Studies on hypnotic effects and GABAergic mechanism of kajime (*Ecklonia cava*) and licorice (*Glycyrrhiza glabra*)

(カジメと甘草の睡眠誘導効果とその作用機構に関する研究)

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CONTENTS

ABBREVIATIONS

ACSF: artificial cerebrospinal fluid

BBB: blood-brain barrier

BECK: 6,6′-bieckol

BT: *n*-butanol

BZD: benzodiazepine

CMC: carboxymethyl cellulose

CNS: central nervous system

CC: column chromatography

CON: control group

DECK: dieckol

DR: dorsal raphe

DV: dependent variable

DZP: diazepam

EA: ethyl acetate

EC: *Ecklonia cava* (kajime)

ECE: *Ecklonia cava* ethanol extract

ECEZ: *Ecklonia cava* enzymatic extract

ECM: *Ecklonia cava* methanol extract

ECK: eckol

EEG: electroencephalogram

EK: *Ecklonia kurome*

EKM: *Ecklonia kurome* methanol extract

EMG: electromyogram

ETN: Eckstolonol

FDRG: fucodiphlorethol G

FFT: fast Fourier transform

FLU: flumazenil

FRF: flavonoid-rich fraction

GABA: γ-aminobutyric acid

GBR: glabrol

GG: *Glycyrrhiza glabra*

GGE: *Glycyrrhiza glabra* ethanol extract

GRAS: generally recognized as safe

HX: *n*-hexane

IC_{50}: half-maximal inhibitory concentration

ILTG: Isoliquiritigenin

i.p.: intraperitoneal injection

IV: independent variable

MeOH: methanol

MW: molecular weight

NREMS: non-rapid eye movement sleep

PG: phloroglucinol

PGE: phloroglucinol equivalents

p.o.: post-oral injection

QE: quercetin equivalents

REMS: rapid eye movement sleep

RSM: response surface methodology

SM: *Salvia miltiorrhiza*

SME: *Salvia miltiorrhiza* ethanol extract

TFC: total flavonoid content

TPRA: triphlorethol A

TPC: total phenol content

VLR: valerian extract

Wake: wakefulness

ZPD: zolpidem

INTRODUCTION

Importance of sleep and insomnia

Sleep is a complex neurological process that is important in mammalian homeostasis, and required for survival [**1**]. In humans, sleep is vital to maintain health and well-being due to its primary function of providing rest and restoring the body's energy levels [**2**]. The importance of sleep is evident from the fact that sleep accounts for one-third of the human lifespan [**3**]. Sleep sustains physical and cognitive performance, the immune system, stable mood, productivity, and quality of life (**Fig. 0-1A**) [**2**, **4**, **5**]. Disorders and deprivation of sleep impair of cognitive and psychological functioning and worsen physical health [**6**]. Obesity and cardiovascular disease are deeply related to sleep disorders [**7-9**].

Although sleep is fundamental to maintain health, insomnia is currently a widespread health complaint, and has become a prevalent and disruptive problem in modern society [**10-12**]. Insomnia is defined as a sleep disorder associated with difficulty initiating sleep, difficulty maintaining sleep, and non-restorative sleep [**13**, **14**]. According to the numerous surveys conducted worldwide, approximately 10–15% of the adult population suffers from chronic insomnia, while an additional 25–35% has transient or occasional insomnia [**15**]. According to a recent survey conducted by the National Sleep Foundation in 2011 [**16**], the majority (87%) of adults in the USA has at least one sleep problem at least a few days a week (**Fig. 0-1B**). The impaired functioning due to insomnia is responsible for the frequent use of healthcare services, reduced productivity, and increased risk of accidents [**17**, **18**]. As a result, the annual overall socioeconomic burden of this disease has been estimated to exceed 100 billion US dollars [**19**]. Therefore, sleep disorders are becoming an important consideration in national health management programs.

Sleep drugs and natural sleep aids

The identification of sleep-related neurotransmitters will lead to the development of new and more effective sleep drugs [**3**]. Currently, the classes of drugs commonly used for the treatment of insomnia include γ-aminobutyric acid type A-benzodiazepine ($GABA_A$-BZD) receptor agonists, antidepressants, and antihistamines (**Table 0-1**) [**11**]. $GABA_A$-BZD receptor agonists are further divided into BZDs (BZD agonists) and non-BZDs. BZDs and non-BZDs bind to $GABA_A$-BZD receptors (to the binding site of $GABA_A$ receptors); however, non-BZDs bind selectively to the $\alpha 1$ subunit of $GABA_A$ receptors [**20**, **21**]. Currently, $GABA_A$-BZD receptor agonists are the most commonly prescribed agents for insomnia. Antidepressants, e.g., trazodone, which show a sedative property as a common adverse effect, have been used to treat insomnia and depression [**22**]. Antihistamines, e.g., diphenhydramine, are used as over-the-counter sleep aids because of their perceived safety and low cost [**23**].

Natural sleep aids, which contain specific constituents or extracts of foods and medicinal plants, have recently become popular as alternative medications to prescription sleep drugs to improve sleep quality and avoid side effects [**24**]. According to the 2002 National Health Interview Survey, more than 1.6 million American adults use alternative medicines to treat insomnia [**25**]. Many insomnia patients prefer natural sleep aids because they think that they have fewer adverse effects and interactions and they do not require a medical prescription [**26**, **27**]. In Western countries, natural sleep aids, e.g., valerian (*Valeriana officinalis*), St. John's wort (*Hypericum perforatum*), passion flower (*Passiflora incarnata*), hops (*Humulus lupulus*), and kava-kava (*Piper methysticum*), are readily available [**28**, **29**]. In particular, the extract of valerian root is the world's top-selling natural sleep aid, and its hypnotic effects have been recognized since the 18^{th} century in Europe [**26**, **30**]. Numerous clinical trials have reported that valerian might improve sleep quality without producing side effects [**30**].

$GABA_A$ receptors

The $GABA_A$-BZD receptor has been considered as the most important target for the development of sedative-hypnotic drugs [**31**, **32**]. Although numerous pharmacological treatments for insomnia are available, the major sleep drugs are BZD and non-BZD agents that act on the sleep-inducing GABAergic pathways [**33**]. Newer hypnotic agents with activity to $GABA_A$-BZD receptors are still under development by pharmaceutical companies. The molecular target of medicinal plants with sedative-hypnotic activity has been identified as the $GABA_A$-BZD receptors [**29**, **34**, **35**]. Therefore, in the present study, the $GABA_A$-BZD receptor was considered as a molecular target for screening hypnotic medicinal plants.

GABA is the major inhibitory neurotransmitter of the central nervous system (CNS), and GABAergic neurotransmission plays a key role in sleep regulation [**36**]. BZDs and non-BZDs stimulate the ability of GABA to cause membrane hyperpolarization by allowing chloride anion (Cl^-) influx (**Fig. 0-2A**) [**20**]. As a result, neurotransmission is inhibited, and these agents subsequently produce sedative-hypnotic, anxiolytic, and anticonvulsant effects [**21**, **37**]. The $GABA_A$ receptor is a pentameric transmembrane protein consisting of 5 subunits that form a central Cl^- channel (**Fig. 0-2B**) [**31**, **38**]. There are 18 different subunits comprising the $GABA_A$ receptor family: α1–6, β1–3, γ1–3, δ, ε1–3, θ, and π [**38**]. Most $GABA_A$ receptors are composed of two α subunits, two β subunits and one γ subunit [**39**, **40**]. $GABA_A$ receptors mediate a wide range of pharmacological effects including sedation, anxiolysis, and muscle relaxation due to the diversity of their subunit composition [**31**, **40**]. The sedative-hypnotic effects of pharmacological compounds are mediated by $GABA_A$ receptors that contain the α1 subunit, and BZDs and non-BZDs bind to these receptors [**40-42**]. Receptors containing the α1 subunit constitute 60% of the $GABA_A$ receptor population and are distributed in the target areas of sleep-promoting pathways, e.g., cerebral cortex, thalamus, and hypothalamus [**31**, **43**, **44**].

Objectives of this study

The characteristics of an ideal sleep drug would include the ability to reduce sleep latency, increase sleep duration, maintain sleep without side effects, and enhance daytime functioning [**45, 46**]. However, the currently available sleep drugs do not adequately fulfill these needs and are associated with side effects and the development of tolerance [**45, 47**]. The use of sedative-hypnotic drugs beyond 4 weeks is generally not recommended because of their various side effects, e.g., impaired cognitive function, memory, and general daytime performance [**48**]. In addition, their long-term administration results in tolerance and dependence [**45-47**]. Therefore, the research and development of novel sleep-promoting agents with ideal properties is ongoing. There has also been a growing demand for a new class of food constituents and natural products with hypnotic activity. In Western countries, studies on the hypnotic effects of medicinal plants have been widely carried out, whereas in Asia, there are only few reports on the hypnotic effects of Asian plants.

The objectives of this study were to find novel hypnotic plants and their active constituents as a source of new sleep drugs or aids from plant resources in Japan and Korea, and to demonstrate the sleep-promoting effects and action mechanisms of hypnotic plant extracts and their active compounds. **Fig. 0-3** shows the schematic overview of the present study. We screened 30 marine and 30 terrestrial plants using the $GABA_A$-BZD receptor binding assay. The marine plant kajime (*Ecklonia cava*, EC) and the land plant licorice (*Glycyrrhiza glabra*, GG) demonstrated high binding activity and were investigated further. To monitor the active compounds and their industrial application, the extraction conditions for EC and GG were optimized using a response surface methodology (RSM). The hypnotic effects of EC and GG extracts were evaluated using the pentobarbital-induced sleep test and analysis of sleep architecture, and their action mechanism were demonstrated. Active phlorotannins and flavonoids were isolated from EC and GG, respectively. Their hypnotic effects, action mechanisms, and potency on GABA-induced currents in neurons were investigated.

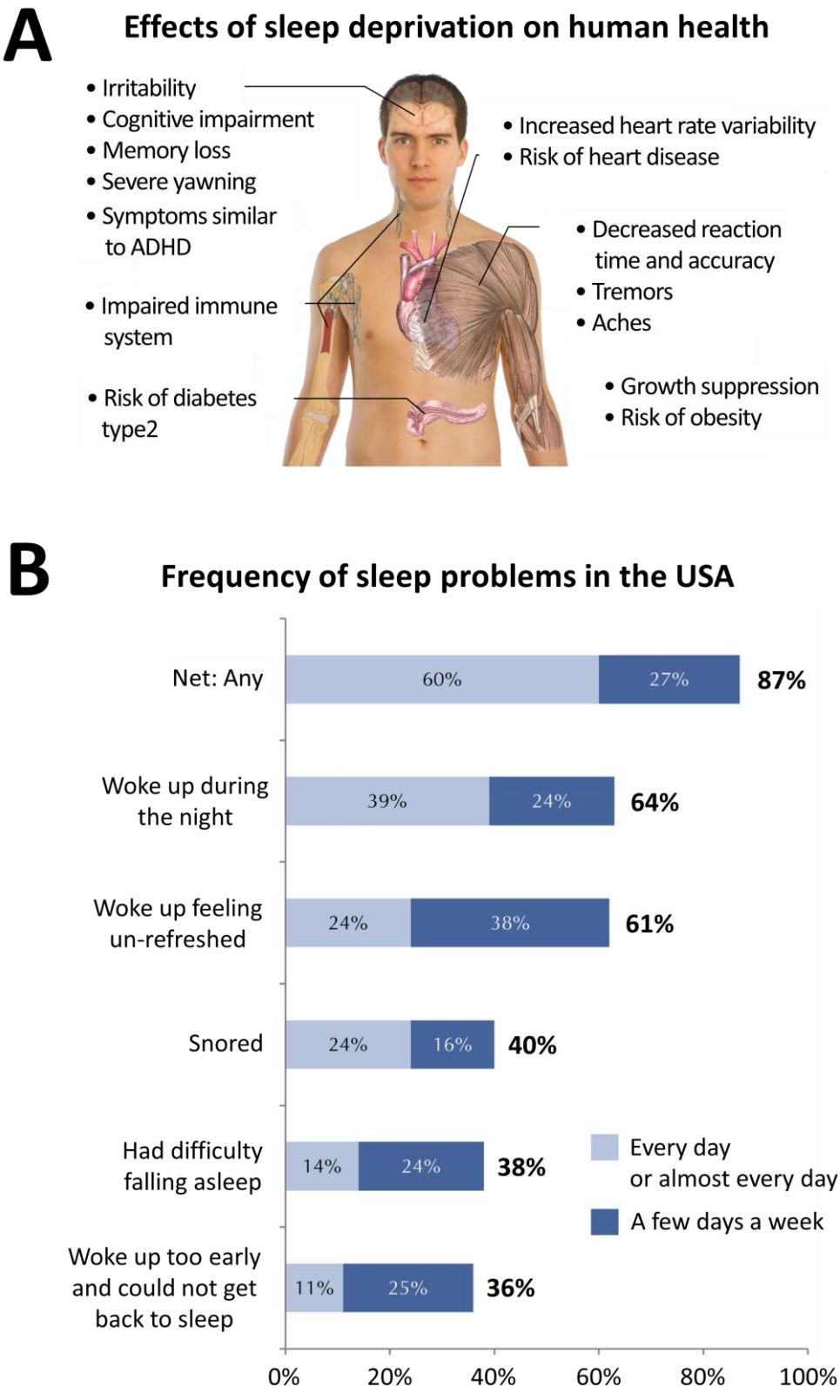


Fig. 0-1. Effects of sleep deprivation on human health (A) and frequency of sleep problems in the USA (B). Sources: (A) http://en.wikipedia.org/wiki/Sleep [**5**]; (B) National Sleep Foundation (USA), 2011 [**16**].

Table 0-1. Comparison of classes of drugs used for the treatment of insomnia

Class	Drug	Mechanism of action	Adverse effects
BZDs	Estazolam Flurazepam Triazolam	CNS effect due to allosteric interactions of BZD receptors with $GABA_A$ receptors facilitating opening of chloride channels	Sedation, confusion, anterograde amnesia
Non-BZDs	Zolpidem Zaleplon	Interact with $GABA_A$ receptor complex at binding domains located near to or allosterically coupled to BZD receptors	Dizziness, somnolence
Anti-depressants	Trazodone	Weak but selective blockade of 5-HT reuptake at the presynaptic neuronal membrane; metabolite blocks CNS 5-HT_2 receptors	Weight changes, sweating, priapism
	Amitriptyline	Blocks reuptake of norepinephrine and 5-HT at the neuronal membrane	Weight gain and bloating
	Mirtazapine	Antagonist at central presynaptic α_2-adrenergic receptors; antagonist at CNS 5-HT_2 and 5-HT_3 receptors	↑Appetite, ↑serum and cholesterol, ↑triglyceride
Anti-histamines	Diphenhydramine	First generation antihistamine that is an antagonist at the $histamine_1$ receptor	Dizziness, somnolence

Abbreviations: BZD, benzodiazepine; CNS, central nervous system; GABA, γ-aminobutyric acid; 5-HT, 5-hydroxytryptamine (serotonin). Source: Borja and Daniel, 2006 [**11**].

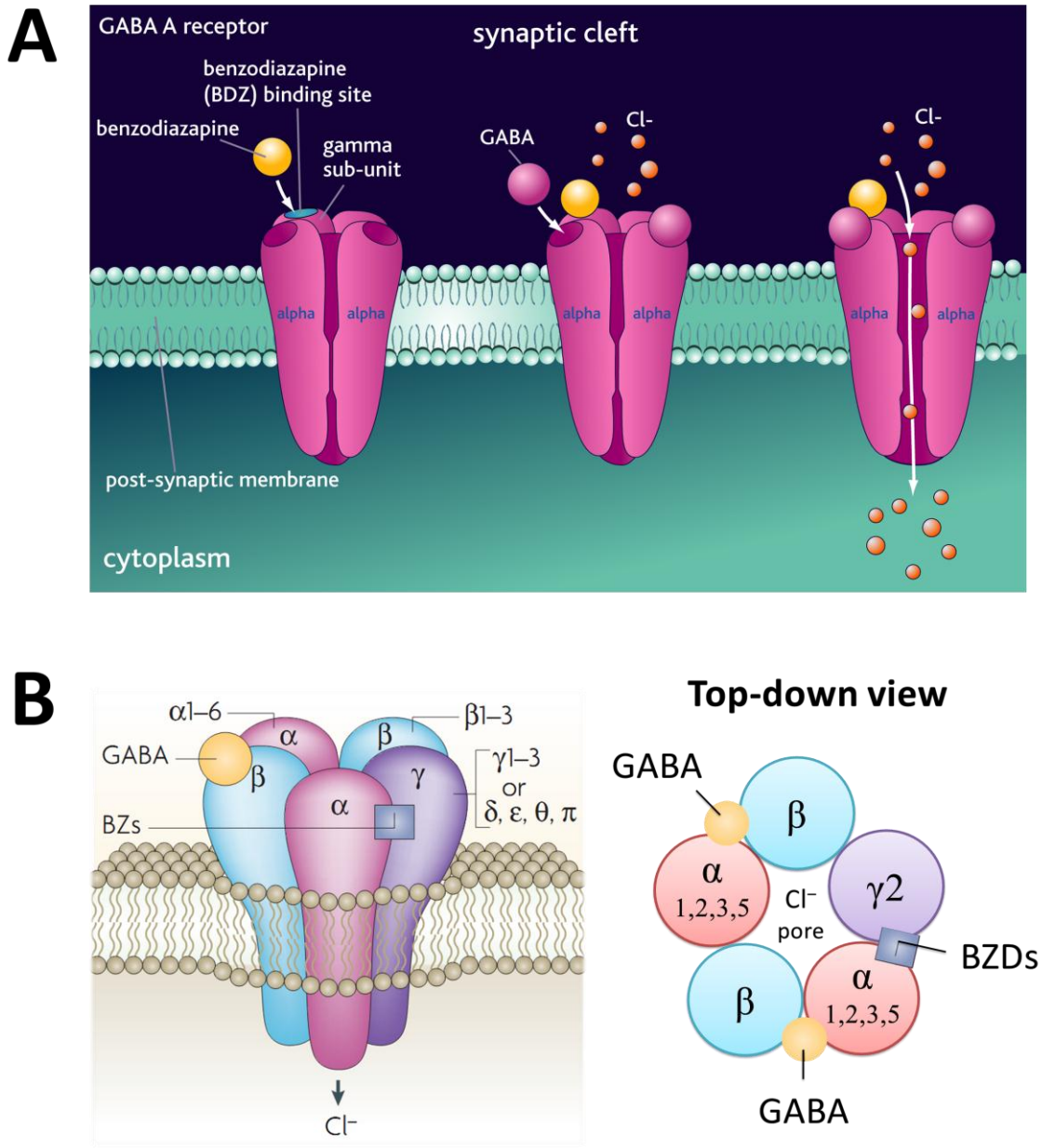


Fig. 0-2. Sedative-hypnotic mechanism of the BZD compounds (A) and structure of the $GABA_A$ receptor (B). Abbreviations: BZ or BZD, benzodiazepine; GABA, γ-aminobutyric acid. Sources: (A) Trevor and Way, 2003 [**20**]; (B) Jacob *et al*., 2008 [**38**].

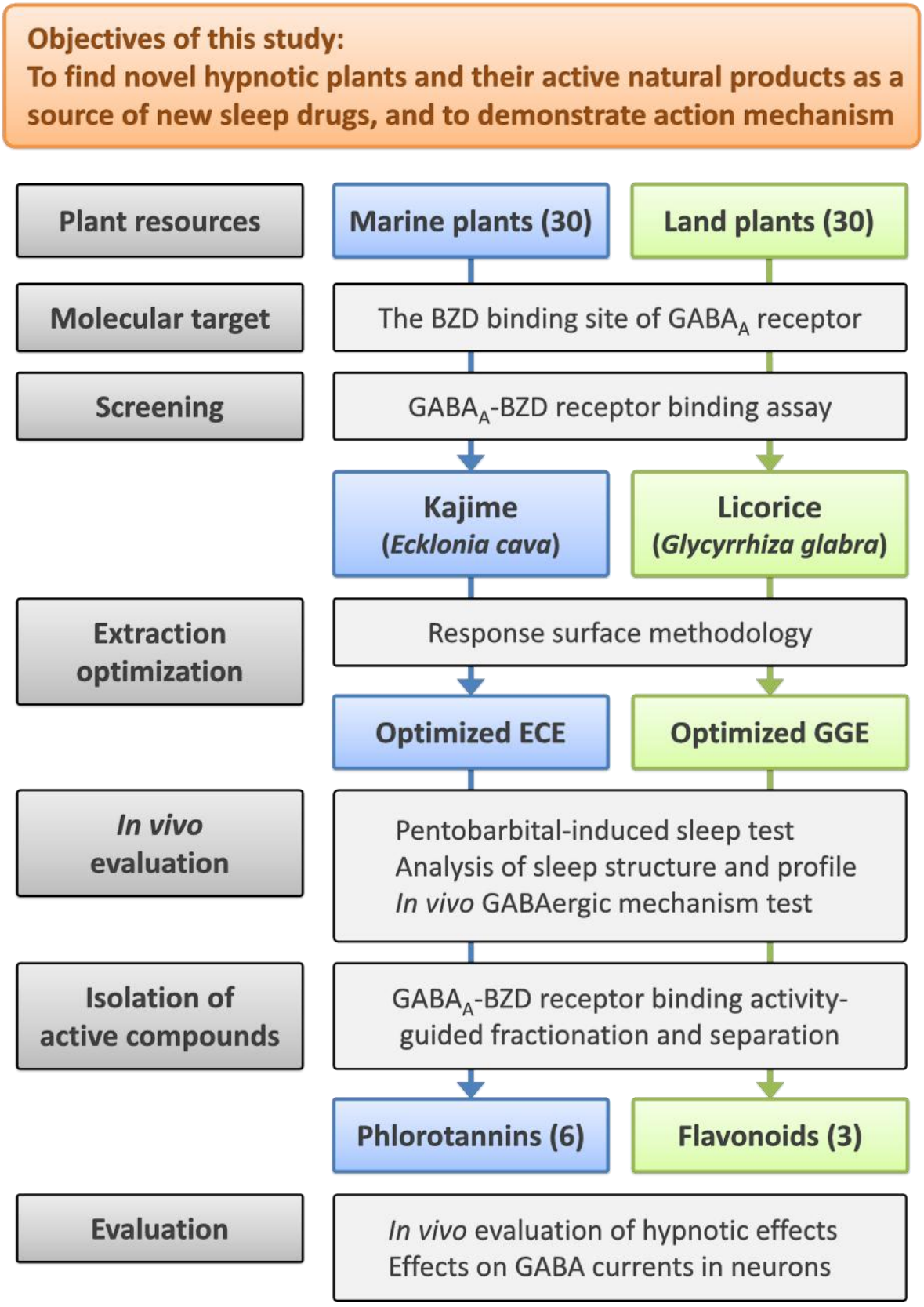


Fig. 0-3. Schematic overview and objectives of this study. Abbreviations: BZD, benzodiazepine; ECE, *Ecklonia cava* ethanol extract; GABA, γ-aminobutyric acid; GGE, *Glycyrrhiza glabra* ethanol extract.

CHAPTER 1: SCREENING OF MARINE AND TERRESTRIAL PLANTS FOR HYPNOTIC ACTIVITY

ABSTRACT

To explore novel sedative-hypnotic plants, 30 marine and 30 terrestrial plants distributed mainly in Japan and Korea were selected. Methanol or ethanol extracts of marine and land plants were screened for their binding activity to the $GABA_A$-BZD receptor, a well-characterized molecular target for sleep drugs. Methanol extracts from the marine plants *Ecklonia cava* (ECM) and *Ecklonia kurome* (EKM) and ethanol extracts from the land plants *Glycyrrhiza glabra* (GGE) and *Salvia miltiorrhiza* (SME) showed good dose-response binding activity, and their IC_{50} values were 0.392, 0.931, 0.093, and 0.137 mg/mL, respectively. To evaluate the hypnotic activity of the plant extracts with high binding activity, a pentobarbital-induced sleep test was adopted. The positive controls diazepam (DZP) and valerian extract (VLR) produced a dose-dependent decrease in sleep latency and increase in sleep duration in mice treated with pentobarbital (45 mg/kg). All plant extracts at 1000 mg/kg (p.o.) exerted significant hypnotic effects. When compared with the hypnotic effect of VLR (1000 mg/kg), ECM and GGE showed potential as hypnotic agents. With the consideration that EC could be used as a functional food, an EC ethanol extract (ECE) was prepared and tested. The binding and hypnotic activities of ECE were similar to those of ECM. The hypnotic effects of EC and GG were demonstrated for the first time in this study. EC (kajime) and GG (licorice) have been widely used as food and medicinal plants in Japan and Korea. Their safety has been also acceptable in the food and pharmaceutical industries. In particular, ECE has the potential to contain novel hypnotic compounds that are different from terrestrial natural products and chemical sleep drugs. Therefore, ECE and GGE were considered suitable for further investigations.

1.1. INTRODUCTION

It has been widely reported that various terrestrial medicinal plants, e.g., valerian and chamomile, have sedative-hypnotic effects based on their positive allosteric modulation of $GABA_A$ receptors [**35**, **49**]. For example, the flavone hispidulin has the ability to stimulate the GABA-induced Cl^- current [**50**]. Hispidulin has positive allosteric properties and permeability across the blood-brain barrier (BBB) in a rat *in situ* perfusion model [**50**]. The chamomile component apigenin, which has been characterized as a $GABA_A$-BZD receptor ligand, exerts anticonvulsant activity [**51**]. Chlorogenic acid [**52**] and epigallocatechin gallate [**53**] exhibit anxiolytic effects by acting as $GABA_A$-BZD receptor agonists. In Western countries, these medicinal plants have been commercialized as natural sleep aids.

In Asia, the identification of suitable natural sleep aids or hypnotic natural products is in high demand due to the limited availability of Western herb preparations. So far, the hypnotic effects of oriental plants, e.g., *Ganoderma lucidum* [**54**], *Schisandra sphenanthera* [**55**], and *Euphoria longana* [**56**], have been reported. Many medicinal plants are used in traditional medicine in Asia for the treatment of insomnia or have the potential to exert hypnotic effects; however, scientific evidences of their effects and precise mechanism of action have not been widely investigated. Therefore, more studies are needed to explore the hypnotic oriental plants. In this study, 30 medicinal land plants with expected hypnotic activity were chosen and screened.

On the other hand, a large number of studies on the hypnotic effects of land plants have been performed, whereas marine plants have not been recognized as a potential source of natural hypnotics. In Japan and Korea, marine plants have long been a key part of the daily diet and have been used in traditional medicine [**57**, **58**]. In particular, in Korea, brown seaweeds are well-known folk medicines that are administered to new mothers after birth [**59**]. Marine plants include various constituents, e.g., phenols, carotenoids, terpenes, and polysaccharides, and reportedly have antitumor, antioxidant, anticoagulant, anti-diabetic, and anti-inflammatory effects [**58**, **60-62**].

Despite a number of reports on the bioactivity and traditional usage of marine plants, their hypnotic activity has not yet been explored. Therefore, it was interesting to determine if seaweeds have similar hypnotic activity as terrestrial plants, e.g., valerian. Marine plants with a sedative-hypnotic effect would represent a great potential as novel sources of sleep drugs because the hypnotic effect of natural products from marine plants has not yet been reported, and marine plants may contain novel hypnotic compounds that are different from those of land plants and chemical sleep drugs.

In the present study, extracts from 30 marine and 30 terrestrial plants were tested. To evaluate the potential of plants as a resource for sedative-hypnotics, their binding activity to $GABA_A$-BZD receptors was screened. The hypnotic effects of the marine plant kajime (*Ecklonia cava*) and the land plant licorice (*Glycyrrhiza glabra*), which demonstrated the highest binding activity, were evaluated using an *in vivo* animal model, and compared with reference sleep drugs and a commercial valerian extract.

1.2. MATERIALS AND METHODS

1.2.1. Drugs and chemicals

Pentobarbital was purchased from Hanlim Pharm. Co. Ltd. (Seoul, Korea). Diazepam (DZP; Myungin Pharm Co. Ltd., Seoul, Korea), a $GABA_A$-BZD agonist, was used as a reference sedative-hypnotic drug. The other reference sleep drug zolpidem (ZPD) was obtained from Korea Food & Drug Administration. For the $GABA_A$-BZD receptor binding assay, the radioligand [^{3}H] flumazenil (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) was used. All other chemicals and reagents were of the highest grade available.

1.2.2. Extracts from marine and land plants

The methanol extracts of marine plants were purchased from the Jeju Bioresource Extract Bank (Jeju, Korea) (**Table 1-1**). According to the extract specification, the marine plant extraction was performed using 80% methanol at room temperature for 24 h. All land terrestrial extracts were purchased from the Plant Extract Bank of Korea (Daejeon, Korea) (**Table 1-2**). According to extraction method of the supplier, dried and powdered plant material (10 g) was extracted with 100 mL of 95% ethanol or methanol at 50°C for 72 h. For preparation of *Ecklonia cava* ethanol extract (ECE), the dried EC powder (Taerim Co., Jeju, Korea) was extracted with 80% ethanol at 50°C for 72 h, and extraction solutions then were filtered and lyophilized.

1.2.3. Animals

To obtain a membrane preparation for the $GABA_A$-BZD receptor binding assay, 200–250 g male Sprague-Dawley (SD) rats were used. In the pentobarbital-induced sleep test, male imprinting control region (ICR) mice weighing 18–22 g were used. All animals were purchased from Koatech Animal Inc. (Pyeongtaek, Korea), and were housed with food and water ad libitum at 24°C at controlled humidity of 55% in a room maintained on a 12 h light/dark cycle (light on at 09:00 AM). All procedures involving animals were conducted in accordance with the guidelines of the Korea Food Research Institutional Animal Care and Use Committee (permission No.: KFRI-M-09118).

1.2.4. $GABA_A$-BZD receptor binding assay

The $GABA_A$-BZD receptor binding assay was modified from the method described by Risa *et al*. [**63**]. The cerebral cortex from 4 male SD rats was homogenized for 10 s in 20 mL of Tris-HCl buffer (30 mM, pH 7.4). The suspension was centrifuged at 27,000 × *g* for 10 min, and the pellet was washed 3 times with Tris-HCl buffer. The washed pellet was homogenized in 20 mL of Tris-HCl buffer, and the suspension was incubated in a water bath (37°C) for 30 min to remove endogenous GABA. Next, the suspension was centrifuged at 27000 × *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 it was used.

The membrane preparation was thawed and washed with 20 mL of Tris-citrate buffer (50 mM, pH 7.1, 0–4°C) 3 times. The pellet was resuspended at a final concentration of 2.5 μg protein in 100 μL binding buffer, and the suspension was used for the binding assay. A membrane suspension (180 μL) was added to 10 μL of a test solution and 10 μL of 1 nM (final concentration) [^{3}H] flumazenil in a 96-well plate. The concentration of [^{3}H] flumazenil was determined by saturation binding curves (data not shown). The solution was mixed and incubated on ice for 40 min. The binding reaction was terminated by rapid filtration onto a 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 and non-specific binding were determined using the binding buffer and DZP (1 μM, final concentration), respectively. The percent displacement of the radioligand binding was determined by the following equation:

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

where DPM, TB, and NSB denote disintegrations per minute, total binding, and non-specific binding, respectively. Half-maximal inhibitory concentration (IC_{50}) values were calculated from the binding displacement curve, which was fitted to a one-site competition-binding model using the Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

1.2.5. Pentobarbital-induced sleep test

All experiments were performed between 01:00 and 05:00 PM, and mice were fasted for 24 h prior to the experiment. For oral administration, all samples were suspended in 0.5% (w/v) carboxymethyl cellulose (CMC)-physiological saline. Test solutions were administered (post-oral injection, p.o.) to mice using a sonde needle 45 min prior to pentobarbital injection. Control mice (0.5% CMC-saline, 10 mL/kg) were tested in parallel with the animals receiving test sample treatment. Following the intraperitoneal injection (i.p.) of pentobarbital (hypnotic dose, 45 mg/kg), the mice were placed in individual cages and observed for measurements of sleep latency and sleep duration. Observers were blinded to the individual treatments. The sleep latency was recorded from the time of pentobarbital injection to the time of sleep onset, and sleeping duration was defined as the difference in time between the loss and recovery of the righting reflex.

1.2.6. Statistical analysis

For multiple comparisons in the pentobarbital-induced sleep test, data were analyzed using one-way ANOVA followed by Dunnett's test. Comparisons between two-group data were analyzed by the unpaired Student's *t*-test. Differences with $p < 0.05$ were considered statistically significant. The significance analysis was performed using the Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA).

Table 1-1. Marine plant extracts (methanol) screened for hypnotic activity

Class	Scientific name	Family	Part	Voucher specimen
Green Seaweeds (Chlorophyta)	*Cladophora wrightiana*	Cladophoraceae	Whole	AC027
	Codium coactum	Codiaceae	Whole	AC012
	Codium contractum	Codiaceae	Whole	AC014
	Codium fragile	Codiaceae	Whole	AC023
	Codium latum	Codiaceae	Whole	AC013
	Codium minus	Codiaceae	Whole	AC002
	Ulva conglobata	Ulvaceae	Whole	AC003
	Ulva pertusa	Ulvaceae	Whole	AC033
Red Seaweeds (Rhodophyta)	*Champia parvula*	Champiaceae	Whole	AR032
	Gelidium amansii	Gelidiaceae	Whole	AR006
	Chondracanthus	Gigartinaceae	Whole	AR031
	Gracilaria verrucosa	Gigartinaceae	Whole	AR001
	Grateloupia filicina	Grateloupiaceae	Whole	AR014
	Polyopes lancifolius	Grateloupiaceae	Whole	AR039
	Grateloupia lanceolata	Halymeniaceae	Whole	AR038
	Polyopes affinis	Halymeniaceae	Whole	AR042
	Hypnea japonica	Hypneaceae	Whole	AR018
	Chondria crassicaulis	Rhodomelaceae	Whole	AR041
	Polysiphonia morrowii	Rhodomelaceae	Whole	AR011
Brown Seaweeds (Phaeophyta)	*Ecklonia cava*	Alariaceae	Whole	AP057
	Ecklonia kurome	Alariaceae	Whole	AP085
	Myelophycus simplex	Asperococcaceae	Whole	AP032
	Leathesia difformis	Corynophloeaceae	Whole	AP046
	Dictyopteris prolifera	Dictyotaceae	Whole	AP028
	Dictyota dichotoma	Sargassaceae	Whole	AP042
	Sargassum horneri	Sargassacea	Whole	AP052
	Sargassum patens	Sargassacea	Whole	AP020
	Petalonia binghamiae	Scytosiphonaeae	Whole	AP033
	Colpomenia sinuosa	Scytosiphonaeae	Whole	AP021
	Ishige okamurae	Shigeaceae	Whole	AP055

Table 1-2. Terrestrial plant extracts (ethanol or methanol) screened for hypnotic activity

Scientific name	Family	Part	Voucher specimen	Extract solvent
Allium victorialis	Amaryllidaceae	Bulb	003-061	Methanol
Panax ginseng C.A. Meyer	Araliaceae	Root	CA03-041	Ethanol
Polygonatum falcatum	Asparagaceae	Root	020-083	Methanol
Ligularia fischeri	Asteraceae	Root	003-016	Methanol
Adenophora triphylla	Campanulaceae	Root	030-081	Methanol
Euonymus alatus	Celastraceae	Leaf	001-213	Methanol
Chrysanthemum boreale	Compositae	Whole	004-039	Methanol
Ixeris dentata	Compositae	Whole	005-062	Methanol
Aster yomena	Compositae	Whole	009-066	Methanol
Sonchus oleraceus	Compositae	Whole	004-035	Methanol
Equisetum arvense	Equisetaceae	Whole	028-052	Methanol
Eucommia ulmoides	Eucommiaceae	Bark	010-019	Methanol
Glycyrrhiza glabra	Fabaceae	Root	CA01-003	Ethanol
Albizzia julibrissin	Fabaceae	Bark	CA03-083	Ethanol
Pueraria thunbergiana	Fabaceae	Root	014-092	Methanol
Agastache rugosa	Labiatae	Flower	032-036	Methanol
Leonurus sibiricus	Lamiaceae	Root	034-074	Methanol
Salvia miltiorrhiza	Lamiaceae	Root	CA01-028	Ethanol
Liriope platyphylla	Liliaceae	Root	023-037	Methanol
Hemerocallis fulva	Liliaceae	Whole	013-064	Methanol
Morus alba	Moraceae	Leaf	007-041	Methanol
Paeonia lactiflora	Paeoniaceae	Root	031-075	Methanol
Poria cocos	Polyporaceae	Whole	CA01-086	Ethanol
Zizyphus jujube	Rhamnaceae	Fruit	CA02-026	Ethanol
Rosa multiflora	Rosaceae	Flower	002-085	Methanol
Saururus chinensis	Saururaceae	Whole	006-042	Methanol
Schizandra chinensis	Schisandraceae	Fruit	CA02-081	Ethanol
Lycium chinense	Solanaceae	Root	018-015	Methanol
Patrinia scabiosaefolia	Sphingidae	Root	012-032	Methanol
Zingiber officinale	Zingiberaceae	Root	CA04-001	Ethanol

1.3. RESULTS AND DISCUSSION

1.3.1. Binding activity of marine and terrestrial plants to $GABA_A$-BZD receptors

Screening of hypnotic plants using a receptor binding assay: The binding of a ligand, e.g., agonist or antagonist, to its receptor is the initial and indispensable step in the cascade of reactions that finally cause pharmacological effects. Therefore, the widely used screening techniques are based on measuring ligand binding [**64**, **65**]. Receptor binding assays using a radio-ligand are an important tool in the search for drug candidates with CNS activity, and are also sensitive and rapid [**66-68**]. The $GABA_A$-BZD receptor binding assay has been widely used to screen for novel drugs and natural products with sedative-hypnotic, anxiolytic, anticonvulsant, and muscle relaxant effects [**63**, **69**, **70**]. These pharmacological properties of the BZDs make them the most important $GABA_A$ receptor-modulating drugs in clinical use [**69**, **71**]. In the present study, the $GABA_A$-BZD receptor binding assay was also adopted to screen the hypnotic plants.

Binding activity of the marine plant extracts: Most of the green and red seaweeds examined did not show any effective binding activity (**Table 1-3**). The most active seaweeds were the brown seaweeds *Ecklonia cava* (EC) and *Ecklonia kurome* (EK). The half maximal inhibitory concentration (IC_{50}) values of the EC methanol extract (ECM) and EK methanol extract (EKM) were 0.392 and 0.931 mg/mL, respectively (**Fig. 1-1A**). The identification of the binding activity of ECM and EKM is the first report to present the potential of marine plants as sources of natural $GABA_A$-BZD receptor ligands.

Binding activity of the terrestrial plant extracts: Thirty terrestrial plants were selected according to their usage in traditional treatments in Korea for sedation or anxiolysis and in consultation with an herb expert. The screening results for the land plants (**Table 1-4**) demonstrated that licorice (*Glycyrrhiza glabra*, GG) and danshen (*Salvia miltiorrhiza*, SM) showed the highest binding activity. The IC_{50} values of the GG ethanol extract (GGE) and SM ethanol extract (SME) were 0.093 and 0.137 mg/mL, respectively, and lower than those of marine plants ECM and EKM (**Fig. 1-1B**).

