Effect of Valerian/Hop Mixture on Sleep-Related Behaviors in Drosophila melanogaster

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The aim of this study was to investigate the sleep-promoting effect of a Valerian/Hops mixture in fruit flies. The HPLC analysis showed that Valerenic acid (1260.53 µg/g of extract) and Xanthohumol (Cascade: 827.49 µg/g, Hallertau: 763.60 µg/g, Saaz: 186.93 µg/g) were contained in Valerian and Hop, respectively. The sleep patterns of fruit flies on the Valerian/Hops were examined in both baseline and caffeine-treated conditions. Total activities of flies significantly decreased in 20 mg/mL Valerian (74%), 10 mg/mL Cascade (25%), and 5 mg/mL Hallertau (11%) during nighttime or daytime compared with the control. Valerian/Cascade mixture showed longer sleeping time (ca. 20%) than control group. This mixture-mediated effect was partly observed in caffeine-treated flies. Valerian/Cascade mixture upregulated mRNA expressions of gamma-aminobutyric acid (GABA) receptors and serotonin receptor, and GABA receptors were more strongly regulated than serotonin receptor. In competitive GABA receptor binding assay, Valerian/Cascade mixture extract showed a higher binding ability on GABA receptor than Valerenic acid or/and Xanthohumol which are estimated to be active compounds in the extract. This study demonstrates that a Valerian/Cascade mixture extract improves sleep-related behaviors, including sleeping time, by modulating GABAergic/serotonergic signaling.

Key words insomnia; sleep-promotion; Valerian; Hop; Drosophila melanogaster

Sleep, occupying one-third of human life, is one of the most important natural states to maintain good health and wellbeing in life.1) Sleep disorders cause noticeable impairments in daytime function or behavior and are accompanied by various problems, such as tiredness, memory problems, accident proneness, lethargy, and other physical or mental impairments.2) It frequently causes clinical problems regardless of gender and age.3) Sleep disorder patients are prone to depression, anxiety and alcohol dependence.4) Insomnia, one sleep disorder, is characterized by difficulty falling and/or staying asleep despite a sufficient opportunity to sleep.5) Adult disease such as cardiovascular disease and obesity was shown to be implicated to insomnia.6) Management of insomnia has usually been achieved through treatment with pharmacological agents, including benzodiazepine, antidepressants, barbiturates, and anti-psychotics.7) However, these medications have shown negative side effects, such as daytime sedation, hangover, and drug dependence.8) Accordingly, many people seek solutions to sleep disorders via natural substances and dietary supplements.7)

Several herbal mixtures, such as Valerian (Valeriana officinalis), Hops (Humulus lupulus L.), chamomile (Matricaria chamomilla), and passion flower (Passiflora), have been known to be effective in sleep disorders and insomnia.8–10) In particular, the root of Valerian extract is traditionally used to treat sleep disorders, anxiety, and nervous afflications in Europe and U.S.A.11) Hops have been known to have sedative effects on activities.12,13) Various kinds of Hops including Cascade, Hallertau, Saaz, Northern Brewer, Sterling, Vanguard and Willamette14) have been known to have different aromatic components. The gamma-aminobutyric acid (GABA) receptors are a kind of receptor that responds to a neurotransmitter called GABA, the major inhibitory compound in the central and peripheral nervous systems (C/PNS).15) GABA receptors have been known to affect cognition, including sleep and wakefulness, by coordinating with glutamatergic processes.16) These receptors are divided into two classes, GABA_A and GABA_B, according to their rate of response to GABA.15) GABA_A receptors have been widely studied as a target site for CNS sedative herbs and many pharmacological medicines.5) Drosophila melanogaster, the fruit fly, has been used as an invertebrate model in many studies. Invertebrates such as D. melanogaster share a similar sleep regulatory mechanism associated with a circadian rest–activity cycle and homeostatic processes with vertebrate systems.7) In particular, sleep patterns of D. melanogaster, unlike vertebrates, are evaluated by multiple factors such as behavior, activity, and electrophysiology,1,18) but fly sleep is modulated by some stimulants and hypnotics that also affect human sleep.3)

