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Hypnotic effects and binding studies for $GABA_A$ and 5-HT_{2C} receptors of traditional medicinal plants used in Asia for insomnia

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ABSTRACT

Aim of the study: Many medicinal plants have been used for treatment of insomnia in Asia. However, scientific evidence and precise mechanism for their sedative-hypnotic activity have not been fully investigated. Thus, we investigated the binding activity of the oriental plant extracts (mainly from Korea and Japan) to the well-known molecular targets for sleep regulation, $GABA_A$ and 5-HT_{2C} receptors. Following the binding assay, sedative-hypnotic effects of the extracts with high affinity were examined in an animal model of sleep.
Materials and methods: Aqueous and ethanol extracts of 15 medicinal plants were tested for binding at the benzodiazepine site of $GABA_A$ receptor and 5-HT site of 5-HT_{2C} receptor. The sedative-hypnotic effects of selected extracts were evaluated by measuring the sleep latency and sleep duration during pentobarbital-induced sleep in mice after oral administration of extracts.
Results: In the $GABA_A$ assay, the ethanol extracts of licorice and danshen displayed concentration-dependent, high affinity binding, whereas in the 5-HT_{2C} assay, the ethanol extracts of ginseng and silk tree showed high affinity. Among these extracts we tested previously uncharacterized licorice and silk tree for hypnotic effects. We found the ethanol extracts of licorice and silk tree significantly decreased sleep latency and increased sleep duration in pentobarbital-induced sleep.
Conclusions: We demonstrate for the first time that licorice and silk tree have the sedative-hypnotic activity possibly by modulating $GABA_A$ and 5-HT_{2C} receptors. We propose that licorice and silk tree might be effective candidates for treatment of insomnia.
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1. Introduction

Insomnia is a widespread health complaint and the most common sleep disorder. Approximately 10–15% of adult population suffer from chronic insomnia, and additional 25–35% have transient or occasional insomnia (Doghramji, 2006). Conventional pharmacological treatments for insomnia include benzodiazepines/non-benzodiazepines (gamma-aminobutyric acid $(GABA)_A$ receptor agonists), antidepressants (serotonin $(5\text{-HT})_2$ receptor antagonists) and antihistamines (Borja and Daniel, 2006). However, the use of these sedative-hypnotic drugs beyond 4 weeks is generally not recommended because of their various side-effects such as impaired cognitive function, memory and general daytime performance (Thomas and Christopher, 2004). In addition, long-term administration results in tolerance and dependence (Fang et al., 2010).

In recent years, herbal sleep aids are becoming popular as an alternative to prescription drugs to improve sleep quality and avoid side-effects (Meletis and Zabriskie, 2008). According to the 2002 National Health Interview Survey, more than 1.6 million American adults use alternative medicines to treat insomnia or sleep problems (Pearson et al., 2006). In western societies, herbal sleep aids such as valerian (*Valeriana officinalis*), St. John's wort (*Hypericum perforatum*), passion flower (*Passiflora incarnata*), hops (*Humulus lupulus*) and kava kava (*Piper methysticum*) are readily available (Meoli et al., 2005). It has been suggested that these herbal extracts

Abbreviations: 5-HT, 5-hydroxytryptamine (serotonin); BZD, benzodiazepine; CMC, carboxymethyl cellulose; CNS, central nervous system; DE, danshen (*Salvia miltiorrhiza* Bunge) ethanol extract; DPM, disintegrations per minute; DZP, diazepam; GABA, gamma-aminobutyric acid; GE, ginseng (*Panax ginseng* C.A. Meyer) ethanol extract; i.p., intraperitoneal injection; LE, licorice (*Glycyrrhiza uralensis* Fischer) ethanol extract; p.o., post-oral injection; SE, silk tree (*Albizzia julibrissin* Durazzini) ethanol extract.

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Table 1
Oriental medicinal plants screened for $GABA_A$-BZD and 5-HT_{2C} receptor binding assays.

Species		Plant part analyzed	Traditional usage for neuropsychology
Common name	Scientific name		
Reishi murshroom	*Ganoderma lucidum* Karsten	Whole	Relaxation
Hoelen	*Poria cocos*	Whole	Treatment of anxiety symptoms
Chinese jujube	*Zizyphus jujuba* Miller	Fruit	Central nervous system (CNS) sedation
Chinese magnolia vine	*Schizandra chinensis* Baillon	Fruit	Inhibition of CNS
Pomegranate	*Punica granatum* L.	Fruit	Appetite improvement
Danshen	*Salvia miltiorrhiza* Bunge	Root	CNS sedation
Gigantic angelica	*Angelica gigas* Nakai	Root	Treatment of insomnia
Licorice	*Glycyrrhiza uralensis* Fischer	Root	CNS sedation
Korean red ginseng[a]	*Panax ginseng* C.A. Meyer	Root	CNS sedation
Ginseng	*Panax ginseng* C.A. Meyer	Root	CNS sedation
Ginger	*Zingiber officinale* Roscoe	Root	CNS sedation
Solomon's seal	*Polygonatum odoratum* var. pluriflorum	Root	CNS sedation
Angelica	*Angelica utilis* Makino	Leaves	Relaxation
Cactus	*Opuntia ficus* indica Mill	Leaves	CNS sedation
Silk tree	*Albizzia julibrissin* Durazzini	Bark	Treatment of anxiety symptoms

[a] Korean red ginseng means the product type air-dried and steamed at 100 °C.

induce sedative-hypnotic effects by regulating neurotransmission such as GABAergic or serotonergic systems in the central nervous system (CNS) (Attele et al., 2000).

In Asia, finding the suitable herbal sleep aids is in high demand due to the limited availability of the western herb preparations. There are many medicinal plants used traditionally for treatment of insomnia in Asia. The sedative-hypnotic effect of oriental plants such as *Ganoderma lucidum* (Chu et al., 2007), *Schisandra sphenanthera* (Huang et al., 2007) and *Euphoria longana* (Ma et al., 2009a) has been reported. However, scientific evidence and precise mechanism for hypnotic activity of those and other oriental medicinal plants have not been fully investigated.