Table 1-3. *In vitro* displacement of [^{3}H] flumazenil binding of the marine plant extracts to the GABA$_A$-BZD receptors

Scientific name	Displacement (%) of [^{3}H] flumazenil binding			
	0.01 mg/mL	0.1 mg/mL	1 mg/mL	10 mg/mL
Cladophora wrightiana	2.7 ± 5.6	−4.1 ± 3.3	18.6 ± 7.1	70.6 ± 5.0
Codium coactum	10.2 ± 3.9	18.1 ± 2.3	26.3 ± 1.8	46.5 ± 4.5
Codium contractum	−3.0 ± 7.7	12.3 ± 5.2	23.5 ± 4.5	42.3 ± 2.0
Codium fragile	10.8 ± 6.9	23.4 ± 3.0	38.3 ± 1.8	55.9 ± 5.6
Codium latum	8.7 ± 2.9	7.6 ± 5.6	19.0 ± 3.8	48.0 ± 4.0
Codium minus	3.5 ± 4.5	15.4 ± 5.2	17.9 ± 2.0	39.2 ± 1.8
Ulva conglobata	2.2 ± 4.7	8.5 ± 4.7	30.8 ± 3.7	86.2 ± 1.9
Ulva pertusa	−0.7 ± 7.3	−6.1 ± 3.2	32.5 ± 5.8	72.5 ± 3.6
Champia parvula	5.0 ± 2.0	6.1 ± 3.0	7.6 ± 2.6	10.4 ± 3.7
Gelidium amansii	−6.1 ± .24	6.4 ± 4.4	25.5 ± 1.4	65.9 ± 1.6
Chondracanthus	1.5 ± 5.3	11.8 ± 6.4	16.3 ± 5.1	21.7 ± 3.3
Gracilaria verrucosa	3.0 ± 1.6	13.9 ± 3.1	20.7 ± 2.3	80.6 ± 4.8
Grateloupia filicina	−10.5 ± 5.6	−2.4 ± 1.9	21.8 ± 5.1	16.9 ± 9.2
Polyopes lancifolius	3.7 ± 8.1	10.1 ± 3.6	15.7 ± 2.6	35.3 ± 3.0
Grateloupia lanceolata	8.2 ± 6.0	17.8 ± 7.2	24.9 ± 2.3	45.7 ± 1.1
Polyopes affinis	8.3 ± 5.3	8.0 ± 3.9	13.8 ± 6.9	7.4 ± 7.4
Hypnea japonica	−3.1 ± 4.8	12.5 ± 2.1	10.1 ± 3.8	57.5 ± 2.7
Chondria crassicaulis	0.9 ± 7.7	−6.1 ± 4.0	7.2 ± 2.1	43.6 ± 0.9
Polysiphonia morrowii	2.3 ± 3.2	15.0 ± 9.1	23.3 ± 4.9	55.0 ± 3.1
Ecklonia cava	7.5 ± 3.5	5.2 ± 6.4	43.1 ± 6.2	91.3 ± 2.3
Ecklonia kurome	1.1 ± 3.9	10.2 ± 3.9	47.1 ± 6.2	90.8 ± 4.0
Myelophycus simplex	−1.7 ± 1.1	17.5 ± 3.4	12.6 ± 5.5	25.1 ± 2.2
Leathesia difformis	4.6 ± 3.0	5.7 ± 7.5	22.0 ± 7.8	21.7 ± 2.3
Dictyopteris prolifera	10.9 ± 6.2	18.5 ± 1.0	31.5 ± 4.9	63.8 ± 4.9
Dictyota dichotoma	5.6 ± 4.0	26.3 ± 14.7	25.7 ± 9.3	72.6 ± 1.6
Sargassum horneri	−5.2 ± 2.5	4.4 ± 3.6	18.8 ± 1.3	70.4 ± 2.4
Sargassum patens	−2.2 ± 3.7	3.9 ± 2.8	15.9 ± 4.7	55.7 ± 1.5
Petalonia binghamiae	−8.5 ± 5.4	−6.9 ± 2.7	12.6 ± 8.5	22.5 ± 4.4
Colpomenia sinuosa	9.4 ± 7.6	16.4 ± 4.7	24.9 ± 2.1	32.6 ± 2.3
Ishige okamurae	1.0 ± 4.9	−8.3 ± 4.1	20.0 ± 2.3	73.8 ± 1.8

Table 1-4. *In vitro* displacement of [^{3}H] flumazenil binding of the terrestrial plant extracts to the GABA$_A$-BZD receptors

Scientific name	Displacement (%) of [^{3}H] flumazenil binding			
	0.01 mg/mL	0.1 mg/mL	1 mg/mL	10 mg/mL
Allium victorialis	2.1 ± 3.4	–4.7 ± 2.4	10.1 ± 4.8	30.2 ± 3.6
Panax ginseng C.A. Meyer	12.5 ± 4.6	10.0 ± 2.2	5.1 ± 6.9	–2.9 ± 1.8
Polygonatum falcatum	–2.2 ± 2.9	–1.3 ± 4.4	2.8 ± 6.6	9.9 ± 5.2
Ligularia fischeri	10.6 ± 5.2	24.8 ± 3.3	30.5 ± 4.9	68.4 ± 7.5
Adenophora triphylla	5.2 ± 3.8	5.8 ± 1.7	15.2 ± 8.3	54.2 ± 5.4
Euonymus alatus	–0.7 ± 4.9	10.6 ± 2.8	22.9 ± 5.3	55.8 ± 1.9
Chrysanthemum boreale	10.8 ± 6.1	25.9 ± 0.5	23.0 ± 6.1	59.1 ± 4.3
Ixeris dentata	–5.7 ± 3.8	34.2 ± 1.8	38.4 ± 3.7	68.7 ± 3.3
Aster yomena	2.5 ± 3.7	10.7 ± 2.7	40.2 ± 1.8	63.9 ± 2.5
Sonchus oleraceus	10.6 ± 7.7	13.4 ± 6.9	30.3 ± 4.0	50.3 ± 5.4
Equisetum arvense	15.5 ± 4.7	25.9 ± 7.4	50.3 ± 2.2	63.5 ± 2.6
Eucommia ulmoides	10.9 ± 3.3	33.7 ± 3.2	45.7 ± 5.5	55.8 ± 1.7
Glycyrrhiza glabra	37.9 ± 3.7	71.6 ± 1.9	90.4 ± 0.2	97.8 ± 1.3
Albizzia julibrissin	–12.2 ± 15.2	–3.2 ± 3.3	–4.8 ± 4.3	16.5 ± 2.3
Pueraria thunbergiana	–5.2 ± 2.9	10.3 ± 1.4	48.7 ± 2.5	88.0 ± 1.6
Agastache rugosa	19.9 ± 5.6	30.7 ± 4.2	42.1 ± 1.3	73.3 ± 4.2
Leonurus sibiricus	10.6 ± 6.2	28.4 ± 5.6	38.7 ± 0.6	63.0 ± 4.0
Salvia miltiorrhiza	8.1 ± 4.6	40.1 ± 6.8	71.8 ± 3.2	90.2 ± 0.7
Liriope platyphylla	–5.8 ± 4.6	20.7 ± 4.6	20.5 ± 1.1	34.8 ± 1.6
Hemerocallis fulva	2.9 ± 2.8	–4.3 ± 2.5	9.6 ± 3.2	5.2 ± 3.0
Morus alba	–0.4 ± 5.1	6.0 ± 3.1	10.6 ± 3.9	10.7 ± 3.8
Paeonia lactiflora	5.5 ± 3.7	10.7 ± 4.0	35.2 ± 1.5	78.3 ± 1.7
Poria cocos	–1.1 ± 6.9	–0.1 ± 6.9	3.1 ± 6.8	6.3 ± 3.5
Zizyphus jujube	18.4 ± 4.7	18.8 ± 3.5	17.1 ± 5.3	26.3 ± 5.0
Rosa multiflora	10.9 ± 6.1	30.2 ± 1.8	60.7 ± 1.0	81.3 ± 3.4
Saururus chinensis	15.2 ± 4.2	25.4 ± 5.2	46.2 ± 4.3	80.3 ± 2.1
Schizandra chinensis	18.8 ± 0.9	18.8 ± 0.3	13.9 ± 0.7	19.2 ± 2.4
Lycium chinense	–4.6 ± 3.9	15.2 ± 2.9	41.8 ± 2.4	47.2 ± 2.1
Patrinia scabiosaefolia	10.2 ± 1.7	24.2 ± 4.0	32.3 ± 3.7	58.7 ± 4.5
Zingiber officinale	20.1 ± 8.8	15.0 ± 5.8	13.9 ± 3.0	46.6 ± 3.7

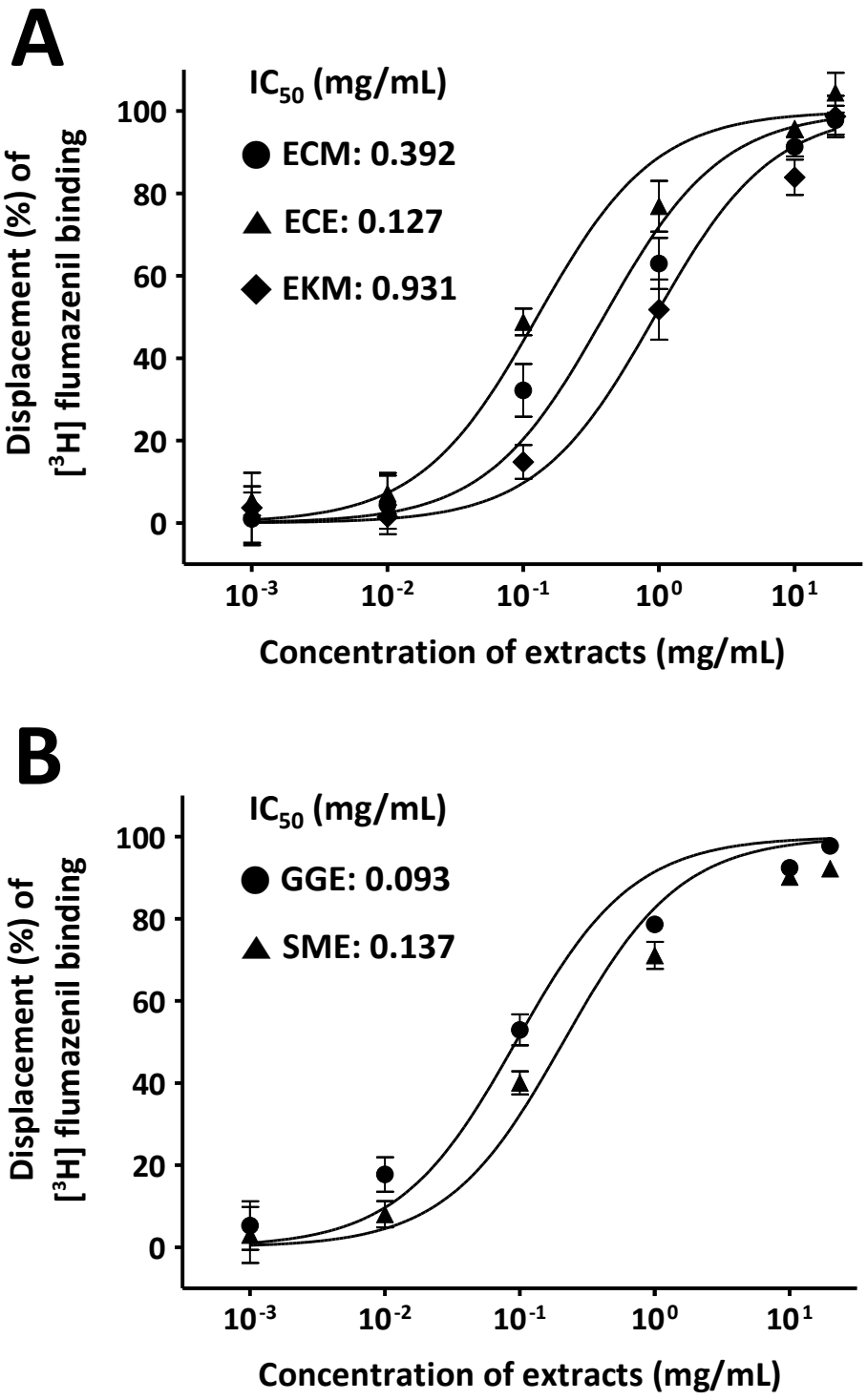


Fig. 1-1. Dose-response curves and half maximal inhibitory concentration (IC_{50}, mg/mL) values of marine (A) and terrestrial (B) plant extracts in the $GABA_A$-BZD receptor binding assay. Each point represents the mean ± SD (*n* = 3). Abbreviations: ECM, *Ecklonia cava* methanol extract; ECE, *Ecklonia cava* ethanol extract; EKM, *Ecklonia kurome* methanol extract; GGE, *Glycyrrhiza glabra* ethanol extract; SME, *Salvia miltiorrhiza* ethanol extract.

1.3.2. In vivo hypnotic effects of the plant extracts with high binding activity

Evaluation of in vivo hypnotic effect: The results of the binding assays suggested that these extracts may contain natural ligands to $GABA_A$-BZD receptors, but could not distinguish between agonists and antagonists [**65**]. It is also important that the unknown active compounds in these plant extracts are able to pass through the BBB to produce their hypnotic activity [**63**]. Therefore, it is necessary to confirm their hypnotic activity using animal model assays [**72**]. To evaluate the hypnotic effects of the plant extracts, the classical pentobarbital-induced sleep test, which is useful to evaluate sedative-hypnotic activity, was used [**56**, **72**].

Hypnotic effects of the reference sleep drugs and valerian extract: To evaluate the hypnotic effects of the marine and terrestrial plant extracts, a positive control test was performed first. The reference sleep drugs diazepam (DZP; 0.25–2 mg/kg) and zolpidem (ZPD; 1.25–10 mg/kg) produced a dose-dependent decrease in sleep latency and an increase in sleep duration in mice treated with pentobarbital (45 mg/kg, i.p.) (**Fig. 1-2**). The concentrations of DZP and ZPD for comparison with marine and land plant extracts were determined as 2 mg/kg (sleep duration: 133.5 min) and 10 mg/kg (119.4 min), respectively. These concentrations of DZP and ZPD as positive controls were used in a large number of previous reports [**72**, **73**]. Valerian is the most famous natural sleep aid worldwide [**26**, **30**]. A commercial valerian extract (VLR; 100–1000 mg/kg) also potentiated pentobarbital-induced sleep in mice in a dose-dependent manner, and showed 111.5 min of sleep duration at 1000 mg/kg.

Hypnotic effects of the marine and terrestrial plant extracts: The oral administration (1000 mg/kg) of ECM, EKM, GGE, and SME exerted a significant hypnotic effect (**Fig. 1-3**). Unlike the binding activity results, the marine plant ECM showed the highest hypnotic activity. For the land plants, GGE ($p < 0.01$) was found to have stronger hypnotic activity than SME ($p < 0.05$). With the consideration that EC could be used as a functional food, an EC ethanol extract (ECE) was prepared. In the $GABA_A$-BZD receptor binding assay, ECE was found to have a lower IC_{50} value (0.127 mg/mL)

than ECM (0.392 mg/mL). ECE (1000 mg/kg) was found to prolong sleep duration for up to 140.5 ± 4.6 min, to a level similar to that induced by DZP (2 mg/kg). These results suggest that ECE and GGE contain active compounds that are able to pass through the BBB to produce hypnotic activity. In particular, ECE and GGE (1000 mg/kg) showed good hypnotic effects compared with DZP (2 mg/kg), ZPD (10 mg/kg), and VLR (1000 mg/kg). When compared with the hypnotic effect of VLR, both ECE and GGE are potential natural sleep aids.

Selection of ECE and GGE as subjects for further investigations: The hypnotic effects of ECE and GGE were demonstrated for the first time. EC (kajime) is an edible brown seaweed that is distributed only in the coastal areas of Korea and Japan, and has been used as a functional food and traditional medicine [**74**]. In particular, EC has great potential as a novel resource of sleep drugs because its active compounds may be different from the natural products isolated from land plants and chemical sleep drugs. GG (licorice) is one of the most widely used medicinal plants worldwide, and has been described as “the grandfather of herbs” [**75-77**]. Therefore, studies on the hypnotic compounds and action mechanisms of GG are significant considering its wide application as a food ingredient and medicinal herb. Both EC [**78**] and GG [**79**] are known to be safe in the food and pharmaceutical industries. For these reasons, ECE and GGE were selected as subjects for further investigations.

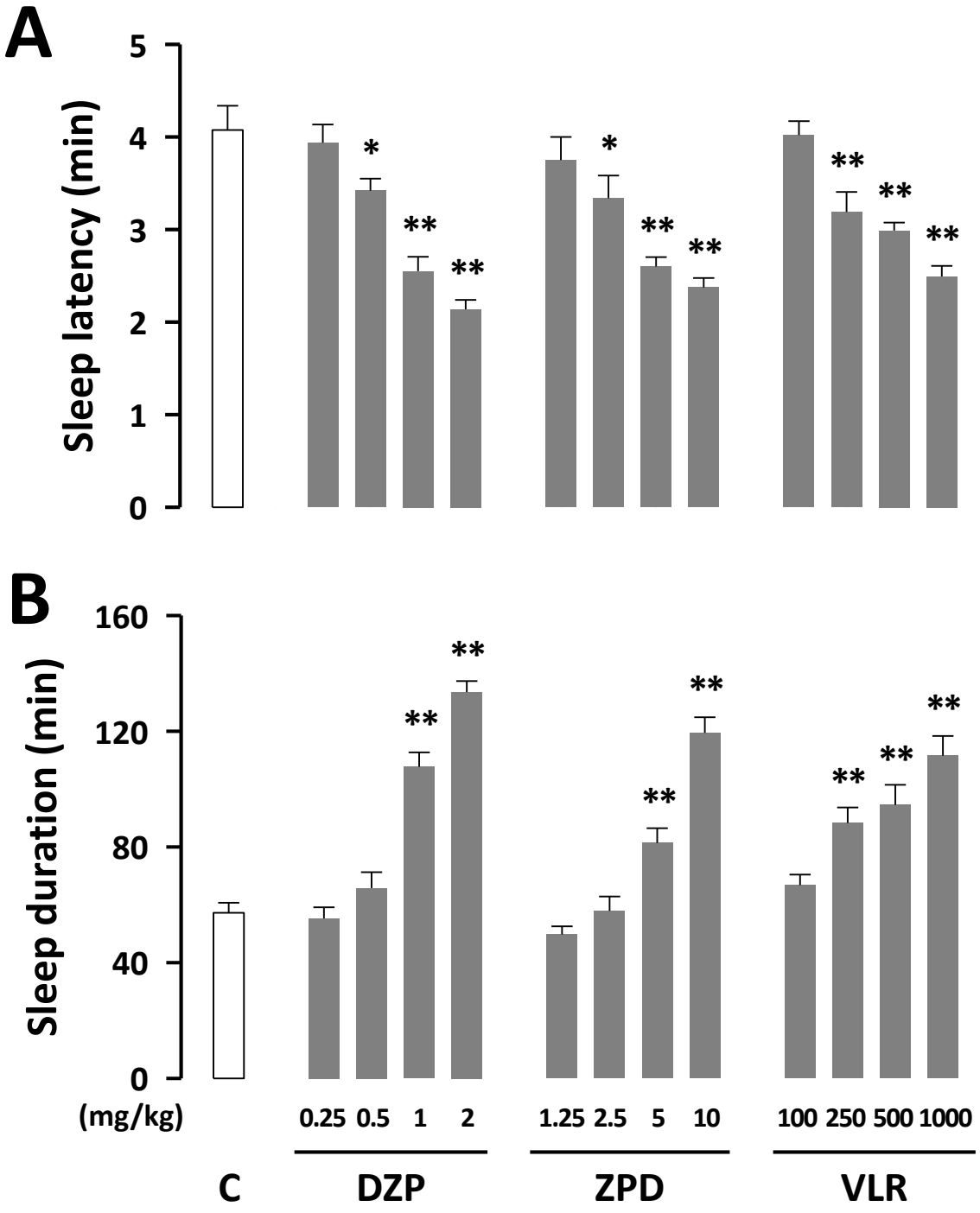


Fig. 1-2. Effects of the reference hypnotic agents diazepam (DZP), zolpidem (ZPD), and commercial valerian extract (VLR) on sleep latency (A) and sleep duration (B) in mice induced by pentobarbital (45 mg/kg). Each column represents the mean ± SEM (n = 10). *p < 0.05, **p < 0.01, significant as compared to the control group (Dunnett's test). Abbreviations: C, control group (0.5% CMC-saline, 10 mL/kg).

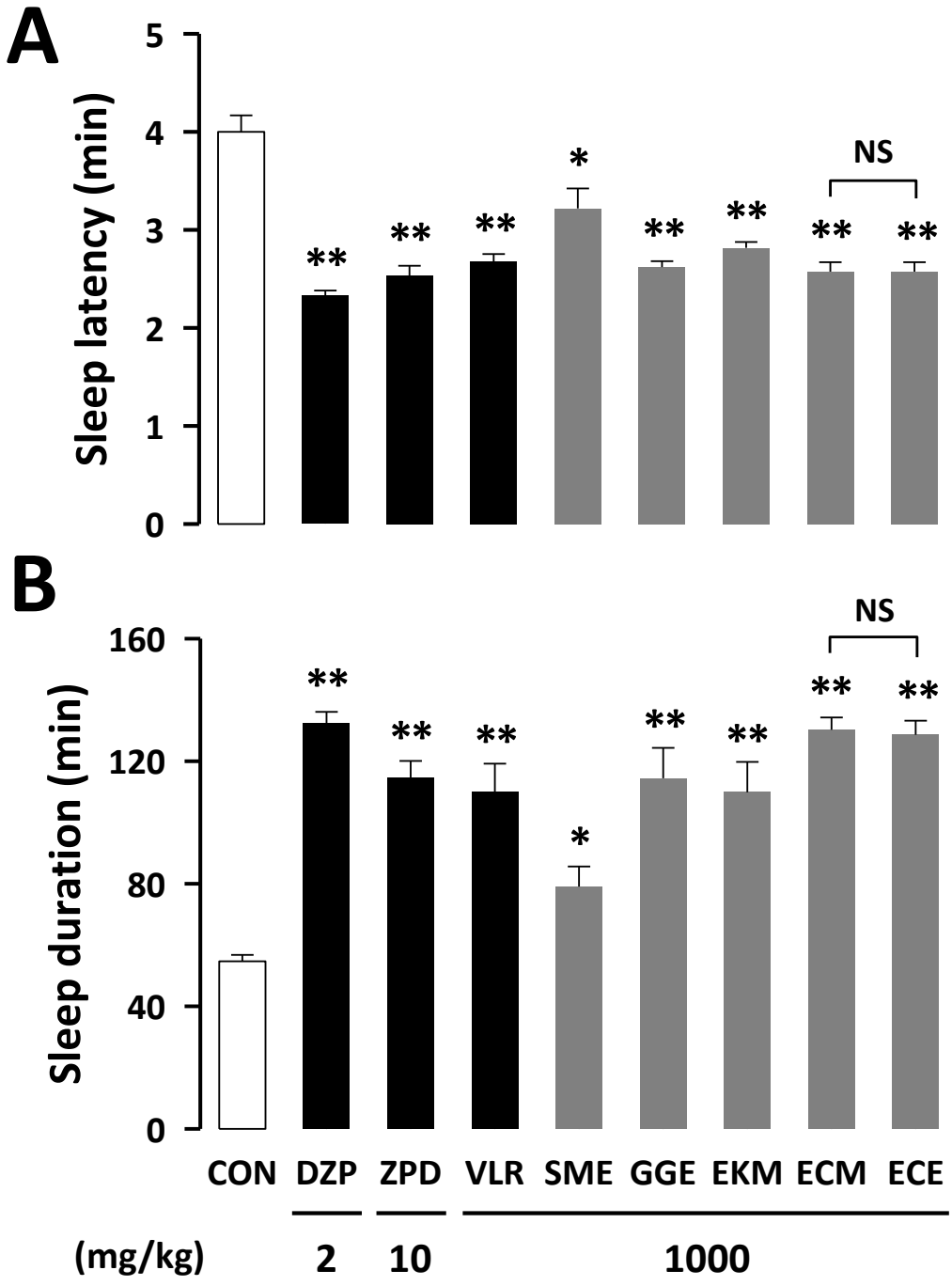


Fig. 1-3. Effects of marine and terrestrial plant extracts with high binding activity on sleep latency (A) and sleep duration (B) in mice induced by pentobarbital (45 mg/kg). Each column represents the mean ± SEM (n = 10). *p < 0.05, **p < 0.01, significant as compared to the control group (Dunnett's test). NS, there was no significant difference between ECM and ECE (unpaired Student's t-test). Abbreviations: CON, control group; DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; ECM, *Ecklonia cava* methanol extract; EKM, *Ecklonia kurome* methanol extract; GGE, *Glycyrrhiza glabra* ethanol extract; SME, *Salvia miltiorrhiza* ethanol extract; VLR, valerian extract; ZPD, zolpidem.

CHAPTER 2: HYPNOTIC EFFECTS AND GABAERGIC MECHANISM OF KAJIME (*Ecklonia cava*) EXTRACT AND ITS PHLOROTANNINS

ABSTRACT

EC (kajime) was selected from the marine plants for further investigations. ECE (100–1000 mg/kg) increased the rate of sleep onset in mice treated with a sub-hypnotic dose of pentobarbital (30 mg/kg) in a dose-dependent manner. To optimize the EC extraction conditions for the highest hypnotic activity and to monitor its active compounds, a response surface methodology was adopted. The optimal conditions were ethanol concentration, 81.6%; extraction time, 52.2 h; and extraction temperature, 43.7°C. In the experiment, there was a high correlation between sleep duration and total phenol content. The optimized ECE (250 and 500 mg/kg) significantly induced the amount of non-rapid eye movement sleep (NREMS) in mice without changes in rapid eye movement sleep (REMS) and delta activity (an indicator of sleep quality). The increase in NREMS by ECE (500 mg/kg) was significant ($p < 0.05$) during the first 2 h after administration. These results suggest that ECE induces NREMS that is similar to physiological sleep. The hypnotic effect of ECE was fully inhibited by flumazenil (FLU; a $GABA_A$-BZD receptor antagonist) like diazepam (DZP; a $GABA_A$-BZD receptor agonist). Therefore, ECE acts as a positive allosteric modulator of $GABA_A$-BZD receptors, similar to DZP. Six active phlorotannins (phenols found only in brown seaweeds) were isolated from ECE using $GABA_A$-BZD receptor binding activity-guided fractionation. Their binding affinities ranged from 1.07–4.42 μM, and were similar with that of the $GABA_A$-BZD ligands previously isolated from land plants. All phlorotannins produced a dose-dependent (5–50 mg/kg) increase in sleep duration and a decrease in sleep latency, and their hypnotic effects were also blocked by FLU. Among the EC phlorotannins, eckstolonol (ETN) and triphlorethol A (TPRA), which demonstrated the strongest hypnotic effect, were further studied using analysis of sleep architecture and an electrophysiological test. ETN and TPRA (50 mg/kg) effectively induced NREMS without decreasing delta activity, unlike the positive control zolpidem (10 mg/kg). ETN and TPRA potentiated GABA-induced currents in neurons. The relative efficacies of ETN and TPRA to the full agonist DZP (100%) were 27.1% and 42.8%, respectively. The partial $GABA_A$-BZD receptor agonists ETN and TPRA might be of particular interest since they could be devoid of the side effects associated with the full agonists, e.g., DZP and ZPD.

2.1. INTRODUCTION

Although the bioactivity of marine plants and their natural products has been reported, there is very little published data on their neurological effects. For example, an *Ulva reticulata* extract [**80**] and phlorotannins from *Ecklonia stolonifera* [**81**] have shown a neuro-protective effect by inhibiting the activity of acetyl and butyryl cholinesterase. Myung *et al*. [**82**] reported that dieckol and phlorofucofuroeckol produced memory-enhancing effects by inhibiting acetylcholinesterase activity. To the best knowledge of the author, the hypnotic effects of marine plants and their natural products have not yet been widely investigated. In this study, the hypnotic effect of EC was demonstrated for the first time, and EC was considered as a subject for further investigations.

EC is popular as an ingredient of functional foods and a traditional medicine in Japan and Korea, and its appearance is shown in **Fig. 2-1A**. In particular, EC is one of the major seaweeds of Jeju Island, Korea, and over 30,000 tons are produced annually in Korea [**83**]. EC reportedly has various biological properties, e.g., antioxidative [**83**, **84**], immune-enhancing [**85**, **86**], anti-allergic [**87**, **88**], anticancer [**89**, **90**], and anti-inflammatory [**91**] effects (**Fig. 2-1B**). An EC polyphenol extract supplement was found to be safe in clinical trials [**78**, **96**]. In addition, EC extract has recently been used as a commercial dietary supplement in the USA due to its antioxidant activity. EC contains a variety of constituents with different biological activity including fucoidans, fucoxanthins, carotenoids, and phlorotannins [**91**, **97**]. In particular, EC contains more phlorotannins than do other brown seaweeds [**98**].

In the present study, the hypnotic effects of ECE were evaluated using the pentobarbital-induced sleep test. For its industrial application and to monitor the active compounds, the extraction conditions for hypnotic activity ware optimized using a response surface methodology (RSM). The effects of ECE on changes in sleep architecture and profile were evaluated, and its mechanism was demonstrated. The isolation of the active compounds from ECE and their sleep-promoting effects and mechanism were also investigated using animal and neuron assays.

A

B

Biological activity	References
Antioxidant activity	Li *et al*., 2009 [**83**]; Kim and Kim, 2010 [**84**]
Immunostimulation	Ahn *et al*., 2008 [**85**]; Ahn *et al*, 2011 [**86**]
Anti-allergy	Le *et al*., 2009 [**87**]; Shim *et al*., 2009 [**88**]
Anticancer	Kong *et al*., 2009 [**89**]; Lee *et al*., 2011 [**90**]
Anti-inflammation	Kim and Bae, 2010 [**91**]
Anti-asthma	Kim *et al*., 2008 [**92**]
Anti-diabetic	Kang *et al*., 2010 [**93**]
Anti-HIV-1 activity	Artan *et al*., 2008 [**94**]
Radioprotection	Park *et al*., 2011 [**95**]

Fig. 2-1. Appearance of the brown seaweed *Ecklonia cava* (EC, kajime) (A) and its biological activity (B).

2.2. MATERIALS AND METHODS

2.2.1. Drugs, chemicals, and animals

Flumazenil (FLU), GABA, and phloroglucinol (PG) were purchased from Sigma-Aldrich (St. Louis, MO, USA). The other compounds were described in Chapter 1 (**1.2.1, page 13**). For analysis of sleep architecture, male C57BL/6N mice (27–30 g) were purchased from Koatech Animal Inc. (Pyeongtaek, Korea). The other animals and their procedures were mentioned in Chapter 1 (**1.2.3, page 13**).

2.2.2. Preparation of EC extract

The preparation of EC ethanol extract (ECE) was explained in Chapter 1 (**1.2.2, page 13**). For preparation of the EC enzymatic extract (ECEZ), dried EC powder (1 kg) was homogenized with 10 L of DW and 10 mL of Celluclast was added (Novo Nordisk, Bagsvaerd, Denmark). The reaction with the enzyme was conducted at 50°C for 24 h. After the enzymatic reaction, the digest was boiled for 10 min at 100°C to inactivate the enzyme. The product was clarified by centrifugation (3000 × g for 20 min) to remove any unhydrolyzed residue. The enzymatic digest was subsequently concentrated and freeze-dried.

2.2.3. $GABA_A$-BZD receptor binding assay

This protocol was described in Chapter 1 (**1.2.4, page 14**). Values of binding affinity (K_i) were calculated by the following equation:

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

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

2.2.4. Pentobarbital-induced sleep test

This protocol was described in Chapter 1 (**1.2.5, page 15**).

2.2.5. Response surface methodology for extraction optimization

Experimental design: Central composite design [**99**] was adopted in the optimization for extraction of the hypnotic compounds from EC. Central composite design matrix consists of 2^3 factorial points, 6 axial points (α = 1.682) and 3 center points (**Table 2-1**). Ethanol concentration (X_1, %), extraction time (X_2, h), and extraction temperature (X_3, °C) were chosen for the independent variable (IV). Sleep duration at 500 mg/kg (Y_1, min), TPC (Y_2, mg PGE/g), and yield (Y_3, %) were selected as the dependent variable (DV) for the combination of IVs.

Analysis of data: For response surface regression procedure, MINITAB software (Ver. 13; Minitab Inc., Harrisburg, Pa, USA) was used. Response optimization was heuristically calculated by desirability function of MINITAB software. For preparation of the most active extract, the DV Y_1 (sleep duration) was considered as a parameter of optimization. The other DVs Y_2 (TPC) and Y_3 (yield) were monitored to find correlations with the hypnotic effect. The response surface plots were developed using Maple software (Ver. 7, Waterloo Maple Inc., Ontario, Canada) and represented a function of 2 IVs while keeping the other 1 IVs.

Table 2-1. Experimental range and levels of the independent variables (IVs) in the central composite design for extraction of hypnotic compounds

Independent variables (IVs)	Symbol	Range and levels				
		–1.682	–1	0	1	+1.682
Ethanol concentration (%, v/v)	X_1	20.0	35.2	57.5	79.8	95.0
Extraction time (h)	X_2	12.0	24.2	42.0	59.8	72.0
Extraction temperature (°C)	X_3	20.0	28.1	40.0	51.9	60.0

2.2.6. Analysis of sleep architecture and profile

Surgical procedure: Under pentobarbital (50 mg/kg, i.p.) anesthesia, C57BL/6N mice were chronically implanted with the head mount (#8201, Pinnacle Technology Inc., Lawrence, KS, USA) equipped

with electroencephalogram (EEG) and electromyogram (EMG) electrodes for polysomnographic recordings (**Fig. 2-2A**). The front edge of the head mount was placed 3.0 mm anterior of bregma of mice skull. Four stainless steel screws were passed through the head mount into four predrilled holes of the skull, and the device was secured with dental cement. Two EMG wires from the head mount were sutured onto the nuchal muscles in the back of the neck. The skin was then sutured around the head mount. After surgery, each mouse was allowed 7 days in an individual transparent barrel for recovery.

Recordings of EEG and EMG: After recovery, the mice were habituated to the recording conditions for 4 days before the sample tests. The samples were administered orally to the mice, and then each mouse was immediately transferred to a soundproof recording chamber and connected to an EEG and EMG recording cable (2 EEG channels and 1 EMG channel) as shown in **Fig. 2-2B**. Recording was started at 09:00 AM, and was continued for 12 h. The time-synchronous digital video was recorded along with EEG and EMG. For evaluation of sleep-promoting effects, the recording was performed for 2 days. The data collected during the first day was served as baseline comparison data (vehicle) for the second experimental day (test article). Cortical EEG and EMG signals were amplified (×100), filtered (low-pass filter: 10 Hz EEG and 10 Hz EMG), digitized at a sampling rate of 200 Hz, and recorded by using the PAL-8200 data acquisition system (Pinnacle Technology Inc.).

Sleep-wake state analysis: The sleep-wake states were automatically classified by 10-s epoch as wakefulness (Wake), rapid eye movement sleep (REMS), and non-REM sleep (NREMS) by SleepSign Ver. 3 software (Kissei Comtec, Nagano, Japan) according to the standard criteria [**100**]. The typical waveform and fast Fourier transform (FFT) spectrum in mice were shown in **Fig. 2-2C**. As a final step, the behavioral observations by video recording also were used to determine the vigilance states. Sleep latency was defined as the time elapsed between sample administration and the first consecutive NREMS episode lasting at least 2 min and not interrupted by more than six 4-s epochs not scored as NREMS. The EEG power spectra were calculated at 0.5-Hz intervals, integrated, and averaged. It could be divided into 3 frequency areas: delta wave (0.65–4 Hz), theta wave (6–10 Hz), and alpha wave (12-14 Hz) [**101**]. The EEG power density of NREMS was normalized as a group by calculating the percentage of each interval from the total EEG power (0–20 Hz) of the individual mouse.

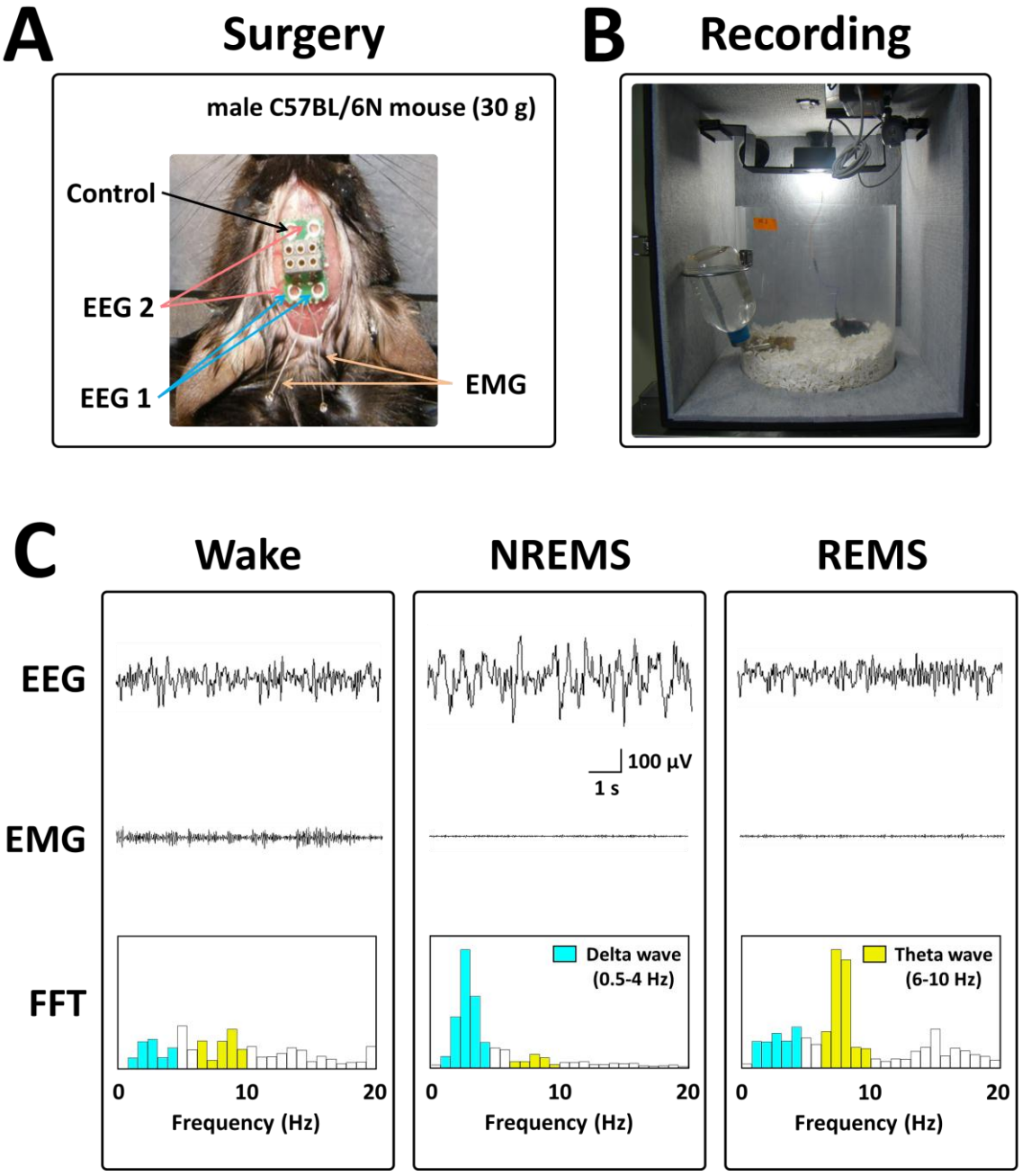


Fig. 2-2. Surgery for EEG and EMG recordings (A). An inside view of the recording chamber (B). Typical EEG, EMG and FFT spectra in mice (C). The EEG and EMG signals and FFT spectra were collected from a vehicle control mouse in this study. Abbreviations: EEG, electroencephalogram; EMG, electromyogram; FFT, fast Fourier transform; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

2.2.7. Isolation and identification of active compounds from ECE

Reagents and instruments for purification and structure determination: SiO_2 (Kiesel gel 60, Merck, Darmstadt, Germany) and ODS (LiChroprep RP-18, Merck) resins were used for column chromatography (CC). Thin-layer chromatography (TLC) analysis was carried out using Kiesel gel 60 F_{254} and RP-18 F_{254S} (Merck) resins, and the compounds were detected using a UV lamp Spectroline Model ENF-240 C/F (Spectronics Corporation, Westbury, NY, USA) and a 10% H_2SO_4 solution. FAB-MS was conducted on a JEOL JMSAX-700 (Tokyo, Japan). ^{1}H-NMR (400 MHz) and ^{13}C-NMR (100 MHz) spectra were recorded on a Varian Unity Inova AS-400 FT-NMR spectrometer (Palo Alto, CA, USA).

