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Hypnotic effects and GABAergic mechanism of licorice (*Glycyrrhiza glabra*) ethanol extract and its major flavonoid constituent glabrol

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ABSTRACT

Licorice (*Glycyrrhiza glabra*, GG) is one of the most frequently used herbal medicines worldwide, and its various biological activities have been widely studied. GG is reported to have neurological properties such as antidepressant, anxiolytic, and anticonvulsant effects. However, its hypnotic effects and the mechanism of GG and its active compounds have not yet been demonstrated. In this study, GG ethanol extract (GGE) dose-dependently potentiated pentobarbital-induced sleep and increased the amount of non-rapid eye movement sleep in mice without decreasing delta activity. The hypnotic effect of GGE was completely inhibited by flumazenil, which is a well-known γ-aminobutyric acid type A-benzodiazepine ($GABA_A$-BZD) receptor antagonist, similar to other $GABA_A$-BZD receptor agonists (e.g., diazepam and zolpidem). The major flavonoid glabrol was isolated from the flavonoid-rich fraction of GGE; it inhibited [^{3}H] flumazenil binding to the $GABA_A$-BZD receptors in rat cerebral cortex membrane with a binding affinity (K_i) of 1.63 μM. The molecular structure and pharmacophore model of glabrol and liquiritigenin indicate that the isoprenyl groups of glabrol may play a key role in binding to $GABA_A$-BZD receptors. Glabrol increased sleep duration and decreased sleep latency in a dose-dependent manner (5, 10, 25, and 50 mg/kg); its hypnotic effect was also blocked by flumazenil. The results imply that GGE and its flavonoid glabrol induce sleep via a positive allosteric modulation of $GABA_A$-BZD receptors.

1. Introduction

The root of licorice (*Glycyrrhiza glabra*, GG) is one of the most frequently used natural medicines in the world, and has been described as 'the grandfather of herbs'.[1,2] It has been used medicinally in both Western and Eastern countries for more than 4000 years.[3,4] GG contains numerous phytochemicals such as triterpenoid saponins, flavonoids, sterols, polysaccharides, and coumarins.[2] GG extract is extensively used in the USA and is considered as 'generally recognized as safe' for use in foods by the FDA.[5,6]

The biological and pharmacological activities of GG have been widely studied as its long history and biologically active constituents are still interesting to many research groups.[7] A large number of clinical and experimental studies report its useful biological properties such as antioxidant, anticancer, immunomodulatory, cardioprotective, and antiinflammatory effects.[2] Several studies have also reported its neurological activities; for example, Dhingra and Sharma[8] reported that aqueous extracts of GG produced antidepressant-like effects in mice undergoing the forced swim and tail suspension tests. GG extract also produced memory-enhancing effects in mice in the plus-maze and passive avoidance paradigm.[9,10] GG ethanol extract (GGE) is also reported to have anxiolytic[11] and anticonvulsant[12] effects. GG is also used as an ingredient of suanzaorentang, which is a very well-known traditional remedy for insomnia in China that also includes *Zizyphus jujuba*, *Poria cocos*,

Abbreviations: BZD, benzodiazepine; CC, column chromatography; CMC, carboxymethyl cellulose; CON, control group; DZP, diazepam; EEG, electroencephalogram; EMG, electromyogram; FLU, flumazenil; FRF, flavonoid-rich fraction; GABA, γ-aminobutyric acid; GG, *Glycyrrhiza glabra*; GGE, *Glycyrrhiza glabra* ethanol extract; ip, intraperitoneal injection; MW, molecular weight; NREMS, non-rapid eye movement sleep; po, post-oral injection; QE, quercetin equivalents; REMS, rapid eye movement sleep; ZPD, zolpidem.

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Ligusticum wallichii, and *Anemarrhena asphodeloides*.[13] Although GG has potential as a sedative-hypnotic plant, its hypnotic effect, mechanism, and active compounds have not been reported previously. Therefore, studying the hypnotic compounds and mechanism of GG extract is important considering its wide application as a food ingredient and medicinal herb.

In the present study, we evaluated the effects of GGE on pentobarbital-induced sleep as well as changes in the sleep architecture and profile in mice. Glabrol was isolated as the major hypnotic compound, and its hypnotic effects, GABAergic mechanism, and pharmacophore modeling were investigated.

2. Results and discussion

2.1. Effects of GGE on pentobarbital-induced sleep in mice

The reference sleep drug diazepam (DZP) significantly (p <0.01) potentiated pentobarbital-induced sleep in mice (Fig. 1). GGE also decreased sleep latency and increased sleep duration in a dose-dependent manner (50, 100, 250, and 500 mg/kg) (Fig. 1). Its hypnotic effect showed a statistically significant difference (p <0.01) at concentrations of 250 and 500 mg/kg.

2.2. Effects of GGE on changes in sleep architecture and profile

To better understand the hypnotic activity of GGE, its effects on sleep–wake regulation and profile in mice were investigated by electroencephalogram (EEG) and electromyogram (EMG). Figure 2A shows the representative EEG and EMG signals and corresponding hypnograms for vehicle, GGE, and DZP during the first 3 h after oral administration. GGE dose-dependently decreased sleep latency (Fig. 2B) and increased the amount of non-rapid eye movement sleep (NREMS) (Fig. 2C) compared with the vehicle. DZP (2 mg/kg) also significantly decreased sleep latency and NREMS duration. However, neither GGE nor DZP affected the duration of rapid eye movement sleep (REMS). It is known that BZD agents such as DZP increase NREMS without altering REMS.[14,15] Figure 3A shows the time courses of the hourly amounts of NREMS and REMS for 12 h after the administration of GGE and DZP. GGE significantly increased the amount of NREMS for the first 2 h (p <0.05). There was no further significant disruption of sleep architecture during the subsequent period. These results indicate that GGE induces NREMS without any adverse effects after sleep induction.[16] DZP also significantly increased NREMS (p <0.01) for the first 2 h; however, during the subsequent period, the hourly NREMS amounts were higher than those of GGE. Both GGE and DZP significantly (p <0.05) decreased the mean duration of wakefulness (Wake) by 57.6% and 58.8%, respectively; however, they did not affect the mean durations of NREMS or REMS (Fig. 3B). The decrease in the mean duration of Wake by GGE without any changes in NREMS or REMS indicates that GGE decreases the maintenance of Wake.[16] GGE did not affect the EEG power density (0–20 Hz) in NREMS, whereas DZP significantly decreased the delta (0.5–4 Hz) activity (p <0.05) (Fig. 3C). These results suggest that GGE induces NREMS in a manner very similar to that of physiological sleep. Delta activity is an indicator of the depth or intensity of NREMS.[17,18] The decrease in the delta activity caused by DZP in humans and rodents is well known.[15,19]

