**Regular Article**

**Romaine Lettuce/Skullcap Mixture Improves Sleep Behavior in Vertebrate Models**

Ki-Bae Hong, Sung Hee Han, Yooheon Park, Hyung Joo Suh, and Hyeon-Son Choi

*Department of Biological Sciences and Environmental Sciences Program, Southern Illinois University–Edwardsville; Edwardsville, IL 62026, U.S.A.*

1 Dongguk University Research Institute of Biotechnology; Goyang 10326, Republic of Korea; 2 Department of Public Health Sciences, Korea University; Seoul 02841, Republic of Korea; and 3 Department of Food Science and Technology, Seoul Women’s University; Seoul 01797, Republic of Korea.

Received April 6, 2018; accepted May 14, 2018

The aim of this study is to investigate the effects of romaine lettuce leaves extract (RE), skullcap root extract (SE), and their mixture on sleep behaviors in vertebrate models. HPLC analysis showed that RE contains luteocuprin (0.02±0.01 mg/g extract), chlorogenic acid (4.05±0.03 mg/g extract), caffeic acid (2.38±0.03 mg/g extract), and chicoric acid (7.02±0.32 mg/g extract) as main phenolic compounds, while SE includes baicalin (99.4±0.5 mg/g extract), baicalein (8.28±0.21 mg/g extract), and wogonin (3.09±0.32 mg/g extract). The mixture of RE (100 mg/g extract) and SE (40 mg/g extract) increased total sleep time by 50.9% compared with the control in pentobarbital-induced sleep model. In electroencephalography (EEG) analysis, RE/SE mixture significantly increased Non-Rapid Eye Movement (NREM), in which delta wave was enhanced by around 40% compared with normal control, leading to the increase of sleep time. In caffeine-induced wake model, RE/SE mixture greatly decreased (53%) caffeine-induced wake time, showing a similar level to normal control. In addition, caffeine-induced decrease of NREM and delta wave was effectively increased with RE/SE mixture; NREM and delta wave increased by 85% and 108%, respectively. Furthermore, RE/SE mixture was shown to bind to a gamma-aminobutyric acid type A (GABA_A)-benzodiazepine (BZD) receptor stronger than RE or SE single extract. Taken together, RE/SE mixture effectively improved sleep behavior with the increase of NREM via GABA_A-BZD receptor binding. RE/SE mixture can be used as an herbal agent for sleep disorders.

Key words romaine lettuce; skullcap; sleep behavior; non-rapid eye movement; gamma-aminobutyric acid (GABA) receptor; rodent model

Humans spend one third of their lifetime sleeping, of which quantity and quality are related to mood stability, productivity, and health. Several studies reported that cognitive performance including learning and memory has been known to depend on adequate sleep. Sleep disorders are closely related to mental and physical impairments in life; the increase of needs on medical treatment for sleep disorders. Although drugs used in the treatment of insomnia show an efficacy, these chemical agents (hypnotics/sedatives) have various side effects such as daytime lethargy, drug dependence, and overdose-derived dangers. Thus, herbal extracts have been studied widely for the treatment of sleep disorders with lowered side effect, and there is accumulating evidence for the possible role of natural compounds on sleep. Several natural herbs have been known to have a positive role in sedation or sleep. Valerian (Valeriana officinalis), hops (Humulus lupulus), St. John’s wort (Hypericum perforatum), camomile (Matricaria chamomilla), lingzhi (Ganoderma lucidum), barley grass, and maca are natural herbs commonly used as sedatives and anti-anxiety agents in Europe, the U.S.A., and Asia. These herbs contain complex mixtures of flavonoids, terpenes, and related substances, and are known to cross the blood–brain barrier (BBB) to influence brain function. The mechanistic action of these compounds include targeting specific receptors such as the γ-aminobutyric acid (GABA) receptor, serotonin receptor, melatonin receptor, histamine receptor, and orexin receptor. The GABAergic system is the most common mechanism of natural substances and compounds in herbs. Natural herbal products have been used both separately and in combination, with the purpose of extending the potential of efficacy. Various herbal extract combinations were shown to be more effective dietary management agents in disease control. Our previous study revealed that leaves of Lactuca sativa varieties (lettuce and romaine lettuce) played an acting in sleep promotion, but the effects of lettuce on sleep architecture and relevant mechanism has yet been studied. In addition, although skullcap-derived flavonoids are reported to bind to GABA receptor, its study on sleep behavior has been limited. Furthermore, the combined effects of both herbal (lettuce and skullcap) extracts on the quantity and quality of sleep have never been reported.