In this study, we evaluated the sleep-promoting effect of a Valerian/Hops mixture using fruit flies. Although several studies have been performed on the sleep-related effects of Valerian and Hops,19,20) systematic study of the sleep-promoting effects of Valerian and/or Hops has been limited. The current study describes the combinational synergetic effect of Valerian and Hops via analysis of several sleep episodes in a Drosophila model, which has never been studied.
MATERIALS AND METHODS

Materials  Valerian root (*Valeriana officinalis*) was purchased from Frontier Co., Ltd. (CA, U.S.A.) and three types of Hops were gifted from Hongcheon Institute of Medicinal Herb (Hongcheon, Korea). Valerian root and Hops were transported to the laboratory in coolers. For HPLC, all solvents used were of HPLC-grade and purchased from Caledon (Caledon, Canada). Xanthohumol and Valerenic acid were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). All other chemicals and reagents were of the highest grade available. Analytic chemical compounds studied in this article are Valerenic acid (PubChem ID 24873924); Xanthohumol (PubChem ID 24902121).

Preparation of Extracts  Valerian roots 40 g were extracted with 1600 mL of 70% ethanol in room temperature by stirring 48 h. Hops 40 g were extracted with 800 mL of 70% ethanol with a Soxhlet apparatus for 3 h. Then, all extracts were filtered by filter paper and evaporated at 40°C using a rotary vacuum evaporator. Valerian and Hops extraction sample were freeze-dried and stored at 4°C.

Fly Stocks  Wild-type *D. melanogaster* Canton-S strain flies were obtained from the Bloomingdon Drosophila Stock Center at Indiana University. The flies were maintained in standard fly bottles containing sucrose medium (sucrose, cornmeal, dried yeast, agar, propionic acid, and *p*-hydroxybenzoic acid methyl ester solution) and raised under a 12:12 h light:dark cycle at 25±1°C in 60% relative humidity (RH). Valerian and/or Hops samples were added to sucrose medium with the indicated concentrations. Prior to sample treatment, 2–5-d-old male flies were collected under anesthesia using CO₂.

HPLC Analysis of Valerenic Acid and Xanthohumol  Valerenic acid from Valerian was determined using previously described HPLC methods.²¹) Agilent HPLC series 1100 (Phenomenex, Torrance, CA, U.S.A.) was used and the Agilent series 1102 Vol. 40, No. 7 (2017) detector (DAD), was used to detect Valerenic acid at 218 nm. The flow rate was adjusted to 1 mL/min. Separation was performed over 20 min with a flow rate of 1.5 mL/min. The analytical column was a Luna C18 (50×4.6 mm, 5 µm reversed phase column maintained at 25°C (Phenomenex, Torrance, CA, U.S.A.).

Xanthohumol from Hop varieties was analyzed using previously described methods.²²) Briefly, a Luna C18 column (4.6×150 mm, 5 µm, Phenomenex, Torrance) was used and the mobile phases consisted in solvent A (0.025% trifluoroacetic acid (TFA) in water), and solvent B (0.025% TFA in acetonitrile). The flow rate was adjusted to 1 mL/min. Separation was performed using gradient elution 25% A and 75% B to 25 min, 5% A and 95% B to 35 min, 65% A and 35% B to 50 min. Detection was performed by UV absorption at 372 nm.

