

Regular Article

Effectiveness of the Sleep Enhancement by Green Romaine Lettuce (*Lactuca sativa*) in a Rodent ModelHyo-Won Kim,^a Hyung Joo Suh,^b Hyeon-Son Choi,^c Ki-Bae Hong,^d and Kyungae Jo^{*b}

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This study was conducted to investigate the effects of the extracts of green romaine lettuce (GRE) on sleep enhancement. GRE contains 1071.7 and 199.2 $\mu\text{g/g}$ of extracts of lactucin and lactucopicrin, respectively, known as sleep enhancement substances. When 100 mg/kg of GRE was administered orally, sleep latency and duration time were significantly increased compared to controls ($p < 0.05$). Rapid eye movement (REM) sleep decreased with 100 mg/kg of GRE administration and non-REM (NREM) sleep also increased. There was no significant difference between REM and NREM among the oral GRE administration groups receiving 100, 120, and 160 mg/kg GRE. In the caffeine-induced insomnia model, total sleep time was significantly increased by 100 mg/kg GRE administration compared to the caffeine-treated group ($p < 0.05$). In addition, GRE inhibited the binding of [³H]-flumazenil in a concentration-dependent manner, and affinity of both lactucin and lactucopicrin to gamma-aminobutyric acid (GABA)_A-benzodiazepine (BDZ) receptor was 80.7% and 55.9%, respectively. Finally, in the pentobarbital-induced sleep mouse model, the sleep enhancement effect of GRE was inhibited by flumazenil, an antagonist of BDZ. Thus, these results demonstrate that GRE acts via a GABAergic mechanism to promote sleep in a rodent model.

Key words *Lactuca sativa*; sleep; lactone; pentobarbital; electroencephalogram; rodent model

INTRODUCTION

Lactuca sativa (lettuce) is a type of lettuce with sturdy, crunchy, and dark green leaves. It is also known as cos or romaine lettuce in North America.¹⁾ Romaine lettuce is used as a savory salad with high fiber and low calories.²⁾ It has traditionally been used in the treatment of insomnia, anxiety, neurosis, dry coughs, and stomach problems.^{1,3)} In particular, the leaves of this plant have been known to promote anticonvulsant and sedative-hypnotic effects and the seeds have been studied in analgesic and anti-inflammatory activity in rats.^{4,5)}

Sleep is a crucial physiological state to maintain normal body function. In particular, sleep has important effects on mental and physical health, promotes brain activity in learning and memory, and supports immune function and hormonal balance.⁶⁾ Sleep proceeds in four stages that include rapid eye movement (REM) and non-rapid eye movement (NREM). REM sleep is also called paradoxical sleep because brain waves demonstrate an awake pattern. In NREM sleep, the delta wavelength is indicated and deep sleep is attained.⁷⁾ During sleep, these stages are repeated 4–5 times in a cycle and all stages are indicated by the different brain waves. Moreover, rodents show the same sleep pattern as humans; thus sleep architecture analysis through an animal model is possible.⁸⁾

Insufficient sleep is one factor causing various mental and physical problems. A lack of sleep has been known to lead to difficulties in daily life, including lethargy and memory failure, which are accompanied by traffic and work-related accidents.⁹⁾ A chronic lack of sleep is involved in the occurrence of various diseases. Illnesses such as cardiovascular

disease, diabetes, obesity, and depression are reported to be linked to insomnia, a sleep disorder.¹⁰⁾ The number of people experiencing insomnia is continually increasing worldwide; approximately one-third of the population are struggling with a sleep disorder.¹⁰⁾

The treatment of insomnia has been performed using pharmacological agents such as benzodiazepine (BDZ). However, sustained use of this drug could cause side effects, including poor job performance, drug resistance/addiction, and depression.¹¹⁾ Recently, nonpharmacologic methods using natural plants have been observed or suggested as an alternative approach with milder adverse effects. Several herbs have been reported to have a sedative or sleep-promoting capacity: valerian (*Valeriana officinalis*), hops (*Humulus lupulus*), German chamomile (*Matricaria recutita*), passionflower (*Passiflora incarnata*), and *Polygonatum sibiricum*.^{12–15)} Our previous study revealed that the mixture of *Lactuca sativa* and skullcap extracts played an active role in sleep promotion.¹⁶⁾ In this study, we focus on the sleep-promoting effect of romaine lettuce and its mechanisms by investigating antagonistic behaviors on sleep-related receptors.