In this study, we investigated the effects of the combination of romaine lettuce extract (RE, Lactuca sativa L.) and skullcap extract (SE, Scutellaria baicalensis) on the quality and quantity of sleep in vertebrates and in a caffeine-induced insomnia model. The Sleep architecture and GABA receptor binding activity of RE or/and SE was also evaluated via electroencephalogram (EEG) analysis and GABA_A-BZD receptor binding assay.

*To whom correspondence should be addressed. e-mail: hschoi@swu.ac.kr*
MATERIALS AND METHODS

Plant Materials  L. sativa leaves and S. baicalensis roots were purchased from Kyeong-dong Oriental Pharmacy (Seoul, Republic of Korea), and each herb was identified by Professor Shin, Department of Food Science and Biotechnology, Kyonggi University, South Korea. Voucher specimens (L. sativa: FSB-2016-10, and S. baicalensis: FSB: 2016-12) were deposited at the same department.

Preparation of Extracts  Dried romaine lettuce leaves and dried skullcap roots were individually extracted twice with 70% ethanol for 4 h at 80°C in a reflux apparatus. After reflux, the extracts were filtered, evaporated in a rotary evaporator, and lyophilized in a freeze dryer (TFD8501, IlisinBioBase Co., Ltd., Seoul, Republic of Korea). The dried RE and SE powders were stored at 5°C until further analysis, and mixed together for further assays.

Identification and Quantification of Flavonoids from Lettuce and Skullcap  An Agilent HPLC series 1100 apparatus (Agilent, Waldbronn, Germany) equipped with a degasser, binary pump, auto-sampler, thermostat, and photodiode array detector (DAD) was used to detect sesquiterpene lactones (STL) at 254 and 320 nm. 13) A diode array detector (DAD) was used to detect sesquiterpene lactones (STL) at 254 and 320 nm. 13) Briefly, a reversed-phase C18 analytical column (4.6×150 mm, 5 µm, Phenomenex) was used with mobile phases A, acidified water (0.2% phosphoric acid, v/v) and B, acetonitrile. A linear gradient elution was programmed as follows: 0–34 min, 15% B; 35–45 min, 100% B; 46–60 min, 15% B. The flow rate was 0.7 mL/min and the injection volume was 20 µL.

The flavonoids in skullcap were quantified via an external standardization method. Briefly, an Agilent eclipse C18 column (4.6×250 mm, 5 µm, Agilent) was used with mobile phases A, acidified water (1.0% acetic acid, v/v) and B, acetonitrile and methanol (7:3, v/v) containing 1.0% acetic acid. A linear gradient elution was programmed as follows: 0 min, 75% A; 10 min, 68% A; 20–35 min, 55% A; 35 min, 52% A; 40–45 min, 75% A. The flow rate was 1.0 mL/min and the injection volume was 10 µL.

Animals  ICR mice (male, 4 weeks) and Sprague-Dawley (SD) rats (male, 8 weeks) were obtained from Orient Bio Inc. (Gyeonggi-do, Republic of Korea). All animals were allowed access to Purina rodent chow and tap water ad libitum, and were housed in acrylic cages at a temperature of 23±5°C, 5% atmospheric humidity with 12-h light/dark cycles, and acclimatized for 1 week prior to use. Vertebrate models were administered with water ad libitum, and were housed in acrylic cages at a temperature of 23±2°C in 50±5% atmospheric humidity with 12-h light/dark cycles, and acclimatized for 1 week prior to use. Vertebrate models were used for the pentobarbital-induced sleep tests, measurements of EEG wave patterns, and GABA_A-BZD receptor binding assay. All experiments were approved by the Ethical Committee and performed according to the guidelines and regulations of the Animal Care committee (KUIACUC-20170307-3, Seoul, Korea) in Korea University.