Extraction and isolation: Isolation of active compounds from ECE was performed by the $GABA_A$-BZD receptor binding activity-guided fractionation, and the fractionation scheme is shown in **Fig. 2-10**. The ECE (98 g) prepared at the optimal conditions was suspended in H_2O (1 L) and partitioned with *n*-hexane (HX, 10 g), ethylacetate (EA, 23 g), *n*-butanol (BT, 11 g), in sequence. The EA fraction, which showed the most binding activity, was applied to a celite CC (Ø 15 cm X 15 cm). The column was eluted using the mixture of $CHCl_3$-methanol (MeOH) (3:1), and elutes were pooled into the 11 sub-fractions (F1–F11) based on TLC. The sub-fraction F3 (2.4 g) was further separated with Sephadex LH-20 CC (Ø 2 cm X 50, 80% MeOH) to get the 19 sub-fractions (FF1–FF19). Six active compounds were isolated FF3 (compound 1, 17 mg), FF7 (compound 2, 24 mg), FF9 (compound 3, 8.5 mg), FF13 (compound 4, 32.4 mg), FF14 (compound 5, 21 mg), and FF19 (compound 6, 252 mg). The purified compounds were identified by comparing ^{1}H-NMR and ^{13}C-NMR data to the literature report. All the isolated compounds were phlorotannins found in brown seaweeds. The structural elucidations of phlorotannins isolated from ECE were summarized as follows.

Triphlorethol A (TPRA, compound 1): yellow amorphous powder (MeOH); FAB/MS *m/z* 373 $[M-1]^-$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 6.05 (1H, d, *J*=2.8 Hz, H-5), 6.00 (2H, d, *J*=2.0 Hz, H-2'',6''), 5.92 (1H, d, *J*=2.0 Hz, H-4''), 5.89 (2H, s, H-3',5'), 5.74 (1H, d, *J*=2.8 Hz, H-3); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 162.37 (C-1''), 160.29 (C-3'',5''), 156.39 (C-4'), 156.14 (C-4), 153.71 (C-6), 152.56 (C-2), 152.07 (C-2',6'), 125.65 (C-1), 124.62 (C-1'), 98.01 (C-3), 97.46 (C-4''), 96.14 (C-3',5'), 95.37 (C-2'',6''), 94.98 (C-5).

Fucodiphlorethol G (FDRG, compound 2): yellow amorphous powder (MeOH); FAB/MS *m/z* 497 $[M-1]^-$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 6.12 (1H, d, *J*=2.8 Hz, H-4''), 6.07 (2H, s, H-3''',5'''), 6.02 (1H, d, *J*=2.8 Hz, H-5),

6.01 (1H, d, *J*=2.8 Hz, H-6''), 5.91 (2H, s, H-3',5'), 5.68 (1H, d, *J*=2.8 Hz, H-3); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 159.48 (C-5''), 159.33 (C-3'',4'''), 159.21 (C-1''), 157.93 (C-4'), 157.51 (C-2), 156.31 (C-2''',6'''), 153.69 (C-4), 152.11 (C-2',6'), 151.97 (C-6), 124.58 (C-1), 124.30 (C-1'), 101.94 (C-2''), 101.76 (C-1'''), 97.98 (C-3), 97.49 (C-4''), 96.66 (C-3''',5''), 96.31 (C-3',5'), 94.42 (C-5), 94.21 (C-6'').

6,6'-Bieckol (BECK, compound 3): yellow amorphous powder (MeOH); FAB/MS *m/z* 741 $[M-1]^-$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 6.12 (2H, s, H-3,3'), 5.06 (2H, s, H-8,8'), 5.95 (2H, d, *J*=1.8 Hz, H-4'',4'''), 5.93 (4H, d, *J*=1.8 Hz, H-2'',2''',6'',6'''); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 161.88 (C-1'), 160.11 (C-3',5'), 152.57 (C-7), 146.92 (C-9), 146.39 (C-2), 143.39 (C-5a), 143.16 (C-4), 138.66 (C-10a), 126.38 (C-1), 125.81 (C-9a), 124.44 (C-4a), 101.25 (C-8), 99.59 (C-3), 99.46 (C-4'), 97.65 (C-6), 95.38 (C-2',6').

Eckol (ECK, compound 4): yellow amorphous powder (MeOH); FAB/MS *m/z* 371 $[M-1]^-$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 6.13 (1H, s, H-3), 5.94 (2H, s, H-6,8), 5.93 (3H, s, H-2',4',6'); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 166.45 (C-1'), 161.89 (C-3',5'), 154.54 (C-7), 147.02 (C-9), 147.09 (C-2), 144.25 (C-5a), 143.36 (C-4), 137.20 (C-10a), 125.61 (C-1), 124.85 (C-9a), 124.55 (C-4a), 99.84 (C-8), 99.37 (C-3), 97.68 (C-4'), 95.76 (C-6), 95.34 (C-2',6').

Eckstolonol (ETN, compound 5): yellow amorphous powder (MeOH); FAB/MS *m/z* 369 $[M-1]^-$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 6.14 (1H, s, H-7), 6.01 (1H, d, *J*=2.8 Hz, H-2), 5.99 (1H, d, *J*=2.8 Hz, H-10), 5.97 (1H, d, *J*=2.8 Hz, H-4), 5.94 (1H, d, *J*=2.8 Hz, H-12); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 154.71 (C-3), 154.34 (C-11), 147.17 (C-1), 146.96 (C-9), 143.75 (C-4a), 143.89 (C-12a), 141.29 (C-6), 139.18 (C-7a), 133.06 (C-13b), 127.84 (C-5a), 124.88 (C-8a), 124.79 (C-13a), 124.47 (C-14a), 99.98 (C-2), 99.86 (C-10), 98.95 (C-7), 95.79 (C-4), 95.68 (C-12).

Dieckol (DECK, compound 6): yellow amorphous powder (MeOH); FAB/MS *m/z* 741 $[M-1]^-$; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 6.15 (1H, s, H-3''), 6.13 (1H, s, H-3), 6.09 (2H, s, H-2''',6'''), 6.06 (1H, d, *J*=2.8 Hz, H-8''), 6.05 (1H, d, *J*=2.8 Hz, H-6''), 5.98 (1H, d, *J*=2.8 Hz, H-8), 5.95 (1H, d, *J*=2.8 Hz, H-6), 5.92 (3H, s, H-2',4',6'); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 162.7 (C-1'), 161.0 (C-3',5'), 158.6 (C-1'''), 156.8 (C-7), 155.3 (C-7''), 153.2 (C-3''',5'''), 148.1 (C-2''), 148.01 (C-2), 147.9 (C-9''), 147.7 (C-9), 145.1 (C-5a''), 145.0 (C-5a), 144.2 (C-4''), 144.1 (C-4), 139.4 (C-10a), 139.3 (C-10a''), 127.3 (C-4'''), 127.0 (C-9a), 126.5 (C-1), 126.4 (C-1''), 125.7 (C-9a''), 125.5 (C-4a''), 125.4 (C-4a), 100.7 (C-8''), 100.6 (C-8), 100.3 (C-3), 100.2 (C-3'''), 98.5 (C-4'), 97.0 (C-2''',6'''), 96.7 (C-6''), 96.6 (C-6), 96.2 (C-2',6').

2.2.8. Determination of total phenol content

The total phenol content (TPC) was determined according to the Folin-Ciocalteu method described by Slinkard and Singelton [**102**]. Dried samples were dissolved in methanol. A 0.5 mL of sample solution was added to 0.5 mL of Folin-Ciocalteu reagent and 6.5 mL of distilled water. After 5 min, 2.5 mL of 10% sodium carbonate was added. Sample solutions were vortexed for 5 s, and incubated in the darkness at room temperature for 60 min. The absorbance of the sample solutions was measured at 765 nm. The calibration curve was prepared with phloroglucinol (Sigma-Aldrich Inc., St. Louis, MO, USA), the basic structural unit of phlorotannins (brown seaweed phenols) [**103**]. TPC was expressed as phloroglucinol equivalents (mg PGE/g).

2.2.9. Analysis of EC phlorotannin constituents

Analysis of phlorotannin compositions was conducted using a Flexar FX-10 UHPLC system (PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) equipped with a 4.6 mm x 150 mm i.d., 5 µm particle size, PerkinElmer AQ C18 column. The sample was then separated for 25 min using a gradient mobile phase consisting of 5% to 100% methanol. The flow rate was set at 0.8 mL/min with an injection volume of 10 µL. The detection wavelength was set to 230 nm. Components were identified and quantified by comparison of their retention times to those of phlorotannin standards under identical analysis conditions and UV spectra using a PDA detector.

2.2.10. Electrical measurements

Neurons from the dorsal raphe (DR) nucleus were acutely dissociated from 2- to 3-week-old SD rats of either sex, according to procedures reported elsewhere [**104**] with some modifications. Briefly, after anesthesia, the brain was resected and placed in ice-cold artificial cerebrospinal fluid (ACSF) composed of the following: NaCl, 125 mM; KCl, 3 mM; KH_2PO_4, 1.2 mM; $MgSO_4$, 1.2 mM; $NaHCO_3$, 25 mM; dextrose, 10 mM; and $CaCl_2$, 2 mM. The solution was then bubbled with 95% O_2 and 5% CO_2. The area containing the DR was cut into coronal slices (300 µm). These slices were pre-incubated for 2–4 h at 31°C in well-bubbled ACSF. For dispersion, the brain slices were transferred to a glass-bottomed perfusion chamber filled with standard external solution containing the following: NaCl, 150 mM; KCl, 5 mM; $MgCl_2$, 1 mM; $CaCl_2$, 2 mM; HEPES, 10

mM; and glucose, 10 mM (pH 7.4). A fire-polished glass pipette with a 100- to 120-mm tip size was mounted in a custom-made vibrator held by a micromanipulator [**105**]. Under the stereomicroscope, the oscillating tip was lowered to the surface of the slices within the DR region. The neurons were dissociated from the upper 100 µm of these slices. After removing the slice, the dispersed neurons were allowed to settle and adhere to the bottom of the chamber; the process is completed within 20 min generally. Electrical measurements were performed in the nystatin-perforated [**106**] patch recordings with modifications. The recording electrodes were filled with a solution composed of the following: KCl, 50 mM; K gluconate, 100 mM; and HEPES, 10 mM (pH 7.2). The final concentration of nystatin was 450 µg/mL. The neurons were visualized with phase-contrast equipment on an inverted microscope (IX70; Olympus, Tokyo, Japan) with a 40× objective and a 10× ocular lens. The current was measured with a patch-clamp MultiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA). All the experiments were performed at room temperature (21–22°C). The drugs were applied with the Y-tube microperfusion system [**107**]. With this technique, the external solution surrounding a neuron could be exchanged within 0.1 s.

2.2.11. Statistical analysis

The statistical analysis was described in Chapter 1 (**1.2.6, page 15**).

2.3. RESULTS AND DISCUSSION

2.3.1. Effects of ECE on pentobarbital-induced sleep in mice

***Hypnotic dose of pentobarbital-induced sleep test*:** With a hypnotic dose of pentobarbital (45 mg/kg), ECE (100–1000 mg/kg) generated a dose-dependent decrease in sleep latency and an increase in sleep duration (**Fig. 2-3**). It showed a significant ($p < 0.01$) hypnotic effect from a concentration of 250 mg/kg. In the range of doses, no adverse effects were observed following ECE administration. Enzymatic extracts of seaweeds have been successfully commercialized due to several advantages, e.g., such as high bioactive compound yield and good water-solubility [**108**, **109**]. Therefore, the hypnotic activity of EC enzyme extract (ECEZ) was also evaluated. ECEZ significantly potentiated pentobarbital-induced sleep; however, its activity was lower than that of ECE.

***Sub-hypnotic dose of pentobarbital-induced sleep test*:** With a sub-hypnotic dose of pentobarbital (30 mg/kg), most of the control mice (83%) did not fall asleep (**Table 2-2**). The sleep drug DZP produced 55.4 ± 4.8 min of sleep duration and a 100% rate of sleep onset. The administration of ECE also increased the rate of sleep onset and prolonged sleep duration in a dose-dependent manner. ECE (1000 mg/kg) significantly ($p < 0.05$) increased sleep duration (38.7 ± 6.3 min) and the rate of sleep onset (92%). This result in the sub-hypnotic dose of pentobarbital-induced sleep test indicates that ECE has a decisive role in sleep induction in mice.

Significance of the study on the hypnotic effects of marine plants: As mentioned above, the hypnotic effects of various land plants have been studied, and a large number of active natural products have also been isolated from them [**49**, **50**]. Phenols from land plants, in particular, have been considered as major sedative-hypnotic compounds [**35**, **110**]. Although marine plants, such as brown seaweeds, are rich in various polyphenols, they have not been considered as a potential source of compounds exerting hypnotic activity. Therefore, the study of the hypnotic effect and mechanism of brown seaweed EC has significance in the analysis of the neurological properties of marine plants.

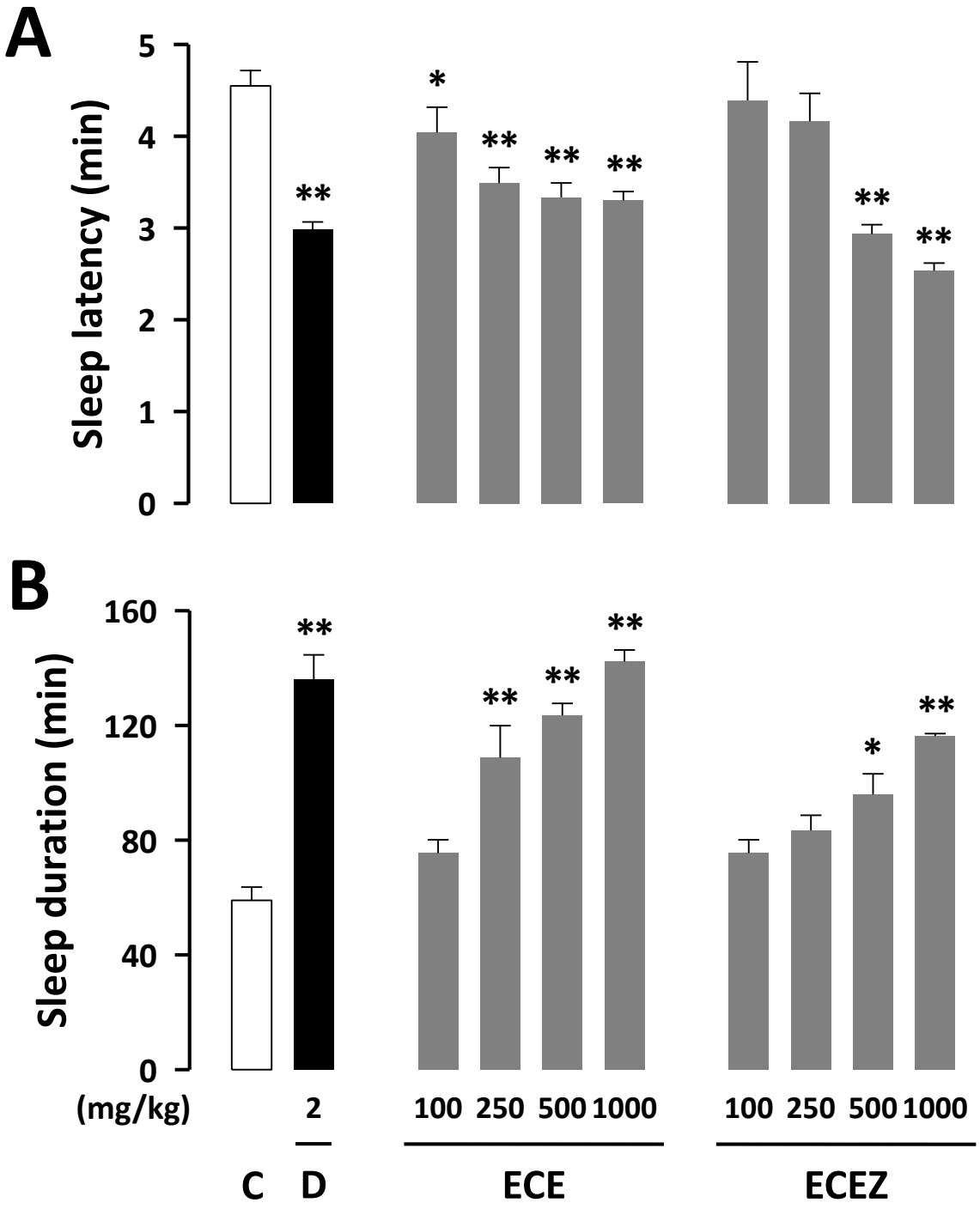


Fig. 2-3. Effects of ECE and ECEZ on sleep latency (A) and sleep duration (B) in mice induced by pentobarbital (45 mg/kg). Each column represents the mean ± SEM (n = 10). *p < 0.05, **p < 0.01, significant as compared to the control group (Dunnett's test). Abbreviations: C, control group (0.5% CMC-saline, 10 mL/kg); D, diazepam; ECE, *Ecklonia cava* ethanol extract; ECEZ, *Ecklonia cava* enzymatic extract.

Table 2-2. Effects of ECE on the rate of sleep onset and sleep duration in mice administered a sub-hypnotic dose of pentobarbital (30 mg/kg)

Groups	Dose (mg/kg)	No. falling asleep / total	Rate of sleep onset (%)	Sleep duration (min)
CON		2 / 12	17	7.3 ± 1.1
DZP	2	12 / 12	100	55.4 ± 4.8**
ECE	100	7 / 12	58	12.7 ± 2.1
	250	8 / 12	67	15.1 ± 4.4
	500	10 / 12	83	19.7 ± 3.4
	1000	11 / 12	92	38.7 ± 6.3*

The rate of sleep onset (%) = no. falling asleep / total no. × 100. Sleep duration is expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, significant as compared to the control group (Dunnett's test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; ECE, *Ecklonia cava* ethanol extract.

2.3.2. Optimization of EC extraction conditions for hypnotic activity

Advantages of extraction optimization by RSM: It is important to improve the bioactivity or yield of products and extracts while minimizing the cost associated with the temperature, processing period, and volume of solvent. In order to obtain the most active ECE and monitor active compounds, an RSM was adopted. RSM is an effective and powerful approach for screening key factors and optimizing processing conditions in the food and chemical industries [**111-113**]. It is also useful to find an indicator compound through correlations between independent variables (IVs, factors).

***Response surface model equations*:** The values of the dependent variables (DVs, responses) for the combination of IVs are given in **Table 2-3**. The response surface model equations for the DVs Y_1 (sleep duration at 500 mg/kg), Y_2 (total phenol content, TPC), and Y_3 (yield) were estimated from response surface regression, as follows:

$$Y_1 = 110.821 + 22.093X_1 + 3.493X_2 - 0.046X_3 - 8.591X_1^2 - 6.116X_2^2 - 4.153X_3^2 + 3.200X_1X_2 + 1.000X_1X_3 + 2.050X_2X_3 \quad (R^2\text{: } 0.913, p\text{-value: } 0.006)$$

$$Y_2 = 191.956 + 82.013X_1 + 1.246X_2 - 3.256X_3 - 34.216X_1^2 - 13.385X_2^2 - 13.638X_3^2 + 5.050X_1X_2 + 0.925X_1X_3 - 1.725X_2X_3 \quad (R^2\text{: } 0.926, p\text{-value: } 0.003)$$

$$Y_3 = 15.016 - 1.515X_1 + 1.116X_2 + 0.005X_3 - 1.778X_1^2 - 0.223X_2^2 + 0.078X_3^2 + 0.188X_1X_2 - 1.513X_1X_3 + 0.263X_2X_3 \quad (R^2\text{: } 0.748, p\text{-value: } 0.142)$$

The coefficient of determination (R^2) indicates that the model equations described the experimental designs adequately [**111**]. The values of R^2 for Y_1 and Y_2 were 0.913 and 0.926, respectively, and significant at the 99% probability level. However, the model equation of Y_3 (yield) was not significant at the 95% probability level.

***The effects of IVs on DVs and response surface 3D plots*:** The 3D plots in **Fig. 2-4** depict the interrelationship between IVs and DVs. The results of the hypnotic effects of ECE and ECEZ showed that the ethanol concentration may be the major factor that determines the degree of hypnotic

activity of the EC preparations. The use of ethanol, which is relatively cheap, reusable, and nontoxic, could lend an environmentally friendly aspect to the low-cost preparation of potentially bioactive extracts from foods and plants [**114**]. As expected, the IV X_1 (ethanol concentration, %) was the most important factor affecting the hypnotic activity of ECE. For all of the DVs, the coefficients of X_1 were significant at the 99% probability level (data not shown). As X_1 was increased from 20% (–1.682) to 95% (+1.682), Y_1 also increased. In previous reports, the ethanol concentration was considered as a major factor (IVs) for the optimization of the extraction of phenolic compounds from plants by RSM [**113-115**]. Phenolic compounds are one of the major hypnotic natural products; therefore, Y_2 (TPC) was monitored together with Y_1 (sleep duration). The values of Y_2 in the experimental design showed a similar tendency as Y_1, and the R^2 value between Y_1 and Y_2 was 0.956. This high correlation between sleep duration and TPC represents the potential of EC phenols as active compounds.

Optimal extraction conditions**:** To obtain ECE with the highest hypnotic activity, only Y_1 (sleep duration) was considered as a parameter during the optimization of the extraction conditions. The optimal conditions (coded and actual values) of X_1 (ethanol concentration), X_2 (extraction time), and X_3 (extraction temperature) were 1.08 (81.6%), 0.57 (52.2 h), and 0.31 (43.7°C), respectively (**Table 2-4**). The predicted value of Y_1 (sleep duration) at the optimal conditions was 126.9 min, and those of Y_2 (TPC) and Y_3 (yield) were 237.8 mg PGE/g and 11.5%, respectively. To verify the accuracy of the predicted value of Y_1, ECE was prepared using the optimal conditions. The sleep duration of the optimized ECE (500 mg/kg) was 130.3 min, and was similar to the predicted value and that of non-optimized ECE (1000 mg/kg).

Table 2-3. Central composite design matrix and response values for extraction of hypnotic compounds from EC (*Ecklonia cava*)

Run order	Coded level			Response		
	X_1	X_2	X_3	Y_1	Y_2	Y_3
Factorial portion						
1	−1	−1	−1	60.9	17.8	11.3
2	1	−1	−1	107.5	201.8	13.0
3	−1	1	−1	60.9	19.2	12.8
4	1	1	-1	117.1	218.8	15.8
5	−1	−1	1	61.5	28.5	14.3
6	1	−1	1	108.9	211.0	10.5
7	−1	1	1	66.5	18.4	17.4
8	1	1	1	129.9	226.3	13.8
Axial portion						
9	−1.682	0	0	64.3	10.4	14.6
10	1.682	0	0	116.7	216.5	3.9
11	0	−1.682	0	93.9	174.5	12.3
12	0	1.682	0	101.1	170.2	15.0
13	0	0	−1.682	109.3	192.6	15.4
14	0	0	1.682	96.8	150.7	13.6
Center portion						
15	0	0	0	115.3	192.5	14.8
16	0	0	0	108.4	187.7	15.5
17	0	0	0	107.4	189.4	15.0

X_1, ethanol concentration (%, v/v); X_2, extraction time (h); X_3, extraction temperature (°C).

Y_1, sleep duration (min, at 500 mg/kg); Y_2, total phenol content (mg PGE/g); Y_3, yield (%, w/w).

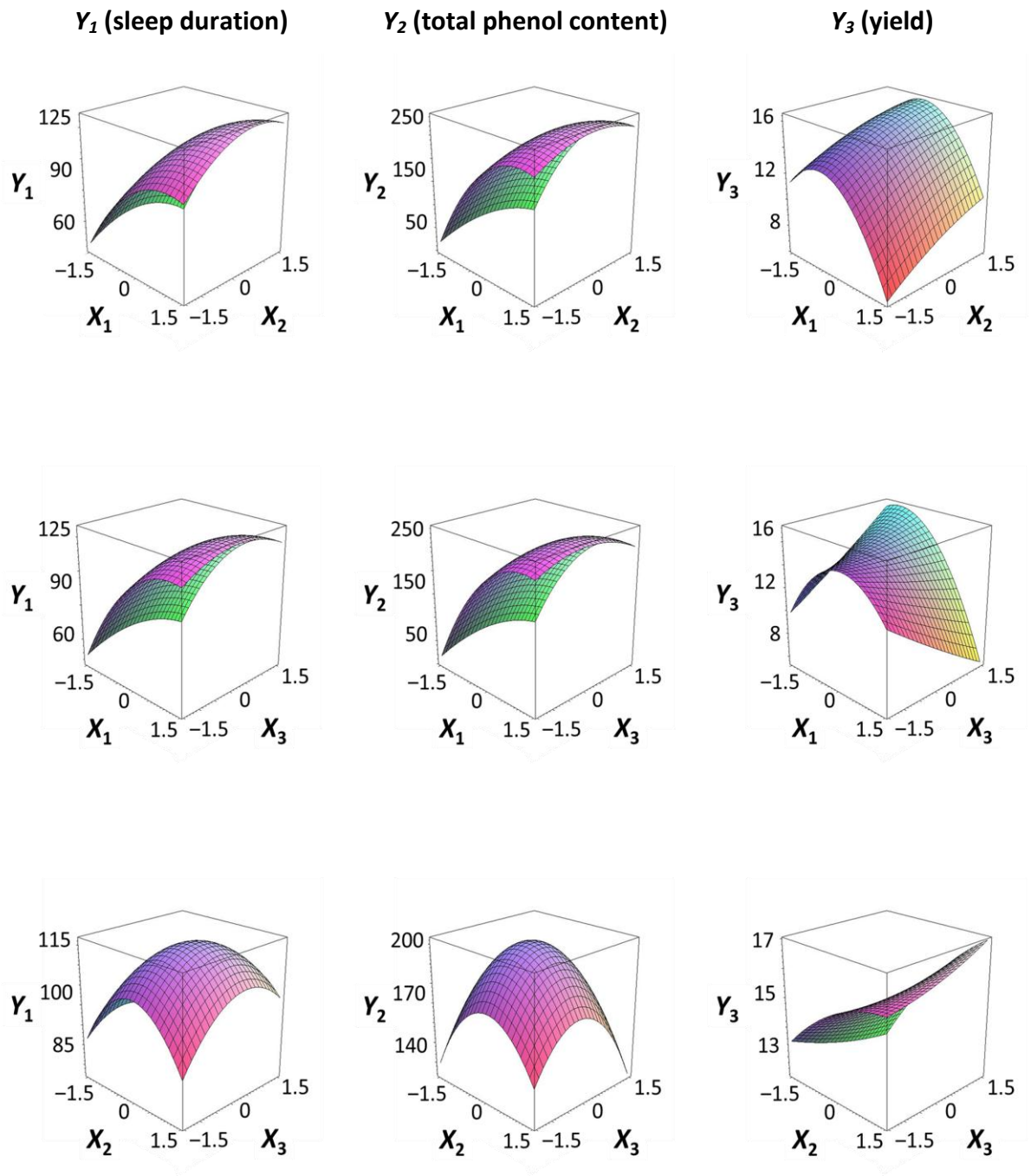
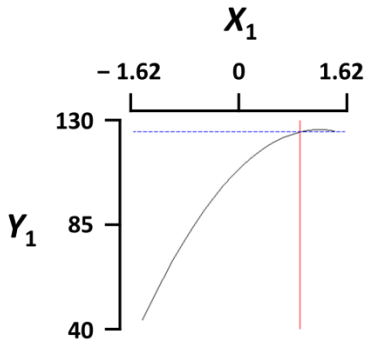
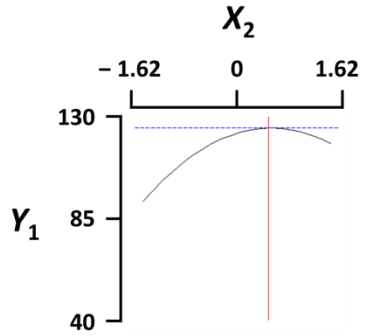
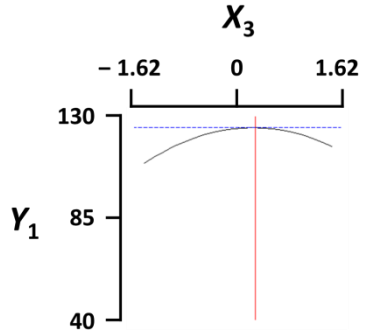


Fig. 2-4. Response surface 3D plots for the extraction of hypnotic compounds from EC (*Ecklonia cava*). X_1, ethanol concentration (%, v/v); X_2, extraction time (h); X_3, extraction temperature (°C). Y_1, sleep duration (min, at 500 mg/kg); Y_2, total phenol content (mg PGE/g); Y_3, yield (%, w/w).

Table 2-4. Optimal conditions and verification of the predicted response values for the extraction of hypnotic compounds from EC (*Ecklonia cava*)

Response			Y_1 (sleep duration at 500 mg/kg)	
Optimal conditions	X_1	Coded value	1.08	
		Actual value (%)	81.6	
	X_2	Coded value	0.57	
		Actual value (h)	52.2	
	X_3	Coded value	0.31	
		Actual value (°C)	43.7	
Predicted value of response Y_1				126.9
Experimental value of response Y_1				130.3

X_1, ethanol concentration (%, v/v); X_2, extraction time (h); X_3, extraction temperature (°C).

Values of Y_2 (TPC) and Y_3 (yield) at the optimal conditions were 237.8 mg PGE/g and 11.5%, respectively.

2.3.3. Effects of ECE on changes in sleep architecture and profile

***Evaluation of sleep-promoting effects by the analysis of sleep architecture and profile*:** The pentobarbital-induced sleep test is useful to evaluate the hypnotic effects, particularly, of a large number of test articles. However, the architecture and quality of sleep and adverse effects cannot be evaluated by this method. To better understand the hypnotic activity of ECE, its effects on sleep-wake regulation and profile were investigated by examining electroencephalogram (EEG) and electromyogram (EMG) recordings in mice. As mentioned in the Methods section, animals have NREMS and REMS. The sleep-wake states are generally characterized as follows: wakefulness (Wake), low-amplitude EEG and high-voltage EMG activity; NREMS, high-amplitude slow or spindle EEG and low-voltage EMG activity; and REMS, low-voltage EEG and EMG activity [**100**, **116**].

***Effects of ECE on sleep latency and the amounts of NREMS and REMS*:** The EEG and EMG signals in mice were recorded for 12 h after the oral administration of ECE (100–500 mg/kg) at 09:00 AM, and its effects were compared with the positive control DZP (2 mg/kg). **Fig. 2-5A** shows the representative EEG and EMG signals and corresponding hypnograms for vehicle, ECE, and DZP. As shown in **Fig. 2-5B**, ECE (250 and 500 mg/kg) significantly ($p < 0.01$) decreased sleep latency compared to vehicle. The administration of DZP also significantly ($p < 0.01$) decreased sleep latency, and was not a significantly different to ECE (500 mg/kg). The short sleep latency in mice administered ECE coincided with its result in the pentobarbital-induced sleep test, and indicates that ECE accelerated the initiation of NREMS. The total time spent in NREMS and REMS for the first 3 h after ECE or DZP administration was calculated (**Fig. 2-5C**). ECE (250 and 500 mg/kg) significantly ($p < 0.05$) increased the total amount of NREMS by 47.8% and 71.4%, respectively. The rate of increase in the amount of NREMS of DZP (2 mg/kg) was 103.8% ($p < 0.01$). However, ECE and DZP did not produce significant changes in the total amount of REMS. BZD agents, e.g., DZP, are known to increase NREMS without changing REMS [**117**, **118**].

***Effects of ECE on the time spent in each sleep stage*: Fig. 2-6** shows the time courses of the amounts of NREMS, REMS, and Wake for 12 h after the administration of ECE and DZP. After the injection of ECE, the amount of NREMS was immediately increased, and the amount of Wake was decreased. These effects of ECE were significant ($p < 0.05$) compared with vehicle for the first 2 h. There was no further significant disruption of sleep architecture during the subsequent period. These results indicate that ECE induce NREMS without causing adverse effects after sleep induction [**101**]. DZP also showed a significant difference ($p < 0.01$) for the first 2 h; however, during the subsequent period, its hourly amount of NREMS was higher than for ECE.

***Effects of ECE on the mean duration of each sleep stage and power density in NREMS*:** To better understand the sleep profile caused by ECE, the mean duration of each sleep stage and EEG power density in NREMS were calculated. ECE and DZP significantly ($p < 0.05$) decreased the mean duration of Wake by 54.0% and 58.8%, respectively; however, they did not affect the mean duration of NREMS and REMS (**Fig. 2-7A and B**). A decrease in the mean duration of Wake by ECE without affecting NREMS and REMS means that ECE decreased the maintenance of Wake [**101**]. ECE did not affect the EEG power density (0–20 Hz) in NREMS compared with vehicle (**Fig. 2-7C**), whereas DZP produced a significant ($p < 0.05$) decrease in delta (0.5–4 Hz) activity, as shown in the inset histogram of **Fig. 2-7D**. Delta activity is an indicator of the depth or intensity of NREMS [**116**, **119**]. The decrease in delta activity caused by DZP in humans and rodents has been reported previously [**118**, **120**]. BZD agents produce an increase in the quantity of sleep (sleep duration), but a decrease in the sleep quality (delta activity) [**116**, **121**]. DZP exerts an increase in beta activity (13–30 Hz), which is the highest EEG frequency generally associated with attention and arousal. Although BZD sleep drugs induce sleep, this increase in beta activity is their own property [**120**, **122**]. In this study, DZP also showed this typical increase in beta activity; however, ECE did not. In summary, ECE decreased sleep latency and increased the amount of NREMS, similar to DZP; however, it did not change the EEG power density, unlike DZP. These results suggest that ECE induces NREMS that is very similar to physiological sleep.

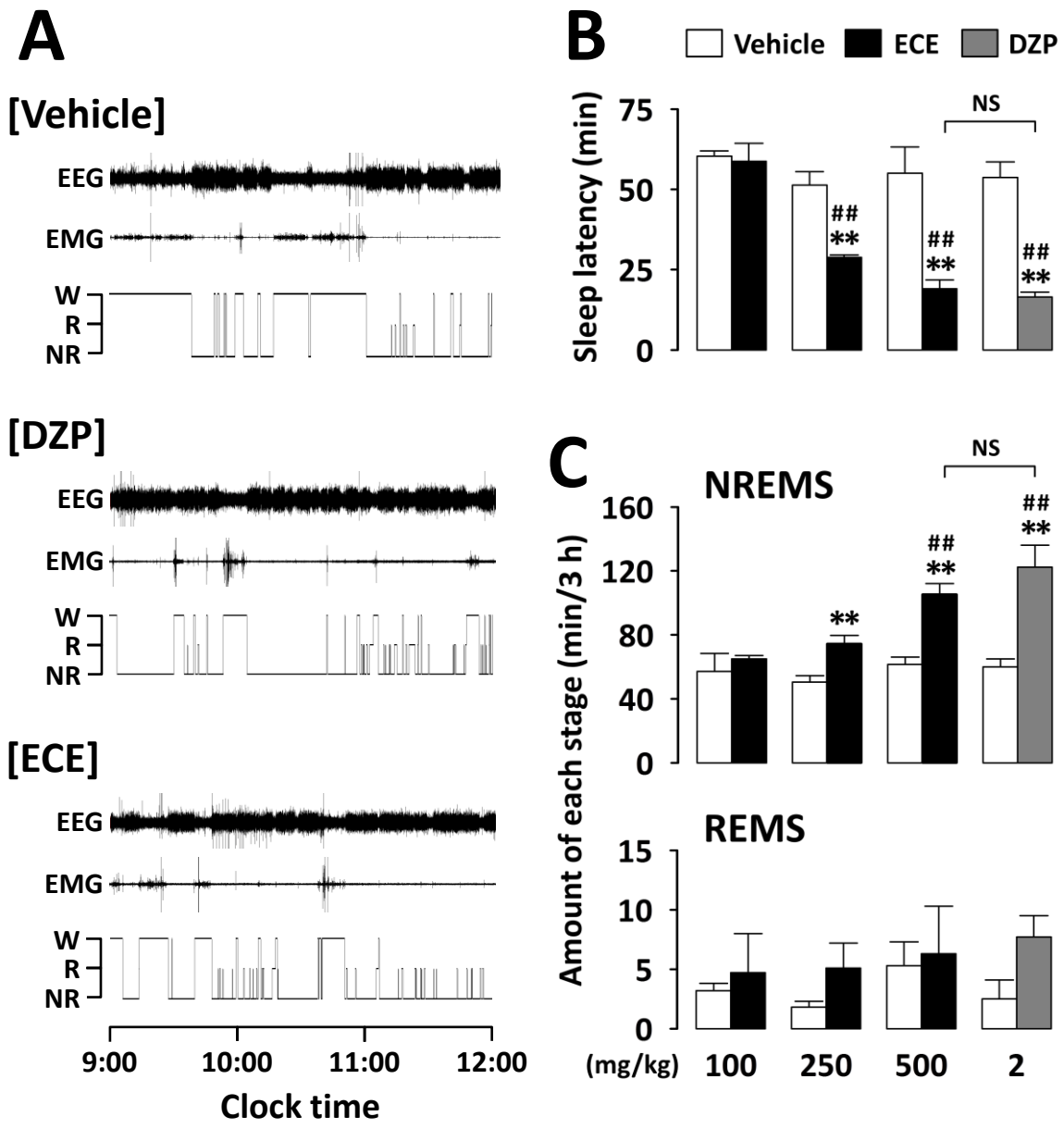


Fig. 2-5. (A) Representative examples of EEG and EMG signals and corresponding hypnograms in a mouse treated with vehicle, ECE, and DZP. (B) Effects of ECE and DZP on sleep latency. (C) Total time spent in NREMS and REMS for 3 h after administration. Each column represents the mean ± SEM (n = 8). **p < 0.01, compared with vehicle (unpaired Student's t-test). $^{\#\#}p$ < 0.01, significant as compared with ECE (100 mg/kg; Dunnett's test). Abbreviations: DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; EEG, electroencephalogram; EMG, electromyogram; NREMS (or NR), non-rapid eye movement sleep; NS, not significant; REMS (or R), rapid eye movement sleep; Wake (or W), wakefulness.