The molecular target of medicinal plants having sedative-hypnotic activity has been mainly focused on the benzodiazepine site of $GABA_A$ ($GABA_A$-BZD) receptor (Abourasheda et al., 2004). GABA, the major inhibitory neurotransmitter in CNS, is essential for the overall balance between neuronal excitation and inhibition (Johnston, 2005). GABAergic neurotransmission plays a key role in sleep regulation, and the BZD binding site on the $GABA_A$ receptor is a target for the most sedative-hypnotics (Bateson, 2006). The BZD agents such as diazepam stimulate the ability of GABA to cause hyperpolarization of membrane by allowing a Cl^- influx. As a result, the inhibition of neurotransmission is achieved, and subsequently these agents produce sedative-hypnotic, anxiolytic and anticonvulsant activities (Smith and Simpson, 2003). The 5-HT_2 receptors (5-HT_{2A}, 5-HT_{2B} and 5-HT_{2C}) have been reported to play a major role in a range of CNS functions including anxiety, depression and sleep (Jerman et al., 2001). These subtypes of 5-HT_2 receptors are coupled to G protein and lead subsequent activation of phospholipase C, induction of phosphoinositide metabolism and increase in intracellular Ca^{2+} concentration (Porter et al., 1999). 5-HT_{2C} receptor is reported to be the potential therapeutic target for sedative-hypnotic (Smith et al., 2002) and anxiolytic (Harada et al., 2006) drugs. The 5-HT_{2C} receptor antagonist, ritanserin increased slow wave sleep in both rats (Kantor et al., 2002) and humans (Viola et al., 2002). In addition, it has been reported that 5-HT_{2C} receptor modulates $GABA_A$ receptor mediated Cl^- currents (Toro et al., 1996; Feng et al., 2001). Therefore, it is important to examine 5-HT_{2C} receptor when screening for sedative-hypnotic herbs, in addition to $GABA_A$-BZD. Although 5-HT_{2C} receptor is the potential target for sedative-hypnotics as well as anxiolytic drugs (Smith et al., 2002), the reports on the binding activity of sedative-hypnotic herbs to 5-HT_{2C} receptor are few.

In the present study, we investigated 15 medicinal plants used traditionally for treatment of insomnia in Asia, mainly in Korea and Japan. Binding affinity of the medicinal plant extracts (water and ethanol) for $GABA_A$ and 5-HT_{2C} receptors was evaluated. The hypnotic activities of the ethanol extracts of licorice (*Glycyrrhiza uralensis* Fischer) and silk tree (*Albizzia julibrissin* Durazzini) with high binding affinity to $GABA_A$ and 5-HT_{2C}, respectively, were evaluated by the pentobarbital-induced sleep test in mice.

2. Materials and methods

2.1. Materials and plant extracts

We selected 15 plants used to treat insomnia in Korea and Japan. Table 1 presents scientific name, traditional usage and used plant part for each plant extract. All plant extracts were purchased from the Plant Extract Bank of Korea (Daejeon, Korea). Voucher specimens are deposited in the Plant Extract Bank of Korea. According to extraction method of the supplier, dried and powdered plant material (10 g) was extracted with 100 mL of distilled water (100 °C, 3 h) and 95% ethanol (50 °C, 72 h), respectively. The aqueous and ethanol extraction solutions were filtered and lyophilized. Diazepam (DZP) (St. Louis, MO, USA) was used as a reference sedative-hypnotic drug. Pentobarbital was purchased from Hanlim Pharm Co. Ltd. (Seoul, Korea).

2.2. Animals

Male ICR mice (Orient Bio Inc., Seongnam, Korea) weighing 18–22 g were housed with food and water *ad libitum* in a temperature (24 °C) and humidity (55%) controlled room on 12 h light/dark cycle (light on at 7:00 am). They were acclimated for 1 week before they were used. To obtain the membrane preparation for the $GABA_A$-BZD receptor binding assay, 200–250 g of male SD rats were used. All procedures involving animals were conducted in accordance with the guidelines for animal experiments at KFRIACUC (Korea Food Research Institutional Animal Care and Use Committee).

2.3. Assay of [^{3}H] flumazenil binding to $GABA_A$-BZD receptor

The $GABA_A$-BZD receptor binding assay was modified from the method described by Risa et al. (2004). Cerebral cortex from four male SD rats (200–250 g) was homogenized for 10 s in 20 mL of Tris–HCl buffer (30 mM, pH 7.4) using Ultra-Turrax (T25; IKA Werke GmbH & Co. KG, Staufen, Germany). The suspension was centrifuged at 27,000 × *g* for 15 min, and the pellet was washed (centrifuged at 27,000 × *g* for 10 min) three times with Tris–HCl buffer. The washed pellet was homogenized in 20 mL of Tris–HCl

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Table 2
In vitro displacement of [^{3}H] flumazenil binding of plant water extracts to the GABA$_A$-BZD receptor in rat cerebral cortex.