Pentobarbital-Induced Sleep Test  The experiments were performed between 13:00 and 17:00, and all mice were fasted for 24 h prior to testing. During oral administration, observers were blinded to individual treatments. There were 14 mice in the RE- and SE-only treatment groups and 15 mice in the RE/SE combination treatment group. RE (80, 100, 120, 140, and 160 mg/kg), SE (40, 50, 80, 100, and 120 mg/kg), and a combination of RE/SE (40, 100, and 120 mg/kg) were dissolved in 0.9% physiological saline. All samples were administered by post-oral injection (p.o.), and pentobarbital (hypnotic dose: 42 mg/kg) was administered by intraperitoneal injection (IP) 45 min later to induce sleep. After the pentobarbital injection, mice were laid down on their backs in individual cages and sleep latency was recorded from this time-point. In addition, sleeping duration was recorded, from the time of loss of righting reflex to recovery and regaining movement. Mice that did not sleep within 15 min of the pentobarbital injection were excluded from the experiment.

Surgical Procedures and Electrophysiological Analysis  Under isoflurane (Troikaa Pharmaceuticals Ltd., Gujarat, India) anesthesia in a mixture of oxygen and nitrous oxide, rats were placed onto a stereotaxic frame (Stoelting Inc., Wood Dale, IL, U.S.A.). A dorsal midline incision was made in the scalp to expose the skull and hemostasis was achieved with sterile cotton-tip applicators. Four holes were bored through the skull and the EEG electrodes were fixed to the skull surface using a mounting screw, socket, and dental cement (AgnThós AB, Lidingö, Sweden). After a 7-d postsurgery recovery period, rats were divided into control and treatment groups (n=8 in each group). Experiments were recorded over 8 h after administration of the herbal extracts (RE only, 100 mg/kg; SE only 40 mg/kg; and combined RE/SE, 140 mg/kg), between 10:00 and 18:00. Caffeine (10 mg/kg) was used in the caffeine-induced insomnia model. EEG activity data were acquired using the Iox2 data acquisition software (version 2.8.0.13, emka Technologies, Paris, France) and EEG spectra were analyzed in 1 Hz bins. Settings for standard bands were as follows: δ wave, 0.5–4; θ wave, 4–9; α wave, 9–12; β wave, 12–30; and γ wave, 30–60 Hz. From the EEG analysis with x/y/z and global acceleration for activity assessment, we calculated wave patterns, wake time, and sleep time from the data recorded at 2-s intervals by fast Fourier transform using the eegAUTO3 software (version. 3.3.0.20, Emka Technologies).

GABA_A-Benzodiazepine Receptor Binding Assay  The GABA_A-BZD receptor binding assay method used was slightly modified from those described by Risa et al. 23) and Kählberg et al. 24) The cerebral cortices of rat brains were homogenized for 10 s in 20 mL Tris–HCl buffer (30 mM, pH 7.4, 0–4°C) and the suspension was centrifuged at 27000×g for 15 min. The pellet was washed three times and homogenized in Tris–HCl buffer. The suspension was incubated in a water bath at 37°C for 30 min, and the suspension was then centrifuged at 27000×g for 10 min. The final pellet was resuspended in Tris–HCl buffer. The suspension was incubated in a water bath at 37°C for 30 min, and the suspension was then centrifuged at 27000×g for 10 min. The final pellet was resuspended in Tris–HCl buffer and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay. Membrane suspension (300 µL) was added to 25 µL [3H]-flumazenil to obtain a final concentration of 33.3 µg protein in 100 µL binding buffer, and this suspension was used in the binding assay.
### Table 1. Main Phenolic Compounds in Lettuce and Skullcap Extracts