MATERIALS AND METHODS

Preparation of Extract Green romaine lettuce leaves were purchased from the local market. Fresh leaves of lettuce were washed with water, dried at 60°C, milled, and stored in the refrigerator. Lettuce leaf powder (100 g) was extracted three times with 70% (v/v) ethanol (500 mL) for 4 h in a Soxhlet apparatus. The combined ethanol extracts were concentrated to 25% of total volume of the extracts using a rotary

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with ice-cold buffer. Radioligands bound to the membrane were counted by a Hidex 300SL counter (Hidex, Finland). For all receptor binding assays, the binding displacement was calculated as follows:

Binding displacement (%)

$$= [1 - (DPM_{GRE} - DPM_{NSB}) / (DPM_{TB} - DPM_{NSB})] \times 100$$

(DPM: disintegrations per minute, TB: total binding, and NSB: nonspecific binding).

Statistical Analysis Experimental results were expressed as means \pm standard error of the mean (S.E.M.). The statistical significance for one-way or two-way ANOVA, followed by *post-hoc* Tukey multiple comparison test, was set at $p < 0.05$ using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago IL, U.S.A.).

RESULTS

Analysis of Lactucin and Lactucopicrin in GRE The content of these bitter compounds was analyzed using HPLC system (Fig. 1). Lactucin and lactucopicrin contents were 1071.7 and 199.2 $\mu\text{g/g}$ of extract, respectively (data not shown). In our previous study,²⁰⁾ the extraction yield of romaine leaf was reported as 37.1%.

Effect of GRE on Sleep Latency and Total Sleep Duration in the Pentobarbital-Induced Sleep of Mice The effects of GRE on sleep latency and duration were investigated in a pentobarbital-induced model (Fig. 2). The sleep latency of pentobarbital-induced mice was not significantly different in the green romaine leaf extract-treated groups compared to the normal group (Fig. 2A). On the other hand, the mice that received a 100 mg/kg dose showed the longest sleep duration (80 min) and the increased duration showed a decreasing trend with dosage, although statistical significance was not observed (Fig. 2). However, dosages more than 100 mg/kg were still shown to be effective in the increase of sleep duration compared with normals. This result indicates that GRE at 100 mg/kg can facilitate the action of pentobarbital-inducing sleep by shortening latency, resulting in extended sleep duration.

Effects of GRE on Sleep Architecture To investigate

the effect of GRE on sleep quality and quantity, sleep architectures were analyzed using EEG for rats receiving green romaine lettuce (Fig. 3). The GRE-administration groups (100, 120, and 160 mg/kg) demonstrated significant increases in total sleep times (Fig. 3E) ($p < 0.05$). In particular, a dose of 100 mg/kg increased sleep time by approximately 14% compared with the normal group. In contrast, wake time, which decreased with GRE administration, was negatively correlated to total sleep time (Fig. 3F). In particular, GRE (100, 120, and 160 mg/kg) increased the duration of delta wave by about 2.1 times (approximately 3.6 h) compared with control group (Fig. 3B). However, the theta wave, which means relatively shallower than delta wave, showed no significant difference in GRE treatments compared to the normal group (Fig. 3C). This result showed that GRE improves the sleep quality by increasing deep sleep. Although GRE administration of 100 mg/kg or more showed similar effects on sleep time and sleep quality (Fig. 3), sleep latency time for the 100 mg/kg dosage was superior to the 120 or 160 mg/kg dosages. Therefore, GRE administration at 100 mg/kg may be effective in improving sleep. This increase in sleep time resulting from GRE was involved in a change of sleep patterns. NREM sleep time increased with GRE administration, while REM decreased (Figs. 3A, D). In particular, 120 mg/kg of GRE increased NREM by approximately 67% compared with normals, while REM sleep time decreased by 50% with the same dosage. Thus, GRE improved total sleep time and quality by increasing NREM, and increased NREM sleep was found to be due to increased delta wave time (Figs. 3A, B).