Behavioral Assays  Valerian and Hops were dissolved in distilled water and mixed in sucrose-agar media (5% sucrose and 1% agar) for the locomotor activity assays. Single treatments of Valerian included 2, 5, 10, and 20 mg/mL concentrations. Single treatments of three types of Hops (Cascade, Hallertau, Saaz) included 2, 5, and 10 mg/mL concentrations. In the Drosophila Activity Monitoring system (DAM; TriKinetics, Waltham, MA, U.S.A.), flies were kept in individual glass tubes for analysis of behavior patterns in each fly. Group activity of flies for single treatment and Valerian/Hops mixture groups was assessed by the Locomotor Activity Monitoring system (LAM, TriKinetics) to provide measures of locomotor activity combined with social behaviors. All the experiments were triplicated (LAM: 10 flies per replicate, DAM: 30 flies per replicate). The Valerian/Hops mixture was composed of Valerian (20 µg/mL) and Hops (10 µg/mL) in sucrose-agar media. Flies were subjected to a 24 h adaptation period in the tubes, and all activities were then recorded every 1 or 30 min for 4–7 d under constant darkness (DD) at 25±1°C. A 0.1% caffeine solution (10 mg/mL) was used as a stimulant in the awake condition.²³) Data were generated by DAM management software (TriKinetics) with controls for environmental stimuli, such as sound and light. The number of infrared detector interruptions at each time interval was recorded and visualized using Actogram J software. Sleep analyses were performed during dark hours of the daily cycle in the control group and caffeine-induced awake groups and compared. Sleep parameters were calculated by summing up all the activity counts recorded in the 12 h dark period (nighttime). Dark phase activity was calculated by summing total activity, and total dark phase sleep was calculated by summing the duration of sleep. Sleep was defined as periods of uninterrupted behavioral immobility and inactivity longer than 5 min (0 counts per min).²⁴) In addition, the number of sleep episodes were counted and summed.²⁵)

**Real-Time PCR**  Total RNA was extracted from the heads of 17–20-d-old flies using TRIzol® reagent (Invitrogen, CA, U.S.A.) while genomic DNA was removed using Direct-zol™ RNA Miniprep (ZYMO Research, CA, U.S.A.) according to the manufacturer’s protocol. All the experiments were triplicated (50 flies per replicate). One microgram of total RNA was reverse transcribed using SuperScript™ III Reverse Transcriptase (Invitrogen) with oligo (dT) as the primer. Real-time quantitative PCR (qRT-PCR) was performed on the resulting cDNA using the Power Taqman PCR Master Mix kit (Applied Biosystems, CA, U.S.A.). Quantitative analyses were conducted using StepOne plus Software V. 2.0 (Applied Biosystems), and results were normalized to a validated control gene, Rpl32 (NM_001144655.3), using the ΔΔct method.²⁶) Information about the target genes used in qRT-PCR is as follows: GABA<sub>A</sub>-R Rdl (NM_001274688.1), GABA<sub>B</sub>-R1 (NM_001259104.1), GABA<sub>B</sub>-R2 (NM_079714.2), and 5-hydroxytryptamine (5-HT)1A (NM_166322.2).