Species		Displacement (%) of [^{3}H] flumazenil binding				
Common name	Scientific name	0.001 mg/mL	0.01 mg/mL	0.1 mg/mL	1 mg/mL	10 mg/mL
Reishi murshroom	*Ganoderma lucidum* Karsten	13.8 ± 12.3	14.5 ± 9.7	11.0 ± 11.1	10.6 ± 10.4	4.5 ± 12.0
Hoelen	*Poria cocos*	3.5 ± 12.6	3.8 ± 8.9	1.7 ± 10.6	4.7 ± 8.6	−6.3 ± 10.8
Chinese jujube	*Zizyphus jujuba* Miller	10.9 ± 18.5	18.8 ± 6.1	14.4 ± 14.4	4.6 ± 16.2	17.2 ± 10.5
Chinese magnolia vine	*Schizandra chinensis* Baillon	22.6 ± 4.8	20.4 ± 14.1	11.8 ± 17.3	16.1 ± 11.1	25.3 ± 12.8
Pomegranate	*Punica granatum* L.	26.9 ± 10.6	11.7 ± 22.3	18.1 ± 17.9	5.0 ± 23.1	12.7 ± 36.1
Danshen	*Salvia miltiorrhiza* Bunge	17.8 ± 15.6	26.7 ± 19.2	20.8 ± 20.9	25.8 ± 18.1	38.2 ± 16.0
Gigantic angelica	*Angelica gigas* Nakai	−0.7 ± 14.5	20.9 ± 7.7	17.0 ± 13.0	4.3 ± 9.9	12.4 ± 13.0
Licorice	*Glycyrrhiza uralensis* Fischer	8.8 ± 9.4	7.6 ± 11.1	11.7 ± 16.4	19.9 ± 13.4	49.6 ± 13.8
Korean red ginseng	*Panax ginseng* C.A. Meyer	8.7 ± 26.6	9.3 ± 18.4	7.2 ± 4.3	−10.5 ± 12.4	7.7 ± 9.4
Ginseng	*Panax ginseng* C.A. Meyer	16.5 ± 6.9	10.2 ± 13.8	5.0 ± 24.3	3.8 ± 1.4	11.3 ± 9.2
Ginger	*Zingiber officinale* Roscoe	25.8 ± 11.0	−7.7 ± 16.6	17.0 ± 25.2	32.5 ± 16.8	36.5 ± 28.9
Solomon's seal	*Polygonatum odoratum* var. pluriflorum	6.6 ± 14.6	11.6 ± 12.3	8.8 ± 15.6	11.0 ± 19.0	4.0 ± 34.5
Angelica	*Angelica utilis* Makino	21.6 ± 15.2	15.9 ± 12.0	12.6 ± 13.3	12.9 ± 23.9	11.1 ± 19.0
Cactus	*Opuntia ficus* indica Mill	18.2 ± 8.8	22.5 ± 6.8	17.5 ± 9.0	14.5 ± 7.5	20.5 ± 5.2
Silk tree	*Albizzia julibrissin* Durazzini	3.1 ± 22.2	20.3 ± 23.0	0.6 ± 16.5	−3.0 ± 23.9	9.6 ± 21.0

buffer, and incubated in a water bath (37 °C) for 30 min to remove endogenous GABA. Then the suspension was centrifuged at 27,000 × *g* for 10 min. The final membrane pellet was resuspended in 30 mL of Tris–HCl buffer and stored in aliquots at −80 °C until the binding assay. The membrane preparation was thawed and washed with 20 mL of Tris–citrate buffer (50 mM, pH 7.1, 0–4 °C). The suspension was centrifuged at 27,000 × *g* at 4 °C for 10 min. Washing and centrifugation of the membrane pellet were repeated twice. The pellet was resuspended at a concentration of 2.5 μg protein in 100 μL binding buffer and then used for the binding assay. Membrane suspension (180 μL) was added to 10 μL of a test solution (10–0.001 mg/mL) and 10 μL of 1 nM (final concentration) of [^{3}H] flumazenil (Ro 15-1788; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) in 96-well plate. The whole solution was mixed and incubated on ice (0–4 °C) for 40 min. The binding reaction was terminated by rapid filtration onto Whatman GF/C glass fiber filter with ice-cold 30 mM Tris–HCl buffer to remove any unbound [^{3}H] flumazenil. The filters were dried at 60 °C for 30 min and suspended in Wallac microbeta plate scintillation fluid. The amount of filter-bound radioactivity was counted using a Wallac 1450 Microbeta liquid scintillation counter (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). Total binding (TB) and non-specific binding (NSB) were determined using binding buffer and clonazepam (1 μM, final concentration), respectively.

2.4. Assay of ^{3}[H] mesulergine binding to 5-HT$_{2C}$ receptor

5-HT$_{2C}$ receptor binding assay was performed using membranes expressing human 5-HT$_{2C}$ receptor (ES-315-M400UA; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA). The 5-HT$_{2C}$ receptor membranes were thawed, and diluted in assay buffer (50 mM Tris, pH 7, 4 mM $CaCl_2$, 0.1% ascorbic acid) at a concentration of 4 μg/180 μL. Membrane suspension (180 μL) was added to 10 μL of a test solution (10–0.001 mg/mL) and 10 μL of 1 nM (final concentration) of [^{3}H] mesulergine (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) in 96-well plate. The mixed solution was incubated at 27 °C for 60 min and then, the incubation was terminated by filtration through GF/C glass fiber filter with ice-cold 50 mM Tris–HCl buffer. Radioactivity was performed as the procedure of the GABA$_A$-BZD receptor binding assay. Non-specific binding was determined using mainserin (100 μM).

2.5. Pentobarbital-induced sleep test in mice

The pentobarbital-induced sleep test was carried out according to the modified method described by Ma et al. (2009a). All experiments were performed between 1:00 and 5:00 pm and mice were fasted for 24 h prior to the experiment. For oral administration, plant extracts were suspended in 0.5% (w/v) carboxymethyl cellulose (CMC)–physiological saline. DZP, a reference sedative-hypnotic drug, was dissolved in 0.5% CMC–saline solution. Plant extracts (100, 250, 500 and 1000 mg/kg) and DZP (2 mg/kg) were administered orally to mice (n = 15) using a sonde niddle, 45 min before pentobarbital injection. Control mice (0.5% CMC–saline 10 mL/kg) were tested in parallel with those animals receiving plant extracts and DZP treatment. Following the intraperitoneal injection (i.p.) of pentobarbital (sub-hypnotic dosage, 30 mg/kg; hypnotic dosage, 45 mg/kg), each mouse was observed for measurement of sleep latency and sleeping time. Observers were blind to the extracts and drugs treatment. The mice lost the righting reflex over 1 min were considered to be asleep. The loss of righting reflex was defined as a failure of the mouse to right itself for at least 10 s after being placed on its back. The sleep latency was recorded from the pentobarbital injection to the sleep onset and sleeping time was defined as the difference of time between loss and recovery of the righting reflex. In the sub-hypnotic dosage of pentobarbital-treated test, the percentage of sleep onset was calculated as follows: sleep onset (%) = no. falling asleep/total no. × 100.