Effect of GRE on Sleep Architecture in a Caffeine-Induced Wake Model Sleep architectures of the green romaine lettuce administration in a caffeine-induced awake model are presented in Fig. 4. GRE dosages of 100 mg/kg, which is considered effective in sleep latency and sleep quality assessment, and 80 mg/kg, slightly lower, were administered orally. Caffeine led to a significant decrease in sleep time and NREM compared with the normal group; in contrast, wake time increased with caffeine administration (Fig. 4). However, GRE co-treatment reversed the effect of caffeine on sleep architecture. NREM increased with the co-treatment of GRE and caffeine, but statistical significance was not observed. Sleep time significantly increased (approximately 15%)

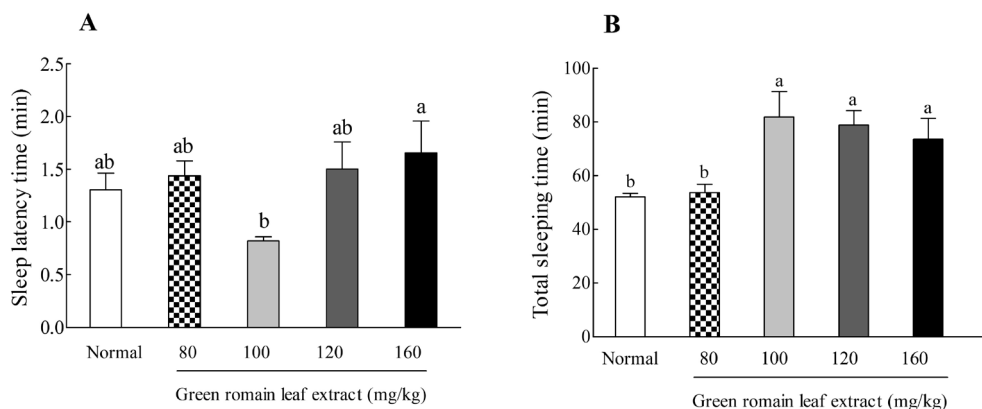


Fig. 2. Effects of Administration of Green Romaine Leaf Extracts (GRE) by Dosage on Sleep Latency Time (A) and Sleep Duration (B) in Mice Intraperitoneal Injected a Hypnotic Dosage of Pentobarbital (42 mg/kg, i.p.)

Mice received pentobarbital 40 min after oral administration of green romaine leaf extracts by dosage. The normal group was administered only 0.9% physiological saline. Each column represents the mean \pm standard error of the mean (S.E.M.) calculated from 8 mice. Different letters indicate significant differences ($p < 0.05$) among samples by one-way ANOVA followed by *post-hoc* Tukey test.