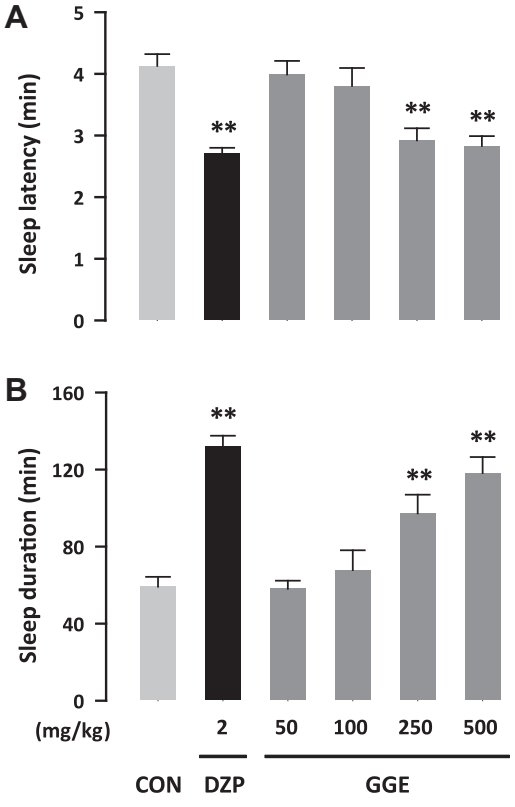

Figure 1. Effects of GGE on sleep latency (A) and sleep duration (B) in mice induced by pentobarbital (45 mg/kg). Each column represents mean ± SEM (n = 10). **p <0.01, significant compared with the control group (Dunnett's test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; GGE, *Glycyrrhiza glabra* ethanol extract.

2.3. GABAergic mechanism of GGE

The $GABA_A$-BZD receptor is considered the most important target for the development of sedative-hypnotic drugs.[20,21] Both BZDs and non-BZD agents stimulate the ability of GABA to hyperpolarize membranes by allowing chloride anion (Cl^-) influx.[22] Consequently, this inhibits neurotransmission; these agents subsequently produce sedative-hypnotic, anxiolytic, and anticonvulsant activities.[23,24] The BZD (e.g., DZP and zolpidem, ZPD) and barbiturate (e.g., pentobarbital) binding sites of $GABA_A$ receptors are the targets of sedative-hypnotic agents that act as positive allosteric modulators.[25] BZDs and barbiturates are known to bind to two different binding sites at $GABA_A$ receptors.[26] Although acting as a modulator, higher doses of barbiturates can directly activate $GABA_A$ receptors and induce sleep.[27] The specific $GABA_A$-BZD receptor antagonist flumazenil (FLU) inhibits the hypnotic activity of $GABA_A$-BZD receptor agonists such as DZP and ZPD by blocking their binding.[25]

The anxiolytic and anticonvulsant effects reported previously[11,12] and the hypnotic effect observed in this study suggests that GGE has the potential to exert positive allosteric modulation of $GABA_A$-BZD receptors. In order to verify the GABAergic mechanism of the hypnotic effect of GGE, the effects of the co-administration of GGE (500 mg/kg), DZP (2 mg/kg), or ZPD (10 mg/kg) with FLU (8 mg/kg) were tested (Fig. 4). As expected, FLU significantly (p <0.01) inhibited the hypnotic effects of DZP and ZPD. The hypnotic activity of GGE was also fully (p <0.01) antagonized by FLU. These results imply that GGE induces sleep via the GABAergic system and that its active compounds act as positive allosteric modulators on $GABA_A$-BZD receptors. GGE (100 mg/kg), DZP (0.5 mg/kg), or ZPD (2.5 mg/kg) did not significantly increase sleep duration in mice (Fig. 5). In contrast, co-administration of GGE with DZP or ZPD significantly (p <0.01) increased sleep duration. The synergic effects of these substances in both sleep latency and sleep duration were fully antagonized with FLU treatment. Verification of the GABAergic mechanism of GGE would also provide evidence regarding the action mechanisms of the anxiolytic[11] and anticonvulsant[12] effects of the GGE in previous reports.