2.6. Statistical analysis

In the binding assay, IC_{50} values (7 different concentrations, 0.0001–20 mg/mL) were calculated with Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA) and displacement binding curves were fitted to a one-site competition binding model. The % displacement of radio-ligand binding was determined as $[1 - (DPM_{sample} - DPM_{NSB})/(DPM_{TB} - DPM_{NSB})] \times 100$. In the pentobarbital-induced sleep experiments, all data were represented as mean ± SEM. Data were analyzed using one-way ANOVA followed by Dunnett's test for multiple comparisons. Differences with $p < 0.05$ were considered statistically significant.

3. Results

3.1. Binding affinity of plant extracts to the GABA$_A$-BZD receptors

Tables 2 and 3 represent percent displacement of [^{3}H] flumazenil binding obtained with five concentrations of aqueous and ethanol extracts of all plants. None of the aqueous extracts of tested plants showed effective binding activity, with less than 90% displacement of [^{3}H] flumazenil binding at 10 mg/mL concentration. The most active extracts were ethanol extracts of licorice (LE, *Glycyrrhiza uralensis* Fischer) and danshen (DE, *Salvia miltiorrhiza*

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Table 3
In vitro displacement of [^{3}H] flumazenil binding of plant ethanol extracts to the GABA$_A$-BZD receptor in rat cerebral cortex.

Species		Displacement (%) of [^{3}H] flumazenil binding				
Common name	Scientific name	0.001 mg/mL	0.01 mg/mL	0.1 mg/mL	1 mg/mL	10 mg/mL
Reishi murshroom	*Ganoderma lucidum* Karsten	13.3 ± 13.9	10.2 ± 17.8	10.4 ± 25.9	18.2 ± 23.7	40.1 ± 15.0
Hoelen	*Poria cocos*	−15.1 ± 6.2	1.1 ± 6.9	−0.1 ± 6.9	3.1 ± 6.8	6.3 ± 3.5
Chinese jujube	*Zizyphus jujuba* Miller	15.5 ± 7.8	18.4 ± 4.7	18.8 ± 3.5	17.1 ± 5.3	26.3 ± 5.0
Chinese magnolia vine	*Schizandra chinensis* Baillon	28.5 ± 3.2	18.8 ± 0.9	18.8 ± 0.3	13.9 ± 0.7	19.2 ± 2.4
Pomegranate	*Punica granatum* L.	21.3 ± 0.7	21.1 ± 1.1	26.5 ± 0.4	21.9 ± 0.3	33.7 ± 1.3
Danshen	*Salvia miltiorrhiza* Bunge	3.0 ± 4.3	8.1 ± 4.6	40.1 ± 6.8	71.8 ± 3.2	90.2 ± 0.7
Gigantic angelica	*Angelica gigas* Nakai	17.9 ± 3.0	17.6 ± 3.1	12.6 ± 10.3	26.3 ± 3.5	67.8 ± 2.0
Licorice	*Glycyrrhiza uralensis* Fischer	1.4 ± 4.6	22.7 ± 6.8	54.0 ± 2.8	81.7 ± 3.3	97.7 ± 0.4
Korean red ginseng	*Panax ginseng* C.A. Meyer	2.0 ± 1.5	−0.6 ± 7.0	−6.6 ± 1.2	−4.4 ± 5.5	−6.8 ± 5.8
Ginseng	*Panax ginseng* C.A. Meyer	6.3 ± 5.3	12.5 ± 4.6	10.0 ± 2.2	5.1 ± 6.9	−2.9 ± 1.8
Ginger	*Zingiber officinale* Roscoe	−6.1 ± 11.6	20.1 ± 8.8	15.0 ± 5.8	13.9 ± 3.0	46.6 ± 3.7
Solomon's seal	*Polygonatum odoratum* var. pluriflorum	26.1 ± 4.4	25.4 ± 1.2	18.9 ± 5.2	21.9 ± 0.9	39.1 ± 5.9
Angelica	*Angelica utilis* Makino	13.5 ± 2.9	9.0 ± 5.3	9.8 ± 8.9	19.8 ± 17.2	45.2 ± 0.4
Cactus	*Opuntia ficus* indica Mill	17.0 ± 4.5	15.2 ± 4.7	13.4 ± 6.8	11.3 ± 3.0	13.9 ± 6.3
Silk tree	*Albizzia julibrissin* Durazzini	−20.7 ± 24.8	−12.2 ± 15.2	−3.2 ± 3.3	−4.8 ± 4.3	16.5 ± 2.3

Bunge), which displaced over 90% of [^{3}H] flumazenil binding at a concentration of 10 mg/mL. The IC$_{50}$ values of LE and DE were 0.064 and 0.137 mg/mL, respectively (Fig. 1).

3.2. Binding affinity of plant extracts to the 5-HT$_{2C}$ receptors

The binding activities of water and ethanol extracts to 5-HT$_{2C}$ receptor are shown as % displacement of [^{3}H] mesulergine (5-HT$_{2C}$ specific agonist) binding in Tables 4 and 5. As in the GABA$_A$-BZD binding assay, none of the aqueous extracts showed an effective binding activity. DE, which showed an effective binding affinity to the GABA$_A$-BZD receptor, did not show any effective binding activity for 5-HT$_{2C}$ receptor. LE had a weak binding activity for 5-HT$_{2C}$ receptor at 10 mg/mL. On the other hand, the ethanol extracts of ginseng (GE, *Panax ginseng* C.A. Meyer), Korean red ginseng and silk tree (SE, *Albizzia julibrissin* Durazzini) showed an effective binding activity. Korean red ginseng which is a steamed preparation of ginseng had a similar binding affinity as GE (fresh ginseng). The IC$_{50}$ values of GE and SE were 0.064 and 0.137 mg/mL, respectively (Fig. 2).

3.3. Direct hypnotic effects of LE and SE compared to pentobarbital and DZP

Administration of hypnotic dose (45 mg/kg, i.p.) of pentobarbital-induced sleeping in all 15 mice as expected.