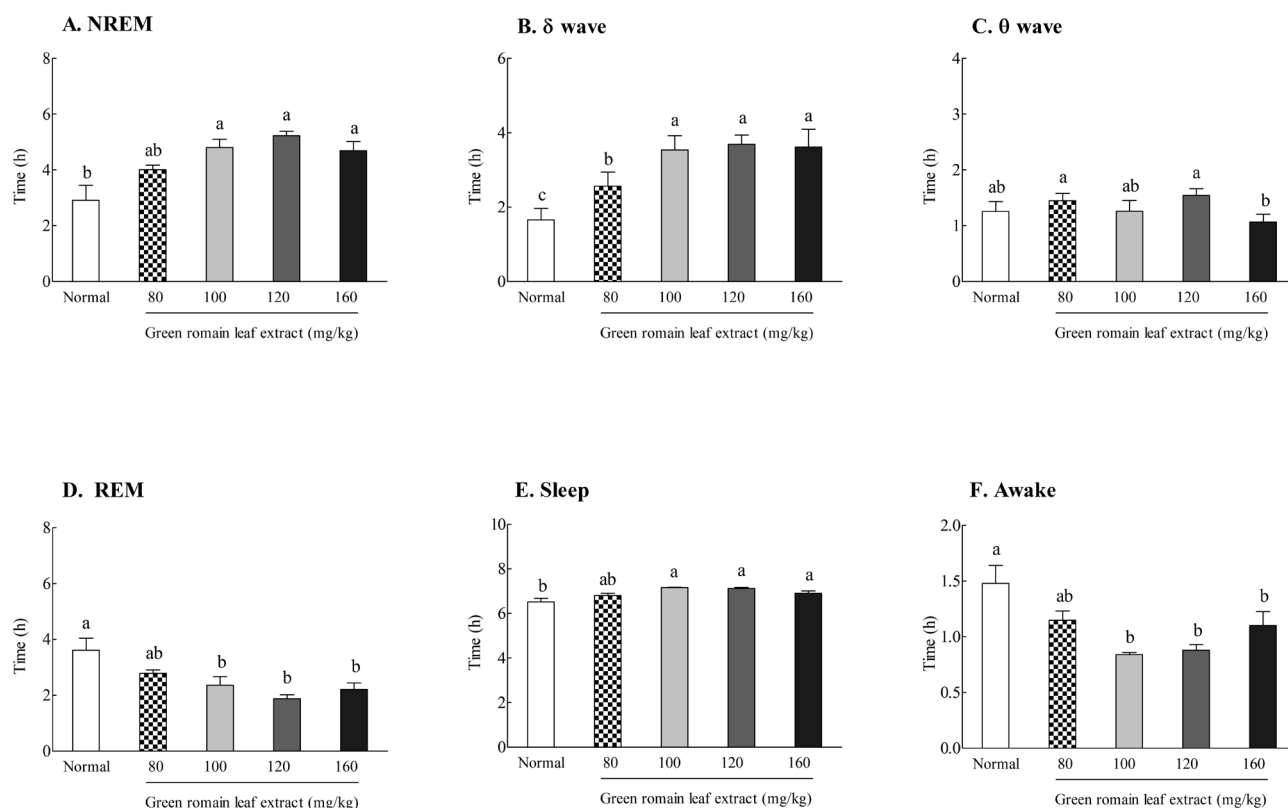


Fig. 3. Effects of Administration of Green Romaine Lettuce Leaf Extracts (GRE) by Dosage on Sleep Quality and Quantity

Values are the mean \pm S.E.M. calculated from 6 rats. The normal group was administered only 0.9% physiological saline. Different letters indicate significant differences ($p < 0.05$) among samples by one-way ANOVA followed by *post-hoc* Tukey test.

with 100 mg/kg of GRE, attaining a normal level. GRE co-treatment decreased wake time by approximately 35% compared with caffeine-only controls (Fig. 4D). However, REM was further increased with GRE co-treatment compared with caffeine-only controls.

GABA_A-Benzodiazepine Receptor-Binding Activity of GRE and Active Compounds Figure 5A shows the GRE-mediated displacement of [³H]-flumazenil binding (%) found in the present study. GRE significantly increased displacement of [³H]-flumazenil from 11.9 to 81.6% in a concentration-dependent manner ($p < 0.05$). The expected lactucin and lactucopicrin as sleep-promoting active ingredients of GRE were exhibited binding activity of 80.7% and 55.9%, respectively, which resulted on significantly higher binding activity ($p < 0.05$). These results suggest that lactucin and lactucopicrin, which are contained in GRE, bind effectively to GABA_A receptor and are an active substance that promotes sleep.

Effects of GABAergic Antagonists on GRE Mediated Sleep Behavior in a Pentobarbital-Induced Sleep Mouse Model At a hypnotic dose of pentobarbital (42 mg/kg, intraperitoneal (i.p.) injection) (Fig. 6), GRE (100 mg/kg) and BDZ significantly decreased the sleep latency, while flumazenil (GABA_A-BDZ receptor antagonist) reversed this results. Flumazenil increased GRE-mediated sleep latency by 37% compared to that in the not treated with flumazenil group (Fig. 6A). In addition, total sleeping time was significantly increased with GRE treatment in a dose-dependent manner. However, flumazenil decreased this GRE-mediated sleeping duration by 14% (Fig. 6B). This result demonstrates that flumazenil eliminates a GRE-mediated sleep promotion effect *in vivo*.