**GABA<sub>A</sub>-Benzodiazepine Receptor Binding Assay**  The GABA<sub>A</sub> receptor binding assay was performed with modification according to the method described by Risa et al.²⁷) and Kahnberg et al.²⁷) The cerebral cortex of male four Sprague–Dawley rats was homogenized for 10 s in 20 mL of Tris–HCl buffer (30 mM, pH 7.4, 0–4°C). The suspension was centrifuged at 0–4°C for 15 min at 27000×g, and the pellet was washed three times with Tris–HCl buffer. The washed pellet was resuspended in 20 mL of Tris–HCl buffer, after which the suspension was incubated in a water bath at 37°C for 30 min, followed by centrifugation at 10 min at 27000×g. The final pellet was suspended in 30 mL of Tris–HCl buffer (50 mM, pH 7.4) and stored in aliquots at −80°C until assayed. The final suspension (membrane suspension) was adjusted with the concentration of 33.3 µg protein in 100 µL Tris-citrate buffer (50 mM, pH 7.1, 0–4°C) to be used for the binding assay. The membrane suspension (300 µL) was added to 25 µL.
test solution and 21 µL of [³H]-flumazenil and incubated on ice for 40 min for the binding, in which its final concentration is 0.8 nM. Samples used to GABAₐ receptor binding assay were Xanthohumol (170.1 µg/mL), Valerenic acid (150.9 µg/mL), Valerenic acid/Xanthohumol mixture, and Valerian/Cascade extract (200 mg/mL) extract. The tested Xanthohumol and Valerenic acid was equivalent to amount in Valerain/Cascade extract (200 mg/mL) extract. The binding was terminated by filtration onto a Whatman GF/B glass fiber filter using a harvester (Brandel Inc., Gaithersburg, MD, U.S.A.) with ice-cold 30 mM Tris–HCl buffer to remove unbound [³H]-flumazenil. The bound samples were counted with 5 mL of liquid scintillation cocktail solution (Aqualight Beta, Hidex Personal Life Science, Turku, Finland) in bottle using a Hidex 300SL counter (Hidex, Turku, Finland). Total and nonspecific bindings were determined using the binding buffer and benzodiazepine (1 µM, final concentration), respectively. The displacement percent of radioligand binding was determined by the following equation (DPM: disintegrations per minute, TB: total binding, and NSB: nonspecific binding).

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\text{Binding displacement (\%)} = \left[1 - \frac{(\text{DPM}_{\text{extr}} - \text{DPM}_{\text{NSB}})}{(\text{DPM}_{\text{TB}} - \text{DPM}_{\text{NSB}})}\right] \times 100
\]

Statistical Analyses All statistical analyses were performed using the Statistical Package for Social Sciences version 12.0 (SPSS Inc., Chicago, IL, U.S.A.). Differences between groups were evaluated by one-way ANOVA and Tukey’s multiple comparison tests. Statistic values of \(p<0.05\) were considered significant. All data are reported as means±standard error of the means (S.E.M.). Student’s \(t\)-tests were also used to analyze differences.

RESULTS

HPLC Analysis for Active Compounds from Valerian and Hops HPLC separation for Valerenic acid and Xanthohumol was shown in Fig. 1. Valerenic acid and Xanthohumol were detected at 10.7 and 18 min, respectively (Fig. 1). Table 1 showed the contents of these compounds from Valerian and Hop varieties. Valerenic acid content of Valerian was 1260.53 µg/g of extract. Xanthohumol contents of Hop

![HPLC Chromatograms of Valerenic Acid (A) and Xanthohumol (B) from Valerian and Hop Varieties](image-url)

**Table 1. Content of Valerenic Acid and Xanthohumol from Valerian and Hop Varieties**

| Compound       | Valerian (µg/g) | Compound       | Hop (µg/g) |
|----------------|----------------|----------------|-------------|
|                |                | Cascade        | Hallertau   | Saaz        |
| Valerenic acid | 1260.53±50.47  |                |             |             |
| Xanthohumol    | 827.49±73.79   | 763.60±56.23   | 186.93±30.38 |

0.05}

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varieties were 186.93 µg/g of extract in Saaz, 763.60 µg/g of extract in Hallertau, 827.49 µg/g of extract in Cascade, respectively. The Cascade Hop had the highest Xanthohumol content among three Hop varieties, and Saaz Hop has the lowest levels of Xanthohumol.