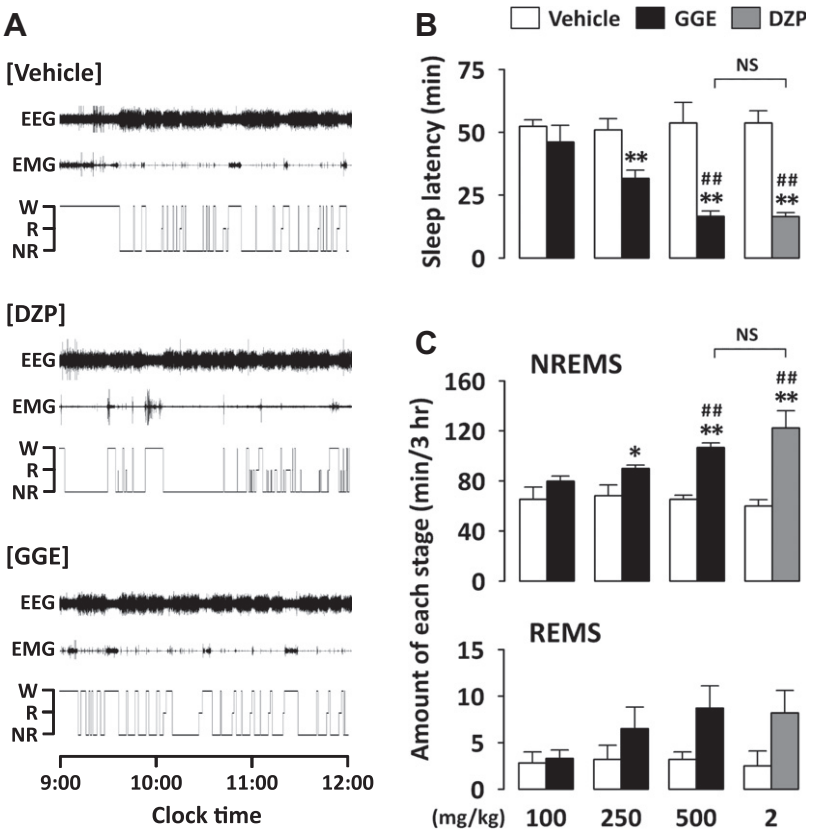

Figure 2. (A) Representative examples of EEG and EMG signals and corresponding hypnograms in mice treated with vehicle, GGE, or DZP. (B) Effects of GGE and DZP on sleep latency. (C) Total time spent in NREMS and REMS for 3 h after drug administration. Each column represents mean ± SEM (n = 8). $^{**}p$ <0.01, compared with vehicle (unpaired Student's t-test). $^{\#\#}p$ <0.01, significant compared with 100 mg/kg GGE (Dunnett's test). Abbreviations: DZP, diazepam; EEG, electroencephalogram; EMG, electromyogram; GGE, *Glycyrrhiza glabra* ethanol extract; NREMS (NR), non-rapid eye movement sleep; NS, not significant; REMS (R), rapid eye movement sleep; Wake (W), wakefulness.

2.4. Isolation of glabrol from the flavonoid-rich fraction of GGE

A large number of flavonoids have hypnotic activity via the positive allosteric modulation of $GABA_A$-BZD receptors.[25,28] With the expectation that GG flavonoids act as hypnotic compounds, the flavonoid-rich fraction (FRF) was prepared with ethyl acetate–*n*-butanol (3:1) from GGE. The total flavonoid content (TFC) value of the FRF was 262.8 mg QE/g, which is 10-fold higher than that of GGE. The residue (H_2O fraction) had a TFC value of 2.3 mg QE/g. FRF at 100 mg/kg (p <0.05) and 200 mg/kg (p <0.01) significantly potentiated the pentobarbital-induced sleep in mice; their hypnotic effects were fully inhibited by FLU the same as for GGE (Fig. 6). However, the residue did not produce a hypnotic effect even at 1000 mg/kg.

Compound **1** was successfully isolated as the major active constituent of GGE from the FRF using $GABA_A$-BZD receptor binding activity-guided fractionation (Fig. 7A). Compound **1** was identified as a flavanone with two hydroxyl groups at C-7 and C-4′, and 2 isoprenyl groups at C-8 and C-3′—that is, glabrol. Glabrol was the main hypnotic constituent of GGE, and its content in GGE was determined to be 2.3 mg/g on the basis of the calibration curve in the HPLC experiment.

2.5. Pharmacophore modeling and binding affinity of glabrol

In order to evaluate the in vitro binding affinity (K_i) of glabrol, for the $GABA_A$-BZD receptor, [^{3}H] flumazenil binding was performed in the receptor membrane preparation from the rat cerebral cortex. Glabrol inhibited [^{3}H] flumazenil binding to the rat cerebral cortex membrane with the binding affinity (K_i) of 1.63 μM (Fig. 7A). To the best of our knowledge, this is the first time glabrol has been characterized as the $GABA_A$-BZD receptor ligand. Several flavonoids bind to $GABA_A$-BZD receptors, which results in anxiolytic, hypnotic, and anticonvulsant effects.[25,29] The phenyl ring, double bond, and carbonyl group at position 4 in the C-ring of flavone (Fig. 7C) are important for its binding affinity though analysis compared with the chemical structures of DZP (Fig. 7B).[29] For example, hispidulin[30] and isoliquiritigenin,[31] which have the phenyl ring, double bond, and carbonyl group at position 4 in the C-ring of flavone, have been demonstrated to be $GABA_A$-BZD receptor ligands with positive allosteric properties (Fig. 7D). Liquiritigenin and liquiritin (Fig. 7E), which have no double bond in the C-ring of flavonoids, are usually isolated from the root of licorice species together with glabrol.[32] In the present study, they were not isolated as active compounds. In addition, the binding affinities of these flavonoids were not calculated at a maximum concentration of 100 μM. Jäger et al.[33] reported that naringenin (Fig. 7E), which has a very similar structure to that of liquiritigenin, has a high IC_{50} value of 2.6 mM and thus has very low activity. Therefore, the results of this study and previous reports[30,31] confirm the importance of the double bond in the C-ring of flavonoids for their binding activity to $GABA_A$-BZD receptors. However, glabrol, which has two isoprenyl groups in addition to the structure of liquiritigenin without the double bond in the C-ring, exhibits binding activity. These results suggest that two isoprenyl group of glabrol play a key role to bind $GABA_A$-BZD receptors and to show sedative-hypnotic effects. Due to the attenuation of polarity by the isoprenyl group, glabrol may bind to the agonist pharmacophores of $GABA_A$-BZD receptors: L1, L2, and L3.[34]