DISCUSSION

Lettuce has been known to contain hypnotic substances that induce sleep. Those components include lactucin and lactucopicrin, which impart a bitter taste.²⁰ Romaine lettuce contains a variety of flavonoids including lactucin and lactucopicrin. Previous studies have reported contents of caftaric acid, chlorogenic acid, chicoric acid, and isochlorogenic acid as 1.9, 1.2, 3.9, and 0.3 mg/g of extract, respectively.²¹ The major constituents found in lettuce species are sesquiterpene lactones, such as lactucin, lactucopicrin, and their derivatives. The sesquiterpene lactone derivatives have been reported to promote various physiological activities such as cytotoxicity, lipid lowering, and anti-inflammatory action.²² Lactucin, 8-deoxylacturin, and lactucopicrin contribute to the bitter taste of lettuce or chicory. Lactucin and lactucopicrin have also been reported as the main active sedative substances in a spontaneous locomotor activity test.²⁰

Green romaine lettuce extract (GRE) was shown to have sleep potentiation activity in a pentobarbital-induced mice sleep model (Fig. 2). Sleep-promoting effects of lettuce were reported in several studies. Ghorbani *et al.*²³ reported that *Lactuca sativum* hydro-alcoholic extract did not affect sleep latency in pentobarbital-induced mice, but did have a sleep-prolonging effect. In addition, our previous studies have confirmed the sleep-promoting effect of green lettuce and we have found that the leaves and seeds of green romaine lettuce were superior to the red lettuce leaves in a pentobarbital-induced sleep test in mice.²¹ GRE may reduce the required use of hypnotic drugs to fall asleep in individuals with sleep

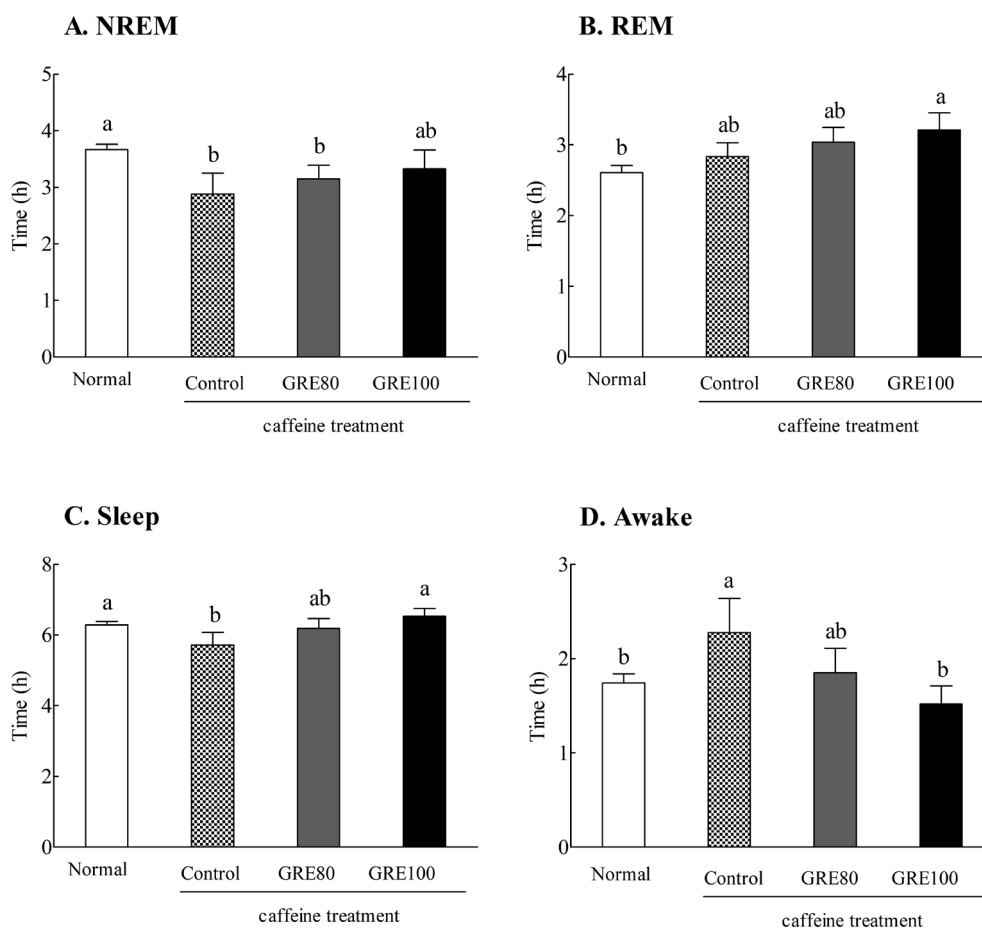


Fig. 4. Effects of Administration of Green Romaine Lettuce Leaf Extract (GRE) on Sleep Quality and Quantity in Caffeine-Induced Wakefulness Rats (Caffeine Dosage of 15 mg/kg)