<table>
<thead>
<tr>
<th>Phenolic compound</th>
<th>Lettuce extract (mg/g extract)</th>
<th>Skullcap extract (mg/g extract)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lactuca</td>
<td>0.02±0.001</td>
<td>0.50±0.001</td>
</tr>
<tr>
<td>Lactopicrin</td>
<td>2.38±0.03</td>
<td>8.28±0.21</td>
</tr>
<tr>
<td>Caffeic acid</td>
<td>4.05±0.03</td>
<td>3.09±0.32</td>
</tr>
<tr>
<td>Chlorogenic acid</td>
<td>7.02±0.32</td>
<td></td>
</tr>
<tr>
<td>Chicoric acid</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baicalin</td>
<td>99.40±0.50</td>
<td>9.45±0.32</td>
</tr>
<tr>
<td>Baicalein</td>
<td>8.28±0.21</td>
<td>7.75±2.37c</td>
</tr>
<tr>
<td>Wogonin</td>
<td>3.09±0.32</td>
<td>5.41±2.93c</td>
</tr>
</tbody>
</table>

### Table 2. Effects of the Two Herbal Extracts on Sleep Latency and Duration in Mice Treated with an Intraperitoneal Injection of Pentobarbital (Hypnotic Dosage, 42 mg/kg)

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Sleep latency (min)</th>
<th>Sleep duration (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal</td>
<td>Romaine lettuce (RE)</td>
</tr>
<tr>
<td>0</td>
<td>1.31±0.16b</td>
<td>1.44±0.14b</td>
</tr>
<tr>
<td>100</td>
<td>1.03±0.04a</td>
<td>8.28±0.21</td>
</tr>
<tr>
<td>120</td>
<td>1.50±0.26ab</td>
<td>78.86±5.41c</td>
</tr>
<tr>
<td>140</td>
<td>1.27±0.16ab</td>
<td>80.89±9.69c</td>
</tr>
<tr>
<td>160</td>
<td>1.66±0.30a</td>
<td>73.63±7.77c</td>
</tr>
</tbody>
</table>

Mice were injected with pentobarbital 45 min after the administration of all treatments. Sleep latency and duration values are shown as means±S.E. (n=13 to 15 per group). Different letters indicate significant differences at p<0.05 according to Tukey’s test.

Results

Phenolic Compounds in Romaine Lettuce/Skullcap Extracts

The main phenolic compounds found in romaine lettuce and skullcap extracts are shown in Table 1. Romaine lettuce extract contained lactopicrin (0.02 mg/g extract), a sequiterpene, but lactucin, another sequiterpene, was not detected. The major flavonoids found in the lettuce extract were caffeic acid (2.38 mg/g extract), chlorogenic acid (4.05 mg/g), and chicoric acid (7.02 mg/g, Table 1), while baicalin, baicalein, and wogonin are main flavonoids from skullcap extract, and their contents were 99.40, 8.28, and 3.09 mg/g, respectively.

Effects of Romaine Lettuce (RE), Skullcap (SE), and the RE/SE Mixture on Pentobarbital-Induced Sleeping Behaviors

The effects of RE and SE on sleep latency and duration were investigated in mice injected with a hypnotic dose of pentobarbital (42 mg/kg, via IP) (Table 2). Oral administration of RE caused a significant changes in sleep latency and in sleep duration. In particular, RE of 100 mg/kg decreased sleep latency by 21.4% (1.03±0.04 min) and increased sleep duration by 48.3% (80.32±9.45 min) compared with control (sleep latency; 1.30±0.16 min, sleep duration; 54.14±1.32 min).

In SE administration, the decrease of sleep latency was not observed in most of treatments, but sleep duration was significantly increased in low doses (40 and 50 mg/kg). SE of 40 and 50 mg/kg increased sleep duration by 26% (75.48±2.69 min) and 40.5% (84.18±2.93 min), respectively, compared with the control (sleep latency; 1.30±0.34 min, sleep duration; 59.91±2.37 min).

In mice treated with the combination of two herbal extracts (Fig. 1), administration of the RE/SE mixture did not significantly reduce sleep latency (Fig. 1A), but sleep duration was significantly increased compared to that in the control group; the mixture (RE 100 mg/kg and SE 40 mg/kg) led to the increase by 50.9% (p<0.05, Fig. 1B).