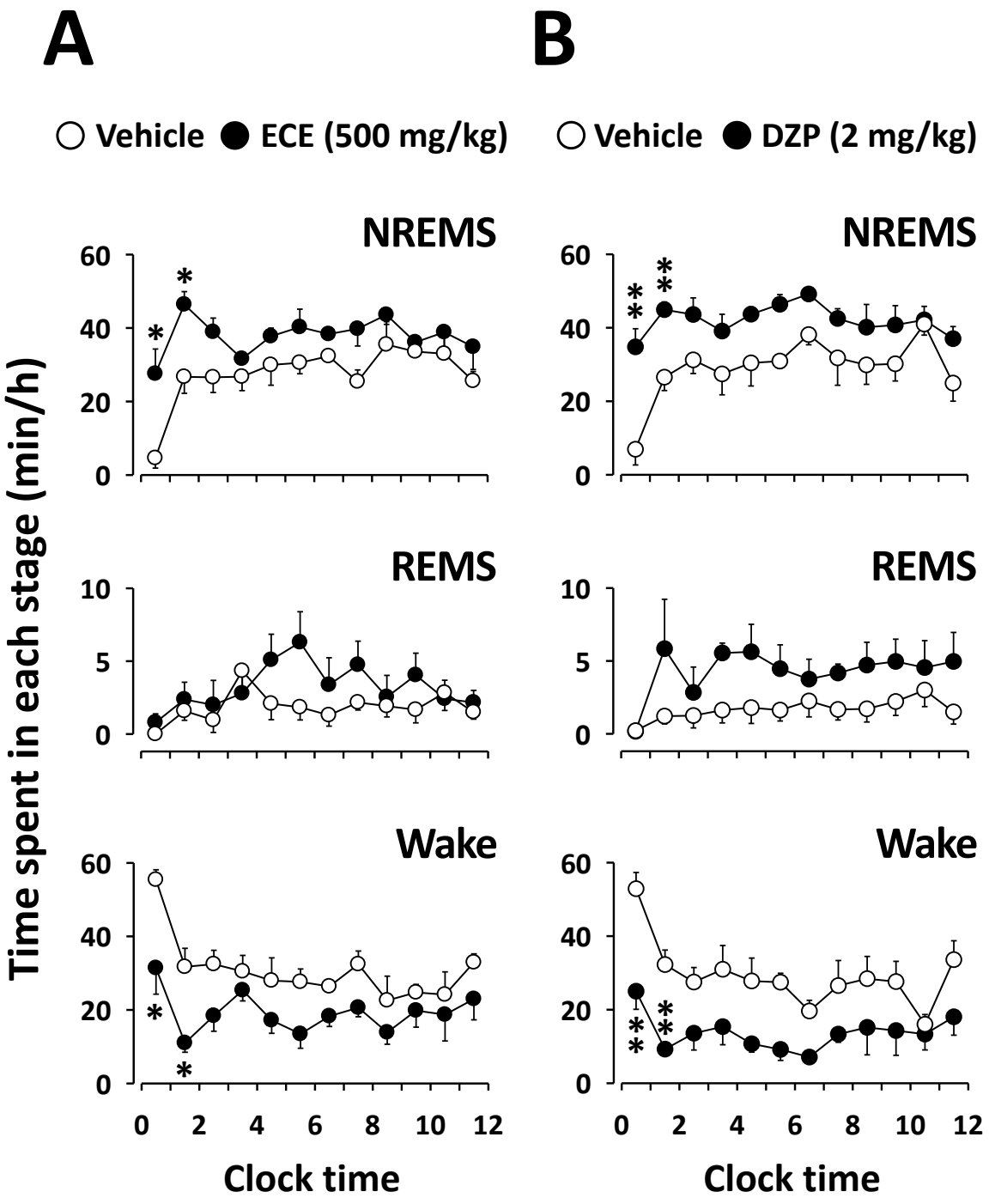


Fig. 2-6. Time courses of NREMS, REMS, and Wake after the administration of ECE (A) and DZP (B). Each circle represents the hourly mean ± SEM (n = 8) of NREMS, REMS, and Wake. *p < 0.05, **p < 0.01, compared with vehicle (unpaired Student's t-test). Abbreviations: DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

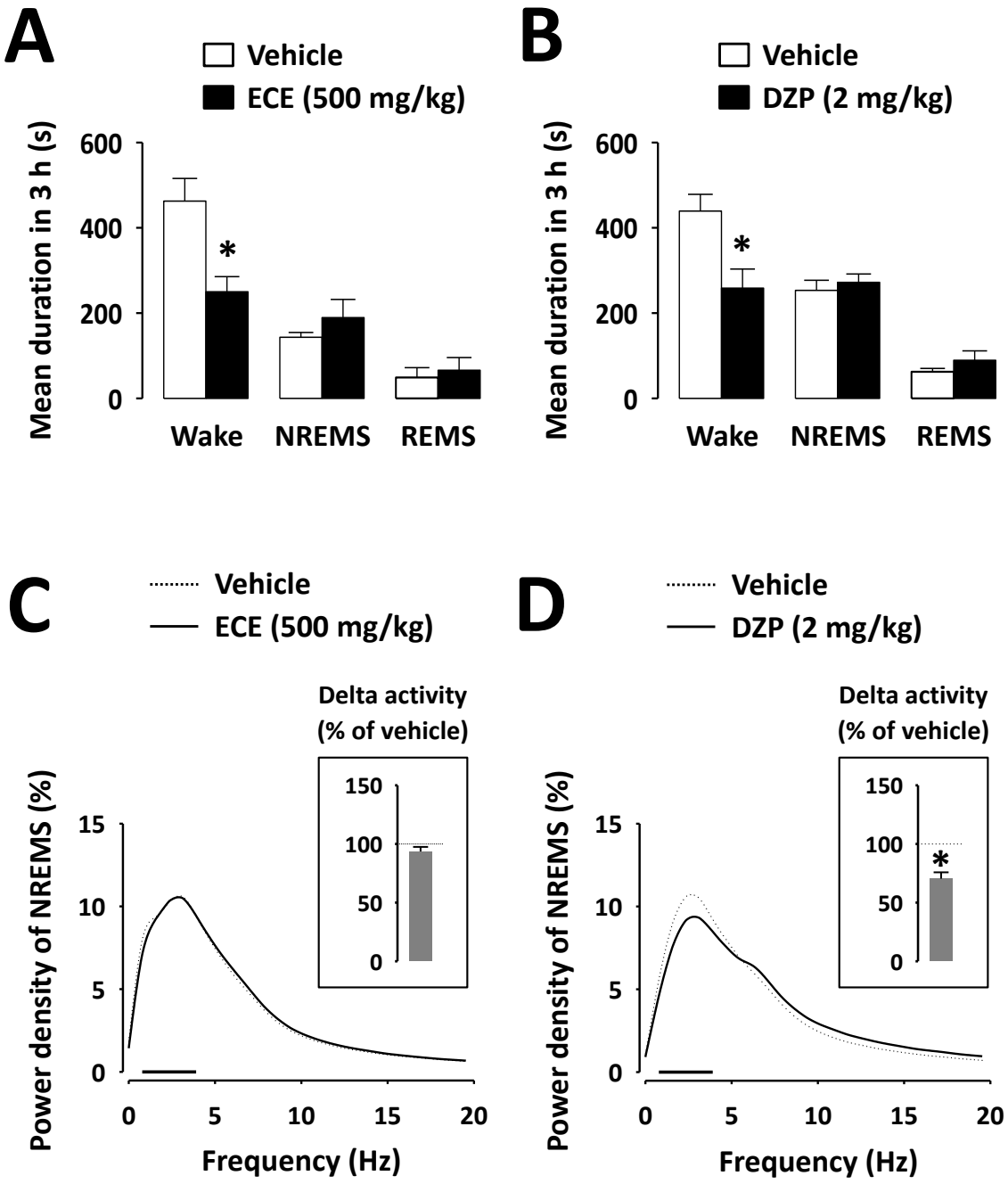


Fig. 2-7. Effects of ECE and DZP on changes in the mean duration of each sleep stage (A and B) and EEG power density in NREMS (C and D). Delta activity in NREMS, an index of sleep intensity, is shown in the inset histogram of (C) and (D). The bar (**—**) represents the range of the delta wave (0.5–4 Hz). *$p < 0.05$, compared with vehicle (unpaired Student's *t*-test). Abbreviations: DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; EEG, electroencephalogram; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

2.3.4. Verification of the in vivo GABAergic mechanism of ECE

Flumazenil (FLU) as an inhibitor of the $GABA_A$-BZD receptor agonist: Analysis of the changes in sleep latency and sleep duration in mice treated by pentobarbital can be a useful tool to investigate the influences of a compound on the GABAergic system [**123**]. The BZD (e.g., DZP and ZPD) and barbiturate (e.g., pentobarbital) binding sites of $GABA_A$ receptors are the targets for sedative-hypnotic agents that act as positive allosteric modulators [**35**]. BZDs and barbiturates are known to bind to 2 different $GABA_A$ receptor binding sites [**124**]. Although acting as a modulator, higher doses of barbiturates can directly activate $GABA_A$ receptors and induce sleep [**125**]. BZD and non-BZD agonists that act on $GABA_A$-BZD receptors are known to potentiate pentobarbital-induced sleep [**124**, **125**]. The specific $GABA_A$-BZD receptor antagonist FLU inhibits the hypnotic activity of $GABA_A$-BZD receptor agonists (DZP and ZPD) by blocking their binding to $GABA_A$ receptors [**35**].

Inhibition of the hypnotic effect of ECE by FLU: In order to verify the GABAergic mechanism of the hypnotic effect of ECE, the effects of ECE (500 mg/kg), DZP (2 mg/kg), and ZPD (10 mg/kg) co-administration with FLU (8 mg/kg) were tested (**Fig. 2-8**). Pretreatment with FLU alone did not affect the changes of sleep latency and sleep duration in mice treated with pentobarbital (45 mg/kg). As expected, FLU significantly ($p < 0.01$) inhibited the hypnotic effects of DZP and ZPD. The hypnotic activity of ECE was also fully ($p < 0.01$) antagonized by FLU. This result implies that ECE induces sleep via the GABAergic system, and its active compounds act as positive allosteric modulators of $GABA_A$-BZD receptors.

Synergic effects of ECE and the $GABA_A$-BZD receptor agonist: Lower doses of ECE (100 mg/kg), DZP (0.5 mg/kg), and ZPD (2.5 mg/kg) did not produce a significant prolongation of sleep duration in mice (**Fig. 2-9**). However, co-administration of ECE with DZP significantly ($p < 0.01$) increased sleep duration. The synergic effects of ECE and DZP were fully blocked by FLU. These inhibitory effects by FLU were also observed in sleep latency. These findings present additional evidence that ECE acts on the same binding site as DZP and ZPD.

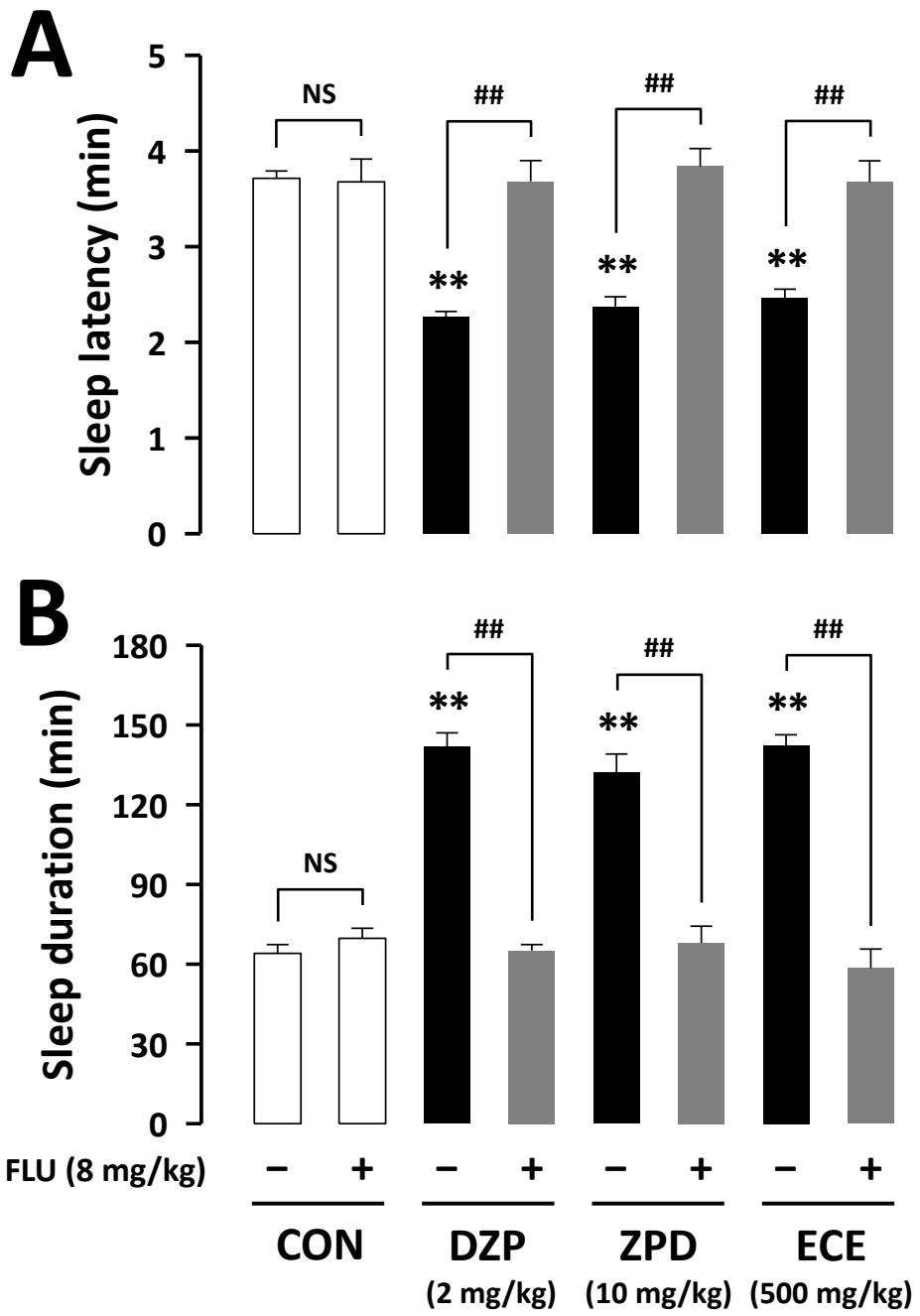


Fig. 2-8. Effects of FLU on the changes in sleep latency (A) and sleep duration (B) in mice treated with DZP, ZPD, and ECE. Mice received pentobarbital (45 mg/kg) at 45 min after the oral administration of the drugs. FLU was administered (i.p.) at 15 min before the oral administration of the drugs. Each column represents the mean ± SEM (n = 10). **p < 0.01, significant as compared to the control group (Dunnett's test). $^{\#\#}p$ < 0.01, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; FLU, flumazenil; NS, not significant; ZPD, zolpidem.

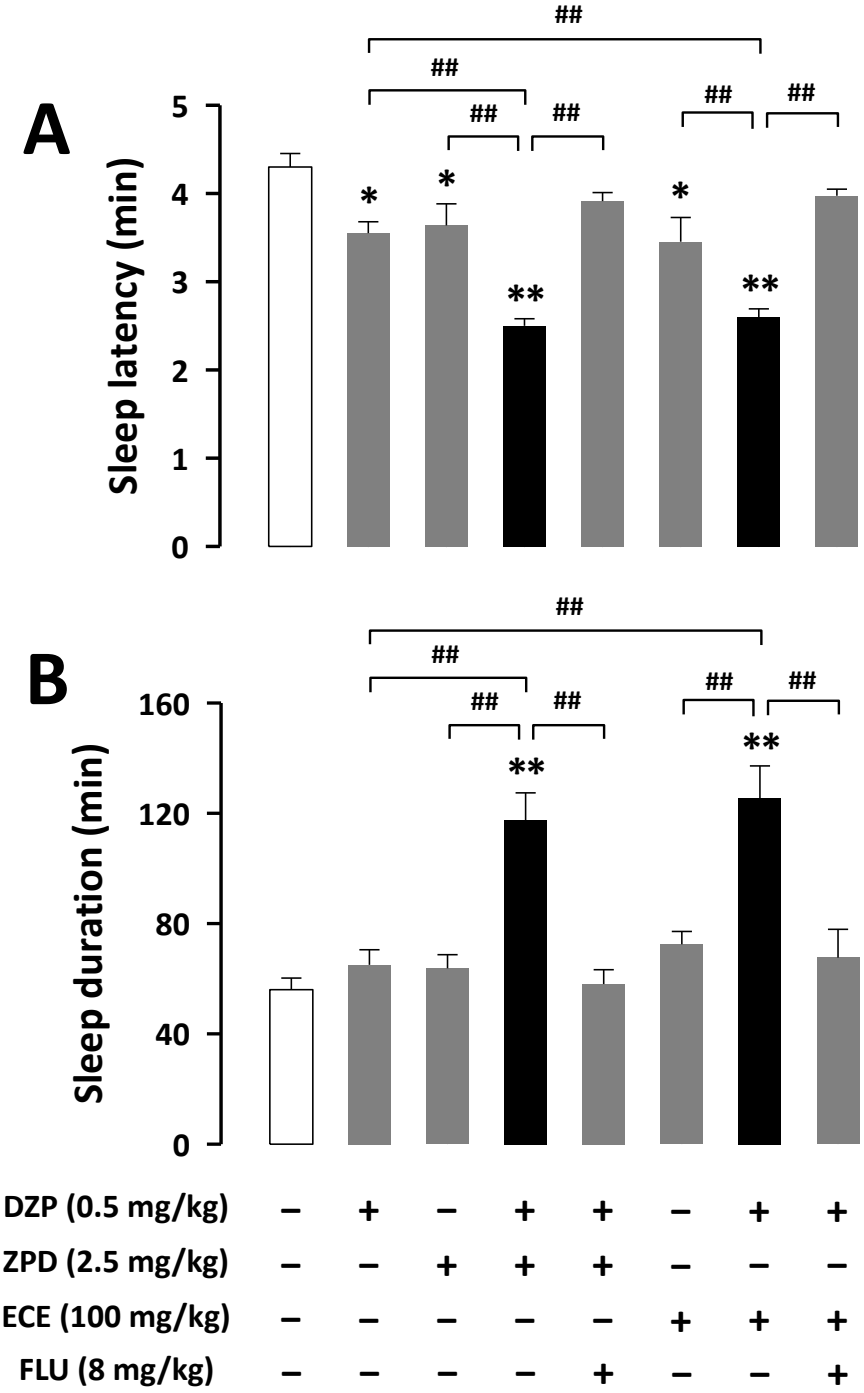


Fig. 2-9. Effects of the co-administration of DZP, ZPD, ECE, and FLU on sleep latency (A) and sleep duration (B) in mice. Mice received pentobarbital (45 mg/kg) at 45 min after the oral administration of ECE and DZP. FLU was administered (i.p.) at 15 min before the oral administration of the drugs. Each column represents the mean ± SEM (n = 10). *p < 0.05, **p < 0.01, significant as compared to the control group (Dunnett's test). $^{\#\#}p$ < 0.01, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: DZP, diazepam; ECE, *Ecklonia cava* ethanol extract; FLU, flumazenil; ZPD, zolpidem.

2.3.5. Isolation of active phlorotannins from ECE

Preparation of the ECE solvent fractions and their activity: To identify active compounds with hypnotic activity, ECE was fractionated with different solvents (**Fig. 2-10**). *n*-Hexane (HX, 10 g), ethylacetate (EA, 23 g), *n*-butanol (BT, 11 g), and H_2O (54 g) fractions were obtained from 98 g of ECE. The EA fraction was found to have the lowest IC_{50} value (0.019 mg/mL) to $GABA_A$-BZD receptors (**Fig. 2-11A**). The hypnotic effects of the ECE solvent fractions showed a similar tendency with their binding activity. The EA fraction (100 mg/mL) significantly ($p < 0.01$) increased sleep duration (**Fig. 2-11C**).

Correlation between TPC and activity in the ECE solvent fractions: According to previous reports on phlorotannins, phenols from brown seaweeds, most phlorotannin compounds were isolated from the EA fraction of brown seaweed extracts [**74, 126, 127**]. The TPC values for the EA, BT, HX, and H_2O fractions were 685.7, 262.6, 102.9, and 30.4 mg PGE/g, respectively. The binding activity and hypnotic effects of the ECE solvent fractions were found to be proportional to their TPC values. In the case of the binding activity, the R^2 value of the non-linear regression was 0.9544 (**Fig. 2-11B**). A good correlation (R^2 = 0.8396) were established between TPC and sleep duration (**Fig. 2-11D**). The hypnotic activity of the EA fraction was significantly ($p < 0.01$) blocked by FLU, similar to ECE (**Fig. 2-11E and F**). The characteristics of the EA fraction as a phlorotannin-rich fraction suggests that the hypnotic effects of ECE might be due to its phlorotannins.

Isolation of active phlorotannins: Six active phlorotannins were successfully isolated from the EA fraction of ECE using $GABA_A$-BZD receptor binding activity-guided fractionation (**Fig. 2-12**). Phlorotannins, which are oligomers and polymers of phloroglucinol (PG, 1,3,5-tri-hydroxybenzene), are an extremely heterogeneous group [**128**]. They are structurally different from the polyphenols of terrestrial plants, which are based on gallic acids or flavones [**128**]. Phlorotannins have only been found to exist within brown seaweeds [**74**], and EC contains more phlorotannins than other brown seaweeds [**98**]. The isolated phlorotannin compounds were eckstolonol (ETN), triphlorethol A (TPRA),

eckol (ECK), fucodiphlorethol G (FDRG), 6,6′-bieckol (BECK), and dieckol (DECK). The isolated phlorotannins were identified from their spectroscopic data including NMR, MS, and IR. ETN was identified as a trimer of PG, eckstolonol, which was polymerized through four ether bonds created by four hydroxyls and four olefin quaternary carbons of PG. TPRA was also identified as a trimer of PG, triphlorethol A, which was linked through two ether bonds generated between a hydroxyl at C-1 and an olefin quaternary carbon at C-2, and between a hydroxyl at C-1′ and an olefin quaternary carbon at C-2′′. ECK was also identified as a trimer of PG, eckol, which was linked through three ether bonds formed by three hydroxyls and three olefin quaternary carbons. FDRG was identified as a tetramer of PG, fucodiphlorethol G, which was formed by linking of TPRA and PG through an C-C bond between C-2′′ of a TPRA and C-1′′′ of a PG. BECK was identified as a hexamer of PG, 6,6′-bieckol, which was formed by the linkage of two ECKs through a C-C bond at C-6 of an eckol and C-6′ of another eckol. DECK was also identified as a hexamer of PG, dieckol, which was created by the linkage of two ECKs through an ether bond between OH-5 of an eckol and C-2′ of another eckol.

Binding affinity of EC phlorotannins to GABA$_A$-BZD receptors: To compare the relative potency of phlorotannins, the binding affinity (K_i) of DZP, a well-known GABA$_A$-BZD receptor ligand, was tested. Its K_i value was 0.012 μM. The range of K_i values for the phlorotannins was from 1.07–4.42 μM (ETN: 1.49, TPRA: 4.42, ECK: 1.07, FDRG: 2.97, BECK: 3.07, DECK 3.36 μM; **Fig. 2-12**). In the present study, phlorotannins were characterized, for the first time, as GABA$_A$-BZD receptor ligands. A number of flavonoids with binding affinity to GABA$_A$-BZD receptors have been isolated from terrestrial plants [**129**]. For example, 6-methylapigenin (*Valeriana wallichii*) [**130**] and hispidulin (*Artemisia herba-alba*) [**131**] were found to have K_i values of 0.5 and 8 μM, respectively. When considering the chemical structure of DZP, the phenyl ring is one of the most important structures to determine its binding affinity to GABA$_A$-BZD receptors [**129**]. The phenyl rings of phlorotannins may play an important role in their binding to L1, L2, and L3, which are characterized as agonist pharmacophores of GABA$_A$-BZD receptors [**132**]; however, a detailed study on the pharmacophore modeling of phlorotannins for GABA$_A$-BZD receptors is needed.

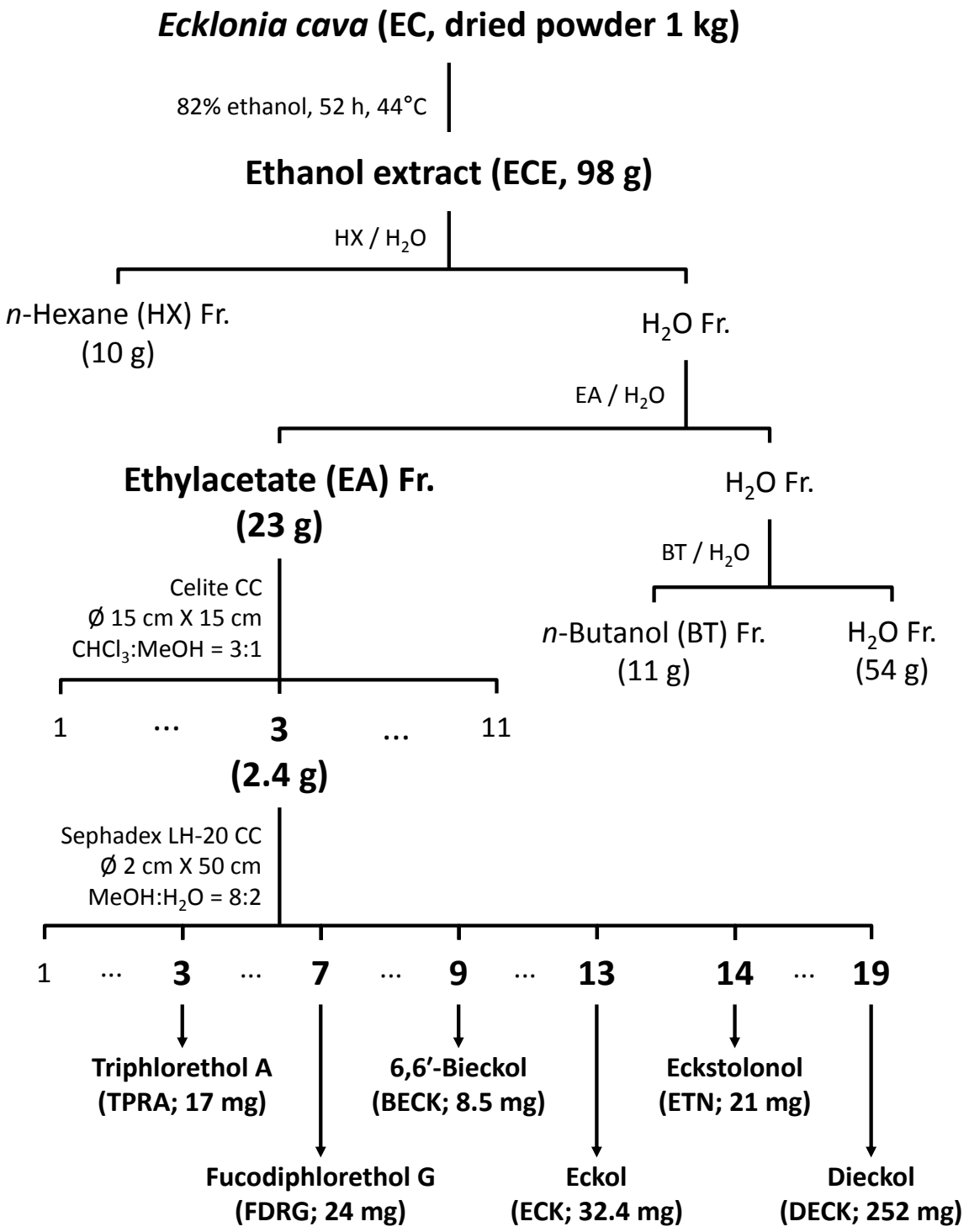


Fig. 2-10. Schematic overview for the fractionation and separation of active phlorotannins from ECE (*Ecklonia cava* ethanol extract).

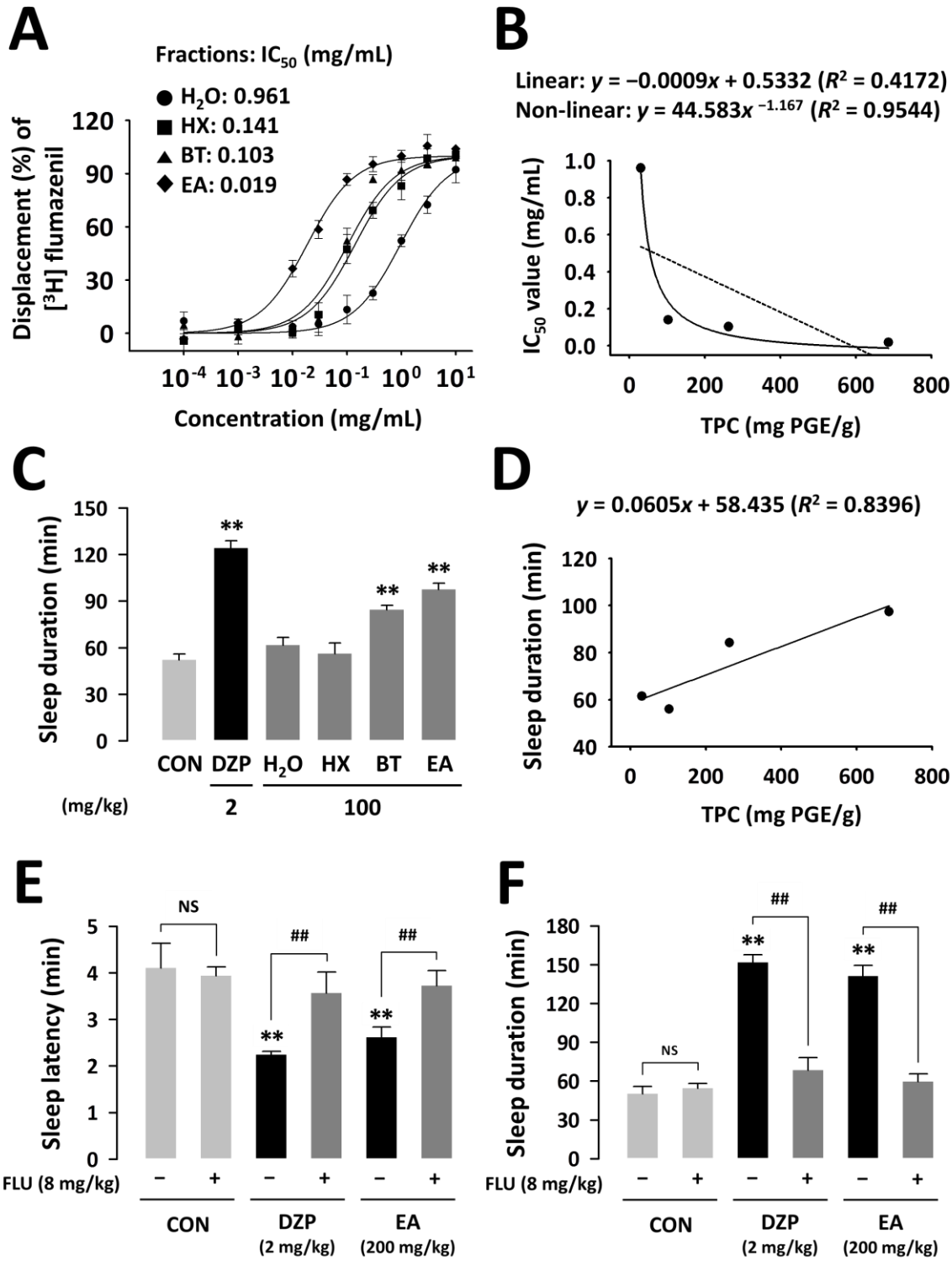
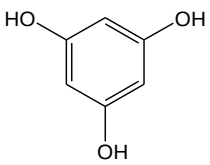


Fig. 2-11. Binding activity (A) of the ECE solvent fractions and its correlation with TPC (B). Effect of the ECE solvent fractions on sleep duration (C) and its correlation with TPC (D). Effects of FLU on the changes in sleep latency (E) and sleep duration (F) in mice treated with the EA fraction. Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); BT, *n*-butanol; DZP, diazepam; EA, ethylacetate; FLU, flumazenil; HX, *n*-hexane; NS, not significant; TPC; total phenol content.

Phloroglucinol (PG)

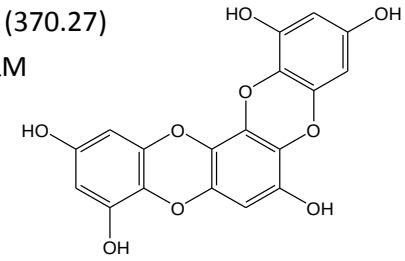
$C_6H_6O_3$ (126.11)



Eckstolonol (ETN)

$C_{18}H_{10}O_9$ (370.27)

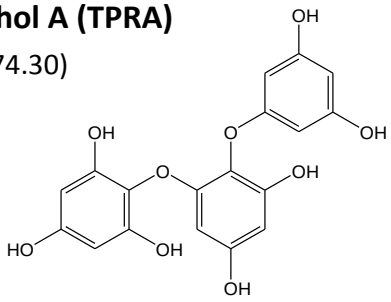
K_i: 1.49 μM



Triphlorethol A (TPRA)

$C_{18}H_{14}O_9$ (374.30)

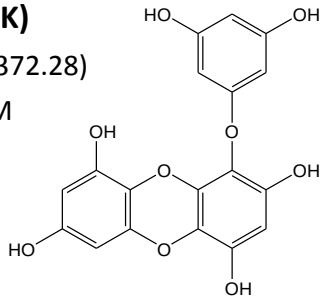
K_i: 4.42 μM



Eckol (ECK)

$C_{18}H_{12}O_9$ (372.28)

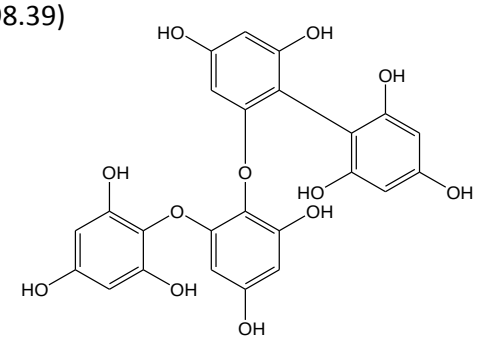
K_i: 1.07 μM



Fucodiphlorethol G (FDRG)

$C_{24}H_{18}O_{12}$ (498.39)

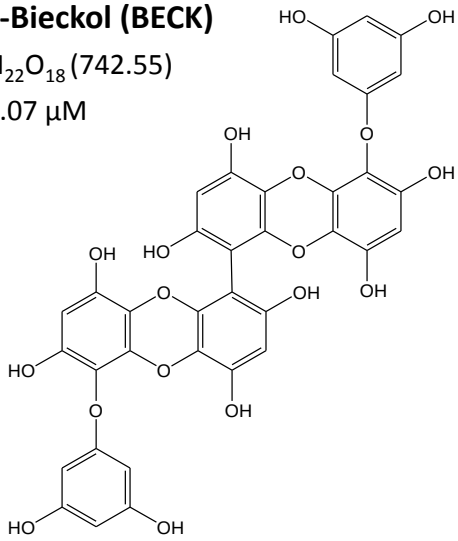
K_i: 2.97 μM



6,6'-Bieckol (BECK)

$C_{36}H_{22}O_{18}$ (742.55)

K_i: 3.07 μM



Dieckol (DECK)

$C_{36}H_{22}O_{18}$ (742.55)

K_i: 3.36 μM

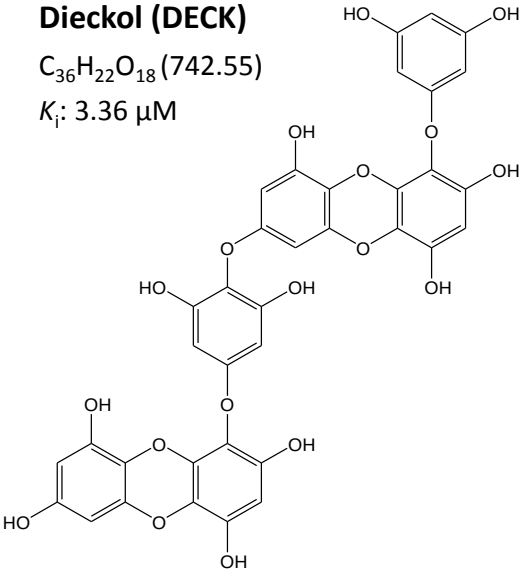


Fig. 2-12. Molecular structure, chemical formula (molecular weight), and binding affinity (K_i) to $GABA_A$-BZD receptors of the active phlorotannins isolated from ECE (*Ecklonia cava* ethanol extract). Phloroglucinol (PG) is shown as the basic unit of phlorotannins.

2.3.6. Hypnotic effects and in vivo mechanism of EC phlorotannins

Hypnotic effects of phlorotannins: Phlorotannins, the phenolics of brown seaweeds, have been extensively studied for their potential health benefits for the past 5 years [**133**]. Japanese and Korean researchers have mainly reported that phlorotannins exhibit various types of biological activity, e.g., antioxidant [**83**, **134**], anti-inflammatory [**126**], anticancer [**135**], anti-diabetic [**93**], and anti-allergic [**136**] effects. Their neurological properties, e.g., neuroprotective [**81**] and memory-enhancing [**82**] effects, were investigated; however, their hypnotic effect has not yet been reported. The hypnotic effect of individual phlorotannins was evaluated. The oral administration of all phlorotannins (5–50 mg/kg) increased sleep duration in a dose-dependent manner (**Fig. 2-13**), and decreased sleep latency (data not shown). Phlorotannins with a molecular weight under 400 (ETN, TPRA, and ECK) showed a better hypnotic effect than those with a molecular weight over 400 (FDRG, BECK, and DECK). The effects of ETN, TPRA, and ECK (50 mg/kg) on sleep duration were comparable to those of DZP (2 mg/kg) and ZPD (10 mg/kg). In this study, the hypnotic effect of PG was also evaluated because it is the basic unit of phlorotannins. Actually, during the isolation of active phlorotannins, PG showed very weak binding activity (42.25% inhibition at 1 mM). However, PG (5–50 mg/kg) potentiated pentobarbital-induced sleep in mice in a dose-dependent manner (data not shown), and showed even better activity than FDRG, BECK, and DECK. In addition, its hypnotic activity was fully inhibited by FLU (data not shown). These interesting results may be explained by its higher BBB permeability and bioavailability. Future studies are needed to demonstrate the detailed pharmacological properties of PG on $GABA_A$-BZD receptors.

