The constituents of licorice (Glycyrrhiza uralensis) differentially suppress nitric oxide production in interleukin-1β–treated hepatocytes

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A B S T R A C T

Licorice (Glycyrrhiza radix) is the roots and stolons of Glycyrrhiza uralensis Fischer or Glycyrrhiza glabra Linnaeus in the Japanese Pharmacopoeia. Glycyrrhiza radix has been widely used as a sweetener and a traditional medicine. A Glycyrrhiza radix extract contains many constituents and has antispasmodic, antitussive, anti-ulcer, and anti-inflammatory effects. However, reports comparing the anti-inflammatory effects of these constituents are very few. Here, we purified several constituents from the roots and stolons of Glycyrrhiza uralensis and examined and compared their anti-inflammatory effects by monitoring the levels of the inflammatory mediator, nitric oxide (NO), in interleukin (IL)-1β–treated rat hepatocytes. From the Glycyrrhiza radix extract, we purified the main constituent glycyrrhizin and the constituents that are characteristic of Glycyrrhiza uralensis (chalcones and flavanones). These constituents suppressed NO production in IL-1β–treated rat hepatocytes, and isoliquiritigenin showed the greatest suppression activity. Isoliquiritigenin, isoliquiritin, and liquiritigenin significantly decreased both protein and mRNA for the inducible nitric oxide synthase. These constituents reduced the levels of mRNAs encoding tumor necrosis factor α and IL-6. In contrast, although glycyrrhizin is abundant, it showed a 100-fold lower potency in NO suppression. Therefore, both glycyrrhizin and the minor constituents (isoliquiritigenin, isoliquiritin, and liquiritigenin) may be responsible for the anti-inflammatory effects of Glycyrrhiza uralensis. It is also implied that these constituents may have a therapeutic potential for inflammatory hepatic disorders.

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1. Introduction

Licorice is as a natural sweetener in foods and a traditional medicine that is used worldwide to treat a variety of diseases, such as peptic ulcer and hepatitis [1]. In the Japanese Pharmacopoeia, licorices are defined as the dried roots and stolons of Glycyrrhiza uralensis Fischer and Glycyrrhiza glabra Linnaeus and are designated Glycyrrhizae radix (kanzo in Japanese) [2]. Glycyrrhiza radix has been used in many formulae of Japanese herbal (Kampo) medicines, such as kanzo and shakuyakukan zoto. Glycyrrhiza radix has a variety of pharmacological properties, including antispasmodic, antitussive, anti-ulcer, and anti-inflammatory effects, and attenuates the adverse effects of other components in the Kampo formulae [3]. Glycyrrhizae radix includes many constituents: triterpene glycosides (mainly, glycyrrhizin), chalcones (e.g., isoliquiritigenin and isoliquiritin), and flavanones (e.g., liquiritigenin and liquoritbin) [4,5]. Glycyrrhizin (also designated glycyr rhizic acid) is a glycoside of glycyr rhyclic acid (also designated glycyrhretic acid) (Fig. 1A) and is abundantly present in the roots and stolons. This saponin is the major sweet constituent and the main bioactive compound in Glycyrrhizae radix [1], which displays hepatoprotective properties in humans and mice [6,7]. The contents of isoliquiritin, liquiritigenin, and liquiritin in G. uralensis are significantly higher than those of G. glabra; therefore, these constituents are characteristic of G. uralensis [4,5]. As far as we searched the literatures to date, there are no reports that compare anti-inflammatory activity of several Glycyrrhizae radix constituents using any types of cells.

The inflammatory mediator nitric oxide (NO) plays a pivotal role in innate immunity and pathophysiology of various diseases, and inducible nitric oxide synthase (iNOS) synthesizes NO in hepatocytes and macrophages [8,9]. Similarly in the hepatocytes in the liver, the iNOS gene is induced by the inflammatory cytokine...
interleukin-1β (IL-1β) in primary cultured rat hepatocytes, and this induction mimics liver injury, such as acute hepatitis [9,10]. As iNOS protein is induced, the NO level linearly increases from 6 h to 12 h after the addition of IL-1β.