Effects of RE, SE, and RE/SE Mixture on Sleep Architecture

The effect of the two herbal extracts and the RE/SE mixture on sleep architecture are presented in Fig. 2. NREM and delta wave, which signifies deep sleep, were significantly increased in the RE/SE mixture-treated group; the durations of the waves increased 17 and 35%, respectively, compared to the control group (Figs. 2A and B, p<0.05).
REM was not significantly changed among the groups (Fig. 2C). RE/SE mixture administration significantly increased total sleep time (14.5%) and significantly reduced wake time (44.4%) compared to that in the control group (Figs. 2D and E, \( p < 0.05 \)). This result showed that RE/SE mixture promotes sleep by increasing NREM and delta wave.

**Effects of RE, SE, and RE/SE Mixture on Sleep Architecture in Caffeine-Induced Sleepless Rats** The effect of the RE/SE mixture on sleep architecture was investigated in a caffeine-induced insomnia model (Fig. 3). Caffeine (10 mg/kg) was used as a stimulant to induce wakefulness. Caffeine administration effectively caused a reduction in sleep times (\( p < 0.01 \)) and an increase in wake times (\( p < 0.01 \)), indicating that caffeine induced wakefulness in rodent model. Co-treatment of caffeine and RE/SE mixture significantly increased NREM and delta wave (\( p < 0.01 \)), and RE or SE single treatment also showed significantly increased NREM and delta wave (\( p < 0.05 \)) (Figs. 3A, B). In particular, RE/SE mixture increased NREM and delta wave by 85% and 108%, respectively, compared with the only caffeine-treated group (Figs. 3A and B, \( p < 0.01 \)). However, REM sleep pattern did not show any significant changes among the groups (Fig. 3C). This RE/SE mixture-mediated increase of NREM led to the increase of total sleep time with the reduced wake time. Total sleep duration of RE/SE mixture was increased by 28% compared with caffeine-treated control, showing a similar level to the normal group (Fig. 3D). In contrast, wake time significantly decreased with RE or/and SE treatments, and RE/SE mixture showed an around 52%-decrease of wake time compared to the caffeine-treated control (Fig. 3E). This result showed that RE/SE mixture improves the sleep duration and sleep quality, which was deteriorated by caffeine, by recovering sleep pattern to the normal level.

**Effects of RE, SE, and RE/SE Mixture on GABA \(_{\text{A}}\)-BZD Receptor Binding Activity** To investigate the sleep-promoting effects via GABAergic mechanism of the RE/SE mixture, we determined the effect of RE/SE mixture on the binding of [\(^{3}H\)] flumazenil, which is a radioligand for GABA receptor from animal (Fig. 4). Dose–response curves and EC\(_{50}\) values for RE, SE, and the RE/SE mixture in the binding assay are shown in Figs. 4A, B, and C. The EC\(_{50}\) values of RE, SE, and the RE/SE mixture were 28.75, 8.09, and 7.47 mg/mL, respectively. This result showed that the binding ability of RE/SE mixture is stronger than the other single samples. In Fig. 4D,
the displacement of [3H] flumazenil obtained with three concentrations of each herbal extract and combined RE/SE mixture is shown. Single herbal extracts showed lower displacement of [3H] flumazenil binding than RE/SE mixture in all tested concentrations. RE/SE mixture replaced [3H] flumazenil binding by over 90% from 50 mg/mL dose, showing a stronger replacement of [3H] flumazenil binding than single herbal extracts. This result showed that RE/SE mixture is more effective in binding to GABA receptor with high affinity.

Fig. 3. Effects of the Two Herbal Extracts and RE/SE Mixture on Sleep Quantity and Quality in Caffeine-Induced Sleepless Rats

Values are means±S.E. (n=40). Different letters indicate significant differences at p<0.05 according to Tukey’s test. RE, lettuce extract; SE, skullcap extract; RE/SE mixture, romaine lettuce extract/skullcap extract mixture; NREM, non-rapid eye movement sleep; δ wave, delta wave; REM, rapid eye movement sleep; sleep, total sleep time; awake, wakefulness.