Verification of the hypnotic mechanism of phlorotannins: The GABAergic mechanism of ECE and its EA fraction was demonstrated by the co-administration of the $GABA_A$-BZD receptor antagonist FLU. We tested whether the hypnotic phlorotannins use the same mechanism of action. The hypnotic effects of all phlorotannins (50 mg/kg) were significantly ($p < 0.01$) blocked by pretreatment with FLU (8 mg/kg), similar to the positive control DZP (**Fig. 2-14**).

Composition of active phlorotannins in the solvent fractions: In the ECE solvent fractions, there was a good correlation between TPC and sleep duration or binding activity. To find the relationship between phlorotannin content (i.e., the total amount of PG, ETN, TPRA, ECK, and DECK) and hypnotic activity, the phlorotannins were quantified using HPLC. The EA (367.42 mg/g) and BT (177.11 mg/g) fractions, which had higher hypnotic effects and binding activity, were found to contain higher levels of phlorotannins than the HX (20.49 mg/g) and H_2O (15.24 mg/g) fractions (**Table 2-5**). Phlorotannin content also had an excellent correlation with sleep duration (R^2 = 0.9682). DECK was the most abundant compound in the EA (274.73 mg/g) and BT (115.00 mg/g) fractions, while PG, ETN, and ECK were not detected in the HX and H_2O fractions.

Effects of ETN and TPRA on changes in sleep architecture and profile: ETN and TPRA, which have relatively better hypnotic activity that the other phlorotannins, were investigated further through the analysis of sleep architecture and profile. Their sleep-promoting effects were compared with the well-known sleep drug zolpidem (ZPD). The non-BZD agent ZPD is structurally different from the BZD agents; however, it acts on the same $GABA_A$-BZD receptor as the BZD agents (DZP) [**119**]. Currently, ZPD is the most widely prescribed sleep drug [**120**], and it is known to be a safer hypnotic than the BZD agents with regard to an absence of withdrawal effects, minimal rebound insomnia, and low tolerance to chronic administration relative to DZP [**119**, **137**]. According to previous reports, ZPD alters sleep architecture by promoting NREMS and reducing delta activity in NREMS, similar to DZP [**138**]. These typical characteristics of ZPD were also observed in the present study (**Fig. 2-15 and 16**). ETN and TPRA (50 mg/kg) significantly ($p < 0.01$) decreased sleep latency (**Fig. 2-15B**), and were not show significantly different to ZPD (10 mg/kg). ETN, TPRA, and ZPD significantly ($p < 0.01$) increased the total amount of NREMS by 54.8%, 52.8%, and 63.5%, respectively, during the first 3 h after administration (**Fig. 2-15C**). The amount of NREMS induced by ETN and TPRA was higher than that of vehicle for the first 2 h after administration; however, the higher amount of NREMS induced by ZPD compared to vehicle continued for 3 h (**Fig. 2-16A**). A significant decrease in the mean duration of Wake, which indicates the decreased maintenance of Wake, was observed for ETN, TPRA,

and ZPD (**Fig. 2-16B**). The changes in delta activity in NREMS are considered to be a physiological indicator of changes in the quality of sleep and the intensity of homeostatic sleep [**139**]. As expected, ZPD significantly ($p < 0.05$) decreased delta activity in NREMS (63.9% relative to the vehicle 100%), whereas neither ETN nor TPRA induced a significant change in delta activity (**Fig. 2-16C**). These results indicate that ETN and TPRA effectively induce NREMS without altering the architecture or profile of physiological sleep. A comparison of the effective concentration of ETN and TPRA indicated that they may function as partial $GABA_A$-BZD receptor agonists. The ideal hypnotic drug should show certain characteristics, e.g., rapid sleep induction and maintenance, and unaltered physiological sleep architecture [**138**]. Awareness of the side effects of BZD agents resulted in the development of non-BZD hypnotics [**139**]. Therefore, these results may suggest that the marine natural products ETN and TPRA could prove to be safer hypnotic or natural sleep aids with fewer side effects than BZD and non-BZD hypnotics. To utilize ETN and TPRA in this context, various investigations on the effects of their chronic administration are required.

Significance of the hypnotic phlorotannins: Natural products from medicinal plants have played an important role in drug discovery [**140**]. In particular, natural products from terrestrial plants have long been a traditional source of drug molecules [**141**]. However, during the past 2 decades, attention has been focused on marine natural products as a source of novel compounds [**142**, **143**]. The study of marine natural products as new anticancer agents from marine animals, e.g., coelenterates and sponges, has been a major focus of research [**141-144**]. In the present study, it was demonstrated, for the first time, that phlorotannins induced sleep via the positive allosteric modulation of $GABA_A$-BZD receptors. Phlorotannins, which are only found in brown seaweeds, have a different structure to phenolics isolated from land plants, which are based on gallic acids or flavones [**128**]. Brown seaweeds have been also consumed as a food in Asia including Japan and Korea. Therefore, the results of the present study show the promising potential of marine natural products as a source of safe and novel sleep drugs and natural sleep aids.

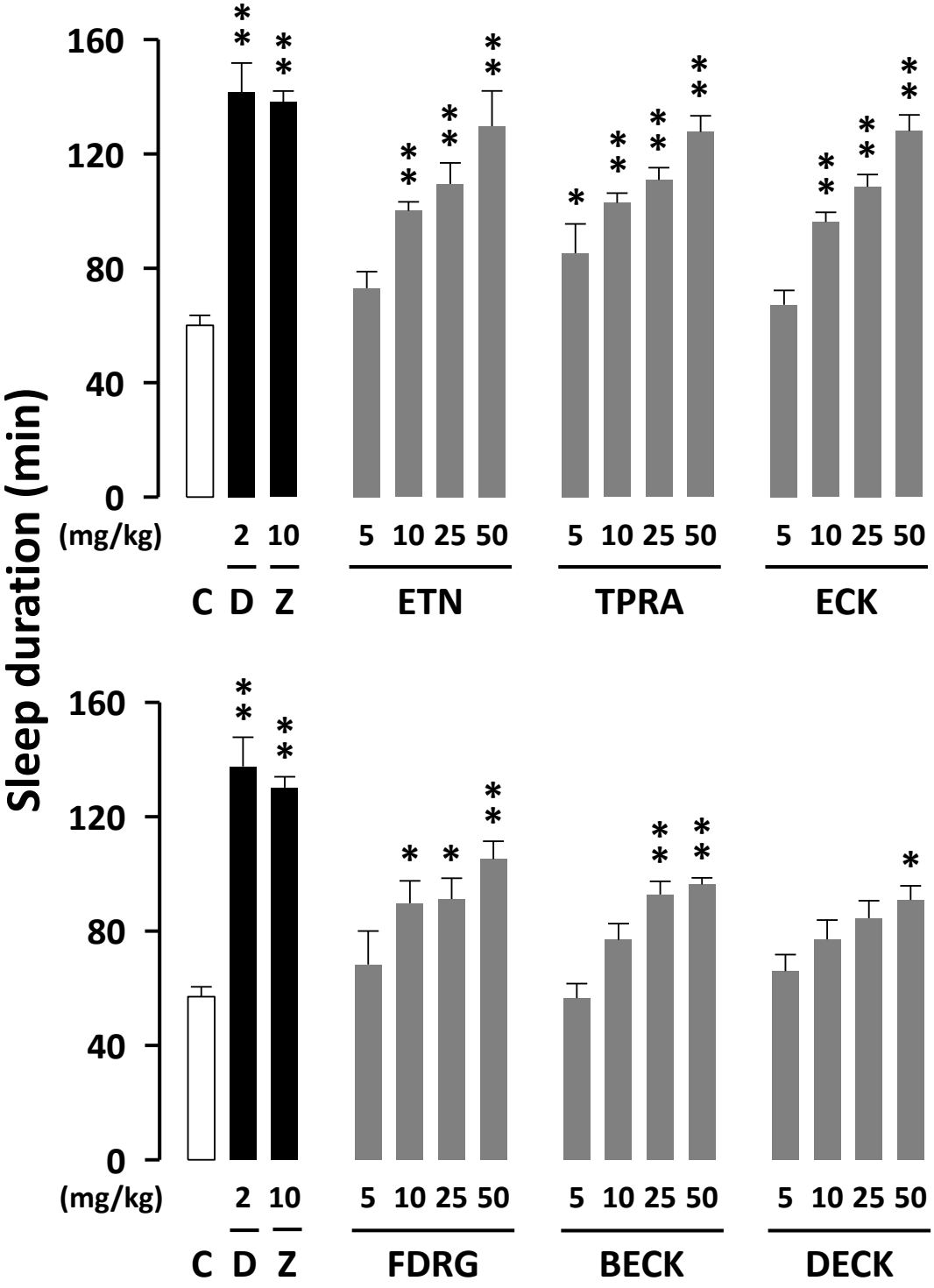


Fig. 2-13. Effects of phlorotannins on sleep duration in mice induced by pentobarbital (45 mg/kg). Each column represents the mean ± SEM (n = 10). *p < 0.05, **p < 0.01, significant as compared to the control group (Dunnett's test). Abbreviations: BECK, 6,6′-bieckol; C, control group (0.5% CMC-saline, 10 mL/kg); DECK, dieckol; D, diazepam; ECK, eckol; ETN, eckstolonol; FDRG, fucodiphlorethol G; TPRA, triphlorethol A; Z, zolpidem.

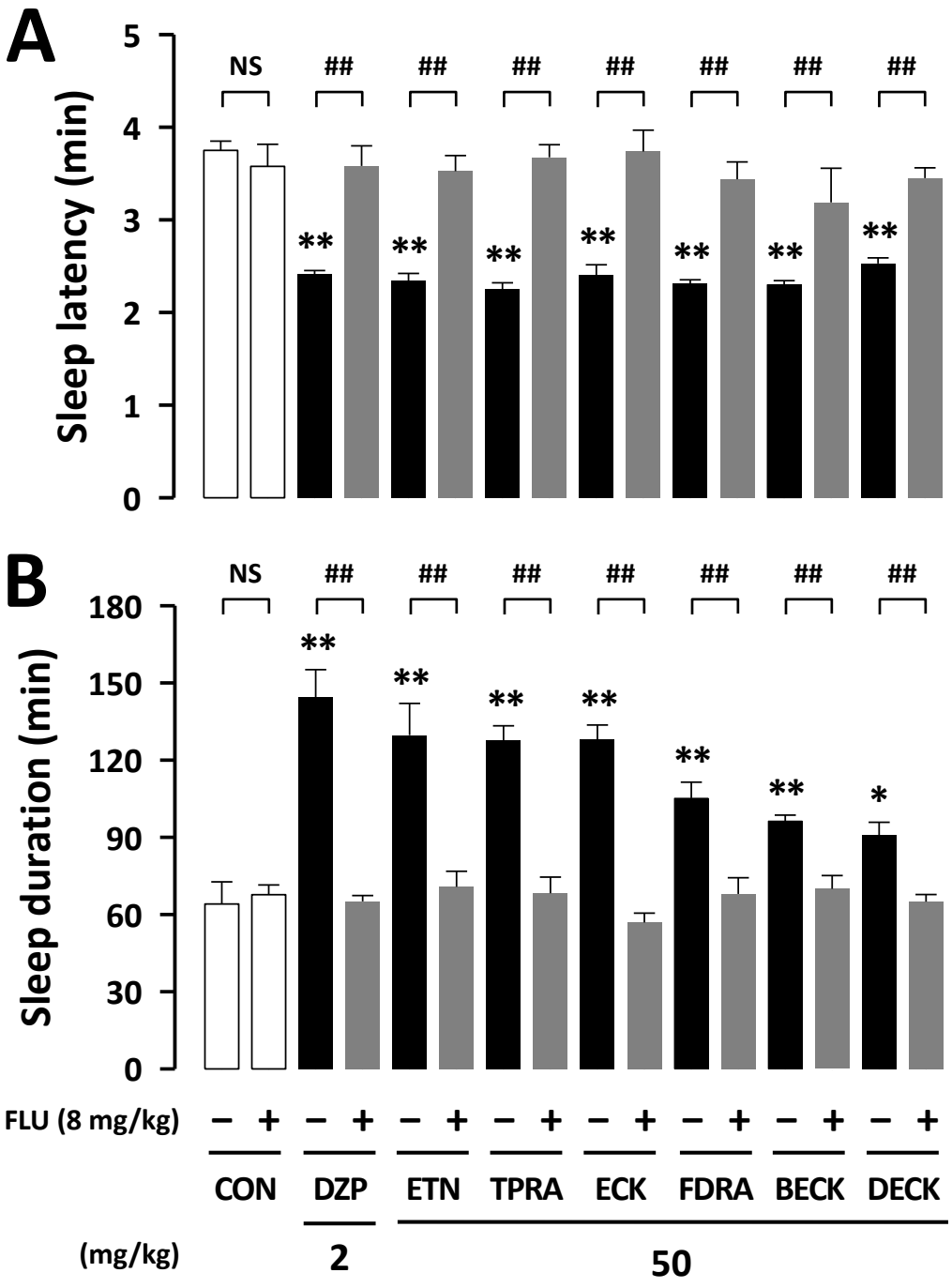


Fig. 2-14. Effects of FLU on the changes in sleep latency (A) and sleep duration (B) in mice treated with phlorotannins. FLU was administered (i.p.) at 15 min before the oral administration of the drugs. Each column represents the mean ± SEM (n = 10). *p < 0.05, **p < 0.01, significant as compared to the control group (Dunnett's test). $^{\#\#}p$ < 0.01, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: BECK, 6,6'-bieckol; CON, control group (0.5% CMC-saline, 10 mL/kg); DECK, dieckol; DZP, diazepam; ECK, eckol; ETN, eckstolonol; FDRG, fucodiphlorethol G; FLU, flumazenil; NS, not significant; TPRA, triphlorethol A.

Table 2-5. Phlorotannin composition (mg/g) of the solvent fractions from ECE (*Ecklonia cava* ethanol extract)

Constituents	H_2O	*n*-Hexane (HX)	*n*-Butanol (BT)	Ethylacetate (EA)
Phloroglucinol (PG)	-	-	-	16.14
Eckstolonol (ETN)	-	-	-	21.83
Triphlorethol A (TPRA)	9.15	11.19	24.56	21.46
Eckol (ECK)	-	-	37.55	33.26
Dieckol (DECK)	6.09	9.31	115.00	273.73
Total	15.24	20.49	171.11	367.42

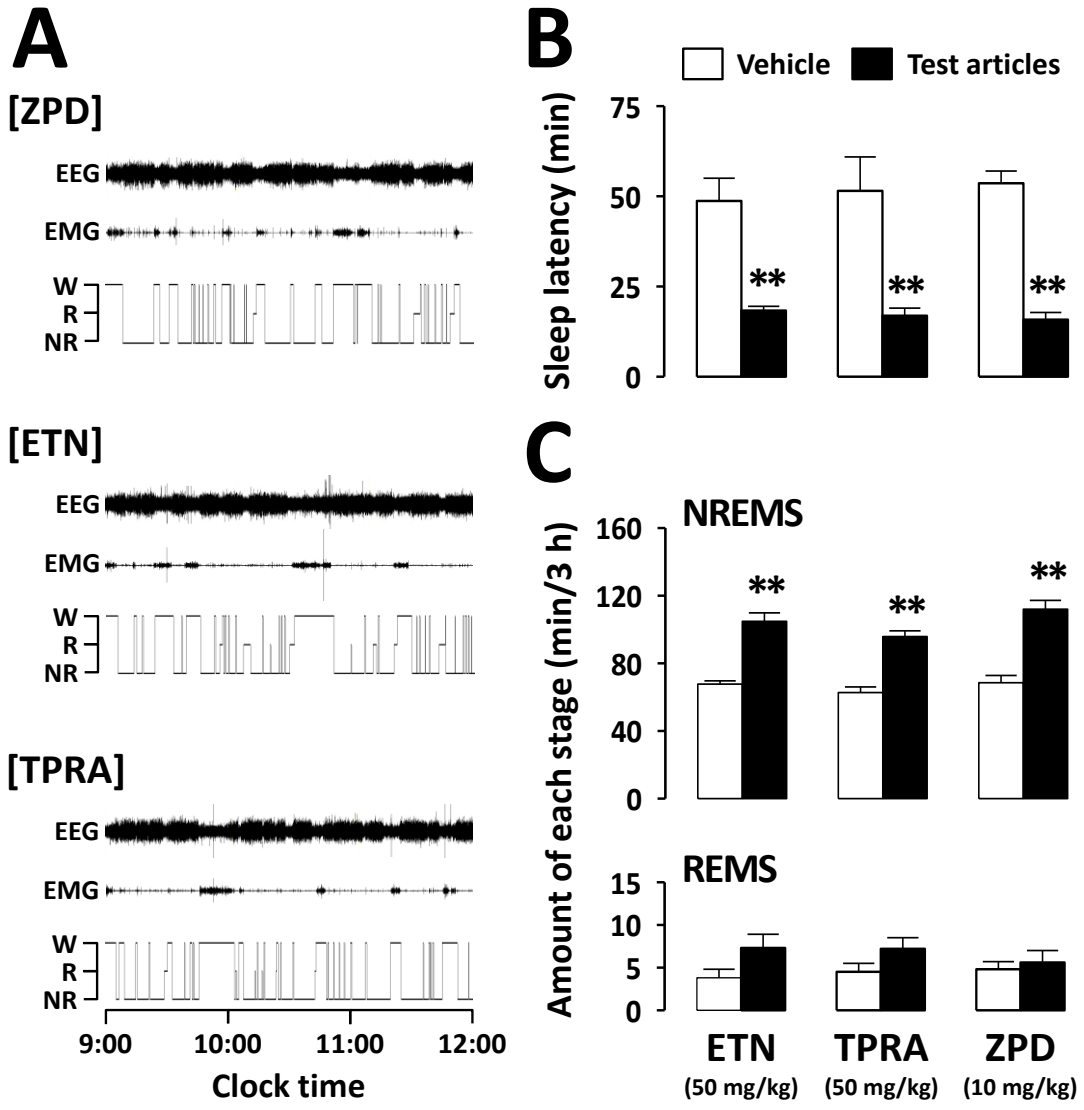


Fig. 2-15. (A) Representative examples of EEG and EMG recordings and corresponding hypnograms in a mouse treated with ETN, TPRA, and ZPD. (B) Effects of ETN, TPRA, and ZPD on sleep latency. (C) Total time spent in NREMS and REMS for 3 h after administration. Each column represents the mean ± SEM (n = 8). **p < 0.01, compared with vehicle (unpaired Student's t-test). Abbreviations: EEG, electroencephalogram; EMG, electromyogram; ETN, eckstolonol; NREMS (or NR), non-rapid eye movement sleep; REMS (or R), rapid eye movement sleep; TPRA, triphlorethol A; Wake (or W), wakefulness; ZPD, zolpidem.

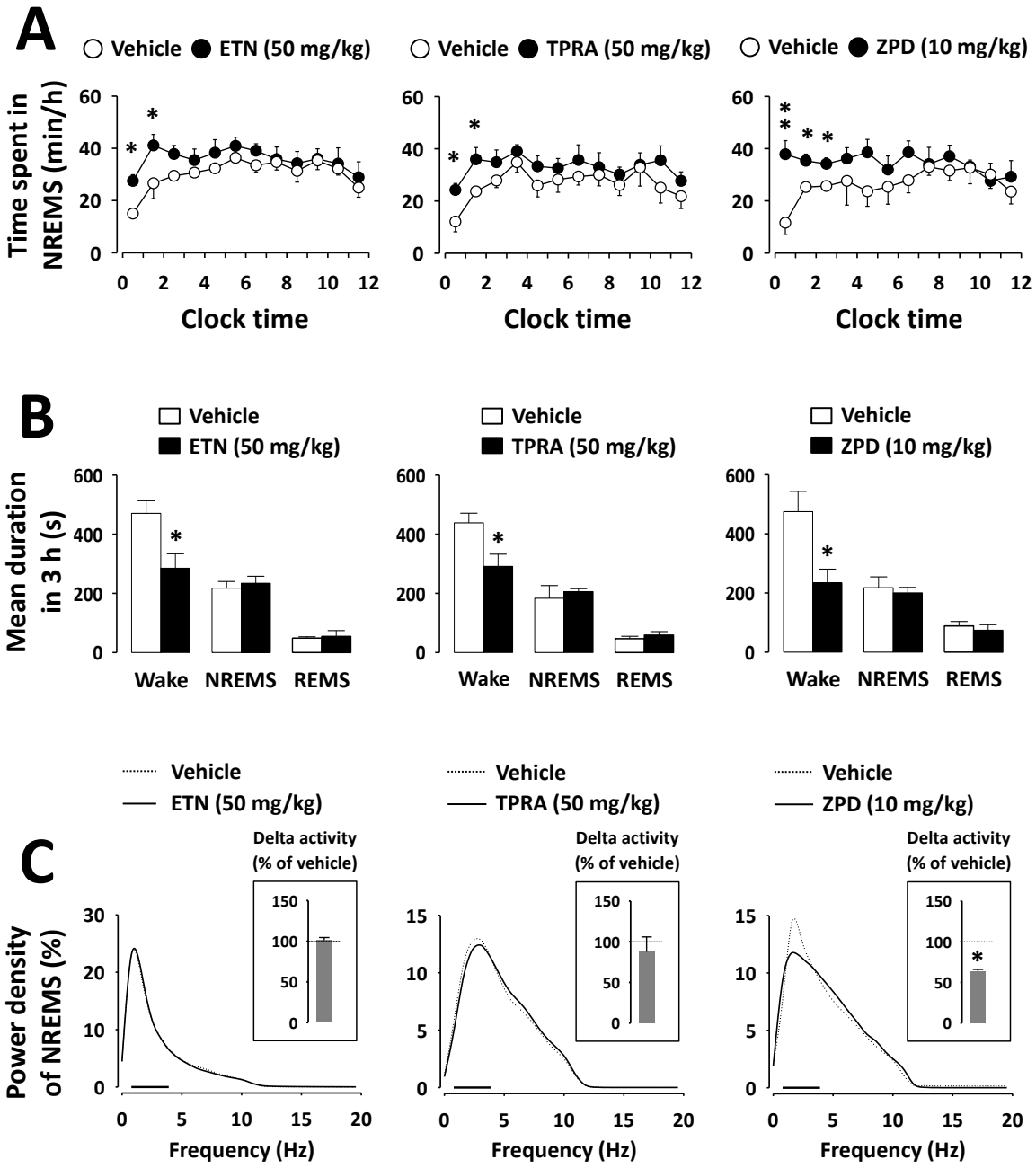


Fig. 2-16. (A) Time courses of NREMS, REMS, and Wake after the administration of ETN, TPRA, and ZPD. (B) Effects of ETN, TPRA, and ZPD on changes in the mean duration of each sleep stage. (C) EEG power density during NREMS for ETN, TPRA, and ZPD. Delta activity in NREMS, as an index of sleep intensity, is shown in the inset histogram. The bar (▬) represents the range of the delta wave (0.5–4 Hz). **p* < 0.05, ***p* < 0.01, compared with vehicle (unpaired Student's *t*-test). Abbreviations: EEG, electroencephalogram; ETN, eckstolonol; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; TPRA, triphlorethol A; Wake, wakefulness; ZPD, zolpidem.

2.3.7. Effects of EC phlorotannins on GABA-induced currents in neurons

The EC phlorotannins ETN and TPRA showed binding affinity to $GABA_A$-BZD receptors, and their hypnotic effects were inhibited by the $GABA_A$-BZD receptor antagonist FLU. These findings support the idea that their hypnotic effects should be attributed to the positive allosteric modulation of $GABA_A$-BZD receptors, similar to the well-known BZD agonists DZP and ZPD. To find additional evidence for the GABAergic mechanism of ETN and TPRA, their positive allosteric modulator action on $GABA_A$-BZD receptors was evaluated in neurons.

A large number of studies on the activation of $GABA_A$ receptors by BZDs via positive allosteric modulation have been performed using the expression of $GABA_A$ receptors in frog oocytes (*Xenopus*) or HEK cells. However, there is little evidence indicating that BZDs may induce sleep by inhibiting the neuronal activity of the arousal centers, e.g., the dorsal raphe (DR). For example, Trulson *et al*. [**145**] reported that the injection of the $GABA_A$-BZD receptor agonist DZP produces a decrease in the discharge rate of DR nucleus neurons at concentration-induced hypnotic states (>10 mg/kg). This study suggests that DZP may induce its hypnotic effect by inhibiting the activity of the DR nucleus. DR nucleus neurons fire tonically during wakefulness, decrease their activity during NREMS, and are almost quiescent during REMS [**146**]. The neuronal activity of the DR nucleus is depressed by tonic GABAergic inhibition in the lateral preoptic area and pontine ventral periaqueductal gray, and this GABAergic inhibition further increases during sleep [**147**]. Previous studies showed that GABAergic inhibition plays a pivotal role in the regulation of DR nucleus neurons' activity and that BZDs may induce their hypnotic effects by potentiating a GABA response in DR nucleus neurons. Therefore, we tested whether ETN and TPRA affect GABA-induced responses in acutely dispersed DR neurons.

The effects of GABA in DR neurons were tested using patch-clamp methods at a holding potential (V_H) of –50 mV and with GABA-evoked currents (I_{GABA}). I_{GABA} increased in a dose-dependent manner from 10^{-6} to 3.0×10^{-3} M. To examine the effects of ETN and TPRA, GABA (2.0×10^{-6} M) was

used to produce a control response, a concentration at which approximately 10% of the maximal value was obtained. As expected from the *in vivo* results, ETN and TPRA potentiated I_{GABA} in a concentration-dependent manner (**Fig. 2-17B**). Representative traces for the effects of ETN, TPRA, and DZP are shown in **Fig. 2-17A**. ETN and TPRA alone did not produce any inward current of DR neurons in the absence of GABA, as observed with DZP. This result means that ETN and TPRA do not act on the GABA binding site of $GABA_A$ receptors. The maximal potentiation values (P_{max}, %) of ETN and TPRA (10^{-8} M) were 145% and 171%, respectively. The P_{max} value of the full agonist DZP (3.0×10^{-7} M) was 266%. The relative efficacies of ETN and TPRA to DZP (100%) were 27.1% and 42.8%, respectively. Remarkably, TPRA showed potentiation (135%) of I_{GABA} at a lower concentration of 10^{-10} M, a concentration at which DZP did not produce activity. These results imply that in spite of its high affinity for $GABA_A$-BDZ receptors, TPRA acts as a partial agonist.

The usage of BDZs is curtailed by their side effects, e.g., next-day sedation, cognitive impairment, and amnesic effects [**148**]. Their long-term administration can also lead to the development of tolerance and dependence [**149**]. These side effects of BZDs have led to the screening of novel compounds that also act on $GABA_A$-BZD receptors [**138**]. During the past 10 years, partial agonists for $GABA_A$-BZD receptors have been widely developed. EVT-201 and NG2-73, which have the characteristics of partial $GABA_A$-BZD receptor agonists, are in phase II clinical trials [**150**]. In a phase II study in adults with primary insomnia, EVT-201 (1.5 and 2.5 mg) doses significantly increased total sleep time, and there was no subjective residual sedation [**150**]. NG2-73 (1 mg) produced a significant decrease in the latency to persistent sleep in a transient insomnia model in healthy volunteers [**151**]. ELB-138, which has an efficacy of 27–43% relative to the P_{max} of DZP, showed anticonvulsant effects [**152**]. These partial $GABA_A$-BZD agonists have a lower propensity to cause side effects, tolerance, and dependence. Therefore, the partial $GABA_A$-BZD agonists, ETN and TPRA might be of particular interest since they could be devoid of the side effects associated with the full agonist sleep drugs, e.g., DZP. In particular, the unique structure of the phlorotannins found only in brown seaweeds will be a source of novel sleep drugs.

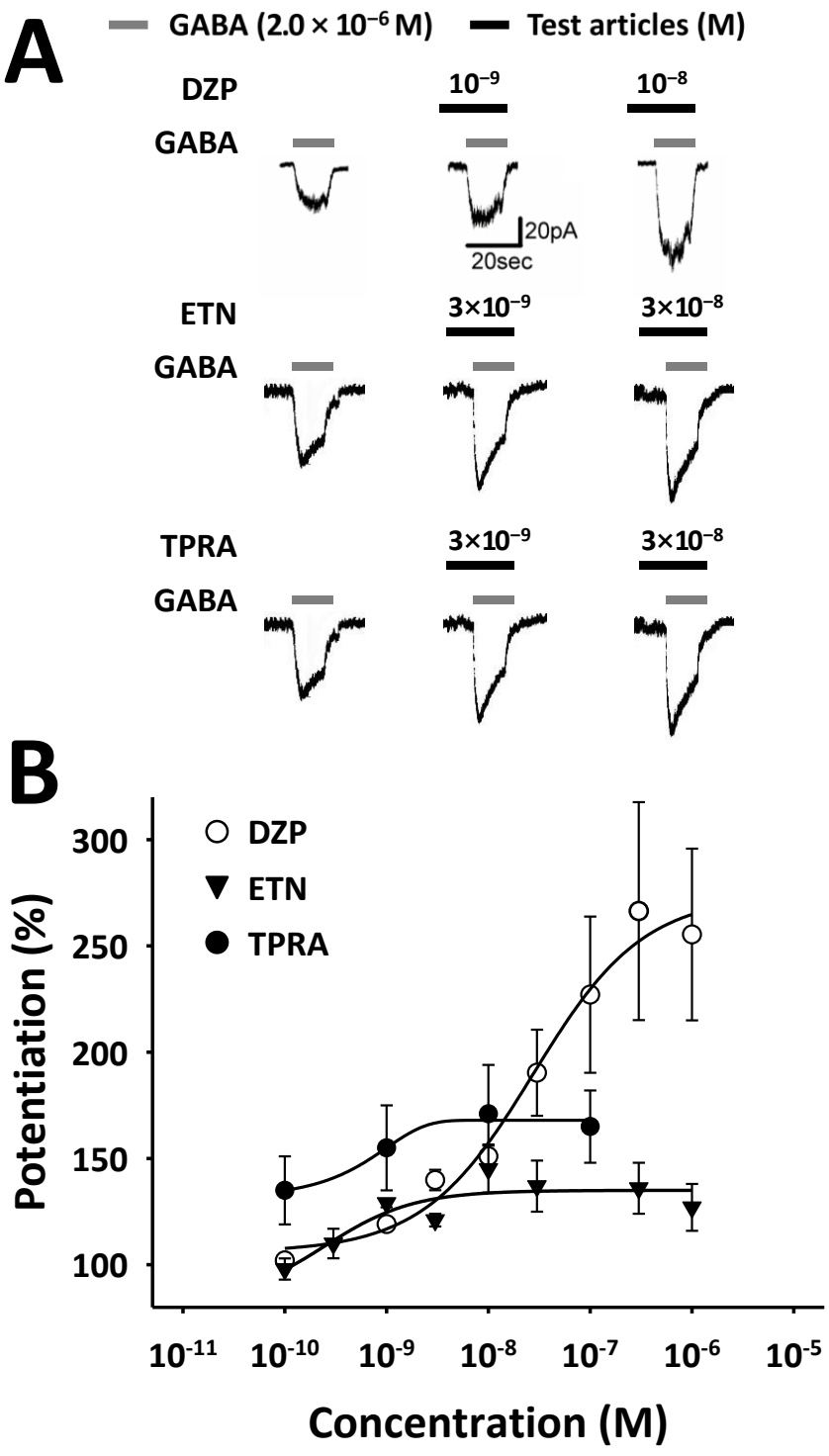


Fig. 2-17. (A) Representative traces for the effects of ETN, TPRA, and DZP on the EC_{10} GABA (2.0×10^{-6} M) response in DR neurons. (B) The dose-dependent potentiation of GABA-induced currents by ETN, TPRA, and DZP. The traces for ETN, TPRA, and DZP were taken from different set of neurons. Each point represents the mean ± SEM (5 or 6 neurons). Abbreviations: DZP, diazepam; ETN, eckstolonol; TPRA, triphlorethol A; DR, dorsal raphe.

CHAPTER 3: HYPNOTIC EFFECTS AND GABAERGIC MECHANISM OF LICORICE (*Glycyrrhiza glabra*) EXTRACT AND ITS FLAVONOIDS

ABSTRACT

Licorice (GG) was further investigated together with kajime (EC). GGE had a decisive role on sleep induction in mice treated with a sub-hypnotic dose of pentobarbital (30 mg/kg). To optimize GG extraction conditions for the highest hypnotic activity and to identify its active compounds, RSM was adopted. The optimal conditions were: ethanol concentration, 79.8%; extraction time, 12.0 h; and extraction temperature, 48.0°C. There was a high correlation between sleep duration and total flavonoid content (TFC). GGE (250 and 500 mg/kg) increased the amounts of NREMS by 47.8% and 71.5%, respectively, without changing in REMS or delta activity. Like ECE, GGE was effective during the first 2 h after administration, and showed sleep architecture and profile that were similar to those of physiological sleep. The hypnotic effect of GGE was fully inhibited by FLU, similar to DZP. This result demonstrated that GGE acts as positive allosteric modulator of $GABA_A$-BZD receptors. Glabridin (GBD), glabrol, and isoliquiritigenin (ILTG) were isolated as active compounds from GGE, and their binding affinities were 0.84, 1.63, and 1.07 μM, respectively. All GG flavonoids increased sleep duration and decreased sleep latency in a dose-dependent manner (5–50 mg/kg), and they were confirmed as the hypnotic compounds of GGE. Inhibition of their hypnotic effects by FLU was also observed, similar to GGE. Among the GG flavonoids, GBD and ILTG, which demonstrated a stronger hypnotic effect, were further studied using sleep architecture analysis and an electrophysiological test. GBD and ILTG (50 mg/kg) significantly increased the duration of NREMS and decreased the duration of Wake, similar to the positive control ZPD (10 mg/kg). However, they did not decrease delta activity, unlike ZPD. GBD and ILTG potentiated GABA-induced currents in DR neurons. ILTG acted as a partial $GABA_A$-BZD agonist (30.7 % of relative efficacy to DZP). Surprisingly, GBD showed a P_{max} value of 581% (3-fold higher that the P_{max} of DZP). This result means that GBD acts as a super $GABA_A$-BZD agonist, at least in DR neurons.

3.1. INTRODUCTION

In the screening stage for the terrestrial plants, GG showed the highest $GABA_A$-BZD receptor binding activity and *in vivo* hypnotic effect, and was then selected as a subject for further investigation. The root of GG (licorice; kanzo in Japanese; gamcho in Korean) is one of the most frequently used natural medicines in the world, and has been described as "the grandfather of herbs" [**77**, **153**]. GG has been used medically in Western and Eastern countries for more than 4000 years [**154**, **155**]. In particular, GG has been widely consumed as food and a herbal medicine in Japan and Korea [**156**]. GG reportedly contains numerous phytochemicals, e.g., triterpenoid saponins, flavonoids, chalcones, sterols, polysaccharides, and coumarins [**153**]. The triterpenoid saponin glycyrrhizin (also known as glycyrrhizic or glycyrrhizinic acid) is the major constituent of GG [**157**]. GG extract and its primary constituent glycyrrhizin are extensively consumed in the USA, and are considered as "Generally Recognized as Safe (GRAS)" for use in foods by the US Food and Drug Administration (FDA) [**158**, **159**].

The biological and pharmacological activity of GG has been widely studied as its long history, and its biologically active constituents are still attractive to many research groups [**156**]. A large number of clinical and experimental studies reported its useful biological properties, e.g., antioxidant, anticancer, immunomodulatory, cardioprotective, and anti-inflammatory effects [**153**]. Several studies reported its neurological activity. For example, Dhingra and Sharma [**160**] reported that the aqueous extract of GG produced antidepressant-like effects in the forced swim test and tail suspension test in mice. A GG extract also showed memory-enhancing effects in the plus-maze and passive avoidance paradigm [**161, 162**]. The anxiolytic [**163**] and anticonvulsant [**164**] effects of GG ethanol extract have been reported. However, the hypnotic effect and precise mechanism of GG and its active compounds have not yet been reported.

As was performed for EC, the extraction conditions for GG were optimized, and its hypnotic effects were evaluated using the animal model assays. The isolation of the active GG compounds and their hypnotic effects were also investigated using animal and neuron assays.

3.2. MATERIALS AND METHODS

3.2.1. Materials and animals

Licorice (*Glycyrrhiza glabra*) was purchased from a local oriental medicine market (Gyeongdong Market) in Seoul of Korea, and it was imported from Uzbekistan. Glabridin, glycyrrhizin, liquiritin (Wako Pure Chemical Industries, Osaka, Japan), liquiritigenin (Extrasynthese, Genay, France), isoliquiritigenin (Sigma-Aldrich Inc., St. Louis, MO, USA), and glycyrrhetinic acid (Shanghai Tauto Biotech Co., Ltd., Shanghai, China) were purchased for *in vitro* or *in vivo* assays. The materials and animals were described in Chapter 2 (**2.2.1, page 29**).

3.2.2. Evaluation assays

The $GABA_A$-BZD receptor binding assay (**2.2.3, page 29**), pentobarbital-induced sleep test (**1.2.5, page 15**), analysis of sleep architecture and profile (**2.2.6, page 30**), and electrical measurements (**2.2.10, page 35**) were described in Chapter 1 or 2.

3.2.3. Response surface methodology and statistical analysis

RSM and statistical analysis were described in Chapter 2 (**2.2.5, page 30**) and Chapter 1 (**1.2.6, page 15**), respectively.