Using the hepatocytes, we demonstrated that NO is a sensitive marker that can be used to monitor inflammatory responses to anti-inflammatory drugs [11]. Japanese herbal medicines [12] and their constituents, such as chlorogenic acid (i.e., 5-O-cafeoylquinic acid) in the flowers and buds of the Japanese honeysuckle (Lonicera japonica) [13] and nobiletin (polymethoxylated flavone) in the peels of Citrus unshiu [14]. Only one paper using primary cultured hepatocytes, which described anti-inflammatory effects of glycyrrhizin, was found in the literatures that we have searched to date [15]. This report suggested NO suppression activity of glycyrrhizin in primary cultured hepatocytes that were isolated from Bacillus Calmette-Guérin (BCG)-vaccinated rats. Hepatoprotective herbal medicines suppress the IL-1β-induced expression of iNOS and inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin-6 (IL-6), in hepatocytes [13,14]. The transcription factor nuclear factor κB (NF-κB) is involved in these processes by regulating the expression of iNOS gene and these inflammatory genes [16–18].

iNOS induction is also regulated via a post-transcriptional mechanism that is mediated by antisense transcripts (asRNAs) [9]. asRNAs are transcribed from the iNOS gene and interact with iNOS mRNA to stabilize iNOS mRNA [9,19–21]. Recently, herbal constituents (e.g., chlorogenic acid [13], nobiletin [14], gomisin N [18], and shisoflavonone A [22]), as well as Campo formulae (e.g., inchinoto [12] and ninjinyoeto [23]), have been reported to decrease the levels of iNOS asRNA, leading to the inhibition of iNOS expression.

In the present study, we purified glycyrrhizin and several constituents that are characteristic of G. uralensis. Next, we examined whether the constituents (aglycone–glycoside pairs) suppressed NO production, as well as iNOS gene expression, in IL-1β-treated hepatocytes. Finally, we investigated which constituent (s) is responsible for the NO suppression activity of Glycyrrhizae radix.

2. Materials and methods

2.1. Materials

Glycyrrhizin, isoliquiritigenin, and liquiritigenin were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan) as standards. Glycyrrhetic acid was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). These compounds (greater than 95% purity) were dissolved in dimethyl sulfoxide.

2.2. Plant materials and extraction

The roots and stolons of G. uralensis Fischer, which were collected from the Inner Mongolia Autonomous Region, China, and identified and authenticated by Dr. Yutaka Yamamoto (Tochimoto Tenkaido Co. Ltd., Osaka, Japan), were purchased from Tochimoto Tenkaido Co. Ltd. A voucher specimen was deposited in the Ritsumeikan Herbarium of Pharmacognosy, Ritsumeikan University, under the Code number RIN-GU-013. The dried roots and stolons of G. uralensis (Glycyrrhiza radix: 502.6 g) were pulverized and extracted by hot water under reflux. The solvent was evaporated under reduced pressure to yield a hot-water extract (160.0 g).

2.3. Isolation of the constituents from a G. uralensis extract

The G. uralensis extract was fractionated on a Diaion HP20 column (Mitsubishi Chemical Corporation, Tokyo, Japan) by the elution stepwise by methanol–water mixture (0, 20, 40, 60, 80, and 100%) to give the fractions (GR-0, 20, 40, 60, 80, and 100%, respectively). The fraction GR-80 showing high NO suppressive activity was further purified by Wakogel C-200 chromatography (Wako Pure Chemical Industries Ltd.) or Cosmosil 75 C18–Prep octadecysilyl chromatography (Nacalai Tesque, Kyoto, Japan). Finally, we purified glycyrrhizin (3850 mg), isoliquiritigenin (4.3 mg), isoliquiritin (234 mg), liquiritigenin (16.4 mg), and liquiritin (8.8 mg). To determine the structures, the 1H- and 13C nuclear magnetic resonance (NMR) spectra, infrared (IR) spectra, and ultraviolet (UV) spectra were analyzed (Supplementary data).