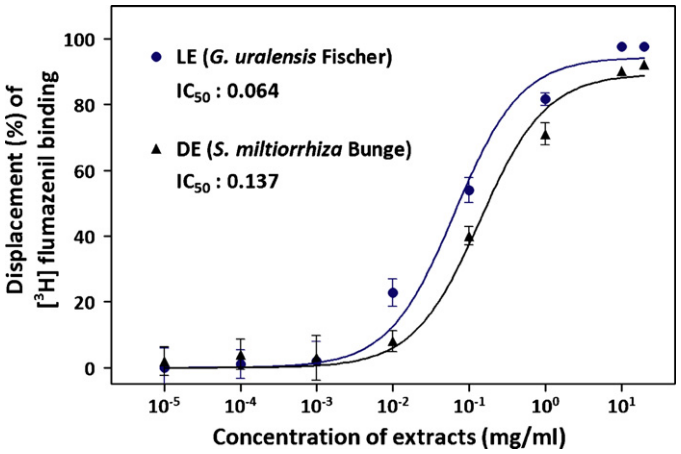

Fig. 1. Dose–response curves and IC$_{50}$ values of LE (licorice, *Glycyrrhiza uralensis* Fischer) and DE (danshen, *Salvia miltiorrhiza* Bunge) in the GABA$_A$-BZD receptor binding assay. Each data point is expressed as mean ± SD (n = 3).

However, all mice treated by sub-hypnotic dose (30 mg/kg, i.p.) of pentobarbital did not fall asleep (Table 6). We found that LE and SE alone did not induce sleep even at a high dose (1000 mg/kg, p.o.). Single administration of DZP (2 mg/kg, p.o.) also did not show any hypnotic effect.

3.4. Effects of LE and SE on sleep with hypnotic dose of pentobarbital

The oral administration of LE and SE produced a dose-dependent decrease in sleep latency and increase in sleep duration in mice treated with hypnotic dose (45 mg/kg) of pentobarbital. LE significantly decreased sleep latency ($p < 0.01$) (Fig. 3A) and increased sleep duration ($p < 0.05$) (Fig. 3B) at a high dose of 1000 mg/kg, compared to the control group. The significant hypnotic activity of SE was observed at 500 ($p < 0.05$) and 1000 ($p < 0.01$) mg/kg (Fig. 3A and B). However, lower doses (100 and 250 mg/kg) of both LE and SE had no significant hypnotic effect. As expected, DZP (2 mg/kg, p.o.) significantly potentiated the pentobarbital-induced sleep in mice (Fig. 3A and B).

3.5. Effects of LE and SE on sleep with sub-hypnotic dose of pentobarbital

The effects of LE and SE on sleep onset and sleeping time induced by sub-hypnotic dose (30 mg/kg) of pentobarbital are shown in Table 7. All 15 mice of control group treated by sub-hypnotic dose of pentobarbital did not fall asleep. The administration of DZP (2 mg/kg, p.o.) showed the highest rate of sleep onset (93%). LE and SE increased the rate of sleep onset and the sleeping time in a dose–response manner, and the sleep onset rates of them were 80 and 67% at 1000 mg/mL, respectively.

3.6. Effects of co-administration of LE and SE on pentobarbital-induced sleep in mice

To investigate the hypnotic activity of LE and SE when administered together, the concentration of each extract was chosen at low dose of 250 mg/kg, at which each extract did not significantly potentiate the pentobarbital-induced sleep. LE and SE alone did not affect both sleep latency and sleep duration (Fig. 4A and B). However, co-administration of LE and SE showed a significant hypnotic activity ($p < 0.05$). The co-administration of LE and DZP or SE and DZP also significantly decreased sleep latency and increased sleep duration, whereas DZP (0.5 mg/kg) alone had no effect (Fig. 4A and B). The most active group was the combination of LE and DZP ($p < 0.01$).

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Table 4
In vitro displacement of [^{3}H] mesulergine binding of plant water extracts to membrane expressing human 5-HT$_{2C}$ receptor.

Species		Displacement (%) of [^{3}H] mesulergine binding				
Common name	Scientific name	0.001 mg/mL	0.01 mg/mL	0.1 mg/mL	1 mg/mL	10 mg/mL
Reishi murshroom	*Ganoderma lucidum* Karsten	40.7 ± 6.7	44.2 ± 0.6	35.3 ± 2.9	24.8 ± 2.1	44.8 ± 1.0
Hoelen	*Poria cocos*	−25.3 ± 8.4	−86.9 ± 11.1	−30.2 ± 5.1	−10.5 ± 6.5	−25.3 ± 5.9
Chinese jujube	*Zizyphus jujuba* Miller	38.4 ± 8.0	28.8 ± 8.3	20.9 ± 8.2	−13.7 ± 5.0	−27.0 ± 7.2
Chinese magnolia vine	*Schizandra chinensis* Baillon	35.7 ± 6.0	47.8 ± 3.3	26.7 ± 8.7	29.1 ± 9.9	50.4 ± 8.8
Pomegranate	*Punica granatum* L.	33.3 ± 5.8	35.4 ± 1.8	35.3 ± 2.1	38.5 ± 6.2	44.6 ± 3.2
Danshen	*Salvia miltiorrhiza* Bunge	6.5 ± 11.9	−14.4 ± 3.2	−6.5 ± 6.4	−16.0 ± 5.8	8.0 ± 6.0
Gigantic angelica	*Angelica gigas* Nakai	−21.7 ± 8.8	−15.7 ± 8.7	−22.8 ± 6.7	−50.1 ± 5.2	−5.6 ± 6.5
Licorice	*Glycyrrhiza uralensis* Fischer	48.4 ± 7.0	28.6 ± 8.3	22.6 ± 8.6	33.7 ± 1.7	67.1 ± 5.7
Korean red ginseng	*Panax ginseng* C.A. Meyer	19.5 ± 0.6	26.1 ± 6.0	28.4 ± 3.7	21.5 ± 4.1	31.3 ± 2.8
Ginseng	*Panax ginseng* C.A. Meyer	40.9 ± 6.2	46.6 ± 0.6	45.5 ± 10.7	31.5 ± 6.5	0.8 ± 9.7
Ginger	*Zingiber officinale* Roscoe	9.0 ± 5.7	−26.7 ± 8.2	−24.3 ± 1.3	−53.1 ± 5.3	−61.7 ± 7.9
Solomon's seal	*Polygonatum odoratum* var. pluriflorum	32.9 ± 7.7	17.0 ± 10.5	14.1 ± 9.9	−15.1 ± 1.7	−12.4 ± 7.9
Angelica	*Angelica utilis* Makino	34.8 ± 2.8	27.2 ± 0.4	20.2 ± 0.5	14.5 ± 9.3	37.6 ± 6.9
Cactus	*Opuntia ficus* indica Mill	32.0 ± 3.3	34.4 ± 4.0	27.5 ± 5.0	45.3 ± 7.3	51.1 ± 6.7
Silk tree	*Albizzia julibrissin* Durazzini	11.4 ± 6.0	21.4 ± 0.9	−11.2 ± 1.5	−39.0 ± 8.9	−49.8 ± 7.8

Table 5
In vitro displacement of [^{3}H] mesulergine binding of plant ethanol extracts to membrane expressing human 5-HT$_{2C}$ receptor.