Fig. 4. Dose–Response Curves and Half-maximal Effective Concentration (EC₅₀) Values for the Two Herbal Extracts and RE/RE Mixture Acting on the GABAₓ-BZD Receptor in Rat Cerebral Cortices

Data values are expressed as means±S.D. (n=3). RE, romaine lettuce extract; SE, skullcap extract; RE/SE mixture, lettuce extract/skullcap extract mixture.
DISCUSSION

Sleep disorder is one of the most common illnesses worldwide and leads to significant impairments in social and occupational functions with consequent daytime symptoms (e.g., sleepiness and fatigue). Food and Drug Administration (FDA)-approved pharmacological treatments for insomnia and sleep disorders are efficacious. However, benzodiazepines, non-benzodiazepines (e.g., zolpidem, zopiclone, and zaleplon), antidepressants (e.g., paroxetine, amitriptyline, and bupropion), and antihistamines (e.g., diphenhydramine, doxepin, and doxylamine) have obvious short- or long-term adverse effects. Although, compared with these pharmacological medications, herbal extracts or natural products produce a relatively weak efficacy in sleep promotion, they can be administered in large doses and have excellent safety profiles.

Over the past few decades, many studies have reported that extracts or active components from natural plants are able to bind to the GABA$_A$-BZD receptor to play a sleep-promoting effect. Lettuce and skullcap have been known to be two of the herbs that possess sedative effects, although systematic study on their sleep-promoting effects and relevant mechanism has been insufficient. Traditionally, lettuce has been suggested to have sedative-hypnotic properties. In particular, lactucin and lactucopicrin from lettuce showed sedative properties in a spontaneous locomotor activity test. Baicalin from skullcap has been reported to bind to the GABA$_A$-BZD receptor, and wogonin and baicalin, which are the other aglycone flavonoids of skullcap, have been known to bind to the benzodiazepine site of the GABA$_A$ receptor much stronger than baicalin. Thus, the skullcap-mediated sleep promoting effect, based on the GABA receptor binding, is recognized to depend on the amount of baicalin or wogonin, aglycone types rather than baicalin in skullcap. However, since skullcap is shown to have baicalin in much higher level than baicalin or wogonin (Table 1), skullcap needs to be processed to enhance aglycones such as baicalein or wogonin which has a much stronger affinity to GABA receptor. For instance, food processing catalyzing the conversion of baicalin to baicalein may be helpful, or extractions to increase quantity of baicalin and wogonin from skullcap would have to be devised in the future. Chang et al. reported that baicalin exhibits biphasic effects on sleep-wake regulation. Ghorbani et al. reported the sleep-enhancing activity of lettuce, in which terpenoids such as lactucin and lactucopicrin, were presumed to be the active component. However, lactucin was not detected in the analysis of current study. It is thought to be due to the small amount of lactucin in romaine lettuce itself and extraction method might affect the lactucin detection. Seo et al. identified lactucin from the extracts of some lettuce cultivars using methanol. However, its level was much lower than the other sesquiterpene such as lactucopicrin. Based on the above reports, lactucopicrin, baicalein, baicalin, and wogonin are recognized to contribute to the sleep-promoting effects of romaine lettuce and skullcap extract mixture. In particular, baicalin, wogonin, and baicalein have been shown to directly exert sleep-promoting effect due to the ability to cross BBB. However, the ability of lactucopicrin for the crossing BBB has not been confirmed, although its sedative effect. Further detailed study would confirm the effect of each compounds or combinations on the sleep behaviors and BBB crossing in the next study.

The present study showed that romaine lettuce also contains the other phenolic compounds (caffeic acid, chlorogenic acid, chicoric acid) (Table 1). In general, inflammation and oxidative stress are related to the high incidence of short sleep. Thus, since these chemicals are known to be antioxidants which can defend oxidative stress and inflammation, they are expected to indirectly affect sleep behavior. Caffeic acid has been shown to maintain or increase oral melatonin level, when melatonin is orally medicated, by decreasing melatonin metabolism. However, chlorogenic acid and its metabolite caffeic acid are controversial in term of sleep. Shinomiya et al. and Ohnishi et al. reported that sleep latency and locomotor activity in rodents was increased by chlorogenic acid, while a recent study showed chlorogenic acid shortened sleep latency. This discrepancy seems to be due to the difference of usual dose. In addition, since these compounds have the ability to cross the BBB, their effect on sleep may depend on the binding sites of their receptors. Further systematic analysis of these phytochemicals would be performed on the sleep behaviors in the future.