2.4. Preparation of primary cultured rat hepatocytes

Male Wistar rats were purchased from Charles River Laboratories Japan Inc. (Yokohama, Japan), housed at 21–23 °C and acclimated. Hepatocytes were isolated from the rat livers by collagenase perfusion [24]. The isolated cells were resuspended in Williams’ E (WE) medium (Sigma-Aldrich Corp., St. Louis, MO, USA), seeded at 1.2 × 10⁵ cells/dish, and incubated at 37 °C for 2 h, after which the medium was replaced. The hepatocytes were incubated at 37 °C overnight and analyzed the next day. All of the animal care and experimental procedures were carried out in accordance with the guidelines and laws of the Japanese government and were approved by the Animal Care Committee of Ritsumeikan University, Biwako-Kusatsu Campus.

2.5. Determination of the NO levels and LDH activity

The hepatocytes were each treated with fraction or a constituent in the presence of 1 nM rat IL-1β (PeproTech, Rocky Hill, NJ, USA) for 8 h. If required, 1400W (Abcam plc., Cambridge, Cambridgeshire, United Kingdom), a selective iNOS inhibitor [25], was added to the medium to a final concentration of 50 nM. The levels of nitrite (a stable metabolite of NO) in the medium were measured using the Griess method [26]. Briefly, 150 μl of the medium or sodium nitrite (standard) was mixed with 150 μl of the Griess reagent [0.5% sulfanilamide, 0.05% N-(1-naphthyl)
3.1. The fractions of a *G. uralensis* extract suppress the NO induction in hepatocytes

We extracted the dried roots and stolons of *G. uralensis* (Glycyrrhizae radix) using hot water, and this extract (GR extract) was subjected to fractionation by hydrophobicity. Because the high content of saponins (mostly glycyrrhizin) inhibited phase separation, the GR extract was not successfully fractionated by the standard ABC method [13] (data not shown). Therefore, we performed Diaion HP-20 chromatography to fractionate the GR extract into six fractions.

Then, we investigated the effects of the resultant fractions on NO induction in IL-1β-treated rat hepatocytes. When each fraction was simultaneously added to the medium with IL-1β, all of the fractions significantly decreased the levels of NO production (Fig. 1B). Three fractions GR-60 (21.2 g), GR-80 (22.0 g) and GR-100 (3.7 g), which were eluted with 60%, 80% and 100% methanol, respectively, effectively suppressed NO production. The LDH activity in the medium was very low (data not shown), suggesting that all of the fractions of the GR extract were not toxic to hepatocytes at the concentrations that are indicated in the figure. Together, these data indicate that the three fractions GR-60, GR-80, and GR-100 may include active compounds that may suppress NO induction.

3.2. Several constituents in a GR extract suppress NO induction in hepatocytes

Next, we tried to isolate the constituents that effectively suppressed NO induction from the fractions showing NO suppression activity. Because the amount of the fraction GR-100 was much smaller, the fractions GR-60 and GR-80 were subjected to thin-layer chromatography. This analysis showed that the major spots of both fractions were similarly developed (data not shown). Therefore, we selected the middle fraction GR-80 and then purified five constituents from this fraction, as described in Supplementary data. Finally, we identified glycyrrhizin and two aglycone-glycoside pairs (isoliquiritigenin and isoliquiritin; and liquiritigenin and liquiritin) by analyzing the NMR and UV spectra (Supplementary data). These constituents except for glycyrrhizin are characteristic of *G. uralensis* [4,5].

To examine whether the constituents in a GR extract affect iNOS gene expression, we added each constituent to the medium for hepatocytes. Among these constituents, isoliquiritigenin significantly suppressed NO induction in the presence of IL-1β in a dose-dependent manner (Fig. 2A). Similarly, isoliquiritin and liquiritigenin significantly suppressed NO induction. When evaluating the LDH activity in the medium, these constituents displayed no cytotoxicity at the concentrations that are indicated in Fig. 2A. When the IC₅₀ values were calculated (Table 1), isoliquiritigenin showed the highest potency in NO suppression activity, with an IC₅₀ value of 11.9 μM. In another assay system using a mouse RAW264.7 macrophage line, an IC₅₀ value of isoliquiritigenin of 7.8 μM was reported [27].