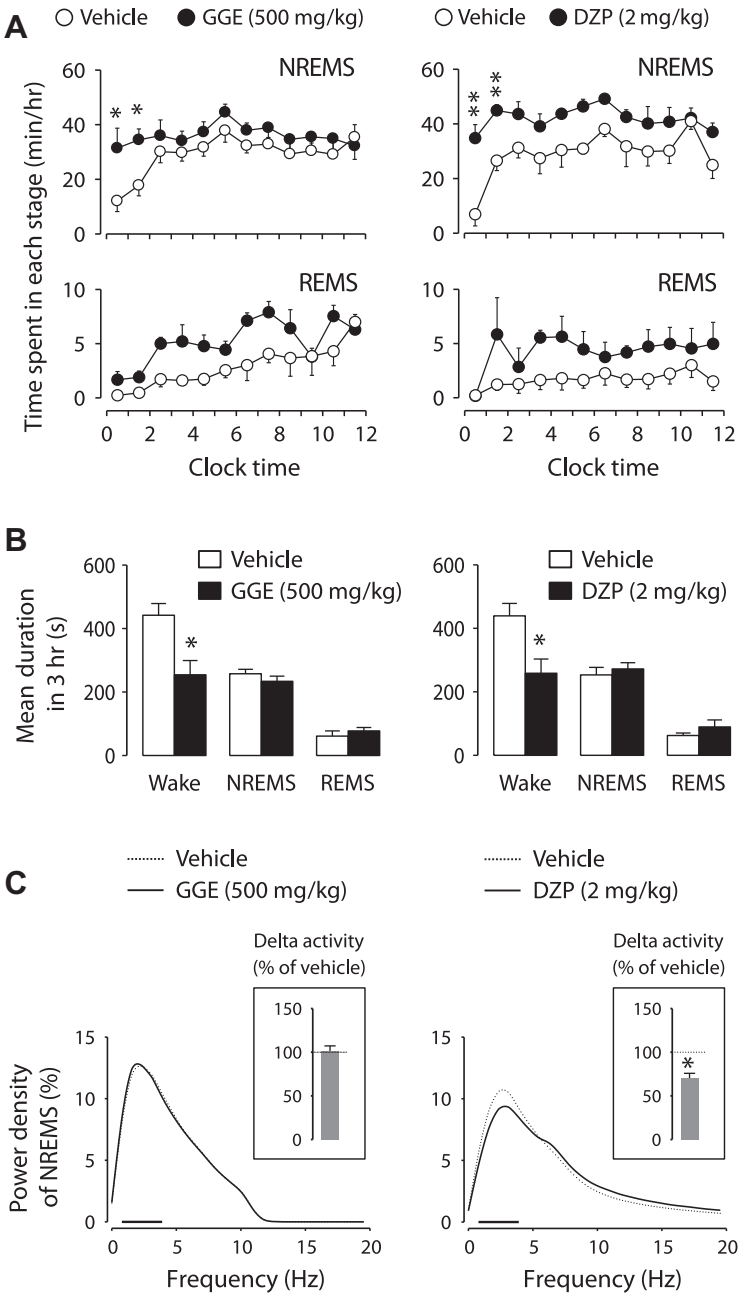

Figure 3. (A) Time courses of NREMS, REMS, and Wake after GGE and DZP administration. (B) Effects of GGE and DZP on the mean durations of each sleep stage. (C) EEG power density during NREMS. Each circle represents the hourly mean ± SEM (n = 8) of NREMS, REMS, and Wake. Delta activity in NREMS (an index of sleep intensity) is shown in the histogram in the square of C. Under bar (—) represents the range of delta wave (0.5–4 Hz). $^{*}p$ <0.05, $^{**}p$ <0.01, compared with vehicle (unpaired Student's t-test). Abbreviations: DZP, diazepam; EEG, electroencephalogram; GGE, *Glycyrrhiza glabra* ethanol extract; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

The better binding affinity of glabrol compared with that of liquiritigenin can be explained by pharmacophore mapping generated with known active $GABA_A$-BZD receptor agonists diazepam, CGS-9896, and diindole (Fig. 8A) using CATALYST/Discovery Studio (version 3.0, Accelrys Inc.). This pharmacophore model, which is similar to that suggested by Zhang et al.,[35] consists of seven features with two hydrogen bond acceptors, one hydrogen bond donor, and four hydrophobic centers. Among these seven features, two hydrogen bond acceptors and two hydrophobic features (H1 and H2) are common among three known agonists: diazepam, CGS-9896, and diindole. The hydrophobic feature H3 is specific to diazepam, and the hydrogen bond donor and hydrophobic feature H4 are absent in diazepam. Thus, these three features are optional for binding affinity. The isoprenyl groups of glabrol were mapped with hydrophobic features H2 and H4 (Fig. 8B). However, three hydrophobic features, H1, H2, and H4, are missing in liquiritigenin (Fig. 8C). These results imply that the isoprenyl group may play a key role in the binding activity of flavonoids to $GABA_A$-BZD receptors.

2.6. Hypnotic effect and GABAergic mechanism of glabrol

Few studies have investigated the bioactivity of glabrol. Until now, there have been few reports about the diacylglycerol acyltransferase[36] and cholesterol acyltransferase[37] inhibitory activities and PPAR-γ ligand-binding activity of glabrol.[38] Glabrol increased

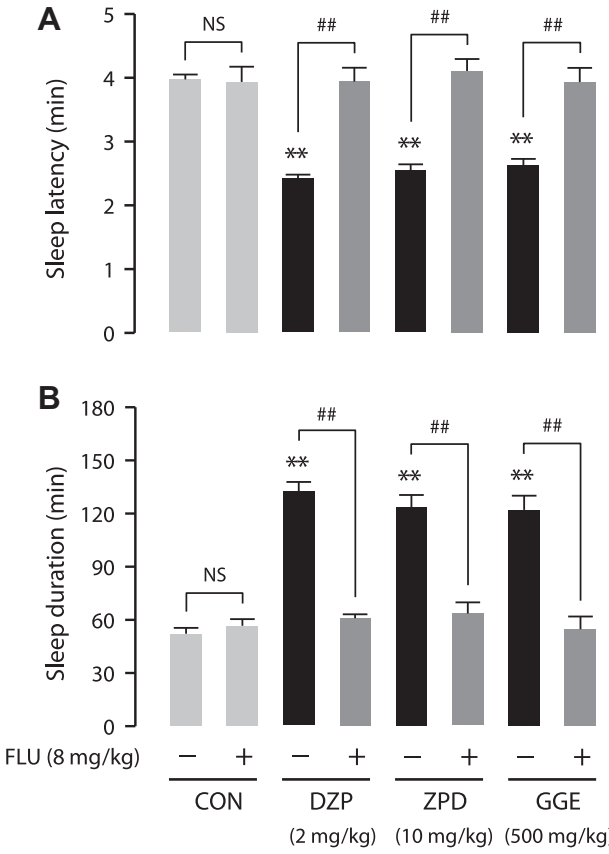

Figure 4. Effects of FLU on the changes in sleep latency (A) and sleep duration (B) in mice treated with DZP, ZPD, and GGE. Mice received 45 mg/kg pentobarbital 45 min after the oral administration of drugs. FLU was administered (ip) 15 min before the oral administration of drugs. Each column represents mean ± SEM (n = 10). $^{**}p$ <0.01, significant compared with the control group (Dunnett's test). $^{\#\#}p$ <0.01, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; FLU, flumazenil; GGE, *Glycyrrhiza glabra* ethanol extract; NS, not significant; ZPD, zolpidem.