Species		Displacement (%) of [^{3}H] mesulergine binding				
Common name	Scientific name	0.001 mg/mL	0.01 mg/mL	0.1 mg/mL	1 mg/mL	10 mg/mL
Reishi murshroom	*Ganoderma lucidum* Karsten	21.0 ± 9.6	5.6 ± 11.6	3.2 ± 6.6	5.5 ± 5.8	2 ± 0.7
Hoelen	*Poria cocos*	16.6 ± 2.9	5.9 ± 1.1	21.0 ± 2.6	12.0 ± 2.2	6.5 ± 0.6
Chinese jujube	*Zizyphus jujuba* Miller	37.8 ± 0.5	28.4 ± 2.8	37.3 ± 2.8	44.2 ± 1.7	39.6 ± 1.8
Chinese magnolia vine	*Schizandra chinensis* Baillon	33.3 ± 5.8	35.4 ± 1.8	35.3 ± 2.1	38.5 ± 6.2	44.6 ± 3.2
Pomegranate	*Punica granatum* L.	−42.9 ± 5.5	−49.7 ± 1.0	−60.0 ± 9.7	−48.6 ± 6.8	8.0 ± 6.0
Danshen	*Salvia miltiorrhiza* Bunge	31.4 ± 0.1	23.3 ± 2.3	22.7 ± 1.6	4.7 ± 8.1	7.5 ± 1.4
Gigantic angelica	*Angelica gigas* Nakai	22.1 ± 9.8	20.1 ± 6.3	4.6 ± 6.9	−1.4 ± 4.0	−6.1 ± 8.4
Licorice	*Glycyrrhiza uralensis* Fischer	14.5 ± 11.0	16.5 ± 7.3	12.2 ± 0.7	9.5 ± 7.8	−14.8 ± 0.1
Korean red ginseng	*Panax ginseng* C.A. Meyer	4.1 ± 10.8	8.7 ± 6.9	5.0 ± 8.6	47.1 ± 7.0	90.1 ± 2.0
Ginseng	*Panax ginseng* C.A. Meyer	4.1 ± 4.3	9.1 ± 2.4	37.8 ± 5.3	56.6 ± 2.2	98.7 ± 0.4
Ginger	*Zingiber officinale* Roscoe	10.8 ± 6.1	7.9 ± 9.2	13.9 ± 8.6	10.7 ± 1.7	−12.0 ± 6.5
Solomon's seal	*Polygonatum odoratum* var. pluriflorum	21.0 ± 8.1	14.8 ± 7.0	22.9 ± 7.6	31.0 ± 3.7	13.5 ± 0.4
Angelica	*Angelica utilis* Makino	21.0 ± 4.6	27.8 ± 0.7	31.3 ± 6.1	45.4 ± 4.2	83.1 ± 0.7
Cactus	*Opuntia ficus* indica Mill	19.6 ± 4.6	28.7 ± 3.4	26.7 ± 5.4	24.0 ± 10.2	37.2 ± 1.0
Silk tree	*Albizzia julibrissin* Durazzini	5.0 ± 5.2	7.2 ± 2.6	25.2 ± 5.3	50.3 ± 2.9	87.9 ± 1.1

4. Discussion

In the present study, 15 medicinal plants used traditionally in Asia, mainly Korea and Japan, for treatment of insomnia were screened, and their water and ethanol extracts were investigated in the GABA$_A$-BZD and 5-HT$_{2C}$ receptor binding assays. We demonstrate for the first time that LE and SE show binding activity to GABA$_A$-BZD and 5-HT$_{2C}$ receptor, and they enhance the pentobarbital-induced sleep in mice. These results suggest that the hypnotic activity of LE and SE may be due to modulation of GABA$_A$ and 5-HT$_{2C}$ receptors, respectively.

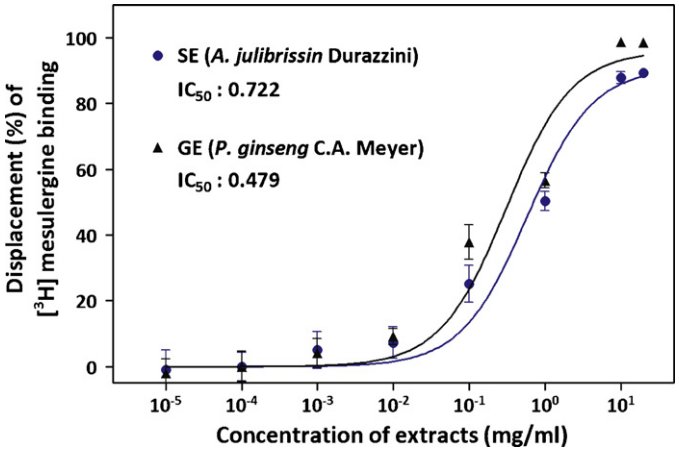

Fig. 2. Dose–response curves and IC$_{50}$ values of SE (silk tree, *Albizzia julibrissin* Durazzini) and GE (ginseng, *Panax ginseng* C.A. Meyer) in the 5-HT$_{2C}$ receptor binding assay. Each data point is expressed as mean ± SD (n = 3).