In contrast, the main constituent glycyrrhizin suppressed NO induction only at a high concentration, showing an IC₅₀ value of 1176 μM, which is approximately 100-fold higher than that of isoliquiritigenin. Then, we examined the degradation of glycyrrhizin by incubating the WE medium containing 1000 μM glycyrrhizin at 37 °C for 8 h. Glycyrrhizin was purified by Diaion HP-20 chromatography and analyzed by high-performance liquid chromatography (HPLC). The content of glycyrrhizin did not change by the incubation (data not shown). Furthermore, when the WE medium containing 1000 μM glycyrrhizin was similarly incubated in the presence of hepatocytes, the content of glycyrrhizin was almost the same as that of the medium without incubation (data not shown). These results suggest that the...
Fig. 2. The G. uralensis constituents suppress iNOS expression in hepatocytes. (A) The effects of the constituents in a GR extract on the induction of NO production. The hepatocytes were treated with IL-1β and/or each constituent for 8 h. The NO levels in the medium were measured in triplicate (mean ± SD). *P < 0.05 and **P < 0.01 versus IL-1β alone. ILG, isoliquiritigenin; IL, isoliquiritin; LG, liquiritigenin. (B) The effects of the constituents on the induction of iNOS protein expression. The hepatocytes were treated with IL-1β and/or each constituent for 8 h. The cell extracts were resolved using SDS-PAGE and immunoblotted with an anti-iNOS or anti-β-tubulin antibody (internal control). (C) The effects of 1400W, a selective iNOS inhibitor, on the levels of NO (upper panel) and iNOS mRNA (lower panel). The hepatocytes were treated with 50 nM 1400W and/or 100 μM isoliquiritin in the presence of 1 nM IL-1β for 8 h (NO) or 4 h (iNOS mRNA). The NO levels in the medium were measured in triplicate (mean ± SD). The total RNA from the cells was analyzed using quantitative RT-PCR to estimate the levels of iNOS mRNA. The mRNA levels were measured in triplicate (mean ± SD), the obtained values were normalized to EF mRNA (internal control), and the value in the presence of IL-1β alone was set at 100%. **P < 0.01.

Table 1
The effects of the G. uralensis constituents on NO induction in rat hepatocytes.

| Constituent | Sugar            | Content [%] | IC50 [μM] | LogP |
|------------|------------------|-------------|------------|------|
| Triterpenes|                  |             |            |      |
| Glycyrrhetinic acid | None | ND      | NA | 5.45 |
| Glycyrrhizin   | Glucuronic acid  | 3.37 ± 1.57 | 1176 ± 482 | 2.74 |
| Chalcones:     |                  |             |            |      |
| Isoliquiritigenin | None | 0.05–0.65 | 11.9 ± 1.5 | 3.04 |
| Isoliquiritin  | Glucose          | 0.32 ± 0.20 | 40.4 ± 7.5 | 0.82 |
| Flavanones:    |                  |             |            |      |
| Liquiritigenin | None            | 0.11 ± 0.12 | 41.2 ± 5.9 | 2.79 |
| Liquiritin     | Glucose          | 1.68 ± 1.06 | NA | 0.43 |

ND, not determined; NA, not applicable due to high cytotoxicity (glycyrrhetinic acid) or low NO suppression activity (liquiritin).

* The contents of isoliquiritigenin (range) [5] and the others (mean ± SD; n = 87) [4] in a G. uralensis extract.

b The half-maximal inhibitory concentration of nitric oxide (NO) production in hepatocytes (mean ± SD; n = 3–5).

c n-Octanol/water partition coefficient as predicted using the ALOGPS 2.1 program [29].

3.3. The G. uralensis constituents inhibit iNOS gene expression in hepatocytes

To further investigate the effect of the active G. uralensis constituents on iNOS gene expression, we examined the expression of the iNOS protein in hepatocytes. As shown in Fig. 2B, western blot analyses indicated that isoliquiritigenin, isoliquiritin, and liquiritigenin decreased the iNOS protein expression that was induced by IL-1β.