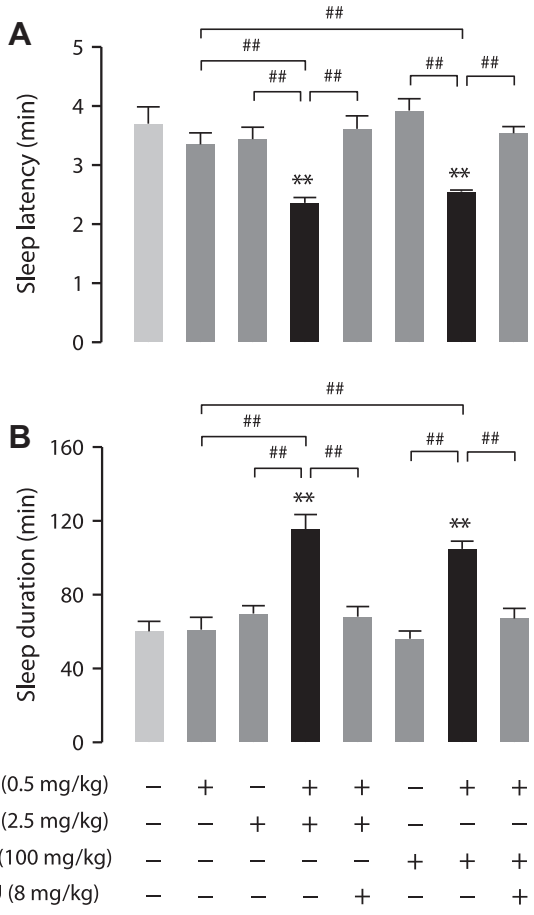

Figure 5. Effects of co-administration of DZP, ZPD, GGE, and FLU on sleep latency (A) and sleep duration (B) in mice. Mice received 45 mg/kg pentobarbital 45 min after the oral administration of drugs. FLU was administered (ip) 15 min before the oral administration of drugs. Each column represents mean ± SEM (n = 10). $^{**}p$ <0.01, significant compared with the control group (Dunnett's test). $^{\#\#}p$ <0.01, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: DZP, diazepam; FLU, flumazenil; GGE, *Glycyrrhiza glabra* ethanol extract; ZPD, zolpidem.

sleep latency and decreased sleep duration in a dose-dependent manner (5–50 mg/kg) (Fig. 9). At a maximum concentration of 50 mg/kg, its hypnotic effect was comparable to that of ZPD (10 mg/kg). The hypnotic effect of glabrol was fully blocked by FLU the same as ZPD (Fig. 9).

3. Conclusion

In summary, we demonstrated that GGE and its active flavonoid glabrol induce sleep via the positive allosteric modulation of $GABA_A$-BZD receptors. In particular, the characteristic of glabrol as a $GABA_A$-BZD receptor ligand with a hypnotic effect was demonstrated for the first time. The importance of the isoprenyl group in binding affinity is significant in the $GABA_A$ receptor pharmacology of flavonoids. Considering the wide applications of GG as a food ingredient and medicinal herb, future studies are needed to isolate other active flavonoids and evaluate their sleep-promoting effects as well as tolerance and dependence.

4. Experimental section

4.1. General experimental procedures

4.1.1. Preparation of plant extract and flavonoid-rich fraction

Licorice (*Glycyrrhiza glabra*) was purchased from a local oriental medicine market (Gyeongdong Market) in Seoul of Korea, which was imported from Uzbekistan. It was identified by Professor Dae-Keun Kim (Woosuk University, Jeonju, Korea). A voucher specimen (KHU-100310) was reserved at the Laboratory of Natural Products Chemistry, Kyung Hee University (Youngin, Korea). The dried and powdered licorice roots (1 kg) were extracted with 80% ethanol (1 L) at 48 °C for 12 h. The extraction solutions were then filtered and lyophilized. The GG ethanol extract (GGE) was further fractionated to prepare the flavonoid-rich fraction (FRF). The powder of GGE (117 g) was suspended in H_2O (1 L), and then extracted successively with ethyl acetate–n-butanol (3:1, 1 L × 2). The organic layer was concentrated in vacuo to afford the FRF (11.5 g). The total flavonoid content (TFC) of the FRF was determined as 252.7 mg QE/g by the method of Moreno et al.[39]

4.1.2. Regents and instruments

SiO_2 (Kiesel gel 60, Merck, Darmstadt, Germany) and ODS (LiChroprep RP-18, Merck) resins were used for column chromatography (CC). TLC analysis was carried out using Kiesel gel 60 F_{254} and RP-18 F_{254S} (Merck) plates, and the spots were detected using a UV lamp Spectroline Model ENF-240 C/F (Spectronics Corporation, Westbury, NY, USA) and a spraying a 10% H_2SO_4 solution followed by heating. Optical rotation was measured on a JASCO P-1010 digital polarimeter (Tokyo, Japan). IR spectrum was obtained using a Perkin Elmer Spectrum One FT-IR spectrometer

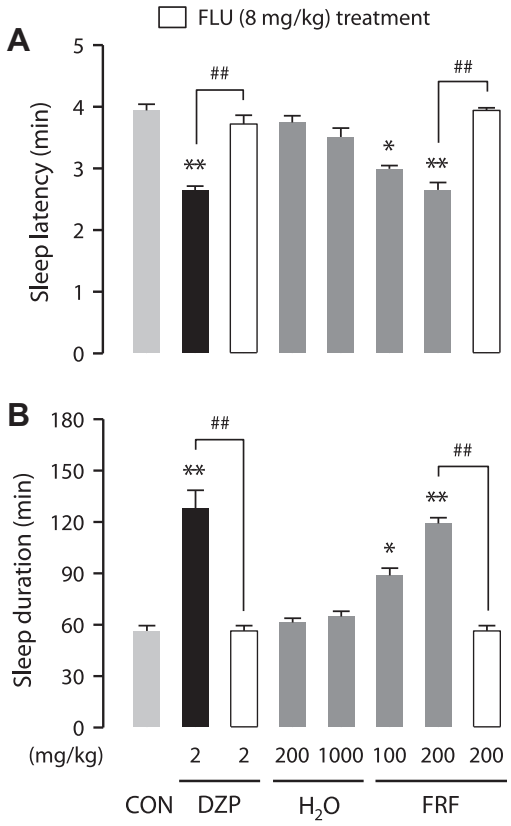

Figure 6. Effects of flavonoid-rich (FRF) and H_2O (residue) fractions from GGE on sleep latency (A) and sleep duration (B) in mice induced by pentobarbital (45 mg/kg). $^{*}p$ <0.05, $^{**}p$ <0.01, significant compared with the control group (Dunnett's test). $^{\#\#}p$ <0.01, significant between FLU treatment and no FLU treatment (unpaired Student's *t*-test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; FLU, flumazenil; GGE (*Glycyrrhiza glabra* ethanol extract).