3.2.4. Isolation and identification of active compounds from GGE

Isolation of active compounds from GGE was performed by the $GABA_A$-BZD receptor binding activity-guided fractionation, and the fractionation scheme is shown in **Fig. 3-9**. As the first step, flavonoid-rich fraction (FRF) with hypnotic activity was prepared from GGE. The powder of GGE (117 g) was suspended in H_2O (1 L), and the GG FRF (11.5 g) then extracted successively with EA-BT (3:1, 1 L × 2). The total flavonoid content (TFC) of the FRF was 252.7 mg QE/g. To isolate the hypnotic compounds, the FRF (11.5 g) was applied to a SiO_2 CC (Ø 8 × 20 cm) and eluted with HX-EA (10:1 → 5:1 → 3:1, 30 L of each). The eluting solutions were monitored by

TLC to produce 23 fractions (F1–F23). The fraction F18 [825 mg, V_e/V_t (elution volume/total volume) 0.82-0.86] was subjected to an ODS CC (Ø 5.0 × 13 cm) and eluted with MeOH-H_2O (5:1, 1.3 L), yielding 19 fractions (F18-1 to F18-19) including a purified compound 2 [glabrol, F18-11, 202 mg, V_e/V_t 0.35-0.36, TLC (ODS F_{254S}) R_f 0.60, MeOH-H_2O = 5:1]. The fraction F18-4 (22 mg, V_e/V_t 0.11-0.15) was purified using a Sephadex LH-20 CC (Ø 2 × 50 cm) eluting with 70% MeOH to ultimately produce a compound 1 [glabridin, F18-4-11, 8.7 mg, V_e/V_t 0.62-0.71, TLC (ODS F_{254S}) R_f 0.50, MeOH-H_2O = 5:1]. The FRF-22 (411 mg) was applied to an ODS CC (Ø 4 × 5 cm) and eluted with MeOH-H_2O (1:1→3:1, 800 mL of each) and produce 11 fractions (FRF-22-1–FRF-22-11). The fraction FRF-22-5 [7 mg, V_e/V_t 0.56-0.64] was subjected to a Sephadex LH-20 CC (Ø 2 × 50 cm) and eluted with 70% MeOH (450 mL), yielding 3 fractions (F22-5-1 to F22-5-3) including a purified compound 3 [isoliquiritigenin, F22-5-1, 4.4 mg, V_e/V_t 0.35-0.36, TLC (SiO_2 F_{254}) R_f 0.60, $CHCl_3$-MeOH = 10:1]. The structural elucidations of flavonoids isolated from GGE were summarized as follows.

Glabridin (GBD, compound 1): white powder (CH_3OH); m.p. 233-235°C; $[\ ]^{20}_D$ = -15° (*c*= 0.10, MeOH); EI/MS *m/z* 324 $[M]^+$; IR (KBr, *v*) 3380, 1606 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 6.88 (1H, d, *J*=8.4 Hz, H-2′), 6.78 (1H, d, *J*=8.4 Hz, H-5), 6.61 (1H, d, *J*=10.0 Hz, H-1″), 6.31 (1H, br s, H-5′), 6.26 (1H, d, *J*=8.4 Hz, H-6), 6.25 (1H, br d, *J*=8.4 Hz, H-3′), 5.57 (1H, d, *J*=10.0 Hz, H-2″), 4.31 (1H, dd, *J*=10.4, 5.2 Hz, H-2*eq*), 4.01 (1H, dd, *J*=10.4, 10.4 Hz, H-2*ax*), 3.40 (1H, m, H-3), 2.96 (1H, dd, *J*=15.6, 11.2 Hz, H-4*ax*), 2.77 (1H, dd, *J*=15.6, 4.8 Hz, H-4*eq*), 1.37 (6H, s, H-4″, 5″); ^{13}C-NMR (100 MHz, CD_3OD, δ_C) 157.86 (C-4′), 157.12 (C-6′), 152.85 (C-7), 150.89 (C-9), 130.17 (C-5), 129.58 (C-2″), 128.63 (C-2′), 119.80 (C-1′), 118.03 (C-1″), 116.03 (C-10), 110.88 (C-8), 109.39 (C-6), 107.52 (C-3′), 103.45 (C-5′), 76.43 (C-3″), 71.38 (C-2), 33.02 (C-3), 31.68 (C-4), 27.97 (C-4″), 27.84 (C-5″).

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

Isoliquiritigenin (ILTG, compound 3): yellow powder (CH_3OH); m.p. 209-210°C; EI/MS *m/z* 256 $[M]^+$; IR(KBr, *v*) 3343, 1629, 1598, 1451, 1367, 1235, 1120 cm^{-1}; ^{1}H-NMR (400 MHz, CD_3OD, δ_H) 7.98 (1H, d, *J*=8.8 Hz, H-6'), 7.76 (1H, d, *J*=15.2 Hz, H-α), 7.63 (1H, d, *J*=15.2 Hz, H-β), 7.61 (2H, d, *J*=8.8 Hz, H-2,6), 6.83 (2H, d, *J*=8.8 Hz, H-3,5), 6.42 (1H, dd, *J*=8.8, 2.4 Hz, H-5'), 6.27 (1H, d, *J*=2.4 Hz, H-3'); ^{13}C-NMR (100 MHz, CD_3OD, δ_H) 193.26 (C=O), 167.36 (C-4'), 166.34 (C-2'), 161.42 (C-4), 145.48 (C-β), 133.24 (C-6'), 131.71 (C-2,6), 127.71 (C-1), 118.22 (C-α), 116.81 (C-3,5), 114.55 (C-1'), 109.09 (C-5'), 103.72 (C-3').

3.2.5. Determination of total flavonoid content

The measurement of total flavonoid content (TFC) was based on the method described by Moreno *et al*. [**165**]. An aliquot of 1 mL of methanol solution (containing 1 mg of samples) was added to test tubes containing 0.1 mL of 10% aluminium nitrate, 0.1 mL of 1 M potassium acetate, and 3.8 mL of methanol. After incubation at room temperature for 40 min, the absorbance of test solutions was measured at 415 nm. TFC was calculated by using the calibration curve (11-point: 0-0.2 mg/L, $y = 14.409x + 0.0253$, $R^2 = 0.9993$) generated with quercetin (Sigma-Aldrich Inc., St. Louis, MO, USA) as a standard. The data were expressed as mg of quercetin equivalents (QE) per 100 g of the sample (mg QE/g).

3.2.6. Analysis of GG flavonoid constituents

Quantification of GBR, GBD, and ILTG in GGE is accomplished using a HPLC (Shimadzu, Tokyo, Japan) equipped with a UV detector (230 nm) and a Phenomenex LUNA 5u C18 column (5 µm, 250 mm x 4.6 mm). Acetonitrile and water with 0.1% formic acid were used as the solvent at a flow rate of 0.6 mL/min. The eluting solvent was programmed as follows: 65% for acetonitrile, increased to 70% at 10 min and 90% at 20 min, then 65% at 22 min. The sample was weighed 8 mg of the concentrated filtrate was dissolved in 1 mL of methanol, after which the sample was allowed to cool room temperature prior to HPLC analysis. Sample solutions (20 µL) were injected into the HPLC. In the calibration curve test, working standard solutions were prepared by diluting the stock solution of each flavonoid to obtain five different concentrations, and were analyzed in five replications. Their values of correlation coefficient (R^2) were over 0.99, and are indicative of an excellent linear relationship between HPLC peak area and concentration of each flavonoid.

3.3. RESULTS AND DISCUSSION

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

***Hypnotic dose of pentobarbital-induced sleep test*:** In the hypnotic dose (45 mg/kg) of pentobarbital-induced sleep test, GGE produced a decrease in sleep latency and an increase in sleep duration in a dose-dependent manner (100–1000 mg/kg) (**Fig. 3-1**). The hypnotic effects of GGE (>500 mg/kg) showed a statistically significant difference ($p < 0.01$) in sleep latency and sleep duration. The aqueous extract of GG, which was prepared at 100°C for 2 h, was evaluated; however, it did not exert a significant hypnotic effect (data not shown).

***Sub-hypnotic dose of pentobarbital-induced sleep test*:** In the hypnotic dose (30 mg/kg) of pentobarbital-induced sleep test, the administration of GGE also increased the rate of sleep onset and prolonged sleep duration (**Table 3-1**). It was found that GGE induces sleep in mice treated with a sub-hypnotic dose of pentobarbital.

Significance of the study on the hypnotic effect of GGE: In previous reports, GG showed anxiolytic [**163**] and anticonvulsant [**164**] effects in animal models; however, the active constituents and precise mechanism were not demonstrated. The extracts or compounds with anxiolytic and anticonvulsant activity may have the potential to produce a hypnotic effect [**37**, **43**]. In the present study, the major GG flavonoid glabridin was isolated as a hypnotic compound, and produced significant anxiolytic activity at >25 mg/kg in an elevated plus-maze test (data not shown). GG has also been used as an ingredient of suanzaorentang, which is a very famous traditional remedy for the treatment of insomnia in China, with *Zizyphus jujuba*, *Poria cocos*, *Ligusticum wallichii*, and *Anemarrhena asphodeloides* [**166**]. However, its hypnotic effect and mechanism of action have not yet been demonstrated. In Japan [**167**] and Korea [**168**], GG has played an important role in the traditional medicine system; therefore, studies on its active constituents and hypnotic mechanism have significance from the scientific and industrial viewpoints.

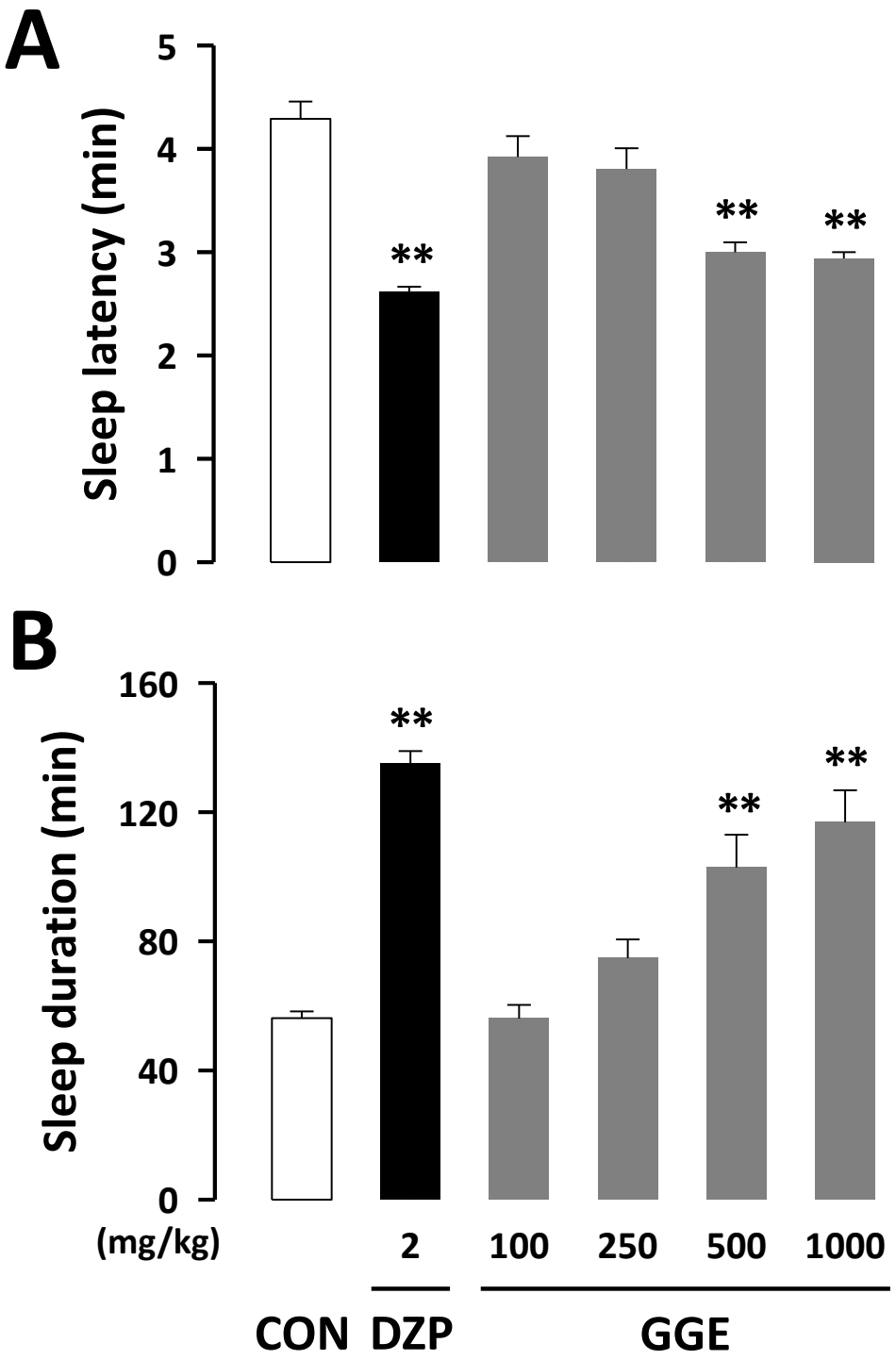


Fig. 3-1. Effects of GGE on sleep latency (A) and sleep duration (B) in mice induced by pentobarbital (45 mg/kg). Each column represents the mean ± SEM (n = 10). **p < 0.01, significant as compared to the control group (Dunnett's test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; GGE, *Glycyrrhiza glabra* ethanol extract.

Table 3-1. Effects of GGE on the rate of sleep onset and sleep duration in mice administered a sub-hypnotic dose (30 mg/kg, i.p.) of pentobarbital

Groups	Dose (mg/kg)	No. falling asleep / total	Rate of sleep onset (%)	Sleep duration (min)
CON		3 / 12	25	8.4 ± 6.2
DZP	2	12 / 12	100	50.7 ± 5.5 **
GGE	100	5 / 12	42	14.1 ± 4.3
	250	6 / 12	50	18.1 ± 5.7
	500	8 / 12	66	22.6 ± 4.8
	1000	10 / 12	83	33.1 ± 5.9 *

The rate of sleep onset (%) = no. falling asleep / total no. × 100. Sleep duration is expressed as the mean ± SEM. *$p < 0.05$, **$p < 0.01$, significant as compared to the control group (Dunnett's test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; GGE, *Glycyrrhiza glabra* ethanol extract.

3.3.2. Optimization of GG extraction conditions for hypnotic activity

Experimental design for the optimization of GG extraction conditions: On the basis of the results for the optimization of EC extraction, TPC was correlated with the hypnotic effects. In the subsequent separation step, the marine phenol phlorotannins were successfully isolated as active compounds. For the optimization of the GG extraction conditions, total flavonoid content (TFC) was monitored as an important factor together with sleep duration. Flavonoids have a range of activity on $GABA_A$-BZD receptors, and are one of the major natural sedative-hypnotic products [**35**, **49**, **110**, **129**, **169**]. In particular, flavonoids are well known as the major active compounds of ethanol extracts from licorice species [**153**, **170-172**]. The ranges and values for the IVs were the same as those for the optimization of EC extraction (**Table 2-1**).

***Response surface model equations*:** The DV (responses) values for the combination of IVs (factors) are given in **Table 3-2**. The ranges for the DVs Y_1 (sleep duration at 500 mg/kg), Y_2 (TFC), and Y_3 (yield) were 51.3–117.5 min, 7.4–32.2 mg QE/g, 3.6–25.4%, respectively (**Table 3-2**). The response surface model equations for the DVs Y_1, Y_2, and Y_3 were estimated from the response surface regression, as follows:

$$Y_1 = 92.548 + 17.056X_1 - 4.263X_2 + 4.445X_3 - 4.173X_1^2 - 1.079X_2^2 - 1.238X_3^2 - 1.700X_1X_2 + 1.250X_1X_3 - 0.225X_2X_3 \quad (R^2\text{: } 0.907, p\text{-value: } 0.007)$$

$$Y_2 = 10.640 + 5.718X_1 - 0.866X_2 - 0.398X_3 + 3.242X_1^2 + 0.106X_2^2 + 0.657X_3^2 - 1.481X_1X_2 - 0.544X_1X_3 - 0.971X_2X_3 \quad (R^2\text{: } 0.921, p\text{-value: } 0.001)$$

$$Y_3 = 21.673 - 4.069X_1 + 1.171X_2 + 0.803X_3 - 3.539X_1^2 - 0.410X_2^2 - 0.162X_3^2 - 0.038X_1X_2 + 1.263X_1X_3 - 0.038X_2X_3 \quad (R^2\text{: } 0.923, p\text{-value: } 0.004)$$

The R^2 values of Y_1, Y_2, and Y_2 were 0.907, 0.921, and 0.923, respectively, and were significant ($p < 0.01$). Unlike the optimization of EC extraction, the response model equation of the DV Y_3 (yield) was significant at the 95% probability level.

***The effects of IVs on DVs and response surface 3D plots*:** For all of DVs, the coefficients of the IV X_1 (ethanol concentration) were significant the 99% probability level (data not shown), and X_1 acted as the significant factor determining the hypnotic activity of GGE. As X_1 was increased from 20% (–1.682) to 95% (+1.682), the DV Y_1 (sleep duration) showed an increasing tendency (**Fig. 3-2**). This tendency was also observed between X_1 and Y_2 (TFC). The values of Y_1 in the experimental design were found to be proportional to Y_2 (R^2 = 0.554). The correlation value was not good; however, the potential of GG flavonoids as hypnotic compounds can be expected.

***Optimal extraction conditions*:** RSM has been successfully adopted to optimize flavonoid extraction conditions from various medicinal plants [**173-174**]. The rich flavonoids in the optimized extracts were associated with the biological activity of the extracts. In the present study, the R^2 values of the model equations were also statistically significant, and correlations between IVs and DVs or IVs and IVs were successfully demonstrated. For the preparation of GGE with the highest hypnotic effect, the heuristically estimated optimal conditions of X_1 (ethanol concentration), X_2 (extraction time), and X_3 (extraction temperature) were 79.8%, 12 h, and 48.0°C, respectively (**Table 3-3**). In previous reports, Gong *et al*. [**173**] and Xiao *et al*. [**174**] obtained 79.7% and 86.2% optimal ethanol concentrations, respectively. The predicted value of Y_1 (sleep duration) at the optimal conditions was 115.9 min, while those of Y_2 (TFC) and Y_3 (yield) were 25.9 mg QE/g and 12.3%, respectively.

***Glycyrrhizin content of the optimized GGE*:** Glycyrrhizin, the principle component of licorice, has been widely used as a sweetening agent in foods and a primary active compound of licorice herbal medicines [**158, 175**]. The licorice extracts and glycyrrhizin are affirmed as "GRAS" for use in foods by the US FDA [**158**]. However, chronic consumption of high amounts of glycyrrhizin may pose a risk for hyperkalemia in humans [**175**]. Glycyrrhizin generally constitutes 10–25% of the licorice root extract [**158, 176**]; however, the optimized GGE contained only 3.4% glycyrrhizin. The licorice ethanol extracts contained less glycyrrhizin than the water extracts [**158**]. When comparing the safety limit (2 $mg \cdot kg^{-1} \cdot day^{-1}$) of glycyrrhizin reported by Van Gelderen *et al*. [**177**], the adverse effects of glycyrrhizin in the optimized extract is thus considered negligible.

Table 3-2. Central composite design matrix and response values for extraction of hypnotic compounds from GG (*Glycyrrhiza glabra*)

Run order	Coded level			Response		
	X_1	X_2	X_3	Y_1	Y_2	Y_3
Factorial portion						
1	−1	−1	−1	69.3	9.8	20.3
2	1	−1	−1	90.9	17.9	11.4
3	−1	1	−1	57.6	9.5	21.0
4	1	1	-1	91.8	17.6	11.7
5	−1	−1	1	69.7	9.8	20.0
6	1	−1	1	115.7	25.9	15.9
7	−1	1	1	76.5	11.5	20.3
8	1	1	1	96.3	15.8	16.3
Axial portion						
9	−1.682	0	0	51.3	7.4	21.0
10	1.682	0	0	117.5	32.2	3.6
11	0	−1.682	0	103.5	11.7	16.9
12	0	1.682	0	82.8	9.9	25.4
13	0	0	−1.682	89.1	13.2	21.0
14	0	0	1.682	96.3	11.5	22.7
Center portion						
15	0	0	0	94.7	10.3	21.1
16	0	0	0	91.3	11.4	22.0
17	0	0	0	90.4	10.2	21.7

X_1, ethanol concentration (%, v/v); X_2, extraction time (h); X_3, extraction temperature (°C).

Y_1, sleep duration (min, at 500 mg/kg); Y_2, total flavonoid content (mg QE/g); Y_3, yield (%, w/w).

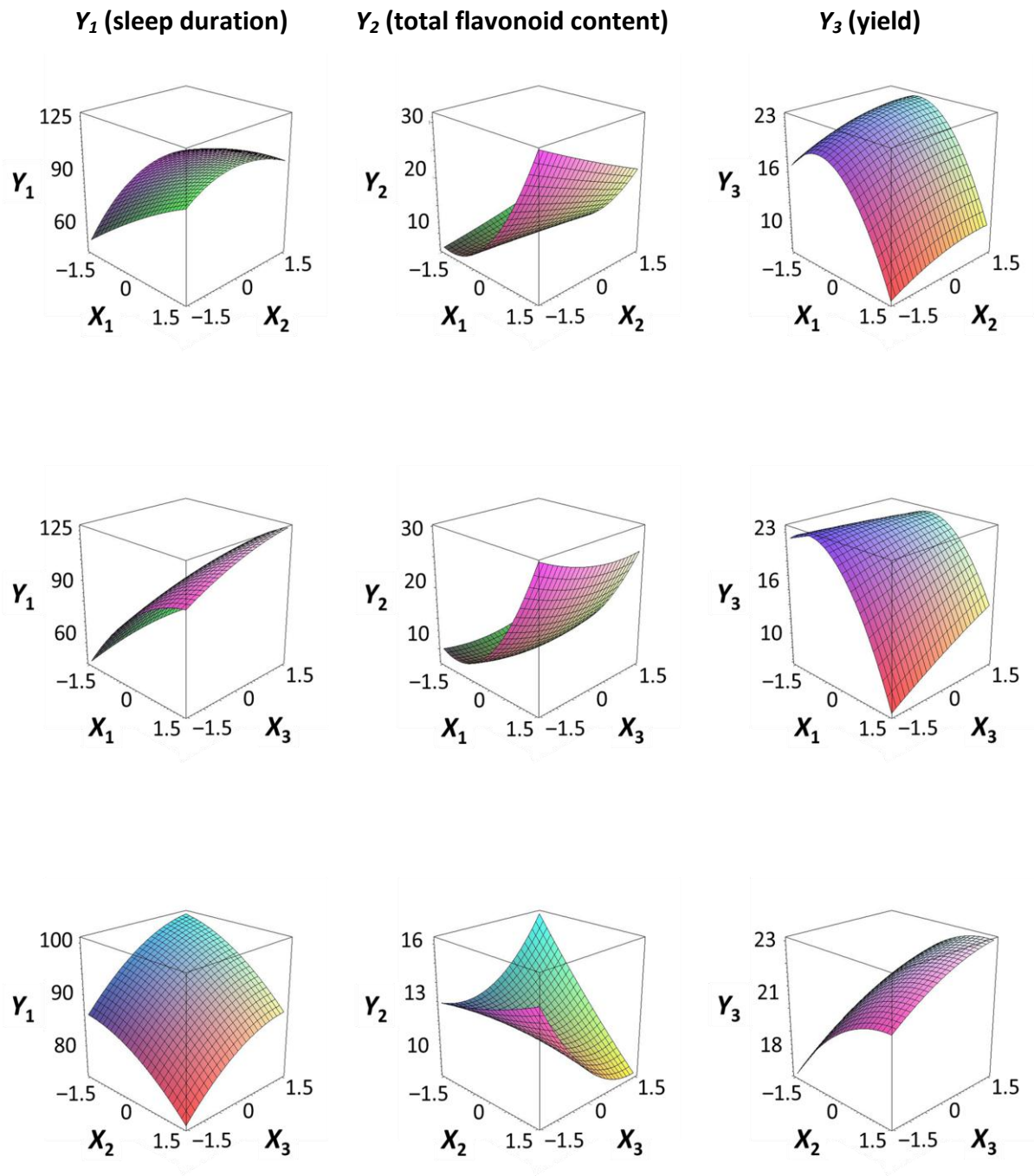
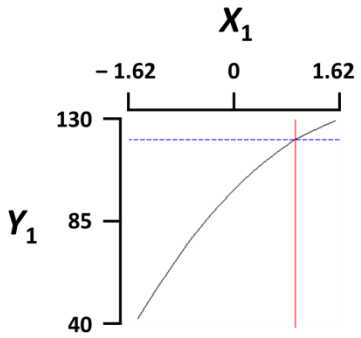
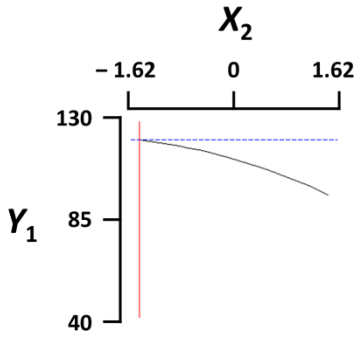
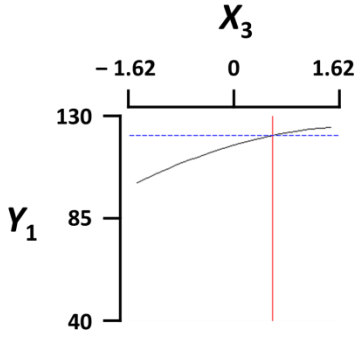


Fig. 3-2. Response surface 3D plots for the extraction of hypnotic compounds from GG (*Glycyrrhiza glabra*). X_1, ethanol concentration (%, v/v); X_2, extraction time (h); X_3, extraction temperature (°C). Y_1, sleep duration (min, at 500 mg/kg); Y_2, total flavonoid content (mg QE/g); Y_3, yield (%, w/w).

Table 3-3. Optimal conditions and verification of the predicted response values for the extraction of hypnotic compounds from GG (*Glycyrrhiza glabra*)

Response			Y_1, sleep duration	
Optimal conditions	X_1	Coded value	1.00	
		Actual value (%)	79.8	
	X_2	Coded value	–1.68	
		Actual value (h)	12.0	
	X_3	Coded value	0.67	
		Actual value (°C)	48.0	
Predicted value of response Y_1				115.9
Experimental value of response Y_1				118.2

X_1, ethanol concentration (%, v/v); X_2, extraction time (h); X_3, extraction temperature (°C).

Values of Y_2, (TFC) and Y_3 (yield) at the optimal conditions were 25.9 mg QE/g and 12.3%, respectively.

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

***Effects of GGE on sleep latency and the amounts of NREMS and REMS*: Fig. 3-3A** shows representative EEG and EMG signals and corresponding hypnograms for vehicle and GGE during the first 3 h after administration. As expected from the results of the pentobarbital-induced sleep test, GGE decreased sleep latency (**Fig. 3-3B**) and increased the amount of NREMS in a dose-dependent manner compared to vehicle (**Fig. 3-3C**; 250 mg/kg: 32.2%, $p < 0.05$; 500 mg/kg: 63.4%, $p < 0.01$). When compared with GGE (100 mg/kg), GGE (500 mg/kg) showed a statistically significant effect ($p < 0.01$). There was no significant change in REMS following GGE administration.

***Effects of GGE on the time spent in each sleep stage*:** The time courses of the hourly amounts of NREMS, REMS, and Wake for 12 h after the injection of GGE (500 mg/kg) and DZP (2 mg/kg) are shown in **Fig. 3-4**. A significant increase in the hourly amount of NREMS by GGE was effective for the first 2 h; however, during the subsequent period, there was no significant change in NREMS. No adverse effects of GGE were observed after sleep induction.

***Effects of GGE on the mean duration of each sleep stage and power density in NREMS*:** GGE significantly ($p < 0.05$) decreased the mean duration of Wake by 57.6%, indicating the decreased maintenance of Wake (**Fig. 3-5A**) [**101**]. Unlike DZP (**Fig. 3-5D**), which produced a reduction of delta activity, an indicator of the quality of NREMS, GGE did not affect delta activity compared to the vehicle control (**Fig. 3-5C**). In the overall EEG power density of GGE, there was no increase in beta activity (13–30 Hz), which is a property of DZP [**120**]. Overall, the effects of GGE on sleep architecture and profile were similar with those of ECE. On the basis of these results, GGE was found to induce NREMS without altering EEG power density and REMS. In addition, these results may suggest that GGE induces sleep that is very similar to physiological sleep. GG is one of the most famous medicinal herbs, and a large amount of GG is consumed worldwide; therefore, GG might provide a useful means for the treatment of insomnia, like the famous valerian and St. John's wort.

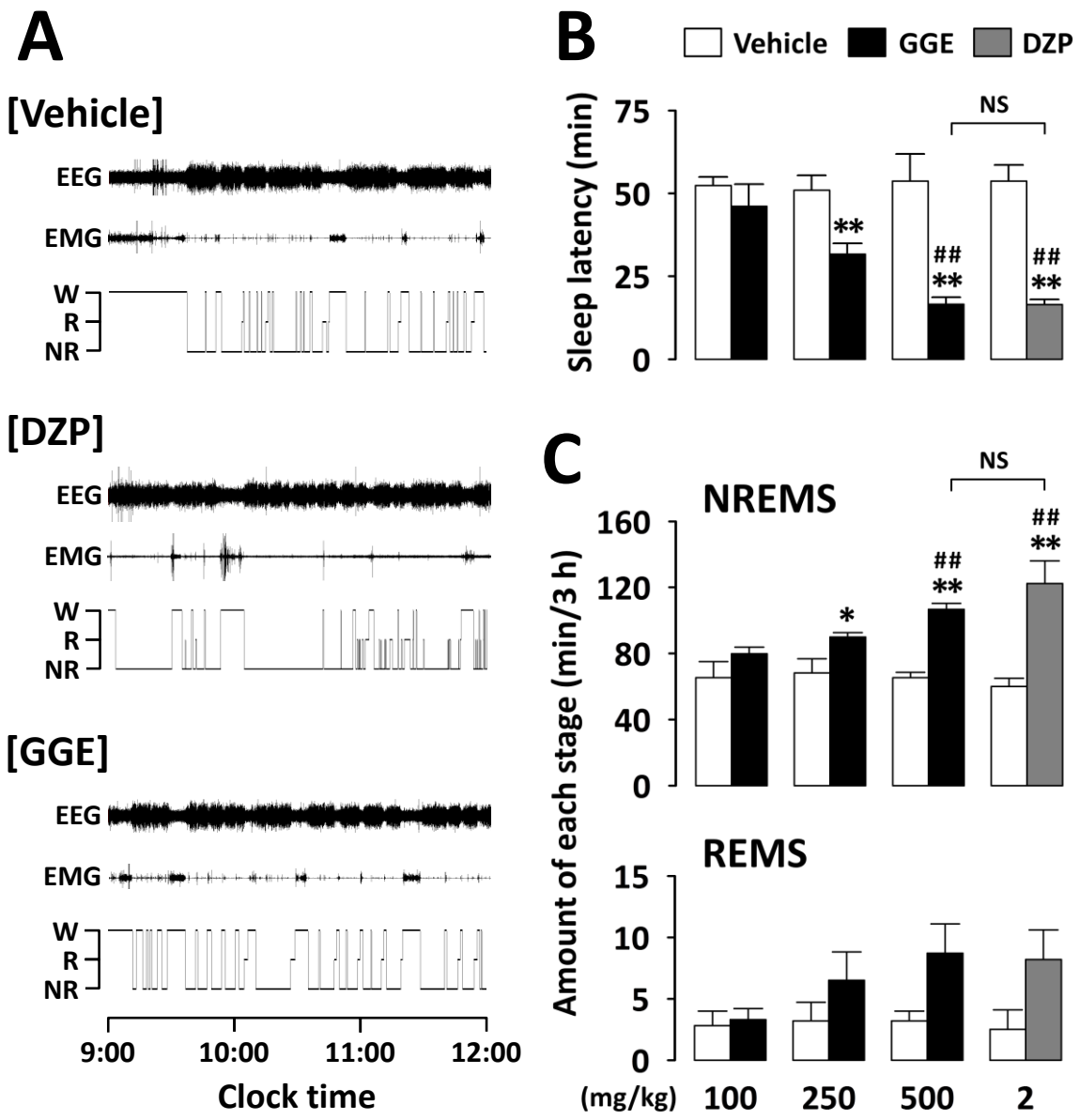


Fig. 3-3. (A) Representative examples of EEG and EMG signals and corresponding hypnograms in a mouse treated with vehicle, GGE, and DZP. (B) Effects of GGE and DZP on sleep latency. (C) Total time spent in NREMS and REMS for 3 h after administration. Each column represents the mean ± SEM (n = 8). *p < 0.05, **p < 0.01, compared with vehicle (unpaired Student's t-test). $^{\#\#}p$ < 0.01, significant as compared with GGE (100 mg/kg; Dunnett's test). Abbreviations: DZP, diazepam; EEG, electroencephalogram; EMG, electromyogram; GGE, *Glycyrrhiza glabra* ethanol extract; NREMS (NR), non-rapid eye movement sleep; NS, not significant; REMS (R), rapid eye movement sleep; Wake (W), wakefulness.

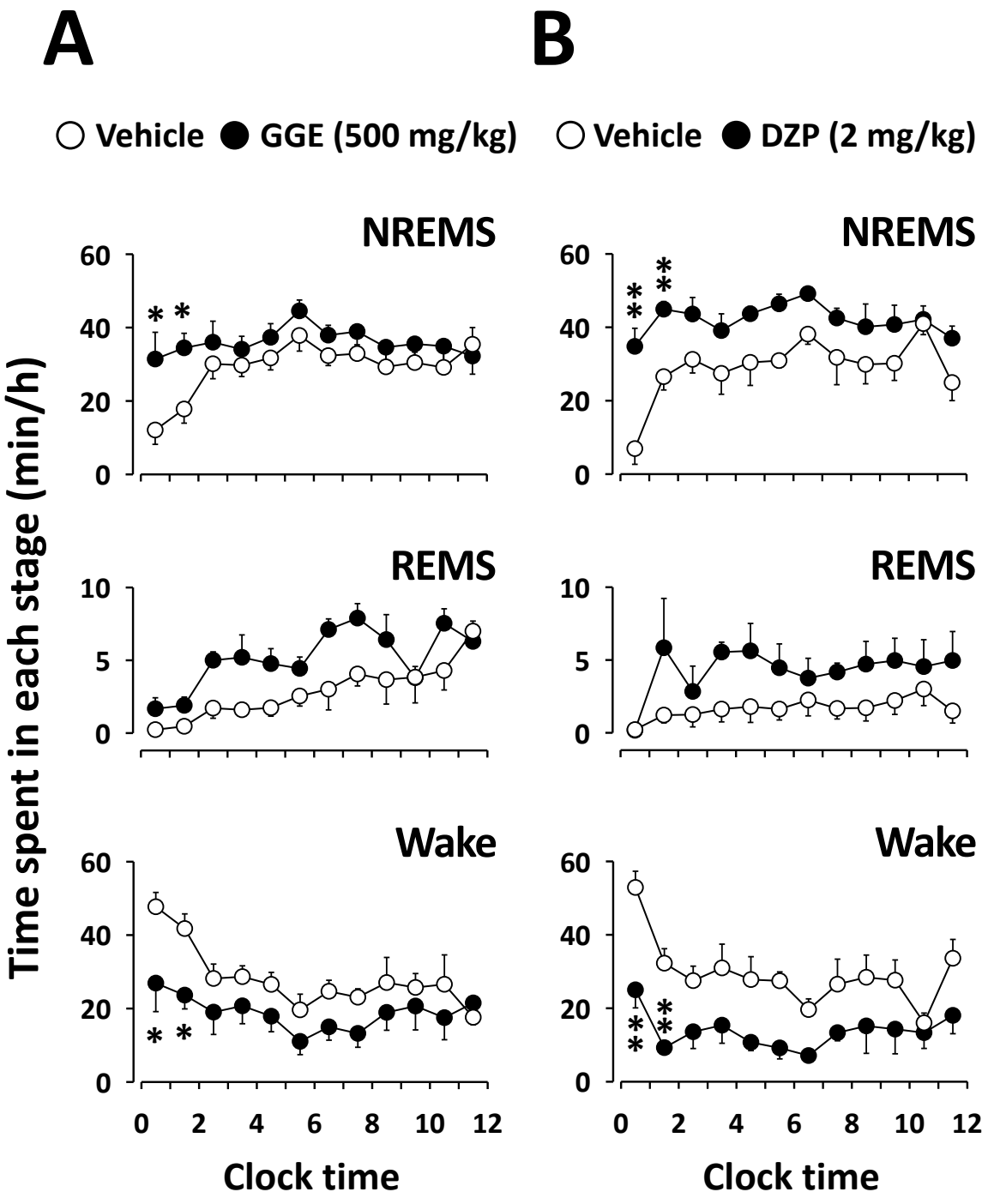


Fig. 3-4. Time courses of NREMS, REMS, and Wake after the administration of GGE (A) and DZP (B). Each circle represents the hourly mean ± SEM (*n* = 8) of NREMS, REMS, and Wake. **p* < 0.05, ***p* < 0.01, compared with vehicle (unpaired Student's *t*-test). Abbreviations: DZP, diazepam; GGE, *Glycyrrhiza glabra* ethanol extract; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

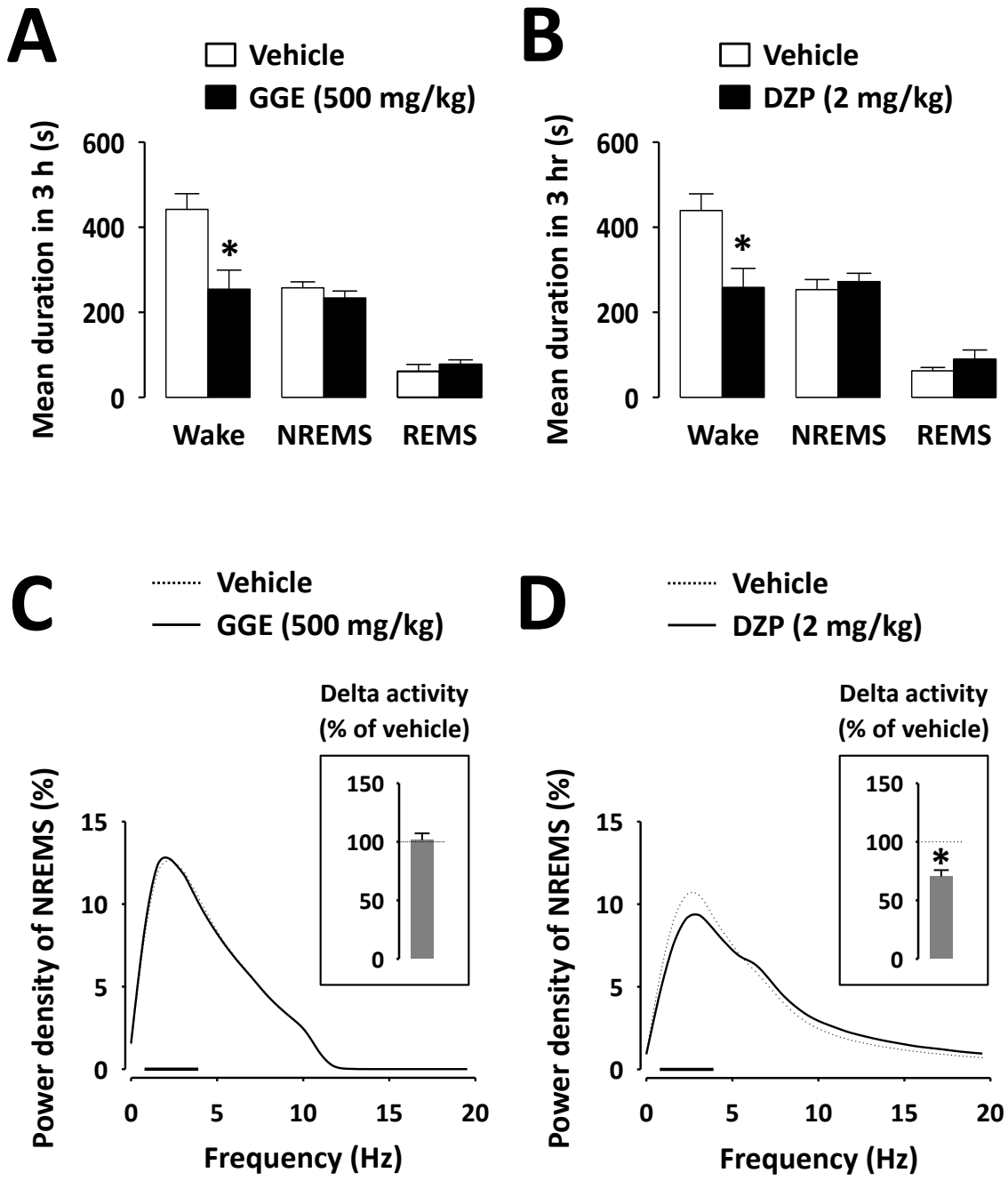


Fig. 3-5. Effects of GGE and DZP on changes in the mean duration of each sleep stage (A and B) and EEG power density during NREMS (C and D). Delta activity in NREMS, an index of sleep intensity, is shown in the inset histogram in (C) and (D). The bar (▬) represents the range of the delta wave (0.5–4 Hz). *$p < 0.05$, compared with vehicle (unpaired Student's *t*-test). Abbreviations: DZP, diazepam; EEG, electroencephalogram; GGE, *Glycyrrhiza glabra* ethanol extract; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness.