Then, we examined a possibility that these constituents inhibited enzyme activity of iNOS protein. Therefore, we added isoliquiritin and/or 1400W [25], which is a selective iNOS inhibitor and does not affect the expression of iNOS mRNA, to the medium and measured the levels of both NO and iNOS mRNA. As shown in Fig. 2C, the addition of 1400W significantly decreased the NO levels, and isoliquiritin also decreased the NO levels, suggesting that 1400W and isoliquiritin showed an additive effect. The addition of 1400W, however, did not decrease in the levels of iNOS mRNA, whereas isoliquiritin significantly reduced the levels of iNOS mRNA (lower panel). Isoliquiritin also decreased iNOS protein (Fig. 2B), although isoliquiritin did not inhibit enzyme activity of iNOS protein (Fig. 2C). Therefore, isoliquiritin may suppress the iNOS gene expression by reducing the iNOS mRNA, resulting in the decrease of iNOS protein.

Quantitative RT-PCR analyses revealed that the constituents including isoliquiritin significantly reduced iNOS mRNA levels in a dose-dependent manner (Fig. 3A), suggesting that these constituents suppressed the induction of iNOS mRNA. Taken together, isoliquiritigenin, isoliquiritin, and liquiritigenin decreased the levels of both iNOS mRNA and protein. These results indicate that the three constituents inhibited the IL-1β-induced expression of the iNOS gene at the transcriptional level.

It is possible that the constituents suppress iNOS expression at the post-transcriptional level because iNOS asRNA interacts with...
and stabilizes iNOS mRNA [9,21]. Therefore, we estimated the levels of iNOS asRNA by quantitative RT-PCR. Isoliquiritigenin, isoliquiritin, and liquiritigenin significantly reduced iNOS asRNA levels (Fig. 3B). Because decreases in iNOS asRNA lead to decreased iNOS mRNA stability, these results indicate that the three constituents may post-transcriptionally regulate iNOS mRNA levels by reducing iNOS asRNA stability.

Both transcriptional and post-transcriptional mechanisms to regulate iNOS expression may be targets of herbal medicines. The Kampo formula *inchinkoto* and herbal constituents, such as gohmisin N and nobiletin, affect the nuclear translocation and activation of NF-κB, leading to the suppression of iNOS gene expression [12,14,18].

### 3.4. The *G. uralensis* constituents suppress inflammatory genes

We examined whether the constituents that were isolated from a *G. uralensis* extract affect the expression of inflammatory genes in hepatocytes. As shown in Figs. 3C and D, the *G. uralensis* constituents decreased the levels of the mRNAs encoding TNF-α and IL-6, whereas the inflammatory cytokine IL-1β induced the expression of these mRNAs. After tissue damage or bacterial infection, IL-6 is an important mediator of an acute phase response in the hepatocytes [28]. Because the constituents down-regulated IL-6 mRNA, these results support that the *G. uralensis* constituents may have anti-inflammatory effects.

NF-κB is believed to regulate the mRNA expression of these inflammatory genes through the NF-κB-binding site(s) in their promoters [16,29]. Indeed, liquiritigenin suppressed iNOS expression by inhibiting NF-κB activation in RAW264.7 macrophages [30,31]. NF-κB also regulates the transcription of iNOS asRNA [9], and the *G. uralensis* constituents decreased the levels of iNOS asRNA (Fig. 3B). Therefore, it is likely that the constituents in a *G. uralensis* extract reduce NF-κB activity, resulting in the inhibition of expression of NF-κB-regulating genes, including not only iNOS but also inflammatory genes in hepatocytes.

### 3.5. The NO suppression activity of the *G. uralensis* constituents

We further compared the effects of the aglycone–glycoside pairs in the *G. uralensis* constituents. An aglycone of glycyrrhizin is glycyrrhetinic acid, which is believed to be an active metabolite [32]. When glycyrrhizin is orally administered, intestinal bacteria hydrolyze it to glycyrrhetinic acid, which is then absorbed in the intestine and transferred to the liver [32]. When glycyrrhetinic acid was added to the medium, it showed toxicity to rat hepatocytes (i.e., LDH activity in the medium) at a concentration of more than 40 μM (data not shown). In contrast, glycyrrhizin did not display cytotoxicity at 1000 μM (data not shown).