Values are the mean \pm S.E.M. calculated from 6 rats. The normal group was administered only 0.9% physiological saline and the control group was treated with 15 mg/kg caffeine. Different letters indicate significant differences ($p < 0.05$) among samples by one-way ANOVA followed by *post-hoc* Tukey test.

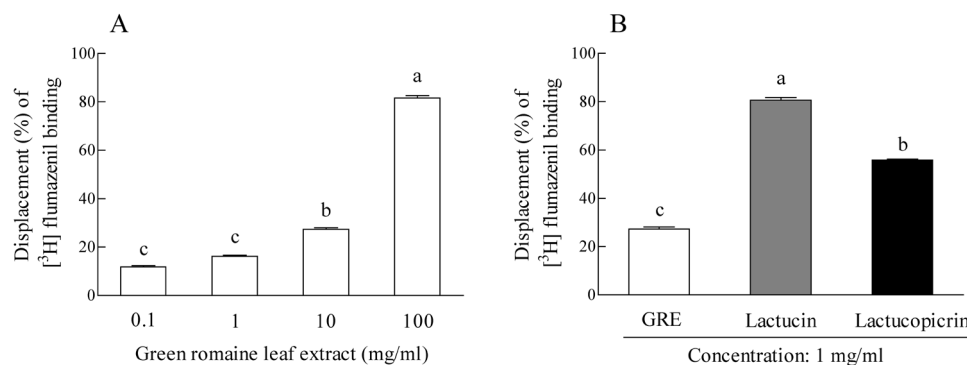


Fig. 5. Displacement of ^3H -Flumazenil Binding of Green Romaine Lettuce Leaf Extract (A), Lactucin and Lactucopicrin (B) in the GABA_A -BDZ Receptor Binding Assay

Each data point is expressed as mean \pm S.E.M. for each group, $n = 3$. Different letters indicate significant differences ($p < 0.05$) among samples by one-way ANOVA followed by *post-hoc* Tukey test.

disorders. This supportive effect of GRE on pentobarbital may contribute to ameliorating the adverse effects derived from an overdose of hypnotic drugs.

Therefore, based on the results of GRE increasing the amount of sleep, the sleep structure was investigated through EEG analysis to evaluate whether GRE could improve sleep quality. As a result, delta waves of NREM and total sleep time were increased by administration of GRE (Fig. 3). The two main sleep types are REM (rapid eye movement) and NREM

(non-rapid eye movement) sleep. REM sleep is characterized by a decrease in muscle activity and cardiac and respiratory rates, with an increase in brain activity, such as active dreams. Sleep begins with NREM sleep and the NREM-REM sleep cycle repeats 4–5 times at 90–120 min intervals per night. NREM, characterized by slow brain waves, is reported to occur in deep sleep, while REM has been described as an active form of shallow sleep.²⁴⁾ The NREM consists of three stages, the first stage is the theta wave, and is in a comfort-

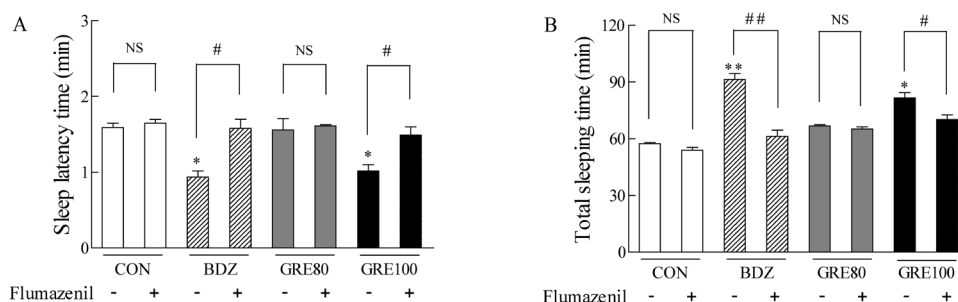


Fig. 6. Effects of Flumazenil on Sleep Latency Time (A) and Total Sleeping Time (B) in Mice Treated with Benzodiazepine (BDZ) or Green Romaine Lettuce Leaf Extract (GRE) at a Hypnotic Dosage of Pentobarbital (42 mg/kg, i.p.)