3.3.4. Verification of the in vivo GABAergic mechanism of GGE

Hypnotic effect of $GABA_A$-BZD receptor ligands: Ligands with agonistic effects to $GABA_A$-BZD receptors produce anxiolytic, hypnotic, and anticonvulsant effects due to their positive allosteric modulation [**69**, **178**]. $GABA_A$-BZD receptor antagonists have a binding affinity, but do not produce hypnotic effects [**179**, **180**]. If the plant extract containing the BZD antagonists has hypnotic activity, it may be able to produce a hypnotic effect due to its active compounds via a non-GABAergic mechanism, e.g., serotonergic and histaminergic systems. Therefore, for the successful isolation of the active compounds, the hypnotic mechanism needs to be verified using well-known drugs.

Inhibition of the hypnotic effect of GGE by FLU: When considering the anxiolytic and anticonvulsant effects described in previous reports [**163**, **164**], the hypnotic and binding activity of GGE observed in the present study has the potential to induce the positive allosteric modulation of $GABA_A$-BZD receptors. To adopt the $GABA_A$-BZD receptor binding assay as a guide for fractionation, its hypnotic mechanism was tested. Pretreatment with FLU (8 mg/kg) was found to significantly ($p < 0.01$) block the hypnotic effects of DZP and ZPD (**Fig. 3-6**). The hypnotic activity of GGE was also fully ($p < 0.01$) inhibited by FLU, similar to the $GABA_A$-BZD receptor agonists DZP and ZPD.

Synergic effects of GGE with DZP and ZPD: GGE (100 mg/kg), DZP (0.5 mg/kg), and ZPD (2.5 mg/kg) did not produce a significant increase in sleep duration in mice (**Fig. 3-7**). However, co-administration of GGE with DZP significantly ($p < 0.01$) increased sleep duration. Their synergic effects on sleep latency and sleep duration were fully antagonized by pretreatment with FLU (8 mg/kg). From these results of their co-administration with FLU, the involvement of a GABAergic mechanism of GGE, similar to DZP and ZPD, was demonstrated. Therefore, the $GABA_A$-BZD receptor binding assay was adopted for the isolation of hypnotic compounds from GGE. Verification of the GABAergic mechanism of GGE would also suggest possible evidence for the mechanisms of action of the anxiolytic and anticonvulsant effects of GG ethanol extracts described in previous reports [**163**, **164**].

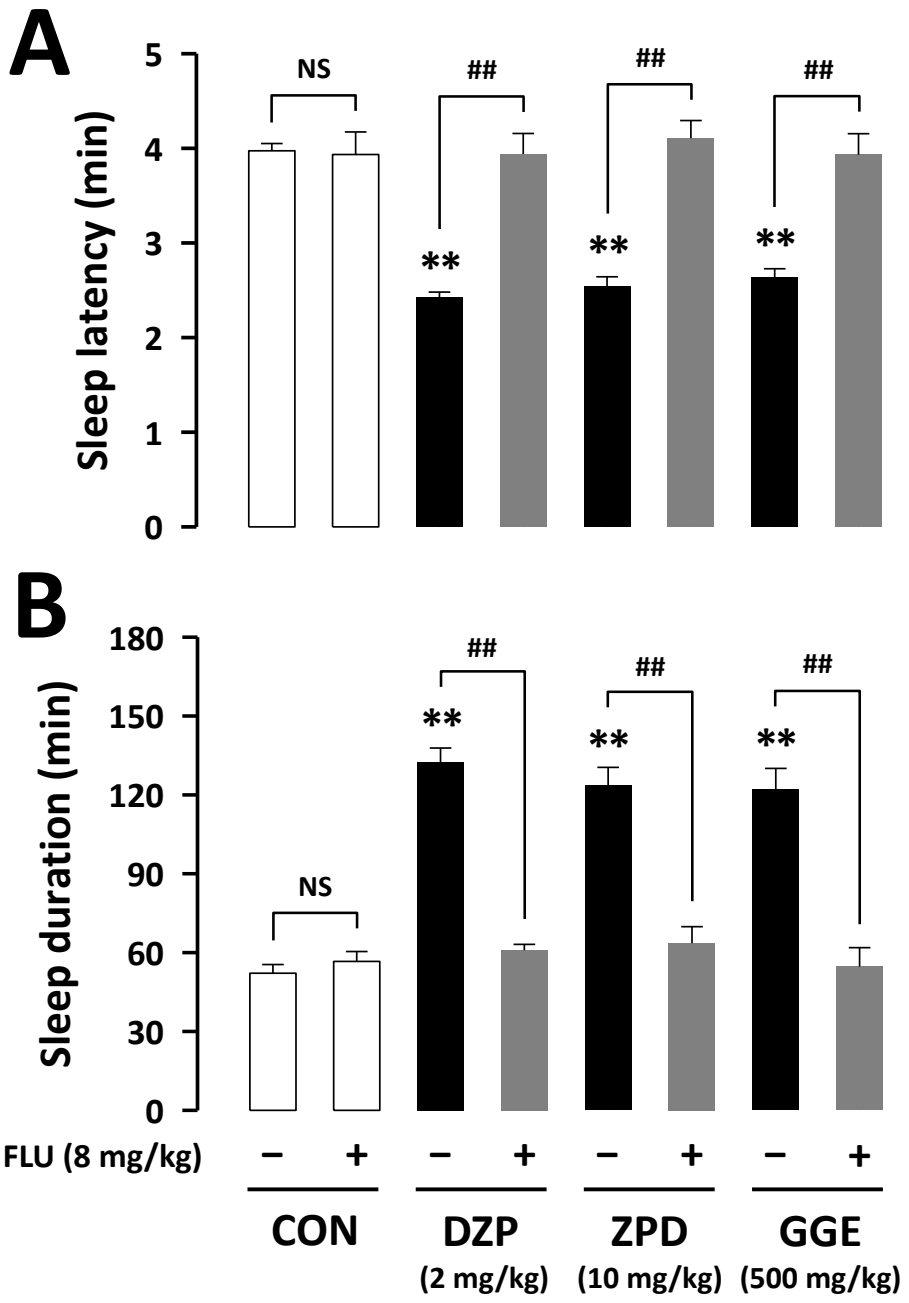


Fig. 3-6. Effects of FLU on the changes in sleep latency (A) and sleep duration (B) in mice treated with DZP, ZPD, and GGE. Mice received pentobarbital (45 mg/kg) at 45 min after the oral administration of the drugs. FLU was administered (i.p.) at 15 min before the oral administration of the drugs. Each column represents the mean ± SEM (n = 10). $^{**}p < 0.01$, significant as compared to the control group (Dunnett's test). $^{\#\#}p < 0.01$, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; FLU, flumazenil; GGE, *Glycyrrhiza glabra* ethanol extract; NS, not significant; ZPD, zolpidem.

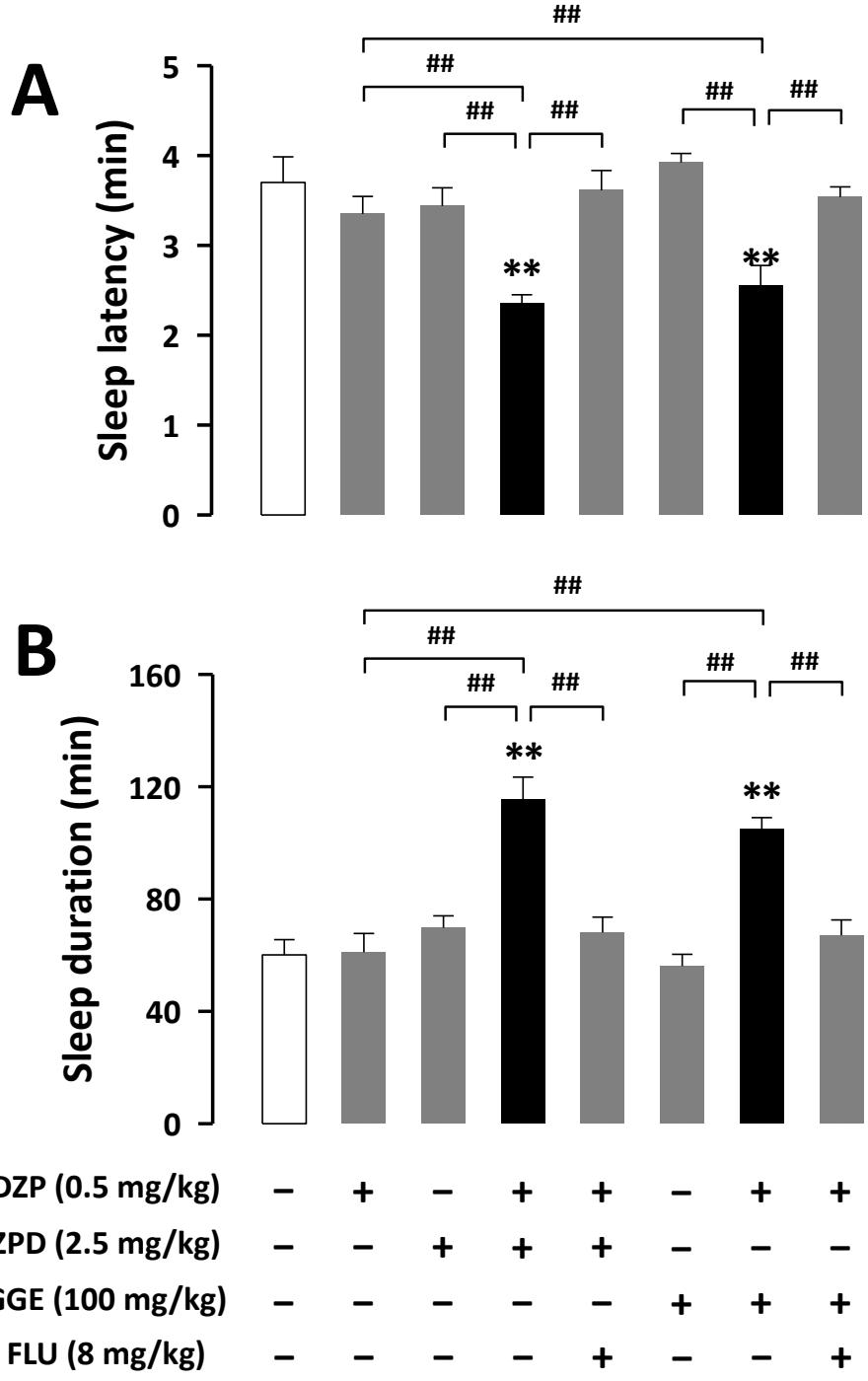


Fig. 3-7. Effects of the co-administration of DZP, ZPD, GGE, and FLU on sleep latency (A) and sleep duration (B) in mice. Mice received pentobarbital (45 mg/kg) at 45 min after the oral administration of ECE and DZP. FLU was administered (i.p.) at 15 min before the oral administration of the drugs. Each column represents the mean ± SEM (n = 10). $^{**}p < 0.01$, significant as compared to the control group (Dunnett's test). $^{\#\#}p < 0.01$, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: DZP, diazepam; FLU, flumazenil; GGE, *Glycyrrhiza glabra* ethanol extract; ZPD, zolpidem.

3.3.5. Isolation of active flavonoids from GGE

Binding and hypnotic activity of the flavonoid-rich faction of GGE: During the optimization of GG extraction, the sleep duration increased as the TFC value increased. A large number of flavonoids have been demonstrated to have hypnotic activity by the positive allosteric modulation of $GABA_A$-BZD receptors in the CNS [**35**, **110**]. With the expectation that GG flavonoids were hypnotic compounds, the flavonoid-rich fraction (FRF) was prepared using EA-BT (3:1) from GGE. The TFC value of the FRF was 262.8 mg QE/g, i.e., 10-fold higher than that of optimized GGE. The residue (H_2O fraction) showed 2.3 mg QE/g of TFC. The FRF from GG showed higher binding activity (0.012 mg/mL) than GGE, whereas the IC_{50} value of the residue was not calculated at a concentration of 10 mg/mL. In the animal test, the FRF at 100 mg/kg ($p < 0.05$) and 200 mg/kg ($p < 0.01$) significantly potentiated pentobarbital-induced sleep in mice, and its hypnotic effect was fully inhibited by FLU, similar to GGE (**Fig. 3-8**). However, the residue did not produce a hypnotic effect even at 1000 mg/kg.

Isolation of active flavonoids: Three flavonoids were successfully isolated from the FRF using $GABA_A$-BZD receptor binding activity-guided fractionation (**Fig. 3-9**). The isolated flavonoids were glabridin (GBD), glabrol (GBR), and isoliquiritigenin (ILTG). These active compounds were suggested to be flavonoids on the basis of their spectroscopic data including NMR, MS, and IR. GBD was identified as an isoflavane, which was substituted with three hydroxyl groups at C-7, C-2′ and C-4′, and had a 2*H*-pyrane ring formed by an isoprenyl group at C-8 and a hydroxyl group at C-7. Therefore, GBD was determined to be glabridin. GBR was identified as a flavanone with two hydroxyl groups at C-7 and C-4′, and two isoprenyl groups at C-8 and C-3′, that is, glabrol. ILTG was identified as a chalcone with α and β carbon atoms in *trans*-configuration and three hydroxyl groups at C-4, C-2′, and C-4′, that is, isoliquiritigenin.

Binding affinity of GG flavonoids to $GABA_A$-BZD receptors: The K_i values of GBD, GBR, and ILTG were 0.84, 1.63, and 1.07 μM, respectively (**Fig. 3-10A**), and were lower than those of EC phlorotannins. To the best knowledge of the author, these flavonoids were characterized as $GABA_A$-BZD receptor ligands for the first time.

In the CNS, the binding of several dietary flavonoids to $GABA_A$-BZD receptors results in anxiolytic, hypnotic, and anticonvulsant effects [**35**, **129**]. Many synthetic derivatives of flavone were also found to be ligands of $GABA_A$-BZD receptors, and resulted in CNS depression in animals [**35**, **69**, **181**]. The phenyl ring, the double bond, and the carbonyl group at position 4 in the C-ring of flavone (**Fig. 3-10C**) are important for its binding affinity through analysis and comparison of the chemical structures of DZP (**Fig. 3-10B**) and flavonoids [**129, 182**]. ILTG has these three structures. ILTG is usually isolated from the root of licorice species together with liquiritigenin [**182**]; however, in this study, liquiritigenin (MW: 256.23) was not isolated as an active compound. The binding affinity of liquiritigenin (Extrasynthese, Genay, France) was not calculated at a maximum concentration of 100 μM (41% displacement at 100 μM). Liquiritin (MW: 418.39), the glycosidic form of liquiritigenin, also did not show effective binding activity. Jäger *et al*. [**183**] reported that naringenin, which has a very similar structure to liquiritigenin, had a high IC_{50} value of 2.6 mM, and thus has very low activity. Therefore, the result of this study confirmed the importance of the double bond in the C-ring of flavonoids for their binding activity to $GABA_A$-BZD receptors. GBR has two isoprenyl groups in addition to the structure of liquiritigenin. GBD also has one isoprenyl group without the double bond and a carbonyl group at position 4 in the C-ring. Due to the attenuation of polarity by the isoprenyl group, these flavonoids may bind to the agonist pharmacophores of $GABA_A$-BZD receptors, i.e., L1, L2, and L3 [**132**]. On the basis of these results, the isoprenyl group may play a key role in the binding activity of flavonoids to $GABA_A$-BZD receptors; however, detailed pharmacophore modeling for GBD and GBR is needed. Glycyrrhizin (MW: 822.93) and its hydrolysate, glycyrrhetinic acid (MW: 470.68), are major constituents of GG together with its flavonoids [**153**, **170**]; however, in this study, they did not show effective binding activity to $GABA_A$-BZD receptors.

Active flavonoid content of GGE: The quantity of GBD, GBR, and ILTG was determined to be 0.3, 2.3, and 0.15 mg in 1 g of GGE according to calibration curves derived from HPLC analysis.

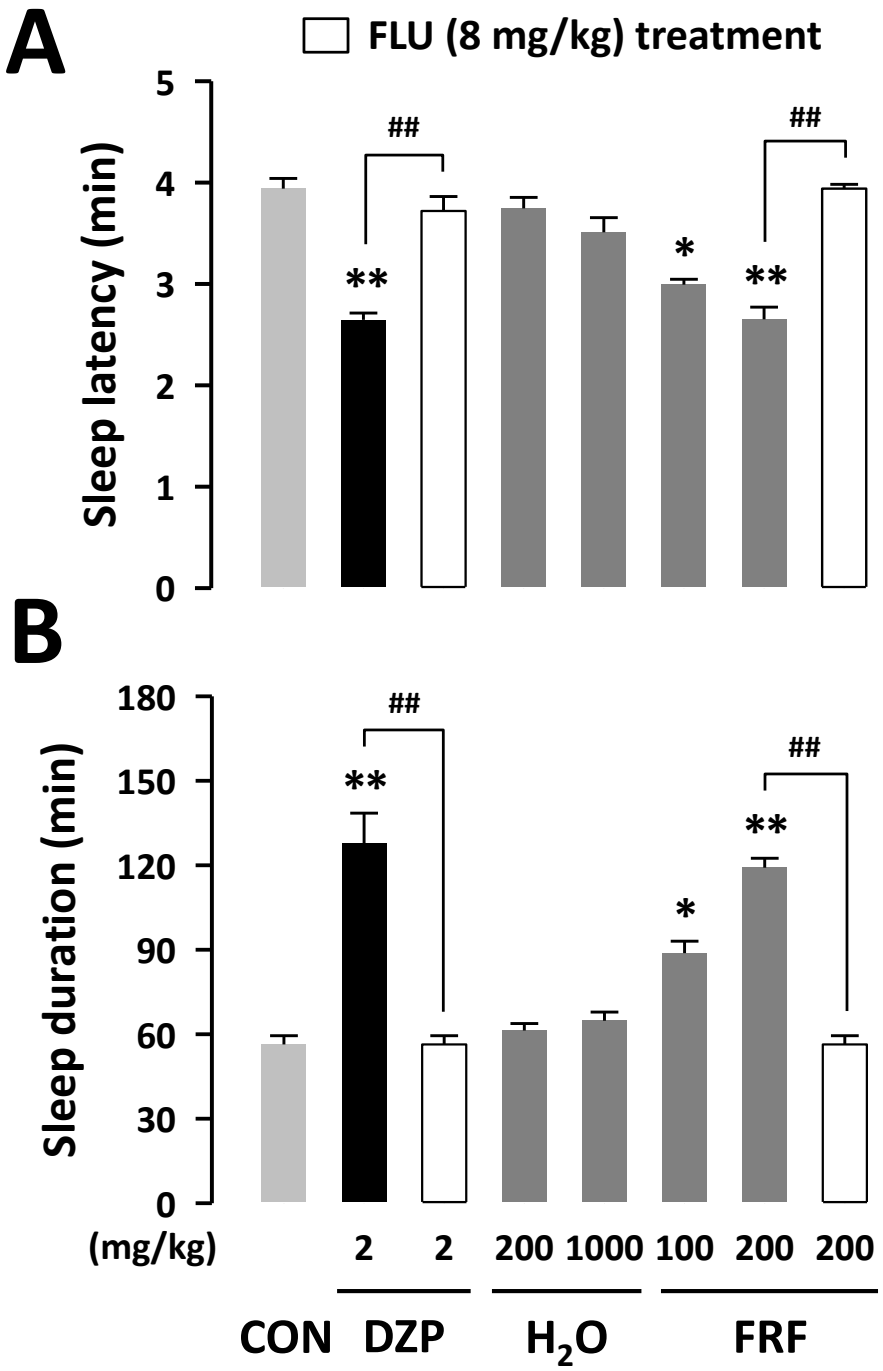


Fig. 3-8. Effects of the flavonoid-rich (FRF) and H_2O (residue) fractions from GGE on sleep latency (A) and sleep duration (B) in mice induced by pentobarbital (45 mg/kg). *p < 0.05, **p < 0.01, significant as compared to the control group (Dunnett's test). $^{\#\#}p$ < 0.01, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; FLU, flumazenil; GGE (*Glycyrrhiza glabra* ethanol extract).

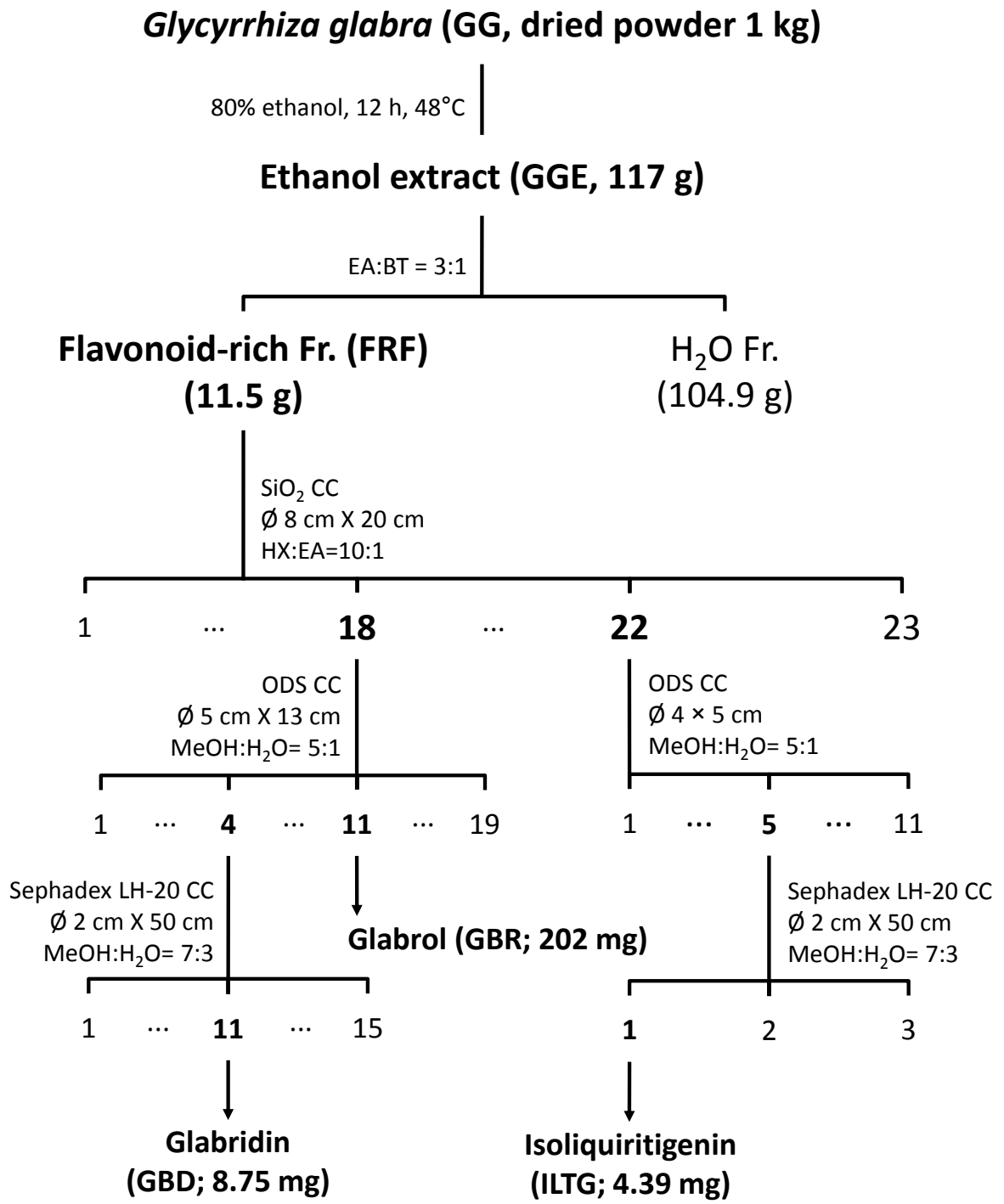


Fig. 3-9. Schematic overview for the fractionation and separation of active flavonoids from GGE (*Glycyrrhiza glabra* ethanol extract).

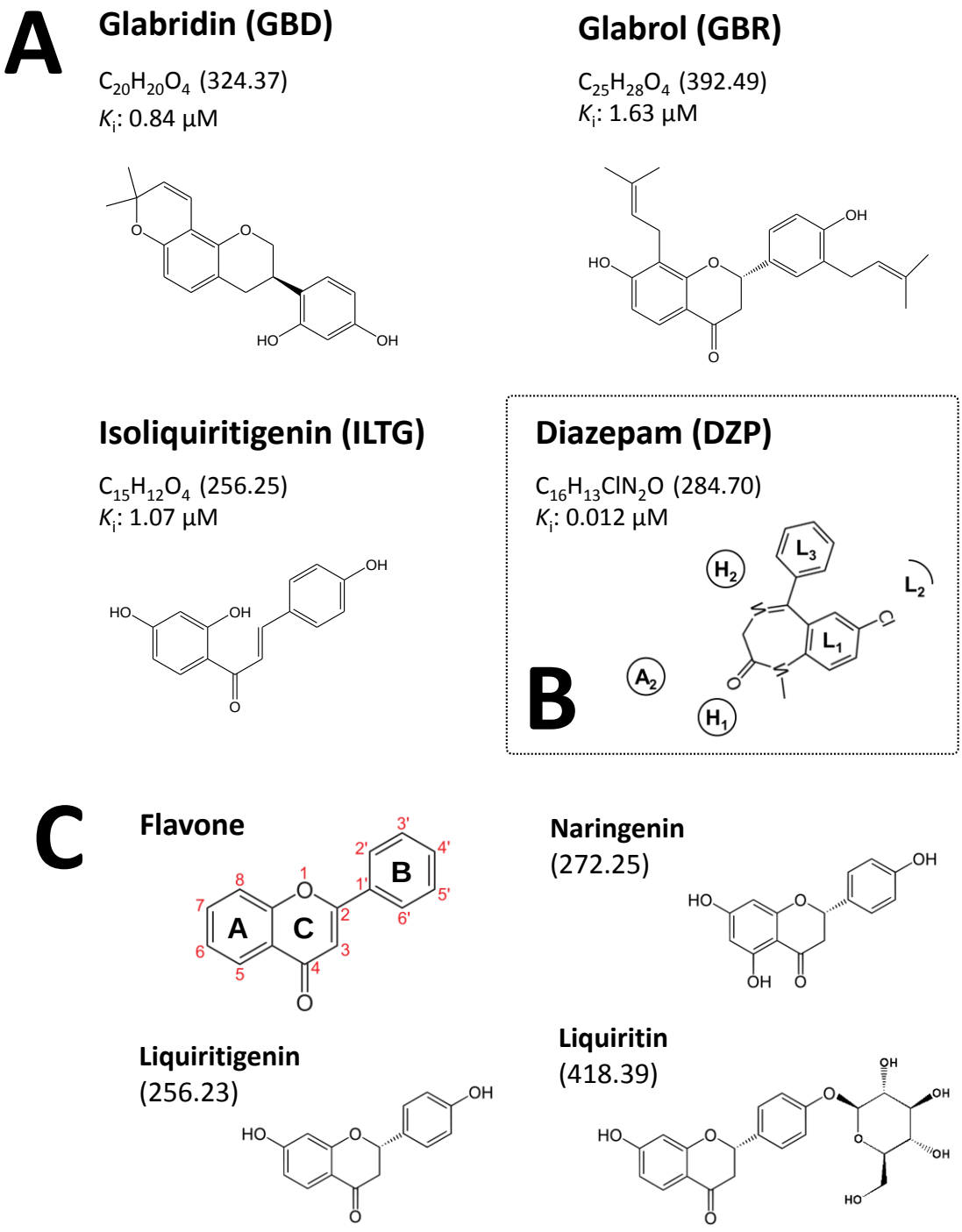


Fig. 3-10. (A) Molecular structure, chemical formula (MW), and binding affinity (K_i) to $GABA_A$-BZD receptors of the active flavonoids from GG (*Glycyrrhiza glabra*). (B) Pharmacophore model for the DZP-sensitive $GABA_A$ receptor. (C) Structures of the other GG flavonoids and flavone. H_1 and H_2 are two hydrogen bond donating sites on the $GABA_A$ receptor. A_2 is a hydrogen bond accepting site. L_1, L_2, and L_3 are three lipophilic pockets. Source: Wang *et al*. [**182**]. Liquiritigenin and liquiritin did not show effective binding activity to $GABA_A$-BZD receptors.

3.3.6. Hypnotic effects and in vivo mechanism of GG flavonoids

Neurological activity of GG flavonoids: Flavonoids are the major active constituents of licorice species [**170**]. In particular, the biological properties of GG flavonoids have been investigated. A wide range of bioactivity, e.g., anti-inflammatory, antitumor, antioxidant, and gastrointestinal effects, has been demonstrated [**153**]; however, only a few reports have described the neurological effects of GG flavonoids. For example, GBD showed a neuroprotective effect via the modulation of multiple pathways involved in apoptosis and neuronal damage [**184**]. Ofir *et al*. [**185**] reported that GBD may be beneficial for mild to moderate depression due to its inhibitory effect on serotonin re-uptake. According to Jamal *et al*. [**186**], ILTG showed anxiolytic effects in the elevated plus-maze test. ILTG also inhibited cocaine-induced dopamine release by modulating $GABA_B$ receptors [**187**]. Liquiritin and isoliquiritin appeared to produce significant antidepressant-like effects in mice by a mechanism that was potentially related to the serotonin and norepinephrine systems [**188**].

Hypnotic effects of GG flavonoids: To evaluate the hypnotic effects of GG flavonoids, GDB and ILTG were purchased from Wako Pure Chemical Industries and Sigma-Aldrich Inc., respectively. Their binding affinities were similar to those of the isolated GDB and ILTG. GBR was obtained from an additional isolation. All GG flavonoids produced an increase in sleep latency and a decrease in sleep duration in a dose-dependent manner (5–50 mg/kg) (**Fig. 3-11**). In particular, GBD and ILTG showed a significance effect at >10 mg/kg. At the maximum concentration (50 mg/kg), the hypnotic effects of GBD, GBR, and ILTG were comparable to those of DZP (2 mg/kg) and ZPD (10 mg/kg), and there was no significant difference among the three GG flavonoids.

Verification of the hypnotic mechanism of GG flavonoids: The hypnotic effects of GGE and its FRF were fully blocked by the BZD antagonist FLU. In addition, none of the three GG flavonoids produced a significant prolongation of sleep duration or reduction of sleep latency under FLU pretreatment (**Fig. 3-12**). From these results, it is demonstrated that the GG flavonoids GBD, GBR, and ILTG induce sleep by the positive allosteric modulation of $GABA_A$-BZD receptors, similar to DZP.

GBD is the species-specific flavonoid constituent of GG, and is not found in other licorice species, e.g., *G. uralensis* and *G. inflata* [**170**]. Among the GG flavonoids, GBD has been the most widely studied in the biological and pharmacological research fields [**184**]. Although GBD has a wide range of bioactivity, its hypnotic effect has not yet been demonstrated. Therefore, its hypnotic effects and GABAergic mechanism would be significant information for the utilization of licorice as medicinal plants. There is growing interest in the valuable pharmacological activity of ILTG [**189**]. ILTG has been found to have various biological properties, e.g., anti-angiogenic [**189**] and anticancer [**190**] effects. Its anxiolytic effect was also reported [**186**]; however, its precise mechanism was not demonstrated. The results of this study can explain that the GABAergic mechanism of ILTG may be the major mechanism of its anxiolytic effect. Studies on the bioactivity of GBR have not been widely conducted. Until now, there are only a few reports describing its diacylglycerol acyltransferase [**191**], and cholesterol acyltransferase [**192**] inhibitory activity.

Effects of GBD and ILTG on changes in sleep architecture and profile: The effects of GBD and ILTG, which have a strong hypnotic effect and are of considerable biological interest, on sleep architecture and profile were evaluated. **Fig. 3-13A** shows representative EEG and EMG signals for GBD and ILTG during the first 3 h after administration. GBD and ILTG (50 mg/kg) significantly ($p < 0.05$ and $p < 0.01$, respectively) decreased sleep latency (**Fig. 3-13B**), and also increased the amount of NREMS by 50.6% and 61.1%, respectively, during the initial 3 h (**Fig. 3-13C**). Their effects on changes in the amount of NREMS were similar to that of the positive control ZPD (63.5%). Neither GBD nor ILTG altered the amount of REMS. ZPD induced a constant increase in the amount of NREMS during the subsequent period (3–12 h); however, GBD and ILTG induced NREMS without further disruption of sleep architecture (**Fig. 3-14A**). They also reduced the mean duration of Wake, and were found to effectively inhibit the maintenance of Wake (**Fig. 3-14B**). Unlike ZPD, GBD and ILTG did not significantly decrease delta activity (**Fig. 3-14C**). From these results, it was found that GBD and ILTG induce sleep that is similar to physiological sleep without a decline in delta activity.

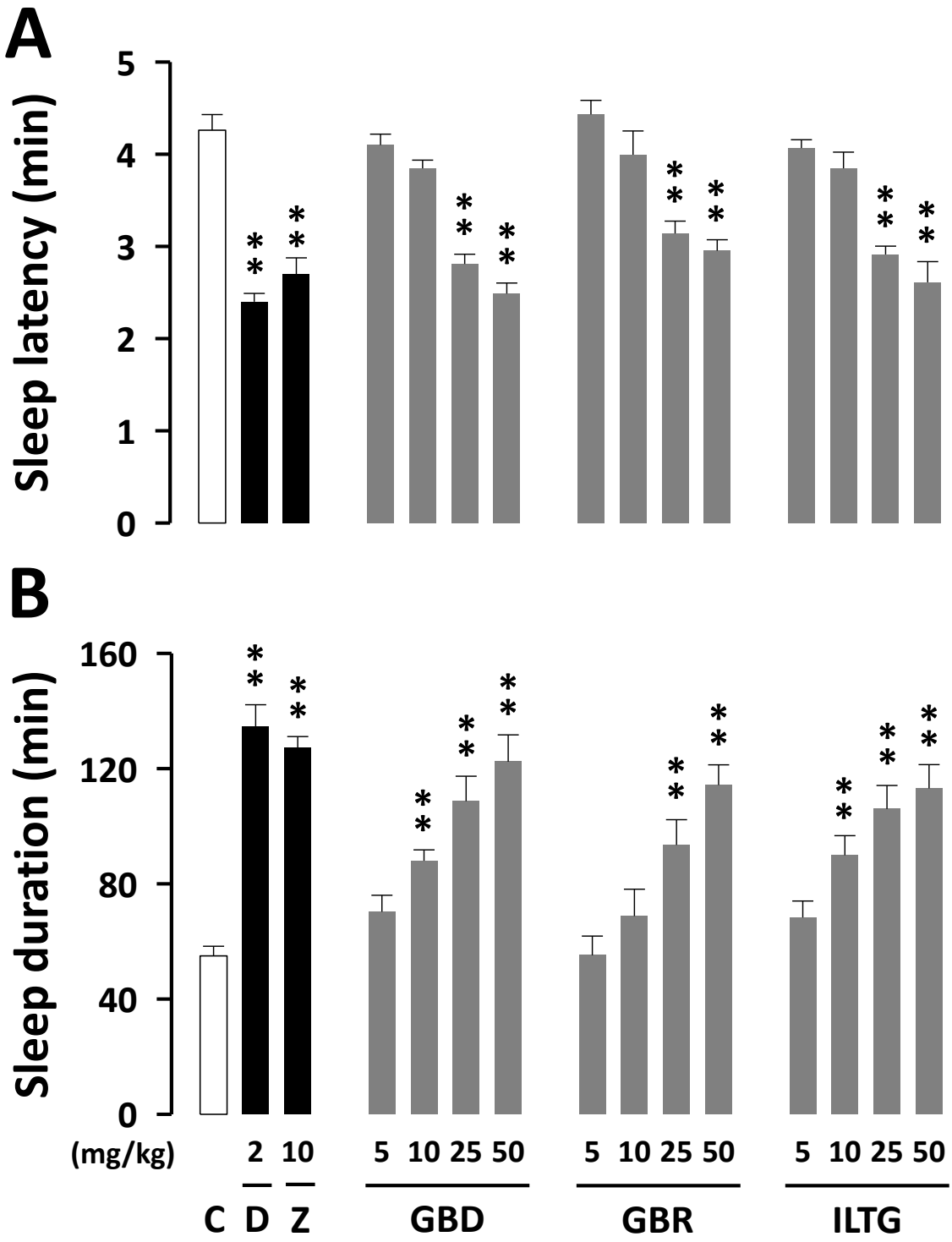


Fig. 3-11. Effects of GBD, GBR, and ILTG on sleep latency (A) and sleep duration (B) in mice induced by pentobarbital (45 mg/kg). Each column represents the mean ± SEM (n = 10). $^{**}p < 0.01$, significant as compared to the control group (Dunnett's test). Abbreviations: C, control group (0.5% CMC-saline, 10 mL/kg); D, diazepam; GBD, glabridin; GBR, glabrol; ILTG, isoliquiritigenin; Z, zolpidem.