The contents of isoliquiritigenin, isoliquiritin, or liquiritigenin were much lower than glycyrrhizin [4,5] but displayed high NO suppression activities, with IC₅₀ values of 11.9–41.2 μM (Table 1). In contrast, although glycyrrhizin is highly abundant (3.37%) in *G. uralensis*, it possessed an IC₅₀ value of 1176 μM in NO suppression. Together with these data, the NO suppression activity of a GR extract can be attributed to the activities of both glycyrrhizin and the three constituents. Because NO is an inflammatory mediator, these constituents may be responsible for the anti-inflammatory effects of *G. uralensis*.

### 3.6. Lipophilicity of the *G. uralensis* constituents

The mechanism how the *G. uralensis* constituents affect intracellular signal transduction remains unclear. When a constituent is
relatively hydrophobic, the plasma membrane may be permeable to the constituent. As a marker of lipophilicity (hydrobicity), we predicted the n-octanol/water partition coefficient (logP) using the ALOOPS 2.1 program [33]. The logP values of the G. uralensis compounds had IC50 values that ranged from 0.82 to 3.04 (Table 1). In contrast, glycyrrhetinic acid, which is a hydrolyzed metabolite of glycyrrhizin and shows toxicity to hepatocytes, had a high logP value, suggesting that glycyrrhetinic acid may be easily absorbed by the cell. Glycyrrhetinic acid, which is hyrolyzed from glycyrrhizin, is almost completely metabolized through glucuronidation and/or sulfation in the liver [34]. Therefore, the concentration of glycyrrhetinic acid in the liver is assumed to be very low; thus, hepatotoxicity may not appear in vivo. Once a constituent enters into the cell, it may interact with various proteins, such as protein kinases. Alternatively, flavonoids from G. uralensis may bind to mRNA or asRNA because flavonoids (i.e., catechins and apigenin) bind to RNA [35,36]. It is possible that these intracellular interactions may affect gene expression.

3.7. The potential use of the G. uralensis constituents for an anti-inflammatory therapy

We compared the NO suppression potency of the G. uralensis constituents with that of other herbal constituents in hepatocytes. The IC50 value of isoliquiritigenin was much lower than that of nootbin (51 μM) [14], suggesting that isoliquiritigenin has high potency in NO suppression. Furthermore, isoliquiritigenin, isoliquritinitrin, and liquiritigenin decreased the mRNA levels of inflammatory cytokines, including TNF-α and IL-6 (Fig. 3C, D). In contrast, an IC50 value of glycyrrhizin was higher than that of chlorogenic acid (652 μM) [13]. Although abundant glycyrrhizin showed much lower potency in NO suppression in hepatocytes (Table 1), its hepatoprotective activities have been reported [67]. Taken together, the anti-inflammatory activity of G. uralensis in hepatocytes may be attributed to the activities of not only glycyrrhizin but also the three constituents. Each constituent may be differentially responsible for a variety of pharmacological effects of G. uralensis.

The G. uralensis constituents have potential values to treat inflammatory diseases, especially viral hepatitis, alcoholic liver diseases, and non-alcoholic steatohepatitis (NASH). Similarly to herbal medicines for prostate cancer [37], future clinical trials are required to examine whether the G. uralensis constituents are used as a complementary and alternative medicine for an anti-inflammatory therapy for inflammatory hepatic disorders.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.bbrep.2015.06.004.