(Buckinghamshire, England). Melting point was obtained using a Fisher–John's Melting Point Apparatus (Fisher Scientific, Miami, FL, USA) with a microscope, and the obtained value was uncorrected. EI-MS was recorded on a JEOL JMS-700 (Tokyo, Japan). ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian Unity Inova AS-400 FT-NMR spectrometer (Palo Alto, CA, USA).

4.1.3. Isolation of glabrol

FRF (11.5 g) was applied to a SiO_2 CC (Ø 8 × 20 cm) and eluted with *n*-hexane–ethyl acetate (10:1→5:1→3:1, 30 L of each). The eluting solutions were monitored by TLC to produce 23 fractions (F1–F23). The fraction F18 [825 mg, V_e/V_t (elution volume/total volume) 0.82–0.86] was subjected to an ODS CC (Ø 5.0 × 13 cm) and eluted with MeOH–H_2O (5:1, 1.3 L), yielding 19 fractions (F18-1 to F18-19) including a purified compound **1** [glabrol, F18-11, 202 mg, V_e/V_t 0.35–0.36, TLC (ODS F_{254S}) R_f 0.60, MeOH–H_2O = 5:1]. Glabrol was identified on the basis of spectroscopic data and chemical evidences. These findings were confirmed by comparison of the physical and spectroscopic data with those reported in the literature.[40] The structural elucidation of glabrol isolated from FRF of GGE was summarized as a follow.

4.1.3.1. Glabrol (compound 1). Yellow powder ($CHCl_3$); mp 106–107 °C (mp = 121–123 °C);[40] $[\alpha]_D^{20}$ −34.5° (*c* = 0.20, $CHCl_3$) {$[\alpha]_D^{24}$ −44.2° (*c* 0.10, $CHCl_3$)};[40] EI/MS *m*/*z* 392 $[M]^+$; IR (KBr, *ν*) 3390, 1662, 1602, 1588, 1516 cm^{-1}; ^{1}H NMR (400 MHz, $CDCl_3$, δ_H) 7.78 (1H, d, *J* = 8.8 Hz, H-5), 7.25 (1H, br s, H-2′), 7.26 (1H, br d, *J* = 8.8 Hz, H-6′), 6.88 (1H, d, *J* = 8.8 Hz, H-5′), 6.58 (1H, d, *J* = 8.8 Hz, H-6), 5.40 (1H, dd, *J* = 13.2, 2.8 Hz, H-2), 5.29 (2H, m, H-2″, 2‴), 3.43 (4H, *J* = 7.6 Hz, H-1″, 1‴), 3.03 (1H, dd, *J* = 16.8, 13.2 Hz, H-3ax), 2.83 (1H, dd, *J* = 16.8, 2.8 Hz, H-3 eq), 1.83 (6H, s, H-4″, 5″), 1.79 (6H, s, H-4‴, 5‴) refer to literature;[40] ^{13}C NMR (100 MHz, $CDCl_3$, δ_C) 191.95 (C-4), 161.42 (C-7), 161.20 (C-9), 154.45 (C-4′), 135.02 (C-3″), 134.84 (C-3‴), 130.99 (C-1′), 127.85 (C-6′), 127.10 (C-3′), 126.40 (C-5), 125.29 (C-2′), 121.26 (C-2″), 121.00 (C-2‴), 115.70 (C-5′), 114.72 (C-8), 114.57 (C-10), 110.46 (C-6), 79.43 (C-2), 44.02 (C-3), 29.77 (C-1‴), 25.89 (C-4″), 25.83 (C-4‴), 22.31 (C-1″), 17.98 (C-5″), 17.92 (C-5‴) refer to literature.[40]

4.1.4. Quantification of glabrol

Quantification of glabrol in GGE is accomplished using a HPLC (Shimadzu, Tokyo, Japan) equipped with a UV detector (230 nm) and a Phenomenex LUNA 5 μ C_{18} column (5 μm, 250 × 4.6 mm). Acetonitrile and water with 0.1% formic acid were used as the eluting solvent at a flow rate of 0.6 mL/min. The eluting solvent was programmed as follows: 65% for acetonitrile, increased to 70% at 10 min and 90% at 20 min, then 65% at 22 min. The sample was weighed 8 mg of the concentrated filtrate was dissolved in 1 mL of methanol, after which the sample was allowed to cool room temperature prior to HPLC analysis. Sample solutions (20 μL) were injected into the HPLC, and glabrol was detected at retention time 14.662 min. In the calibration curve test, the linear regression equation for glabrol was $y = 61{,}666{,}281.3333x + 649{,}942.7917$ ($R^2 = 0.9994$) where *y* is the peak area and *x* is the concentration of glabrol.

4.2. In vitro and in vivo biological assays

4.2.1. Plant material, drugs, and animals

Liquiritigenin (Extrasynthese, Genay, France) was purchased for the binding assay. Pentobarbital was purchased from Hanlim Pharm. Co., Ltd (Seoul, Korea). Diazepam (DZP; Myungin Pharm. Co., Ltd, Seoul, Korea) and zolpidem (ZPD; Korea Food & Drug Administration) were used as reference sedative-hypnotic drugs ($GABA_A$-BZD agonists). Flumazenil (FLU), a $GABA_A$-BZD receptor antagonist, was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). For the $GABA_A$-BZD receptor-binding assay, the radioligand [^{3}H] flumazenil (Ro 15-1788; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) was used. All other chemicals and reagents were of the highest grade available.

To obtain a membrane preparation for the $GABA_A$-BZD receptor-binding assay, 200–250 g male SD (Sprague Dawley) rats were used. For evaluation of the hypnotic effects, male ICR (18–22 g) and male C57BL/6 N mice (27–30 g) were used. All animals were purchased from Koatech Animal Inc. (Pyeongtaek, Korea), and were housed with food and water ad libitum at 24 °C at controlled humidity of 55% in a room maintained on a 12 h light/dark cycle (light on at 9:00 AM). All procedures involving animals were conducted in accordance with the animal care and use guidelines of the Korea Food Research Institutional Animal Care and Use Committee (permission number: KFRI-M-09118).

