998). Further, we observed that isoliquiritigenin was more potent than glabridin in inhibiting PGE$_2$ production, but exerted moderate inhibitory effect towards TXB$_2$ production. Similar study revealed that isoliquiritigenin (isolated from roots of *Glycyrrhiza uralensis*) at 0.8 and 1.6 μM inhibited LPS induced COX-2 protein expression in RAW 264.7 macrophages (Kim et al. 2008). According to our results, glycyrrhizin up to a concentration of 50 μg/ml (59.5 μM) failed to exert inhibitory effect on PGE$_2$ levels. However, significant inhibition of PGE$_2$ production was observed at higher concentrations (100 and 1000 μg/ml) of glycyrrhizin without affecting the protein synthesis (Ohuchi et al. 1981). On comparing the PGE$_2$ inhibitory activity of an extract and the phytoconstituents, isoliquiritigenin and glabridin exhibited two-fold higher potency than the extract. Thus, we conclude that the COX inhibitory effect of *G. glabra* is attributed to glabridin and isoliquiritigenin.

5-LOX is the major isozyme of LOX pathway, which is involved in the biosynthesis of LTs (Leukotrienes), one of the potent inflammatory mediators (Charlier and Michaux 2003). Herein, we observed that increase in A23187 elicited LTB$_4$ production in differentiated HL-60 cells was dose-dependently inhibited by *G. glabra* extract and its constituent glabridin. Apparently, glabridin showed three-

fold higher potency than the extract. However, glycyrrhizin and isoliquiritigenin at the non-cytotoxic concentrations failed to show any inhibitory effect. Based on the results we deduce that the inhibitory effect of *G. glabra* on LTB$_4$ production is influenced by glabridin. Overall, glycyrrhizin did not show any inhibitory activity against COX and LOX products. Our results are in agreement with the study conducted by Inoue et al. (1986) in cell free system. Hence, the anti-inflammatory activity of glycyrrhizin is due to other mechanisms such as by inhibiting the generation of ROS by neutrophils (Akamatsu et al. 1991), inhibiting the development of histamine-serotonin and bradykinin induced oedema and decreased vascular permeability (Nasyrov and Lazareva 1980). Overall, GutGard^{TM} might pose a better candidate to counter acute and chronic inflammatory state without exhibiting marked ulcerogenic risk, contrary to classical NSAIDs since it possesses dual inhibitory property against COX and LOX products.

In summary, we demonstrate that *G. glabra* and its major active principle glabridin exhibited significant inhibition of PGE$_2$, TXB$_2$, and LTB$_4$ production, while isoliquiritigenin actively suppressed the PGE$_2$ and TXB$_2$ levels and glycyrrhizin did not demonstrate any inhibitory effect in this study. Based on the inhibitory activity towards both COX and LOX products, *G. glabra* could be designated as a dual inhibitor. Further, we speculate that the inhibitory propensity of *G. glabra* extract towards COX and LOX products is mainly attributed to glabridin and isoliquiritigenin and is not influenced by glycyrrhizin. As a novel finding, our study demonstrates that *G. glabra* inhibits A23187 induced TXB$_2$ and LTB$_4$ production. Thus, an extended comprehension of the underlying molecular mechanisms involved might lead to improved phytomedical treatment of inflammation allied disorders at an early phase.

Acknowledgement

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