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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

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ABSTRACT

The primary objective was to investigate whether seaweeds have hypnotic activity. Methanol extracts of 30 seaweeds were screened for their binding activity at the GABA type A–benzodiazepine ($GABA_A$–BZD) receptor, a well-characterised molecular target for sedative–hypnotics. The most active seaweed was *Ecklonia cava* Kjellman (ECK). An ethanol extract of ECK (ECK-E) significantly potentiated pentobarbital-induced sleep in mice. In four solvent fractions separated from ECK-E, hypnotic activity was proportional to contents of total phenols and total phlorotannins, known as seaweed polyphenols. Major phlorotannins of the ethyl acetate (EtOAc) fraction with the highest activity were eckol, eckstolonol, dieckol, and triphlorethol-A, and their K_i (binding affinity, μM) values for [^{3}H]-flumazenil binding were 1.070, 1.491, 3.072, and 4.419, respectively. Hypnotic effects of ECK-E and the EtOAc fraction were fully inhibited by flumazenil, a specific $GABA_A$–BZD receptor antagonist. These results imply that phlorotannins of ECK induce sleep by positive allosteric modulation of the $GABA_A$–BZD receptor.

1. Introduction

Sleep is vital to maintain human health and well-being (Krueger et al., 2008). Sleep deprivation leads to impairments in cognitive function, the immune system, and quality of life (Imeri & Opp, 2009). Insomnia is currently a widespread health complaint and represents the most common sleep disorder worldwide. Approximately 10–15% of the adult population suffers from chronic insomnia, and an additional 25–35% have transient or occasional insomnia (Doghramji, 2006). Conventional pharmacological treatments for insomnia include benzodiazepines (BZDs), antidepressants, and antihistamines (Borja & Daniel, 2006). However, the use of these sedative–hypnotics is generally not recommended beyond 4 weeks because of their various side effects, which can include impairment of cognitive function, memory, and general daytime performance (Thomas & Christopher, 2004). In addition, long-term administration typically results in tolerance and dependence (Fang et al., 2010).

Natural sleep aids, which contain specific constituents of foods and herbal plants, have recently become popular as alternatives to prescription sedative–hypnotics to improve sleep quality and avoid side effects (Meletis & Zabriskie, 2008). According to the 2002 National Health Interview Survey (Pearson, Johnson, & Nahin, 2006), more than 1.6 million American adults use alternative sleep aids to treat insomnia. Therefore, there has been a growing demand for a new class of food constituents and natural products with hypnotic effects.

It has been widely reported that polyphenols (mainly flavonoids) of terrestrial plants such as valerian (*Valeriana officinalis*), chamomile (*Matricaria recutita*), and kava-kava (*Piper methysticum*) have sedative–hypnotic effects based on positive allosteric modulation of $GABA_A$ receptors (Johnston, 2005). For example, it has been reported that hispidulin, a flavone, has the ability to

Abbreviations: BBB, blood–brain barrier; BZD, benzodiazepine; CMC, carboxymethyl cellulose; CON, control group; DZP, diazepam; ECK, *Ecklonia cava* Kjellman; ECK-E, ECK ethanol extract; EtOAc, ethyl acetate; GABA, gamma-aminobutyric acid; i.p., intraperitoneal injection; PGE, phloroglucinol equivalents; p.o., post-oral injection; TPC, total phenol content.

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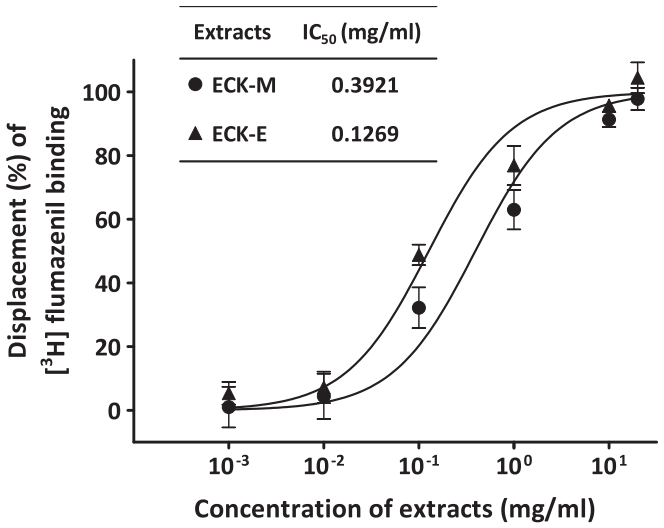


Fig. 1. Dose–response curves and IC_{50} values of methanol (ECK-M) and ethanol (ECK-E) extracts of ECK (*Ecklonia cava* Kjellman) in the $GABA_A$–BZD receptor-binding assay. Each data point is expressed as mean ± SD ($n = 3$).