4.2.2. $GABA_A$-BZD receptor binding assay

The $GABA_A$-BZD receptor binding assay was a modification of the method described by Risa et al.[41] and Kahnberg et al.[42] The receptor membrane was prepared from the cerebral cortex of SD rats.[41] In a 96-well plate, a membrane suspension (180 μL) was added to 10 μL of a test solution and 10 μL of [^{3}H] flumazenil to obtain a final concentration of 1 nM. The solution was mixed and incubated on ice for 40 min. The binding reaction was terminated by rapid filtration with ice-cold 30 mM Tris–HCl buffer. The amount of filter-bound radioactivity was determined by conventional liquid scintillation counting. Total and nonspecific bindings

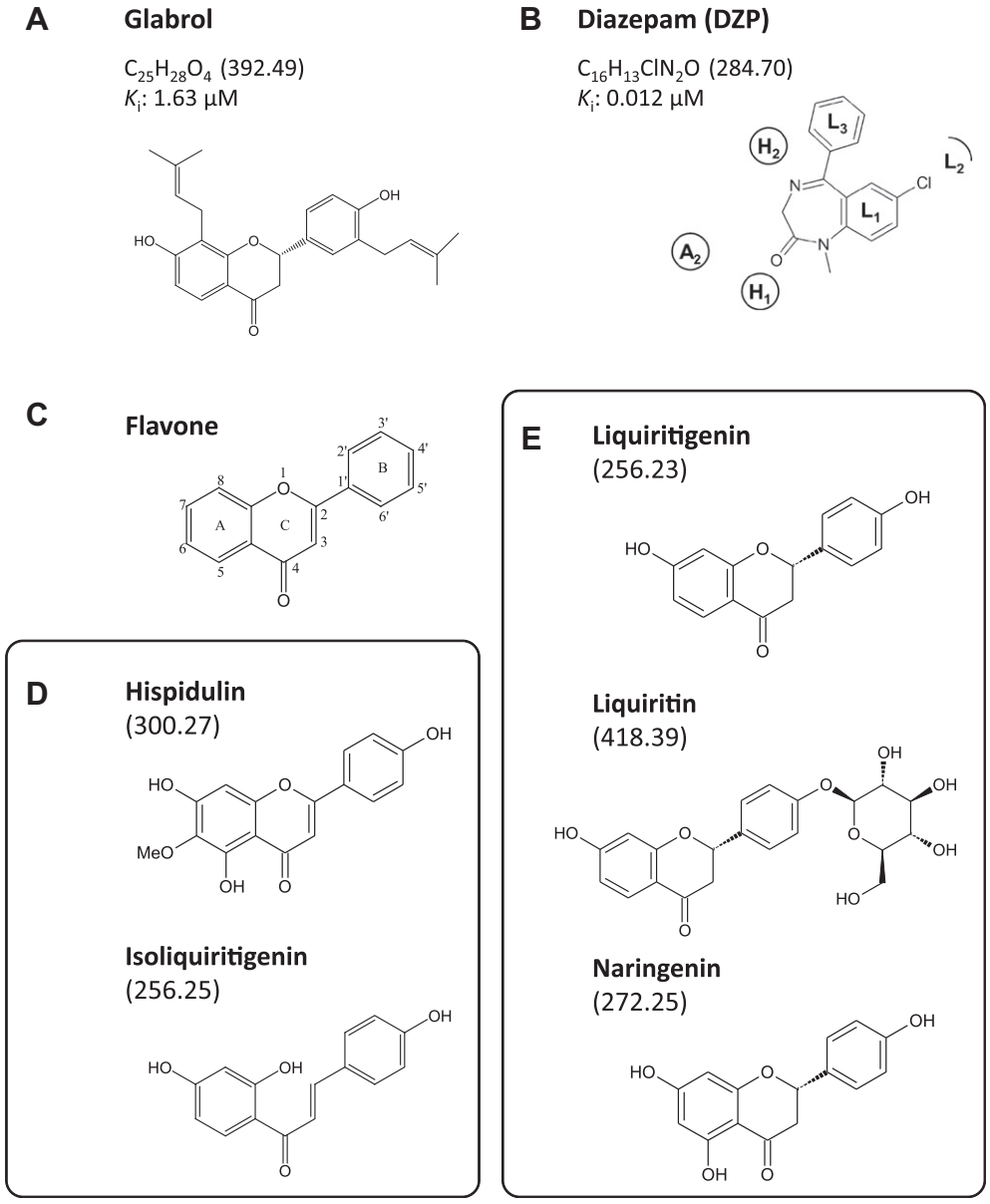

Figure 7. Molecular structure, chemical formula (MW), and binding affinity (K_i) to the $GABA_A$-BZD receptors of glabrol (A) and DZP (B). Structures of flavone (C), sedative flavonoids (hispidulin and isoliquiritigenin) (D), several flavonoids having the similar structure with glabrol (liquiritigenin, liquiritin, and naringenin) (E). H_1 and H_2 are two hydrogen bond-donating sites on the $GABA_A$ receptor; A_2 is a hydrogen bond-accepting site; and L_1, L_2, and L_3 are three lipophilic pockets.

were determined using the binding buffer and DZP (1 μM, final concentration), respectively. Values of binding affinity (K_i) were calculated by the following equation:

$$K_i = \frac{IC_{50}}{1 + [L]/K_d}$$

where [*L*] denotes the concentration of the radio-ligand ([^{3}H] flumazenil) used and K_d denotes the competitor-ligand dissociation equilibrium constant for [^{3}H] flumazenil. The K_d value is 1.6 nM.

4.2.3. Pentobarbital-induced sleep test

All the experiments were performed between 1:00 and 5:00 PM, and the mice were fasted for 24 h before the experiment. For oral administration, all the samples were suspended in 0.5% (w/v) CMC-saline. The test solutions were administered (po) to the mice 45 min before the pentobarbital injection. The control mice (0.5% CMC-saline, 10 mL/kg) were tested in parallel with the animals receiving the test sample treatment. After the administration of pentobarbital (45 mg/kg, ip), mice were placed in individual cages and observed for measurements of sleep latency and duration. The observers were blinded to the individual treatments. The sleep latency was recorded from the time of pentobarbital injection to the time of sleep onset, and sleep duration was defined as the difference in time between the loss and the recovery of the righting reflex.