Effects of Valerian or Hops on Locomotor Activity

Actograms were used to visualize the effects of Valerian or Hops on locomotor activity (Fig. 2A). For Valerian extract, locomotor activity decreased during all phases in a dose-dependent manner, and the activity in the 20 µg/mL Valerian group was greatly reduced, showing a calm state with the decreased black areas; the activities during both night and daytime were significantly decreased by 54 and 78%, respectively, by 20 µg/mL Valerian treatment (p < 0.05) (Figs. 2B, C). In addition, lower doses of Valerian (5, 10 µg/mL) produced a significant decrease in locomotor activity compared to the control group during daytime. This result indicates that Valerian effectively displayed a sedative function that favored sleep promotion. Among tested Hops, Cascade extract produced a dose-dependent significant decrease in movement activity during nighttime; 10 µg/mL Cascade showed a decrease of activity by 25% compared to the control group (Fig. 3D). During daytime, 5 µg/mL Hallertau only showed a small reduction in activity, but the higher dose (10 µg/mL) did not have any effect in decreasing activity (Fig. 3E). This result showed that Cascade was the only effective Hop showing sedative effects in fruit flies. Accordingly, Valerian and Cascade were used as mixture samples in subsequent experiments.

Effect of Valerian/Cascade Mixture on Sleep Behavior
The effects of Valerian, Cascade, and a Valerian/Cascade mixture on dark phase activity, number of sleep bouts, and total dark phase sleep were examined (Fig. 4). With the DAM system for the individual sleep behavior, total movement of flies seemed to decrease in the Valerian- and Valerian/Cascade mixture-treated groups, although it was not statistically significant (Fig. 4A). Valerian- or Cascade-treated groups and the Valerian/Cascade mixture group showed significantly higher levels of sleep bouts, which are an interruption of sleep, compared to the control (Fig. 4B). The mixture extract showed lower sleep bouts than single extract, but still higher than normal control. For total nighttime sleep duration, Valerian single treatment seems to increase sleep time compared to the control, but statistical significance was not observed (Fig. 4C). Cascade or mixture treatment also did not show the significant difference in total nighttime sleep compared to the control. In the LAM system including social behavioral activity, movement activity in the Valerian/Cascade mixture group appeared to decrease, although it was not statistically significant (Fig. 4D). On the other hand, Valerian/Cascade mixture-treated flies demonstrated a significant increase in total nighttime sleep.

Fig. 2. Effects of Valerian on Locomotor Activity in Fruit Flies

This experiment was performed under constant darkness (DD) for 5d (3d: adaptation with normal diet, 5d: experiment with treatments of Valerian extract in sucrose agar media). (A) Typical actograms of individual control flies (n=20) and flies exposed to Valerian (n=16) by dose. Average activity in a 30 min interval was calculated over 5d. Black/white bars on top of the actograms indicate dark (22:00 to 10:00) and light (10:00 to 22:00) phases. (B) Activity during dark phases and (C) activity during light phases. Values indicate the mean±S.E.M. for each group. Symbols indicate statistically significant differences versus Control (*p<0.05, **p<0.01).
sleep (Fig. 4E). This result showed that the Valerian/Cascade mixture had a sleep-promoting effect in social behavioral condition.

**Effect of a Valerian/Cascade Mixture on Sleep Behavior in a Caffeine-Induced Awake Model**

Dark phase activity and sleep bouts in the caffeine-fed group significantly increased compared to the control group while total sleep time showed a significant decrease (Figs. 5A–C). Administration of the Valerian/Cascade mixture showed a marked reduction in total movement activity (Fig. 5A). Number of sleep bouts also significantly decreased with the mixture compared to the caffeine-treated group (Fig. 5B). Meanwhile, total sleep time of the mixture group in the dark phase was significantly increased compared to the caffeine only group (Fig. 5C). In LAM system, total movement of the Valerian/Cascade mixture flies was similar level to normal group, which is slightly lower than caffeine only group (Fig. 5D). In addition, dark phase sleep time of the mixture group exhibited a significant increase compared with caffeine only-exposed group (Fig. 5E). Collectively, this result showed that the Valerian/Cascade mixture was effective in sleep promotion in caffeine induced-awake model.