References

[1] M.N. Asl, H. Hosseinzadeh, Review of pharmacological effects of Glycyrrhiza sp. and its bioactive compounds, Phytother. Res. 22 (2008) 709–724.
[2] The Committee on the Japanese Pharmacopoeia, The Japanese Pharmacopoeia, 16th ed., The Ministry of Health, Labour and Welfare, Japan, 2011.
[3] K. Takagi, The pharmacological action of Glycyrrhiza radix, J. Tradit. Sino-Jpn. Med. 2 (1981) 34–37.
[4] R. Kondo, M. Shiba, R. Nakamura, et al., Constituent properties of licorices derived from Glycyrrhiza uralensis, G. glabra, and G. inflata identified by genetic information, Biopharm. Bull. 30 (2007) 1271–1277.
[5] S. Zhi, R. Sugiya, J. Batikhuu, et al., Survey of Glycyrrhiza radix resources in Mongolia: chemical assessment of the underground part of Glycyrrhiza uralensis and comparison with Chinese Glycyrrhiza radix, J. Nat. Med. 63 (2009) 137–146.
[6] P. Muriel, Y. Rivera-Espinoza, Beneficial drugs for liver diseases, J. Appl. Toxicol. 29 (2009) 93–103.
[7] N. Tamakura, K. Abe, K. Wake, et al., Hepatic protection by glycyrrhizin and inhibition of iNOS expression in concanavalin A-induced liver injury in mice, Inflamm. Res. 58 (2009) 593–599.
[8] M. Colafronti, H. Suzuki, The dual personal option of NO, Trends Pharmacol. Sci. 13 (1992) 249–252.
[9] K. Matsui, M. Nishizawa, T. Ozaki, et al., Natural antisense transcript stabilizes inducible nitric oxide synthase messenger RNA in rat hepatocytes, Hepatology 41 (2005) 686–697.
[10] H. Kitade, K. Sakitani, K. Inoue, et al., Interleukin 1beta markedly stimulates nitric oxide formation in the absence of other cytokines or lipopolysaccharide in primary cultured rat hepatocytes but not in Kupfer cells, Hepatology 23 (1996) 797–802.
[11] H. Inaba, E. Yoshigai, T. Okuyama, et al., Antipyretic and anti-inflammatory activities of liquiritin, liquiritigenin and glycyrrhetinic acid from Glycyrrhiza uralensis, Phytother. Res. 22 (2008) 1036–1042.
[12] T. Matsuura, M. Kairori, Y. Araki, et al., Japanese herbal medicine, isinchikoto inhibits inducible nitric oxide synthase induction in interleukin-1β-stimulated hepatocytes, Hepatol. Res. 42 (2012) 76–90.
[13] N. Ohno, E. Yoshigai, T. Okuyama, et al., Chlorogenic acid from the Japanese herb medicine Kinginokita (Plos Lonicere japonica) suppresses the expression of inducible nitric oxide synthase in rat hepatocytes, HOAJ Biol. 1 (2012) 2, http://dx.doi.org/10.7243/2050-0874-1-2.
[14] E. Yoshigai, T. Machida, T. Okuyama, et al., Citrus nobiletin suppresses inducible nitric oxide synthase gene expression in interleukin-1β-treated hepatocytes, Biochem. Biophys. Res. Commun. 439 (2013) 54–59.
[15] Q.Z. Zheng, Y.J. Lou, Pathologic characteristics of immunologic injury in primary cultured rat hepatocytes and protective effect of glycyrrhizin in vitro, Acta Pharmacol. Sin. 24 (2003) 771–777.
[16] T. Lawrence, The nuclear factor NF-κB pathway in inflammation, Cold Spring Harb. Perspect. Biol. 1 (2009) a001651.
[17] K. Sakitani, M. Nishizawa, K. Inoue, et al., Synergistic regulation of inducible nitric oxide synthase gene by CCAAT/enhancer-binding protein β and nuclear factor kβ in hepatocytes, Gene Cells 3 (1998) 321–330.
[18] Y. Takimoto, H.Y. Qian, E. Yoshigai, et al., Synergistic action of indole carbothioic acid (Schisandra chinensis) on the induction of nitric oxide synthase gene via C/EBP and NF-κB in rat hepatocytes, Nitric Oxide 28 (2013) 47–56.