4.2.4. Analysis of sleep architecture and profile

Under pentobarbital (50 mg/kg, ip) anesthesia, C57BL/6 N mice were chronically implanted with the head mount (#8201, Pinnacle Technology Inc., Lawrence, KS, USA) equipped with electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnographic recordings. The front edge of the head mount was placed 3.0 mm anterior of bregma of mice skull. Four stainless steel screws were passed through the head mount into four predrilled

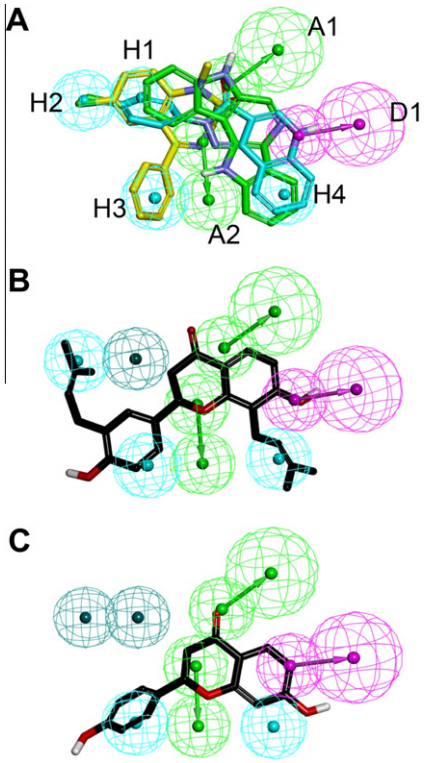

Figure 8. (A) The superimposition of a 7-feature pharmacophore model with diazepam (yellow), CGS-9896 (cyan), and diindole (green). (B) The pharmacophore mapping with glabrol. (C) The pharmacophore mapping with liquiritigenin. H1–H4 represent hydrophobic, A1 and A2 represent a hydrogen bond acceptor, and D represents a hydrogen bond donor.

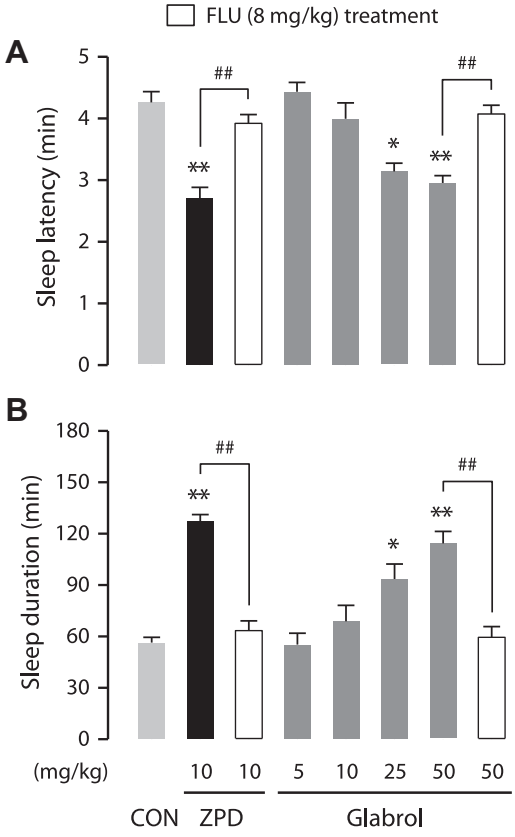

Figure 9. Effects of glabrol on sleep latency (A) and sleep duration (B) in mice treated with pentobarbital (45 mg/kg). Mice received 45 mg/kg pentobarbital 45 min after the oral administration of drugs. FLU was administered (ip) 15 min before the oral administration of drugs. Each column represents mean ± SEM (n = 10). $^{*}p$ <0.05, $^{**}p$ <0.01, significant compared with the control group (Dunnett's test). $^{\#\#}p$ <0.01, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; FLU, flumazenil.

holes of the skull, and the device was secured with dental cement. Two EMG wires from the head mount were sutured onto the nuchal muscles in the back of the neck. After surgery, each mouse was allowed 7 days in an individual transparent barrel for recovery.

After recovery, the mice were habituated to the recording conditions for 4 days before the sample tests. The samples were administered orally to the mice, and then each mouse was immediately transferred to a soundproof recording chamber and connected to an EEG and EMG recording cable (two EEG channels and one EMG channel). Recording was started at 09:00 AM, and was continued for 12 h. The time-synchronous digital video was recorded along with EEG and EMG. For evaluation of sleep-promoting effects, the recording was performed for 2 days. The data collected during the first day was served as baseline comparison data (vehicle) for the second experimental day (test article). Cortical EEG and EMG signals were amplified (×100), filtered (low-pass filter: 10 Hz EEG and 10 Hz EMG), digitized at a sampling rate of 200 Hz, and recorded by using the PAL-8200 data acquisition system (Pinnacle Technology Inc.).

The sleep–wake states were automatically classified by 10-s epoch as wakefulness (Wake), rapid eye movement sleep (REMS), and non-REM sleep (NREMS) by SleepSign Ver. 3 software (Kissei Comtec, Nagano, Japan) according to the standard criteria.[43] Sleep latency was defined as the time elapsed between sample administration and the first consecutive NREMS episode lasting at least 2 min and not interrupted by more than six 4-s epochs not scored as NREMS. The EEG power spectra were calculated at 0.5-Hz intervals, integrated, and averaged. It could be divided into three frequency areas: delta wave (0.65–4 Hz), theta wave (6–10 Hz), and alpha wave (12–14 Hz).[16] The EEG power density of NREMS was normalized as a group by calculating the percentage of each interval from the total EEG power (0–20 Hz) of the individual mouse.

4.3. Pharmacophore modeling

A combined pharmacophore model with known active $GABA_A$-BZD receptors agonists, diazepam, CGS-9896 and diindole was generated using CATALYST/Discovery Studio (version 3.0, Accelrys, Inc. San Diego). Possible conformers of each compound were generated by best conformer generation method with a minimum energy range of 20 kcal/mol. As a consequence of various trials conducted to optimize the feature-based alignment for pharmacophore generation, we identified the best alignment when the weight factors were given to diazepam, CGS-9896 and diindole with the principal values of 2, 2 and 1, respectively. The value 2 ensures that all the chemical features in the most active compound are considered for building hypothesis. The principal value of 1 labels a molecule as moderately active. After alignment of templates based on common feature, the pharmacophore model was generated by average-feature mapping because H3, D1 and H4 were not common. This model composed of seven features, two hydrogen bond acceptors, one hydrogen bond donor and four hydrophobic features. The glabrol and liquiritigenin with the maximum omitted feature one and two were successfully mapped to pharmacophore model using best-flexible search option.

4.4. Statistical analysis

For multiple comparisons, data were analyzed using one-way ANOVA followed by Dunnett's test. Comparisons between two-group data were analyzed by the unpaired Student's t-test.

Differences with $p < 0.05$ were considered statistically significant. The significance analysis was performed using the Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

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