**Effects of Valerian/Cascade Mixture on mRNA Levels of Neurotransmitter Signalings**

Transcript levels of Resistant to dieldrin (Rdl), which is a *Drosophila* GABA<sub>A</sub> receptor, in Valerian, Cascade, and Valerian/Cascade mixture groups were significantly increased compared to the control group (Fig. 6A). The Valerian/Cascade mixture increased Rdl mRNA expression by over 50% compared to control group (Fig. 6A). In addition, mRNA levels of GABA<sub>B</sub> receptor 1 (GABA<sub>B</sub>-R1) also showed a significant increase in the Valerian/Cascade mixture group (Fig. 6B). However, the mRNA levels of these receptors in the single treatment groups decreased or remained similar to controls. Moreover, for GABA<sub>B</sub> receptor 2 (GABA<sub>B</sub>-R2) and 5-hydroxytryptamine receptor 1A (5-HT1A) mRNA expressions, the Valerian/Cascade mixture group expressed a higher level than the control group, but significance was not observed (Figs. 6C, D).

**Binding Effect of Valerian/Cascade Extract on GABA<sub>A</sub>-Benzodiazepine Receptor**

The binding capacity on the GABA<sub>A</sub>-benzodiazepine (BDZ) receptor was measured in order to determine whether the sleep activity of Valerian and Cascade extract was directly associated with GABA<sub>A</sub>-benzodiazepine receptor. The mixture extract showed binding activity of 90%, proving that sleep promoting effect of the extract is resulted from the binding to GABA<sub>A</sub>-BDZ receptor (Table 1).
Fig. 4. Effects of Valerian, Cascade and Valerian/Cascade Mixture on Sleep Behavior in Fruit Flies

This experiment was performed under constant darkness (DD) for 8 d (3 d: adaptation, 5 d: experiment). (A) Dark phase activity, (B) number of sleep bouts, and (C) duration of dark phase sleep of the control group (sucrose-agar media group), 20 mg/mL Valerian, 10 mg/mL Cascade, and the Valerian/Cascade mixture treatment group using the Drosophila Activity Monitoring (DAM) system. (D) Dark phase activity and (E) amount of dark phase sleep of the control group (sucrose-agar media group), 20 mg/mL Valerian, 10 mg/mL Cascade, and Valerian/Cascade mixture (20, 10 mg/mL) treatment group using the Locomotor Activity Monitoring (LAM) system. Values represent the means±S.E.M. for each group. Different letters indicate significant differences.

Fig. 5. Effect of Valerian/Cascade Mixture on Caffeine-Induced Wakefulness in Fruit Flies

This experiment was performed under constant darkness (DD) for 4 d (3 d: adaptation, 4 d: experiment). (A) Dark phase activity, (B) number of sleep bouts, and (C) amount of dark phase sleep of the control group (sucrose-agar media group) 20 mg/mL Valerian, 10 mg/mL Cascade, and the Valerian/Cascade mixture (20, 10 mg/mL) with the 10 mg/mL caffeine treatment group using the Drosophila Activity Monitoring (DAM) system. (D) Dark phase activity and (E) amount of dark phase sleep of the control group (sucrose-agar media group), 20 mg/mL Valerian, 10 mg/mL Cascade, and the Valerian/Cascade mixture with 10 mg/mL caffeine treatment group using the Locomotor Activity Monitor (LAM) system. Values represent the means±S.E.M. for each group. Different letters indicate significant differences.
In order to determine whether the effect of the extract was derived from Valerenic acid and Xanthohumol, each compound and its mixture were examined on the binding capacity. Valerenic acid and Xanthohumol showed binding capacity of 43.9 and 24.0% on GABA_A-benzodiazepine receptor, respectively, which is lower capacity than the extract. The mixture of Valerenic acid and Xanthohumol showed slightly higher binding capacity (57.8%) compared to single use of Valerenic acid or Xanthohumol, but its capacity was still lower level than the extract.