Various studies have described the hypnotic effects of two or more combined herbal extracts to achieve possible additive or synergistic effects. Consumption of four different herbal preparations (lavender oil, and extracts of hops, lemon balm, and oat) resulted in significant time- and dose-dependent increases in the middle frequencies of the EEG power spectrum of human brains. In addition, Zhou et al., reported several effective combinations of herbs using the multifactor dimensionality reduction (MDR) method, in which herbal combinations are more effective in treating insomnia than individual herbs in their previous study. In current study, RE/SE mixture also showed a stronger sleep-promotion effect than single herbal treatment. This additive effect indicates that sleep-promoting substances derived from the two herbs are multiple.

Sleep occurs via periodical cycles of two stages, NREM and REM. REM is an active type of shallow sleep, and NREM is relaxed and deeper type of sleep, in which the deepest delta wave is included. Sleep quality has been known to be determined by the amount of these two sleep types. In particular, recovery system in the body is effectively operated during delta wave stage. Our data showed RE/SE mixture effectively increase NREM, in which the increase of delta wave was obvious, but REM was not significantly affected (Fig. 3). This result indicated that RE/SE mixture improves the sleep quality by enhancing NREM and delta wave, leading to the increase of total sleep time. This RE/SE mixture-mediated beneficial effect on sleep quality and quality was observed more obviously in caffeine-induced alertness model. RE/SE mixture restored the caffeine-induced decrease of NREM and total sleep time into the normal level (Fig. 4). This result indicates that RE/SE mixture can offset the adverse effect of caffeine on the sleep quality and quality. Several studies reported the effect of natural products on caffeine-induced sleep disturbance. A recent study showed that co-administration of rice bran supplement (RBS) counteracted caffeine-induced sleep disturbances via the histaminergic system.

We also investigated the effects of the two herbal extracts (RE or SE) and the RE/SE mixture on GABA$_A$-BZD receptor binding activity. SE was shown to bind to GABA$_A$-BZD receptor more easily than RE, and binding affinity of...
RE/SE mixture was stronger than each single herb treatment (SE or RE) (Fig. 4). This result is compatible with the fact that RE/SE mixture was more effective in the increase of sleep duration and NREM than single herbal extracts (Figs. 2–4). GABA receptor has been known to play a key role in circadian rhythmic activities by controlling arousal and relaxation. 47) Many insomnia drugs such as benzodiazepine have targeted GABA receptors. 48) The sleep-promoting effects of several natural herbs also have been involved in the response to GABA receptors. 49,50) Licorice (Glycyrrhiza glabra, GG) has been shown to allosterically modulate GABA<sub>A</sub>-benzodiazepine receptors in a dose-dependent manner. 48) A recent study showed that Valerian/cascade mixture upregulates the GABA receptor in mRNA level. 51) However, RE/SE mixture did not regulate gene expression levels of GABA receptors in our experiments (data not shown). Instead, the mixture showed a direct binding activity to GABA receptor (Fig. 4). This result indicates that sleep-promoting effect of RE/SE mixture may associated with the affinity to GABA receptor in biochemical levels regardless of genetic regulation of GABA receptor. Along with GABA receptor, serotonin (5-hydroxytryptamine, 5-HT) receptor is another neurotransmitter protein that is involved in the physiology of the sleep cycle in both vertebrates and invertebrates. 52) Binding activity of RE/SE mixture on the other neurotransmitter systems including serotonin receptor would be evaluated to determine the detailed mechanism of RE/SE mixture on sleep promotion.

In conclusion, the RE/SE mixture promotes sleep quality and quantity by binding to the GABA<sub>A</sub>-BZD receptor to a greater extent than individual herbal extracts (RE or SE). This result suggests that RE/SE mixture could be used in the treatment for insomnia and sleep disorders.

Acknowledgments This research was supported by the Ministry of Trade, Industry and Energy (MOTIE) and the Korea Institute for Advancement of Technology (KIAT) through the Encouragement Program for the Industries of Economic Cooperation Region.

Conflict of Interest The authors declare no conflict of interest.

REFERENCES