stimulate a GABA-induced Cl^- current. Hispidulin has positive allosteric properties and permeability across the blood–brain barrier (BBB) in a rat *in situ* perfusion model (Kavvadias et al., 2004). Apigenin, a chamomile component that has been characterised as a $GABA_A$–BZD receptor ligand, has hypnotic and anticonvulsant activities (Viola et al., 1995). Chlorogenic acid (Bouayed, Rammal, Dicko, Younos, & Soulimani, 2007) and epigallocatechin gallate (Vignes et al., 2006) exhibit anxiolytic effects by acting as $GABA_A$–BZD receptor agonists. The search for molecular targets of polyphenols derived from terrestrial plants has been mainly focused on the $GABA_A$–BZD receptor (Johnston, 2005). GABAergic neurotransmission plays a key role in sleep regulation, and the BZD-binding site on the $GABA_A$ receptor is a target for the most sedative–hypnotics (Bateson, 2006). BZD agents such as diazepam (DZP) stimulate the ability of GABA to cause hyperpolarization of membranes by allowing a Cl^- influx (Johnston, 2005).

Although a large number of studies on hypnotic effects of terrestrial plant constituents have been performed, seaweeds have not been recognised as a potential source of natural hypnotics. In Japan and Korea, seaweeds have long been a key part of the daily diet and have been used in treatments in traditional medicine (Fitton, 2003; Smit, 2004). In particular, in Korea, brown seaweeds are well known as a folk medicine administered to new mothers after birth (Moon & Kim, 1999). Seaweeds include various constituents such as phenols, carotenoids, terpenes, and polysaccharides, and were reported to have antitumour, antioxidant, antibacterial, anticoagulant, anti-diabetic and anti-inflammatory activities (Smit, 2004; Blunt et al., 2007; Wang, Jonsdottir, & Olafsdottir, 2009; Cho, Lee, Kang, Won, & You, 2011; O'sullivan et al., 2011; Felix et al., 2011). Despite a number of reports on bioactivities and uses of seaweeds in traditional medicines, their hypnotic activities have not yet been explored. Therefore, we were interested to determine if seaweeds have hypnotic activity similar to that of terrestrial plants such as valerian and chamomile.

In the present study, 30 seaweeds were tested including eight green seaweeds, 11 red seaweeds, and 11 brown seaweeds, that are found in the coastal areas of Japan and Korea. In order to evaluate the potential of seaweeds as a resource for sedative–hypnotics, their binding activity to the $GABA_A$–BZD receptor were screened. *Ecklonia cava* Kjellman (ECK) was identified as the seaweed with the highest binding activity. The phlorotannin composition of ECK was analysed, and the binding affinity of major phlorotannin constituents to the $GABA_A$–BZD receptors was

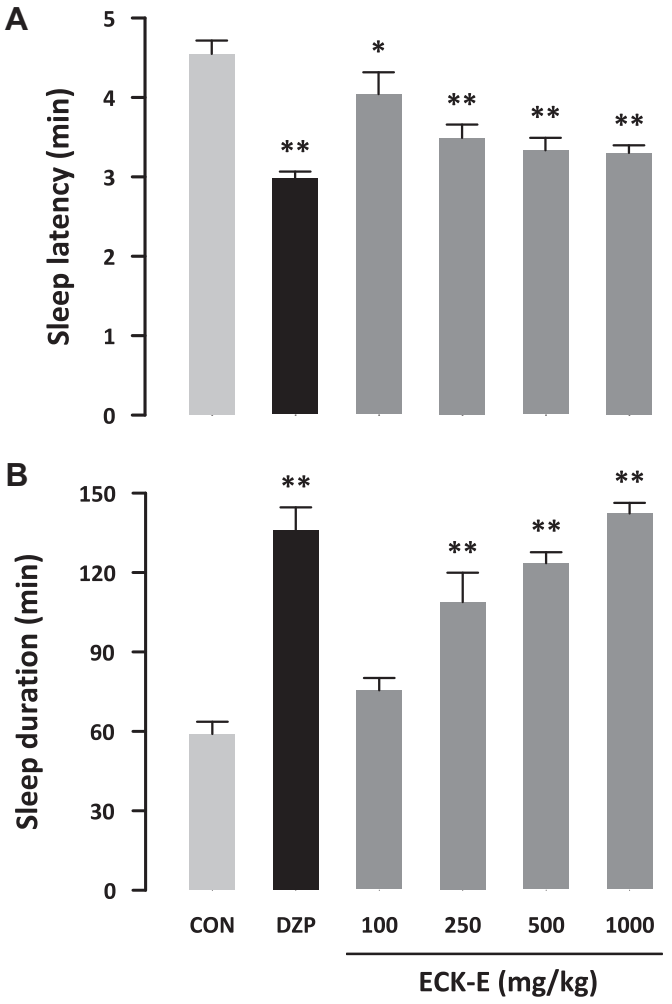


Fig. 2. Effects of ECK-E on sleep latency (A) and sleep duration (B) in mice administered a hypnotic dose (45 mg/kg, i.p.) of pentobarbital. Mice received pentobarbital 45 min after oral administration (p.o.) of CON (0.5% CMC-saline, 10 ml/kg), DZP (2 mg/kg), and ECK-E. Each column represents mean ± SEM ($n = 10$). $^*p < 0.05$, $^{**}p < 0.01$, significant as compared to the control group (Dunnett's test). *Abbreviations:* CON, control group; DZP, diazepam; ECK-E, *Ecklonia cava* Kjellman ethanol extract.

evaluated. Hypnotic effects of ECK and its action mechanism were investigated using an *in vivo* animal model.