**DISCUSSION**

Insomnia is prevalent among people; over 30% of the world’s population have symptoms of insomnia, and 10% of the people are known to have severe chronic insomnia. Many studies have shown that insomnia causes not only risks of mental or physical health but also poor quality of life, increased occupational errors, and increased industrial accidents. Although pharmaceutical treatments have been suggested, the demand on natural product-targeting study has been increased owing to drug-mediated side effects. This study showed the potential of a Valerian and Cascade mixture as a sleep-promoting agent in a *Drosophila* model. Valerian (*Valeriana officinalis*) is recognized for its sedative and soothing medicinal properties and is frequently used to ease symptoms of insomnia. Recent studies showed that Valerian extract influences sleep through various factors in rats and humans, but its efficacy on sleep has not been demonstrated in a *Drosophila* model. Hop, the flower of Hop plant *Humulus lupulus*, has also been used as a dietary supplement for mood and sleep disturbances. Several studies reported the sedative effects of Hops, but a systematic assessment on sleep has rarely been done. In current study, sedative effect of Valerian and Cascade (a kind of Hop) on the locomotor activity was identified (Figs. 2, 3), but sleep enhancing effect of each extract was not observed as a significant increase compared to the normal control in DAM and LAM system, respectively (Figs. 4C, E). The mixture of both extracts was shown to have a significant sleep-promoting effect in normal condition of *Drosophila* in LAM system (Fig. 4E). These results showed that the mixture has combinational or synergistic effect on...
the sleep promotion, and previous reported sedative effects of both plants\textsuperscript{19,20} can be connected to the sleep-promoting effect by mixture of two extracts. However, the mixture-mediated increase of sleep time was only observed in LAM system, not DAM system (Figs. 4C, E). Therefore, more populations need to be analyzed for minute examination of the sleep capacity in DAM system.

Caffeine is the most widely used material to induce physical wakefulness. Caffeine functions as an antagonist for the adenosine 2A subunit.\textsuperscript{31} Moreover, caffeine affects arousal by modulating protein kinase A (PKA) and cAMP in flies.\textsuperscript{32} Thus, caffeine is an appropriate method to enhance arousal in a \textit{Drosophila} model. The Valerian/Cascade mixture extract treatment exhibited less movement activity and longer sleep time than caffeine-only treated group in the caffeine-induced awake condition (Figs. 5A, C). Unlike normal condition, sleep-promoting effect of the mixture was observed in both DAM and LAM systems (Figs. 5C, E). Thus, this result indicated that Valerian/Cascade mixture extract can play more important role in sleep promotion on caffeine-induced awake conditions than normal condition.

A recent study showed the possibility of Valerian extract in the treatment of insomnia patients,\textsuperscript{33} supporting our data. Although the main sleep-promoting compound in Valerian remains elusive, it has been suggested that Valerenic acid, Valepotriates, and their derivatives contribute to the sedative effect.\textsuperscript{34} Among them, Valerenic acid has been believed to be one of the major quantitative compounds for hypnotic effect of Valerian.\textsuperscript{35} In particular, it has been known to bind directly to GABA\textsubscript{A} receptors that favor sleep.\textsuperscript{35} A recent study also reported that Valerian-mediated anxiolytic effect is originated from the Valeronic acid.\textsuperscript{36} Our HPLC analysis showed Valerenic acid contained in Valerian extract with 1260.53 µg/g. Based on these studies, Valerenic acid from the mixture is expected to partially contribute to sleep-promoting effect of Valerian/Hop mixture. In addition, a sedative effect of Hops has been shown to be due to Xanthohumol.\textsuperscript{37} Our data showed that Cascade contained the highest content of Xanthohumol (827.49 µg/g, Table 1), and 10 mg/mL Cascade treatment produced a significant decline in locomotor activity during subjective nighttime (Figs. 3A, D), while Saaz, another Hop, has the lowest Xanthohumol level (Table 1), and has no any sedative effect (Figs. 3C, D). In particular, Xanthohumol was also shown to bind to GABA\textsubscript{A} receptor,\textsuperscript{38} of which activation inhibits central nervous system, providing sedative effect.