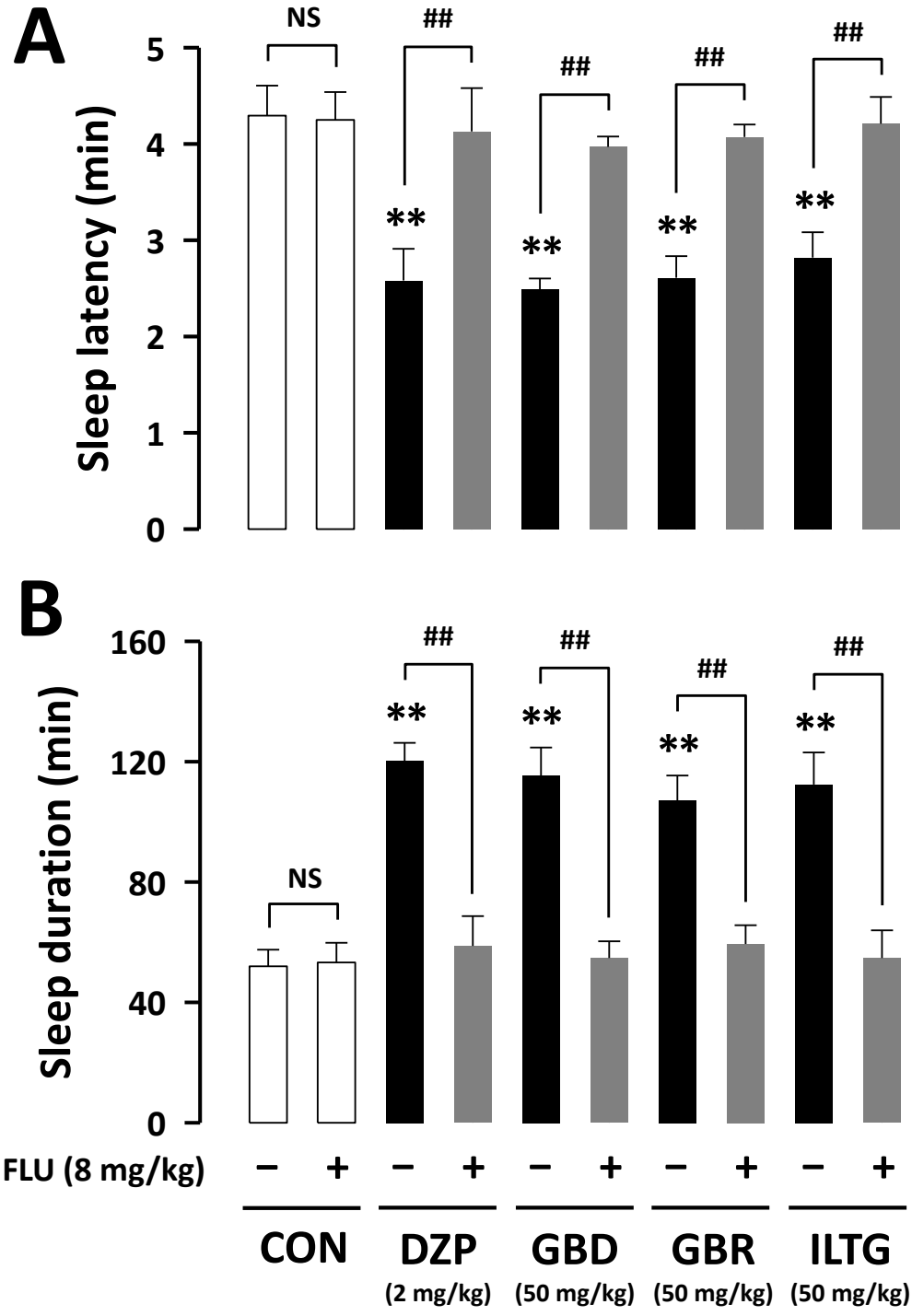


Fig. 3-12. Effects of FLU on the changes in sleep latency (A) and sleep duration (B) in mice treated with GBD, GBR, and ILTG. FLU was administered (i.p.) at 15 min before the oral administration of the drugs. Each column represents the mean ± SEM (n = 10). $^{**}p < 0.01$, significant as compared to the control group (Dunnett's test). $^{\#\#}p < 0.01$, significant between FLU treatment and no FLU treatment (unpaired Student's t-test). Abbreviations: CON, control group (0.5% CMC-saline, 10 mL/kg); DZP, diazepam; FLU, flumazenil; GBD, glabridin; GBR, glabrol; ILTG, isoliquiritigenin; NS, not significant.

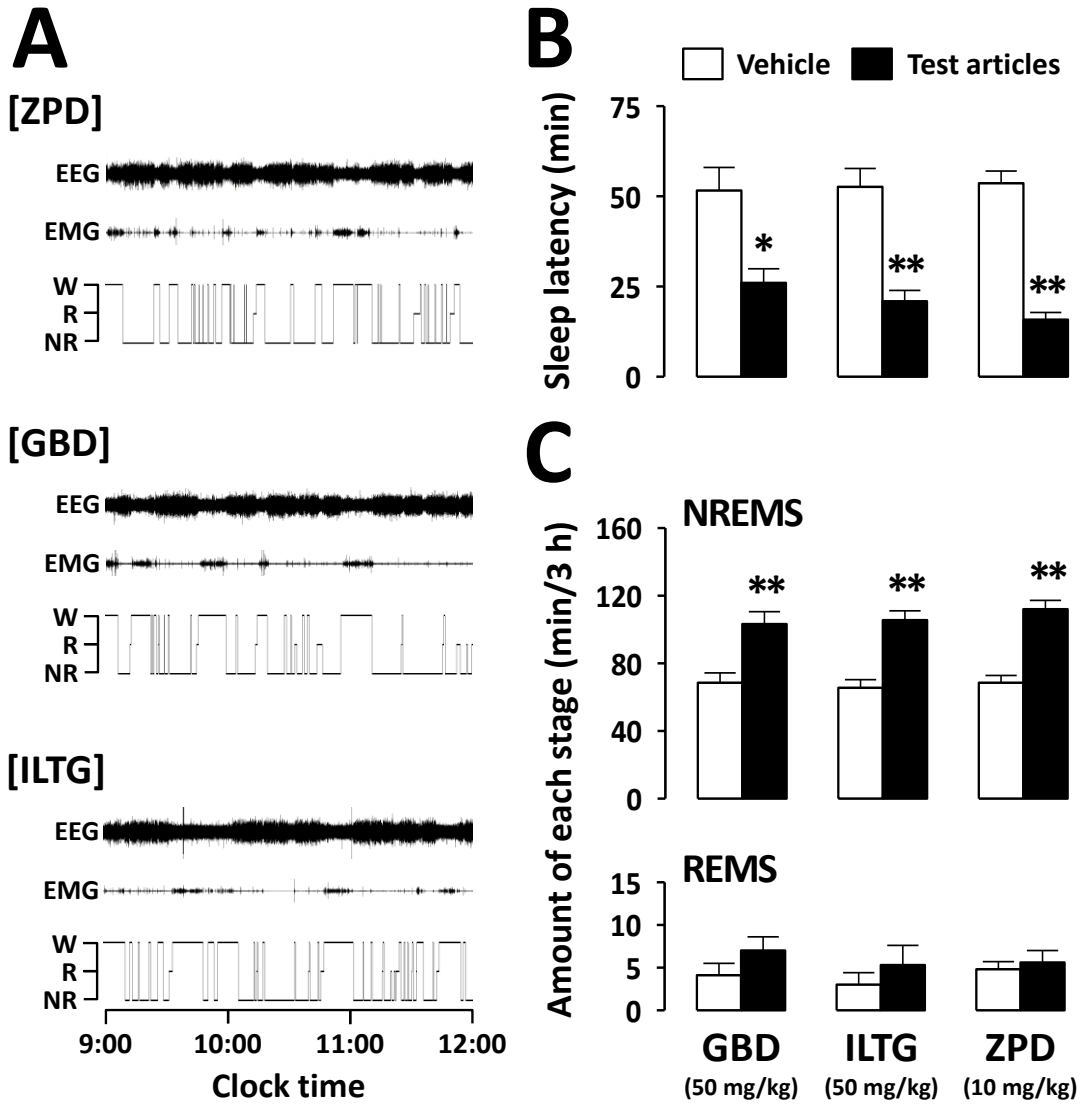


Fig. 3-13. (A) Representative examples of EEG and EMG recordings and corresponding hypnograms in a mouse treated with GBD, ILTG, and ZPD. (B) Effects of GBD, ILTG, and ZPD on sleep latency. (C) Total time spent in NREMS and REMS for 3 h after administration. Each column represents the mean ± SEM (n = 8). *p < 0.05, **p < 0.01, compared with vehicle (unpaired Student's t-test). Abbreviations: EEG, electroencephalogram; EMG, electromyogram; GBD, glabridin; ILTG, isoliquiritigenin; NREMS (or NR), non-rapid eye movement sleep; REMS (or R), rapid eye movement sleep; Wake (or W), wakefulness; ZPD, zolpidem.

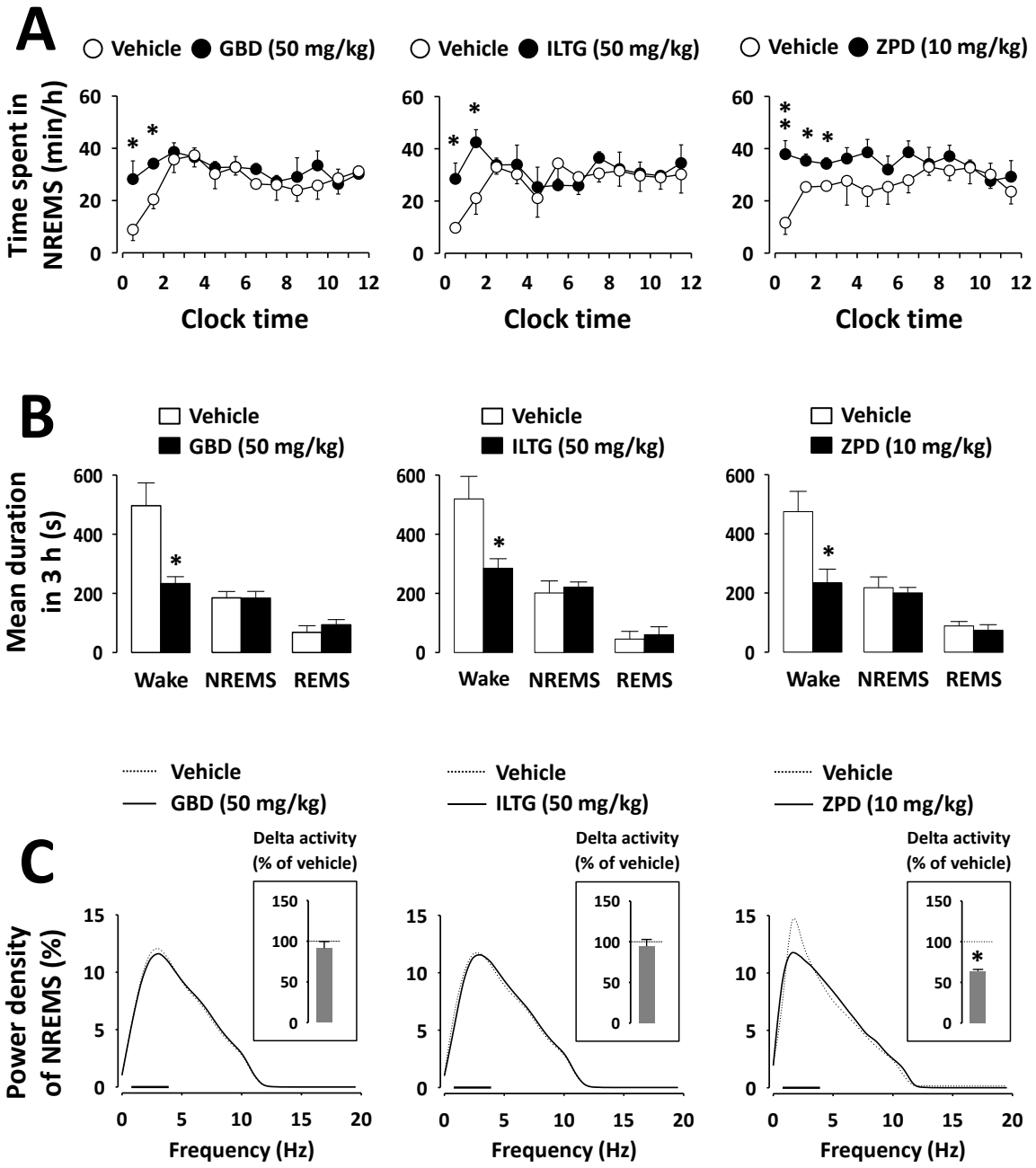


Fig. 3-14. (A) Time courses of NREMS, REMS, and Wake after the administration of GBD, ILTG, and ZPD. (B) Effects of GBD, ILTG and ZPD on changes in the mean duration of each sleep stage. (C) EEG power density during NREMS. Delta activity in NREMS, as an index of sleep intensity is shown in the inset histogram. The bar (▬) represents the range of the delta wave (0.5–4 Hz). *$p < 0.05$, **$p < 0.01$, compared with vehicle (unpaired Student's *t*-test). Abbreviations: EEG, electroencephalogram; GBD, glabridin; ILTG, isoliquiritigenin; NREMS, non-rapid eye movement sleep; REMS, rapid eye movement sleep; Wake, wakefulness; ZPD, zolpidem.

3.3.7. Effects of GG flavonoids on GABA-induced currents in neurons

The results of the binding affinity assays and inhibition of the hypnotic effect of GBD and ILTG by FLU imply that they induce sleep by the positive allosteric modulation of $GABA_A$-BZD receptors. To find more evidence for the GABAergic mechanism of GBD and ILTG, their potentiation on GABA-induced current responses in acutely dispersed DR neurons was investigated. To examine the effects of GBD and ILTG, GABA (2.0×10^{-6} M) was used to produce a control response (10% of the maximal potency). **Fig. 3-15A** shows the representative traces of the effects of GBD, ILTG, and DZP. None of these three drugs produced any currents in DR neurons. ILTG potentiated I_{GABA} in a concentration-dependent manner (**Fig. 3-15B**). The Hill coefficients for ILTG and DZP were 0.45 and 0.74, respectively. ILTG showed a lower P_{max} value than DZP, and the P_{max} values were 151% and 266% after the addition of ILTG (10^{-5} M) and DZP (3.0×10^{-7} M), respectively. However, ILTG had a 65-fold higher affinity for $GABA_A$-BZD receptors than DZP. The K_D values for ILTG and DZP were 4.0×10^{-10} M and 2.6×10^{-8} M, respectively. These results indicate that ILTG acts as a partial agonist (30.7% of the P_{max} of the full agonist DZP), similar to the phlorotannins ETN and TPRA. In the experiment for GBD, a surprising and unique result was observed. GDB remarkably potentiated the GABA-induced currents, and its P_{max} value was 581% (3-fold of the P_{max} of DZP) (**Fig. 3-15C**). The BZD agent DZP and the non-BZD agent ZPD are well known as full agonists of $GABA_A$-BZD receptors [**148-150**, **193**]. According to previous reports on the efficacy of natural products, this phenomenon is unique. Although GBD showed a great efficacy to $GABA_A$-BZD receptors, its hypnotic effect was similar to that of ILTG in the pentobarbital-induced sleep test and analysis of sleep architecture. One possible reason for these results is that there is a difference between the *in vitro* and *in vivo* evaluation models. Therefore, further investigations are needed to evaluate the activity of the GBD metabolites. The partial $GABA_A$-BZD receptor agonist ILTG showed potential as a novel hypnotic, as well as ETN and TPRA. In particular, a precise understanding of the GABAergic mechanism of GBD may present a significant advance in $GABA_A$ receptor pharmacology.

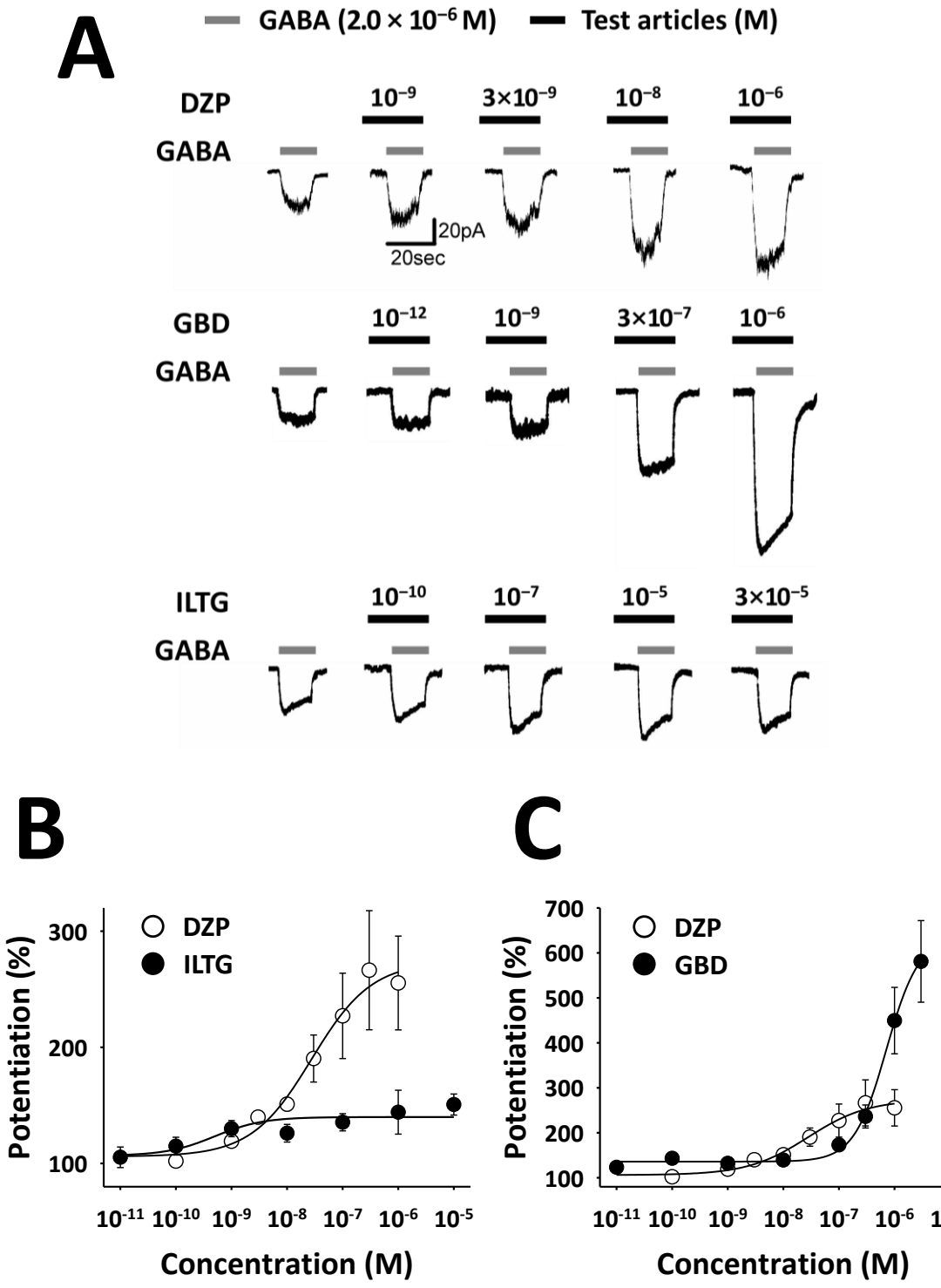


Fig. 3-15. (A) Representative traces of the effects of GBD, ILTG, and DZP on the EC_{10} GABA (2.0×10^{-6}) response in DR neurons. (B) and (C) The dose-dependent potentiation of the GABA response by ILTG and GBD. The traces for ILTG and DZP were taken from different set of neurons. Each point represents the mean ± SEM (5 or 6 neurons). Abbreviations: DZP, diazepam; GBD, glabridin, ILTG, Isoliquiritigenin; DR, dorsal raphe.

SUMMARY AND PERSPECTIVE

Sleep is vital to maintain health and well-being due to its primary function of providing rest and restoring the body's energy levels. Therefore, deprivation and disorders of sleep impair physical and cognitive performance, immunity, mood, and quality of life. However, insomnia is currently a widespread health complaint, and has become a prevalent and disruptive problem worldwide. According to a survey conducted by the National Sleep Foundation in 2011, most adults (87%) in the USA suffer from at least one sleep problem at least a few days a week. For the treatment of insomnia, $GABA_A$-BZD receptor agonists are the most commonly prescribed sleep drug. Recently, natural sleep aids have become popular as alternatives to prescription sleep drugs to improve sleep quality and avoid side effects. Many insomnia patients prefer natural sleep aids due to their low level of side effects and no requirement for a medical prescription. Valerian extract is the most famous herbal sleep aid worldwide, and numerous studies have reported that it improves sleep quality without producing side effects.

In Asia, there are many medicinal plants with potential hypnotic activity; however, scientific evidence for their effects and mechanisms of action have been not widely investigated relative to Western countries. Therefore, the objectives of this study were to identify novel hypnotic plants and their active compounds as a source of new sleep drugs or aids from plant resources, and to investigate their sleep-promoting effects and action mechanisms. In the present study, 30 marine and 30 land plants distributed mainly in Japan and Korea were screened. In particular, a study of the hypnotic effects of marine plants and their natural products was performed for the first time in this study. To evaluate their potential as hypnotic plants, binding activity to the γ-aminobutyric acid type A-benzodiazepine ($GABA_A$-BZD) receptor, a well-known molecular target for sleep drugs, was screened. Their hypnotic effects were evaluated using the pentobarbital-induced sleep test and

analysis of sleep architecture and profile. Investigations for the isolation of active compounds and their mechanisms were performed.

In the screening stage, the most active plant extracts were ECE (*Ecklonia cava* ethanol extract; kajime) in the marine plants and GGE (*Glycyrrhiza glabra* ethanol extract; licorice) in the terrestrial plants, and their binding activities were 0.392 and 0.093 mg/mL, respectively. The oral administration of ECE and GGE (1000 mg/kg) significantly ($p < 0.01$) decreased sleep latency and increased sleep duration in mice treated with pentobarbital (45 mg/kg). Their hypnotic effects were similar with the positive controls diazepam (DZP; 2 mg/kg) and valerian extract (1000 mg/kg). ECE and GGE were considered as subjects for further investigations.

ECE and GGE increased the rate of sleep onset and prolonged sleep duration in mice treated with a sub-hypnotic dose of pentobarbital (30 mg/kg) in a dose-dependent manner (100–1000 mg/kg). They were found to play a decisive role in sleep induction in the mice.

To optimize the EC and GG extraction conditions for the highest hypnotic activity and to monitor their active compounds, response surface methodology (RSM) was adopted. The optimal conditions to maximize hypnotic activity were estimated as follows: (EC and GG, respectively) ethanol concentration, 81.6% and 79.8%; extraction time, 52.2 h and 12.0 h; extraction temperature, 43.7°C and 48.0°C. Phenols, including flavonoids, were identified as natural hypnotic compounds in a large number of previous reports. Therefore, in RSM, the total phenol content (TPC) of EC and total flavonoid content (TFC) of GG were monitored together with their hypnotic effect. The hypnotic effects of ECE and GGE were proportional to their TPC and TFC, respectively. The phenols in EC and the flavonoids in GG showed their potential as active compounds.

To better understand the hypnotic activity of ECE and GGE, their effects on sleep architecture and profile were evaluated by analyzing electroencephalogram (EEG) and electromyogram recordings in mice. The optimized ECE and GGE increased the amounts of non-rapid eye movement sleep (NREMS) in a dose-dependent manner (100–500 mg/kg), and their rate of increase in the amount of NREMS at 500 mg/kg was 71.4% and 63.4%, respectively (the positive

control DZP at 2 mg/kg: 103.8%). ECE and GGE effectively induced NREMS during the first 2 h after their administration, and there was no further disruption of sleep architecture during the subsequent period. DZP, ECE, and GGE did not significantly change rapid eye movement sleep (REMS). The BZD agent DZP significantly decreased delta (0.5–4 Hz) activity, which is an indicator of the quality or intensity of NREMS. However, ECE and GGE induced NREMS that was very similar to physiological sleep without changing delta activity.

ECE and GGE demonstrated binding activity and *in vivo* hypnotic effects; therefore, they may have the potential to induce sleep via the positive allosteric modulation of $GABA_A$-BZD receptors. To verify their GABAergic mechanism, the effects of the well-known $GABA_A$-BZD receptor antagonist flumazenil (FLU) on the hypnotic effects of ECE and GGE were investigated. As expected, the hypnotic effect of the $GABA_A$-BZD receptor agonist DZP was fully inhibited by FLU. Similar to DZP, a significant inhibition of the hypnotic effects of ECE and GGE by FLU was also observed. Therefore, they were found to act as the $GABA_A$-BZD receptor agonists like DZP.

During the isolation of the active compounds, 6 phlorotannins and 3 flavonoids were obtained from ECE and GGE, respectively. The active EC phlorotannins (binding affinity, μM) were eckstolonol (ETN; 1.49), triphlorethol A (TPRA; 4.42), eckol (1.07), fucodiphlorethol G (2.97), 6,6′-bieckol (3.07), and dieckol (3.36). The active GG flavonoids were glabridin (GBD; 0.84), glabrol (1.63), and isoliquiritigenin (ILTG; 1.07). The range of binding affinity of EC and GG was similar with that of $GABA_A$-BZD receptor ligands previously isolated from land plants.

All EC phlorotannins and GG flavonoids potentiated pentobarbital-induced sleep in mice in a dose-dependent manner (5–50 mg/kg), and their hypnotic effects were significantly inhibited by FLU. ETN and TPRA of EC and GBD and ILTG of GG, which exerted strong hypnotic activity and are of biological interest were further investigated by analyzing sleep architecture and profile. All active EC and GG compounds (50 mg/kg) significantly increased the amount of NREMS during the first 3 h after their administration, and did not change REMS, similar to the positive control ZPD (10 mg/kg). ZPD significantly decreased delta activity compared with vehicle; however, none of the EC and GG

compounds produced a significant change in delta activity. All of the active EC and GG compounds were found to induce NREMS that was similar to physiological sleep, unlike ZPD.

To generate more evidence for the GABAergic mechanism of the active EC and GG compounds, their potentiation on GABA-induced currents in dorsal raphe (DR) neurons was evaluated. The maximum potentiation (P_{max}) values of ETN, TPRA, and ILTG were 145% (relative efficacy to DZP: 27.1%), 171% (42.8%), and 151% (30.7%), respectively. They were found to act as partial agonists of $GABA_A$-BZD receptors relative to the well-known full agonist DZP (100%). Surprisingly, GBD showed a P_{max} value of 581% (3-fold higher than the P_{max} of DZP).

In the present study, it was demonstrated for the first time that EC and GG have hypnotic effects that originate from their phlorotannins and flavonoids, which have the characteristics of $GABA_A$-BZD receptor agonists (positive allosteric modulators). Considering their *in vitro* and *in vivo* hypnotic effects, ECE and GGE should prove to be useful for developing natural sleep aids. Natural substances represent a rich diversity in chemical structure that can lead to the development of new therapeutic agents [**35**]. Their active phlorotannins and flavonoids with pharmacological properties of partial or super $GABA_A$-BZD agonists showed potential as a source of novel sleep drugs. In particular, this study is the first report describing the GABAergic mechanism of marine natural products with hypnotic activity. The partial $GABA_A$-BZD agonists ETN and TPRA might be of particular interest since they could be devoid of the side effects associated with full agonists, e.g., DZP and ZPD.

There are over 10,000 species of seaweeds worldwide, and most of these are underutilized or unused. Their effects on neurological activity have been not widely investigated. Our study proposes that marine plants have the potential to be applied to the treatment of neuropsychiatric disorders. Actually, in the screening stage, some marine plants showed high binding activity to histamine or serotonin receptors. Studies on the effects of marine plants on neurological activity may have great potential from the scientific and industrial viewpoints.

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ABSTRACT (IN JAPANESE)

論文の内容の要旨

睡眠は健康を維持する上で最も重要な生理現象のひとつである。睡眠不足または睡眠障害は免疫力、心血管機能、認知機能および心身の安定を低下させ、生活の質にも影響を与える。このように睡眠が重要であるにもかかわらず、世界中で不眠症の人は約30%にも上ると言われている。2011年に行われた米国国立睡眠財団のアンケート調査によると、米国成人の87%が少なくとも数日間に一度は睡眠障害を経験している。睡眠障害は国民の健康の低下だけでなく、社会的にも大きな経済的損失を生み出すので、睡眠は国民の保健の中で最も重要な問題と認識されている。

不眠症の治療のために様々な睡眠剤が開発されているが、その中で代表的な睡眠剤としてγ-aminobutyric acid type A-benzodiazepine($GABA_A$-BZD) receptorをターゲットとしたものがある。また、世界的に有名な製薬会社らは、副作用がなく効果の高い理想的な睡眠剤の開発のために努力をしている。最近は睡眠剤の副作用と依存性のために睡眠の天然サプリメントが愛用されているが、この天然サプリメントの代表的なものとしてはvalerian、St. John's wort、kava-kavaなどがある。米国およびヨーロッパでは睡眠の天然サプリメントが数多く市販されており、植物および食品成分の睡眠誘導効果に対する研究が活発になされている。

日本や韓国を始めとするアジアでは、不眠症の治療のため、伝統的に利用されてきた数多くの生薬があるが、これらの活性成分と作用機構に対する研究は欧米に比べればそれほど多くはない。そこで本研究では、日本と韓国の植物資源から睡眠誘導効果を持つ新たな植物を探索するとともに、それらから活性成分を同定し、その作用機構を明らかにすることを目的とした。睡眠を誘導する新たな天然物を発見し、その作用機構を明らかにすることは、副作用のない睡眠剤の開発の出発点になると思われる。

本研究では抽出物バンクから購入した30種類の陸上植物と30種類の海藻類の抽出物について、睡眠誘導作用の分子ターゲットである$GABA_A$-BZD receptorに対する結合活性をまず調べた。特に、海藻類および海洋天然物の睡眠誘導効果に対する研究はまだ行われておらず、本研究が最初の研究例である。

それぞれ30種類の海藻類および陸上植物の抽出物を調べた結果、海藻類ではカジメ(*Ecklonia cava*; IC_{50}: 0.392 mg/mL)、陸上植物では甘草(*Glycyrrhiza glabra*; IC_{50}: 0.093 mg/mL)が最も高い結合活性を示した。カジメからのエタノール抽出物(ECE)および甘草からのエタノール抽出物(GGE)は1000 mg/kgの濃度でマウスの入眠時間の減少と睡眠時間の増加を示し、既に良く知られている睡眠剤であるdiazepam(DZP; 2 mg/kg)および天然の睡眠サプリメントであるvalerianからの抽出物(1000 mg/kg)とほとんど同じ効果を示した。

次に、ECEおよびGGEについてもっと詳しく調べるために睡眠誘導効果および睡眠構造の変化に及ぼす影響を検討した。産業的活用と活性成分探索を目的としてresponse surface methodology(RSM)による最適化を行い、睡眠誘導活性が最大になる抽出条件を設定した。ECEおよびGGEの活性成分は$GABA_A$-BZD receptorに対する結合活性試験により追跡分離し、動物および神経細胞を用いた活性測定実験より睡眠誘導効果と作用機構を明らかにした。

ECEとGGEは濃度に依存して(100–1000 mg/kg)マウスの睡眠時間の増加と入眠時間の減少を誘導した。特に、睡眠を誘導しない濃度(30 mg/kg)のpentobarbitalで処理したマウスに、ECEとGGEを投与することで睡眠時間を増加させ、睡眠を誘導する役割を示すことが明らかになった。

RSMにより得られた、睡眠誘導効果を最大化するカジメおよび甘草の抽出条件は次のようである。＜カジメ(ECE)＞: エタノール濃度　81.6%、抽出時間　52.2h、抽出温度　43.7℃。＜甘草(GGE)＞: エタノール濃度　79.8%、抽出時間　12.0h、 抽出温度　48.0℃。

最適の条件下で抽出されたECEとGGEは、500 mg/kgの濃度でラットの睡眠誘導時間が其々130.3および118.2 minとなり、既存の抽出物バンクの条件で製造された抽出物より高い睡眠誘導効果を示した。

Flavonoidを含めたphenol成分は睡眠を誘導する代表的な植物成分の一つであることが知られている。本研究の結果でも、甘草の場合はtotal flavonoid content(TFC)、カジメの場合はtotal phenol content(TPC)成分が、ECEおよびGGEの予想の活性成分として示された。すなわち、ECEとGGEの睡眠誘導効果はTPCとTFCに比例する傾向があり、これらが活性成分である可能性を示した。

ECEおよびGGEが睡眠の構造および質に及ぼす影響をマウスの脳波(electroencephalogram,

EEG)および筋電圖(electromyogram)を計ることにより調べた。ECEおよびGGEは濃度に依存し、NREMS(non-rapid eye movement sleep)を増加させた。ECEおよびGGEは共に500 mg/kgの濃度で、対照群であるDZP 2 mg/kgを投与したものとほとんど同じ効果を示したが、REMS(rapid eye movement sleep)の変化には影響を及ぼさなかった。ECEは投与後2時間の間、効果的にNREMSを誘導したが、その後は対照群と類似した睡眠構造を示した。睡眠剤であるDZPは、睡眠の質を表すEEG powder densityのdelta wave(0.5–4 Hz)の活性を有意的に減少させたが、これはDZPの一般的な特徴としてよく知られている。しかし、ECEは一般対照群のdelta活性とほとんど同じ水準を示し、睡眠の質の低下がなく、正常状態の生理的睡眠を誘導することが示唆された。

$GABA_A$-BZD receptorに対する結合活性試験によって見出されたECEおよびGGEが睡眠効果を示したので、これらは生体中でも$GABA_A$-BZD receptorに直接作用する可能性があると思われた。そこで、$GABA_A$-BZD receptorの抑制剤であるflumazenil(FLU)を利用し、ECEとGGEの*in vivo*実験を行った。$GABA_A$-BZD receptorのagonistであるDZPの睡眠誘導効果はFLUよって抑制される。ECEとGGEもDZPと同様にFLUを投与したマウスで睡眠誘導効果が認められなかったので、DZPと同じ$GABA_A$-BZD receptorのagonistとしての作用していることが明らかになった。

一方、$GABA_A$-BZD receptorの結合活性の分析により、次のような6種のECE phlorotanninと3種のGGE flavonoidを活性成分として分離同定した。＜ECE＞: eckstolonol(ETN)、triphlorethol A(TPRA)、 eckol、fucodiphlorethol G、6,6’-bieckol、dieckol、phloroglucinol。＜GGE＞: glabridin(GBD)、isoliquiritigenin(ILTG)、glabrol。ECEおよびGGE中に見出されたこれらの活性成分は、すべて5–50 mg/kgの範囲で濃度依存的に睡眠誘導効果を示した。またFLUによってその効果が抑制されたことから、これらの成分は$GABA_A$-BZD receptorを活性化させ、睡眠を誘導することが確認された。次に、睡眠効果が最も高かったECE中のETNとTPRA、およびGGE中のGBDとILTGが睡眠の構造と質に及ぼす影響について調べた。これらの全ての活性成分は50 mg/kgの濃度で有意にNREMSを増加させたが、REMSの変化は認められなかった。特に、ETNとTPRAはECEと同様にdelta活性には変化のないNREMSを誘導した。

活性成分のこれ以外の機能を調べるために神経細胞のGABA-induced currentに対する影響

をfull agonistであるDZPと比較しながら実験を行った。ETN、TPRAおよびILTGは、DZPに比較して各々27.1%、42.8%および30.7%の活性を示し、$GABA_A$-BZD receptorに対しpartial agonistとして作用することが分かった。GBDの場合はDZPに比べて200%の高い活性を示すなど、特別な薬理学的な特徴を示した。

以上、本研究ではカジメおよび甘草の睡眠誘導効果とその有効成分を明らかにするとともに、これらの活性成分の作用機構についても検討した。見出された活性成分のうち、睡眠誘導効果を表す活性成分であることが認められたETN、TPRAおよびILTGは、副作用がなく、長期間摂取できる天然睡眠サプリメントとして利用できる可能性を示した。特に、海洋天然物であるETNおよびTPRAは褐藻類のみに含まれている特殊なphenol成分であり、本研究により始めて睡眠誘導効果があることが明らかになった。本研究により得られた結果は、副作用のない新たな睡眠剤の開発に役立つものと考えられる。

PUBLICATIONS AND PRESENTATIONS

Publications

1. **Cho S**, Shimizu M, Lee CJ, Han DS, Jung CK, Jo JH, Kim YM. Hypnotic effects and binding studies for $GABA_A$ and 5-HT_{2C} receptors of traditional medicinal plants used in Asia for insomnia. *J*. *Ethnopharmacol*. 2010; 132: 225–232.
2. **Cho S**, Kim S, Jin Z, Han D, Baek NI, JH J, Cho CW, Yang H, Park JH, Shimizu M, Jin YH. Isoliquiritigenin, a chalcone compound, is a positive allosteric modulator of $GABA_A$ receptors and shows hypnotic effects. *Biochem*. *Biophys*. *Res*. *Commun*. 2011; 413: 637–642.
3. **Cho S**, Han D, Kim SB, Yoon M, Yang H, Jin YH, Jo J, Lee SH, Jeon YJ, Shimizu M. Depressive effects on central nervous system and underlying mechanism of the enzymatic extract and its phlorotannin-rich fraction from *Ecklonia cava* edible brown seaweed. *Biosci*. *Biotechnol*. *Biochem*. 2012; 76: 163–168.
4. **Cho S**, Yang HJ, Jeon YJ, Lee CJ, Jin YH, Baek NI, Kim DS, Kang SM, Yoon MS, Yong HI, Shimizu M, Han D. Phlorotannins of the edible brown seaweed *Ecklonia cava* Kjellman induce sleep via positive allosteric modulation of gamma-aminobutyric acid type A-benzodiazepine receptor: A novel neurological activity of seaweed polyphenols. *Food Chem*. 2012; 132: 1133–1142.
5. **Cho S**, Yoon M, Kim D, Kim J, Yang H, Lee C, Kim I, Shimizu M, Han, D. Effect of the licorice flavonoid isoliquiritigenin on the sleep architecture and profile in mice. *Food Sci. Biotechnol*. 2012 (*in press*).
6. **Cho S**, Park J, Pae A, Han D, Kim D, Cho N, No K, Yang H, Yoon M, Lee C, Shimizu M, Baek NI. Hypnotic effects and GABAergic mechanism of licorice (*Glycyrrhiza glabra*) ethanol extract and its major flavonoid constituent glabrol. *Bioorg. Med. Chem.* 2012 (*in press*).
7. **Cho S**, Pae A, Yoon M, Yang H, Cho N, No K, Baek NI, Park JH, Shimizu M, Han D. Isoflavan glabridin: Positive allosteric modulators of $GABA_A$ receptors with sedative-hypnotic effects. *Biochem*. *Pharmacol*. 2012 (*in preparation*).
8. **Cho S**, Kim S, Yang H, Jin Z, Yoon M, Han D, Baek NI, Park JH, Jin YH, Shimizu M. A Novel natural hypnotic: GABAergic mechanism of the marine natural product phlorotannins and pharmacological comparison with diazepam and zolpidem. *Sleep*. 2012 (*in preparation*).

Oral presentations

1. July 9. 2011. Korean Society of Sleep Research (*in Korea*)
   Title: Development of natural sleep aids from Korean foods and plants
2. November 22. 2011. International Conference on Food Factors (*in Taiwan*)
   Title: A novel neurological activity of seaweed polyphenols (*Young Investigator Award*)

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