4.1. LE and SE show high affinity to GABA$_A$-BZD receptor

In the GABA$_A$-BZD binding assay, LE and DE showed an effective concentration-dependent binding activity. The danshen root has been used for the treatment of coronary and cerebrovascular diseases, sleep disorders, hepatitis and carbuncles (Imanshahidi and Hosseinzadeh, 2006). It has been already reported that ten diterpenoids isolated from the danshen root displaced the binding of [^{3}H] flunitrazepam to GABA$_A$-BZD receptors (Lee et al., 1991). Among these compounds, miltirone had the highest binding activity (IC$_{50}$ = 0.3 μM) and was orally active in animal models as a tranquillizer. According to a recent report (Fang et al., 2010),

Table 6
Sleep-inducing effects of pentobarbital, DZP and plant extracts in mice.

Groups	Dose (mg/kg)	No. falling asleep/total	Sleep duration (min)
Pentobarbital	30	0/15	–
	45	15/15	39.6 ± 3.9
DZP	2	0/15	–
LE	1000	0/15	–
SE	1000	0/15	–

DZP: diazepam, LE: licorice ethanol extract, SE: silk tree ethanol extract. Data of sleep duration represent the mean ± SEM (n = 15).

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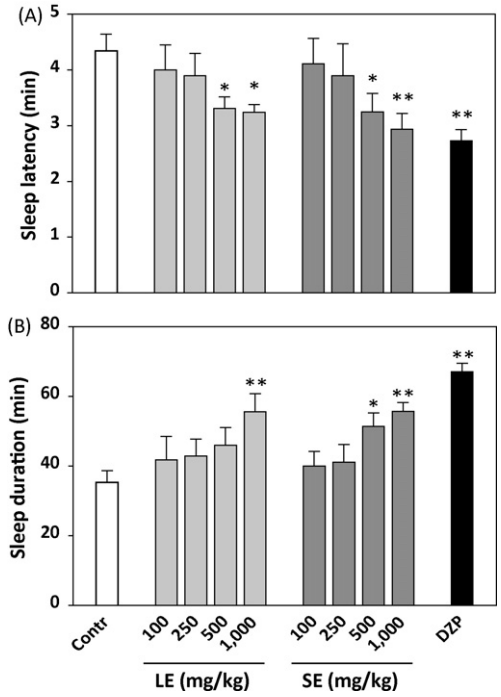

Fig. 3. Effects of LE (licorice, *Glycyrrhiza uralensis* Fischer) and SE (silk tree, *Albizzia julibrissin* Durazzini) on sleep latency (A) and sleep duration (B) in mice induced by hypnotic dose (45 mg/kg, i.p.) of pentobarbital. Mice received pentobarbital 45 min after administration of all samples. DZP: diazepam (2 mg/kg, p.o.), the reference sedative-hypnotic drug. Contr: control (0.5% CMC–saline 10 mL/kg, p.o.). Each column represents the mean ± SEM ($n = 15$). $^{*}p < 0.05$, $^{**}p < 0.01$, significant as compared to the control group (Dunnett's test).

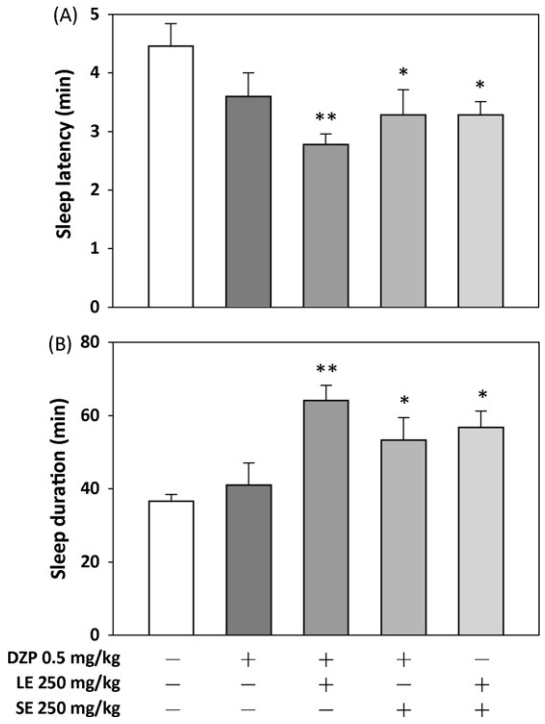

Fig. 4. Effects of combination of LE (licorice, *Glycyrrhiza uralensis* Fischer) and SE (silk tree, *Albizzia julibrissin* Durazzini) and their co-administration with DZP on sleep latency (A) and sleep duration (B) in mice induced by hypnotic dose (45 mg/kg, i.p.) of pentobarbital. Mice received pentobarbital 45 min after administration of all samples. DZP: diazepam (2 mg/kg, p.o.), the reference sedative-hypnotic drug. Contr: control (0.5% CMC–saline 10 mL/kg, p.o.). Each column represents the mean ± SEM ($n = 15$). $^{*}p < 0.05$, $^{**}p < 0.01$, significant as compared to the control group (Dunnett's test).

administration of an ether extract (600 mg/kg) of danshen significantly decreased sleep latency and increased sleep duration in mice treated with pentobarbital.

LE has been used as an ingredient of suanzaorentang, which is a very famous traditional remedy for treatment of insomnia in China, with zizyphi spinosi semen, poria cocos, ligusticum wallichii, anemarrhenae rhizome (Yi et al., 2007). Licorice is a famous medicinal plant with a long history in Asia, and has useful pharmacological properties such as antiinflammatory, antiviral, antimicrobial, antioxidative and anticancer activities (Asl and Hosseinzadeh, 2008). The root of licorice contains triterpenoid saponins (mostly glycyrrhizin), flavonoids (liquiritin, liquiritigenin, isoliquiritin, isoliquiritigenin and rhamnoliquiritin) and isoflavones (glabridin, galbrene, glabrone and shinpterocarpin) (Mae et al., 2003). Natural flavonoids are known to have a range of activities on $GABA_A$ receptor (Johnston, 2005). Apigenin, hispidulin and cirsimaritin competitively inhibited the binding of [^{3}H] flumazenil to the $GABA_A$-BZD receptor (Kavvadias et al., 2003). It has been reported that hispidulin stimulated the GABA-induced Cl^- current indicating positive allosteric properties, and had permeability across the blood–brain barrier (BBB) in a rat *in situ* perfusion model (Kavvadias et al., 2004). Based on these reports, the binding activity of LE to the $GABA_A$-BZD receptor may be attributed to flavonoids such as liquiritin, liquiritigenin, isoliquiritin and isoliquiritigenin.