GABA plays an important role in many rhythmic activities by modulating arousal and relaxation. Thus, many insomnia medications, including benzodiazepine, have targeted GABA receptors.\textsuperscript{39} Several natural herbs have been known to regulate sleep via GABA receptors.\textsuperscript{35,39,40} Licorice (\textit{Glycyrrhiza glabra}, GG) has been a frequently used herb for insomnia both in western and eastern countries for a long time.\textsuperscript{39} The sedation-hypnotic effect of GG extract was evaluated by pentobarbital-induced sleep tests in mice, and GG has been shown to allosterically modulate GABA\textsubscript{A}-benzodiazepine receptors in a dose-dependent manner.\textsuperscript{39} Along with GABAergic neurons, serotonin (5-hydroxytryptamine, 5-HT) is another neurotransmitter that is involved in the physiology of the sleep cycle in both vertebrates and invertebrates.\textsuperscript{41} A previous study showed that serotonin could modulate sleep/wakefulness in \textit{Drosophila}.\textsuperscript{42} Our data showed that a Valerian/Cascade mixture upregulates both the GABA receptor and serotonin receptor (Fig. 6). Particularly, this mixture was shown to regulate GABA receptors more strongly than serotonin receptors (Fig. 6).

Although Valerenic acid and Xanthohumol were expected to be major principles for the sleep promoting effect of Valerian/Cascade mixture extract, the analysis of each compounds on the competitive GABA-BDZ receptor binding assay did not show that both compounds are major active principles for the effect of the mixture (Table 2). Binding activity of each compound or combination on GABA receptor-BDZ is much lower than that of the extract (Table 2). This result indicated that there are another active components besides Valerenic acid and Xanthohumol in Valerian and Cascade, which can bind to GABA\textsubscript{A}-BDZ receptor to promote sleeping. However, based on the binding activity of Valerenic acid and Xanthohumol on the GABA\textsubscript{A}-BDZ receptor, both compounds were shown to partly contribute to the sleeping activity of Valerian and Cascade extract. In addition, the mixture of both compounds did not show the proportional increase of binding activity even if the activity is slightly higher than that of each compound (Table 2). This data indicated that Valerenic acid and Xanthohumol may share the binding sites of GABA-BDZ receptor. Therefore, the studies on the other compounds besides Valerenic acid and Xanthohumol would be performed on the sleep promotion or GABA-BDZ receptor binding capacity in the future. Many constituents have been isolated and reported from Valerian.\textsuperscript{43} Compounds such as Valerenal, Valerenol, and Hydroxyl valerenic acid, which were known to have sedative effect, are worthy of analysis for the sleep promotion effect. In case of Hop including Cascade, several compounds such as β-hitter acid, linalool, and geraniol were shown to have potential for the sleep promoting effect.\textsuperscript{44} Current study describes the effect of the Valerian-Hop mixture extract on the locomotor activity and sleep durations to determine sleep promoting capacity of the Valerian/Cascade mixture extract. However, its effect on sleep patterns including rapid eye movement (REM) and non-rapid eye movement (NREM), and the effect on sleep induction were not handled in this study. Since these sleep-related data would tell more details including effects of the Valerian/Cascade mixture extract on the quality of sleep, studies using mammalian models would be performed in the next.

Our results demonstrated that a Valerian/Cascade mixture extract promotes sleeping with the reduction of dark phase activity compared to the control condition or single administration of the extracts in fruit fly model. This sleep-promoting effect of the mixture occurred via regulation of neuromodulator signaling components such as Rdl, GABA\textsubscript{A} receptors, and 5-HT1A receptors. Valerenic acid and Xanthohumol in the mixture extract were shown to contribute to the sleep promoting effect of the mixture extract, but the other constituents from the extracts were recognized to play an additive role in enhancing the sleeping.

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Conflict of Interest  The authors declare no conflict of interest.

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