Values are presented as means \pm S.E.M. for each group. Symbols indicate significant differences according to two-way ANOVA followed by *post-hoc* Tukey test: versus the control group at * $p < 0.05$ and ** $p < 0.01$; versus the treated without flumazenil group at # $p < 0.05$ and ## $p < 0.01$. NS, not significant.

able wake state. Eye movements occur in second stages, and the third stage is characterized by slow delta waves.⁷⁾ In particular, the body is known to restore tissue during slow sleep, where delta waves are generated, and to strengthen the immune system.⁷⁾ Therefore, we have found that GRE can promote change in healthy sleep architecture.

GRE also exerted a sleep-promoting effect during caffeine-induced insomnia (Fig. 4). Caffeine is a widely used vegetable alkaloid known as a stimulant.²⁵⁾ Caffeine is a methylxanthine compound that increases alertness, induces cortical activation, and reduces fatigue. It blocks the action of the adenosine receptors in the basal forebrain, inducing arousal and reducing slow-wave sleep activity in the cortex.²⁶⁾ Thus, caffeine administration has been used to induce insomnia models in rats.^{27,28)} Our data show that GRE can recover sleep behaviors that are negatively regulated by caffeine (Fig. 4). GRE may alleviate adverse effects of caffeine derived from coffee beverages and drugs that disturb normal sleep.

The ability to modulate GABA_A receptors has been studied to explore molecular mechanisms underlying these traditional sleep remedies. GABA_A receptors are a viable target when searching for natural anxiolytic or sedative components from plants. Research has indicated that many herbal extracts are ligands of the GABA_A receptors in the central nervous system (CNS) and bind to the benzodiazepine binding site, resulting in sleep modulating actions.^{15,29)} Flumazenil, known as an antagonist of benzodiazepine, is a water-soluble imidazobenzodiazepine that acts antagonistically with benzodiazepine in the gamma-amino-butyric acid type A (GABA_A) receptors in the CNS.³⁰⁾ Therefore, by treating with the ³H-labeled flumazenil, we investigated whether GABA_A-BDZ receptor response is involved in a GRE-mediated sleep promoting effects, and as a result demonstrate that GRE effectively binds to GABA_A receptors to promote sleep time (Fig. 5A). Green romaine lettuce is expected to contain various active compounds that can affect sleep modulating actions. In particular, lactucin showed a high binding affinity (80.7 \pm 2.3%) of the GABA_A-BDZ receptor, and lactucopicrin also binds to the GABA_A-BDZ receptor (55.9 \pm 0.7%). These results suggest that lactucin and lactucopicrin contained in GRE promotes sleep *via* GABAergic mechanism (Fig. 5B). In addition, in order to confirm this mechanism *in vivo*, the effect of flumazenil on the sleep activity of GRE in the pentobarbital-induced sleep model was evaluated. As a result, the sleep enhancement of GRE was completely inhibited by the flumazenil, a specific antagonist of GABA_A-BDZ receptor (Fig. 6). Therefore, in future study, iso-

lation of active compounds from green romaine lettuce should provide important information such as the mode of action of romaine lettuce.

Therefore, the results of this study showed that green romaine lettuce contained 1071.7 and 199.2 μ g/g of lactucin and lactucopicrin extract, respectively. GRE effectively reduced sleep latency and increased sleep time in a pentobarbital-induced sleep model. GRE, in particular, increased sleep time by enhancing NREM in sleep architecture. This GRE-mediated increase of NREM and sleep time was also observed in a caffeine-induced wakefulness model. In a receptor binding assay, the interaction of GRE with GABA receptors was indicated as the primary mechanism by which these sleep-promoting effects occur in rodent models. Taken together, our findings describe that lactucin and lactucopicrin from green romaine lettuce extract are attributed to the quality and quantity of sleep and this result suggests that green romaine leaf extract could be used as sleeping agent.

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Conflict of Interest The authors declare no conflict of interest.

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