[19] M. Nishizawa, T. Okumura, Y. Ikeda, et al., Post-transcriptional inducible gene regulation by natural antisense RNA, Front. Biosci. (Landmark Ed.) 20 (2015) 1–36.
[20] H. Yoshida, A.H. Kwon, K. Habara, et al., Edaravone inhibits the induction of iNOS gene expression at transcriptional and posttranscriptional steps in murine macrophages, Shock 30 (2008) 734–739.
[21] E. Yoshigai, T. Hara, Y. Araki, et al., Natural antisense transcript-targeted regulation of inducible nitric oxide synthase mRNA levels, Nitric Oxide 30 (2013) 9–16.
[22] A. Nalajima, Y. Yamamoto, N. Yoshinaka, et al., A new flavanone and other flavonoids from green perilla leaf extract inhibit inducible nitric oxide production in interleukin 1β-treated hepatocytes, Biosci. Biotechnol. Biochem. 79 (2015) 138–146.
[23] Y. Tanaka, M. Kairori, H. Miki, et al., Japanese Kampo medicine, ninjinyoeito, induces tumor necrosis factor α secretion from rat hepatocytes, Nature 368 (1994) 339–341.
[24] T. Kamakani, H. Kitade, Y. Hiramatsu, et al., Stimulation of glycogen degradation by prostaglandin E2 in primary cultured rat hepatocytes, Prostaglandins 45 (1993) 459–474.
[25] L.L. Thomsen, J.M. Scott, P. Topley, et al., Selective inhibition of inducible nitric oxide synthase inhibitors tumor growth in vivo: studies with 1400W, a novel inhibitor, Cancer Res. 57 (1997) 3300–3304.
[26] L.C. Green, D.A. Wagner, J. Glogowski, et al., Analysis of nitrate, nitrite, and [15N]nitrate in biological fluids, Anal. Biochem. 126 (1982) 131–138.
[27] T. Takahashi, T. Takasaki, M. Iigo, et al., Isoliquiritigenin, a flavonoid from licorice, reduces prostaglandin E2 and nitric oxide, causes apoptosis, and suppresses aberrant crypt foci development, Cancer Sci. 95 (2004) 448–453.
[28] M. Kopf, H. Baumann, G. Freer, et al., Impaired immune and acute-phase responses in interleukin-6-deficient mice, Nature 368 (1994) 339–342.
[29] Y. Yoshigai, T. Hara, H. Inaba, et al., Interleukin 1β induces tumor necrosis factor α secretion from rat hepatocytes, Hepatol. Res. 44 (2014) 571–583.
[30] J.Y. Kim, S.J. Park, K.J. Yun, et al., Isoliquiritigenin isolated from the roots of Glycyrrhiza uralensis inhibits LPS-induced iNOS and COX-2 expression via the attenuation of NF-κB in RAW 264.7 macrophages, Eur. J. Pharmacol. 583 (2008) 175–184.
[31] Y.W. Kim, R.J. Zhao, S.J. Park, et al., Anti-inflammatory effects of licoritigenin as a consequence of the inhibition of NF-κB-dependent iNOS and
proinflammatory cytokines production, Br. J. Pharmacol. 154 (2008) 165–173. T. Akao, T. Hayashi, K. Kobashi, et al., Intestinal bacterial hydrolysis is indispensable to absorption of 18beta-glycyrrhetic acid after oral administration of glycyrrhizin in rats, J. Pharm. Pharmacol. 46 (1994) 135–137.

[33] I.V. Tetko, V.Y. Tanchuk, Application of associative neural networks for prediction of lipophilicity in ALOGPS 2.1 program, J. Chem. Inf. Comput. Sci. 42 (2002) 1136–1145.

[34] J. Jing, W. Ren, X. Chen, et al., Glucuronide-sulfate diconjugate as a novel metabolite of glycyrrhetic acid in rat bile, Drug Metab. Pharmacokinet. 23 (2008) 175–180.

[35] T. Kuzuhara, Y. Sei, K. Yamaguchi, et al., DNA and RNA as new binding targets of green tea catechins, J. Biol. Chem. 281 (2006) 17446–17456.

[36] S. Nafisi, A. Shadaloi, A. Feizbakhsh, et al., RNA binding to antioxidant flavonoids, J. Photochem. Photobiol. B 94 (2009) 1–7.

[37] S.J. Klempner, G. Bubley, Complementary and alternative medicines in prostate cancer: from bench to bedside? Oncologist 17 (2012) 830–837.