4.2. SE and GE showed high affinity to 5-HT_2 receptor

In the 5-HT_{2C} receptor binding assay, ethanol extracts of SE, GE and Korean red ginseng showed an effective concentration-dependent binding activity. Ginseng is one of the most commonly

Table 7
Effects of LE (licorice, *Glycyrrhiza uralensis* Fischer) and SE (silk tree, *Albizzia julibrissin* Durazzini) on sleep onset and sleep duration in mice induced by sub-hypnotic dose (30 mg/kg, i.p.) of pentobarbital.

Groups	Dose (mg/kg)	No. falling asleep/total	Sleep onset (%)	Sleep duration (min)
Control		0/15	0	0.0 ± 0.0
DZP	2	14/15	93	22.3 ± 3.6***
LE	250	5/15	33	7.4 ± 1.3
	500	8/15	53	16.3 ± 4.5**
	1000	12/15	80	26.6 ± 3.7***
SE	250	3/15	20	9.5 ± 1.9
	500	6/15	40	12.2 ± 2.8
	1000	10/15	67	16.8 ± 4.1**

Mice received pentobarbital 45 min after administration of all samples. If the mouse did not lose righting reflex in 60 min after treatment, the sleep duration was recorded as 0 min. DZP: diazepam (2 mg/kg, p.o.), the reference sedative-hypnotic drug. Contr: control (0.5% CMC–saline 10 mL/kg, p.o.). Sleep onset (%) = no. falling asleep/total no. × 100. Data represent the mean ± SEM ($n = 15$).

** $p < 0.01$, significant as compared to the control group (Dunnett's test).

*** $p < 0.001$, significant as compared to the control group (Dunnett's test).

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used herbal medicines in Korea and has long been used traditionally for treatment of psychiatric diseases including anxiety, depression and insomnia (Xiang et al., 2008). Ginseng saponins administered at a high dose was shown to prolong the pentobarbital-induced sleep duration (Jung and Jin, 1996). The ethanol extract of Korean red ginseng was also shown to increase total sleep and non-rapid eye movement sleep and decrease wakefulness (Ma et al., 2009b). Constituents of ginseng include saponins (ginsenosides), polysaccharides, peptides and fatty acids. Ginsenosides are generally believed to have pharmacologically important role. Future experiments are needed to investigate the possible mechanism of the sedative-hypnotic effect of ginseng.

Silk tree is widely distributed in Asia, and has been used as a folk medicine for treatment of insomnia and calming the mind in Korea (Kim et al., 2007). Saponins, phenolic glycosides and triterpenes were isolated from the stem bark of silk tree (Kinjo et al., 1992; Chen and Zhang, 1997). The hypnotic effect of a flavonol glycoside isolated from the flower of silk tree was previously observed in the pentobarbital-induced sleep test (Kang et al., 2000). However, the hypnotic effects of the stem bark of silk tree have not been reported yet. Therefore, flavonol glycosides could be the active compound that is responsible for sedative-hypnotic effect of silk tree stem bark, and this needs further investigation.

4.3. Hypnotic effects of LE and SE

The results of the binding assays provide a possibility that LE, DE, SE, and GE contain natural ligands which bind to the $GABA_A$-BZD and 5-HT_{2C} receptors. It is important that the active compounds are able to pass the BBB to produce the hypnotic activity (Risa et al., 2004). The classical method of the pentobarbital-induced sleep test is useful to evaluate the sedative-hypnotic activity (Ma et al., 2009a; Fang et al., 2010). To confirm the hypnotic activity of LE and SE, we tested them in the pentobarbital-induced sleep test in mice. We investigated the hypnotic effect of LE and SE in particular because these have not been characterized in the past. We demonstrated that LE and SE potentiated the sedative-hypnotic effects of pentobarbital in mice by decreasing sleep latency and increasing sleep duration. In the sub-hypnotic dose of pentobarbital-induced sleep test, they also increased the rate of sleep onset and sleep duration. These results suggest that LE and SE contain active compounds that can be effective as a sleep aid. In the previous studies, antidepressant and anxiolytic activities of LE and SE have been reported. Wang et al. (2008) reported that liquiritin and isoliquiritin from licorice produced antidepressant-like activity in the forced swimming test and tail suspension test in mice. The stem bark of silk tree had anxiolytic-like (Kim et al., 2004) and antidepressant-like (Kim et al., 2007) activities via the 5-HT_{1A} receptor system.

To investigate the hypnotic activity of combination of LE and SE, the low doses (250 mg/kg) of LE and SE were chosen, which did not significantly enhance the pentobarbital-induced sleep in the dose-dependent test (Fig. 3). Synergic effect of LE and SE with 0.5 mg/kg of DZP not showing hypnotic activity was also tested. Co-administration of LE and SE significantly potentiated the pentobarbital-induced sleep ($p < 0.05$). Administration of LE and SE with DZP also showed significant hypnotic activity at $p < 0.01$ and $p < 0.05$, respectively. The hypnotic effect of co-administration of LE and DZP can be explained by that constituent of LE acts as an allosteric agonist such as DZP on the $GABA_A$ receptor. Synergic effect of SE and DZP suggests that hypnosis induced by activation of $GABA_A$ receptor be able to potentiated via modulation of 5-HT_{2C} receptor. Although many preclinical and clinical studies have shown that 5-HT_{2C} antagonist may be useful in the treatment of anxiety and sleep disorder, its precise mechanism still remain unclear (Matteo et al., 2000).

In conclusions, we show that LE and SE display effective binding activities to $GABA_A$-BZD and 5-HT_{2C} receptors, respectively, and produce hypnotic activity in the pentobarbital-induced sleep. Therefore, LE and SE or their combination preparation might provide a useful means for the treatment of insomnia. Future studies are needed to isolate and identify the active compounds of LE and SE that cause the sedative-hypnotic activity. Knowing the precise mechanism of how these extracts function and at what molecular targets they act should give us insights into how to develop better sleep aids based on the natural products.

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