2. Materials and methods

2.1. 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. Flumazenil, a $GABA_A$–BZD receptor antagonist, was purchased from Sigma–Aldrich Inc. (St. Louis, MO, USA). For the $GABA_A$–BZD receptor-binding assay, the radioligand [^{3}H]-flumazenil (Ro 15-1788; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) was used. All other chemicals and reagents were of the highest grade available.

2.2. Seaweed extracts

The methanol extracts of 30 seaweeds were purchased from the Jeju Bioresource Extract Bank of the Jeju Technopark (Jeju, Korea).

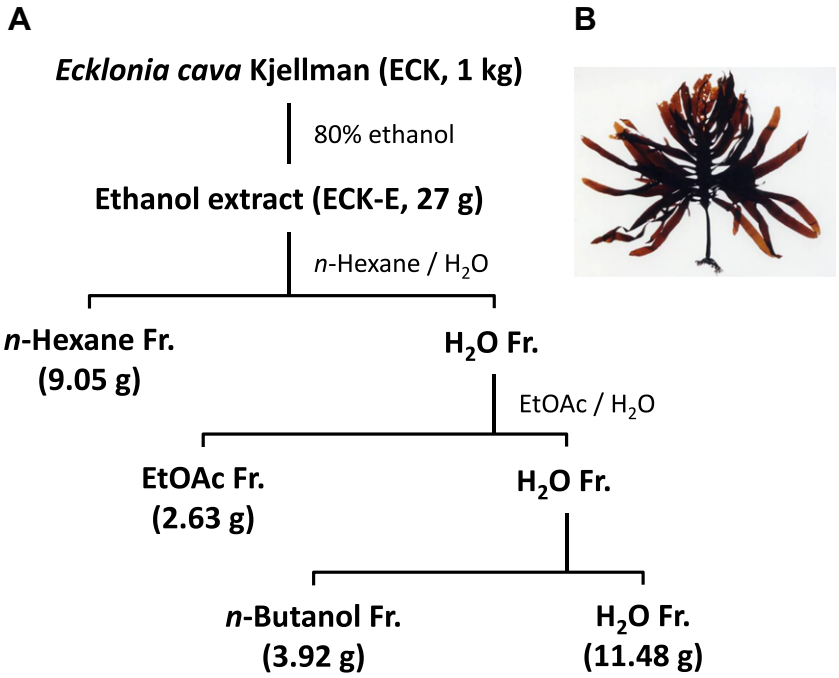


Fig. 3. Scheme for solvent fractionation from ECK-E (A) and a photograph of ECK (B). *Abbreviations:* ECK, *Ecklonia cava* Kjellman; ECK-E, ECK ethanol extract.

Voucher specimens are deposited in the Jeju Bioresource Extract Bank. According to the extract specification, all seaweed extractions were performed using 80% methanol at room temperature for 24 h.

2.3. Preparation of ethanol extract and solvent fractions from ECK

In order to evaluate the hypnotic effects of ECK with the highest binding activity to the $GABA_A$–BZD receptor, we prepared ECK-E. With the consideration that ECK could be used as a functional food, ethanol was selected as an extraction solvent. Dried ECK was purchased from Taerim Co. Ltd. (Jeju, Korea). Whole dried ECK was extracted with 80% ethanol at 50 °C for 3 days. The extraction solutions were then filtered and lyophilised. ECK-E was further fractionated with different solvents to search for the active compounds (Fig. 3). ECK-E was suspended in distilled water, and partitioned with *n*-hexane, EtOAc (ethyl acetate), *n*-butanol, and distilled water in sequence. All solvent fractions were evaporated to dryness.

2.4. Animals

To obtain a membrane preparation for the $GABA_A$–BZD receptor-binding assay, 200–250 g male SD (Sprague Dawley) rats were used. In the pentobarbital-induced sleep test, male ICR (Imprinting Control Region) 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 9:00 AM). All procedures involving animals were conducted in accordance with the animal care and use guidelines of the Korea Food Research Institutional Animal Care and Use Committee (Permission No.: KFRI-M-09118).

2.5. $GABA_A$–BZD receptor-binding assay

The $GABA_A$–BZD receptor binding assay was modified from the method described by Risa et al. (2004). The cerebral cortex from four male SD rats was homogenised 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 three times with Tris–HCl buffer. The washed pellet was homogenised 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 27,000*g* for 10 min. The final membrane pellet was resuspended in 30 ml of Tris–HCl buffer and stored in aliquots at −80 °C until it was used in the binding assay. The membrane preparation was thawed and washed with 20 ml of Tris–citrate buffer (50 mM, pH 7.1, 0–4 °C) three 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 solution was mixed and incubated on ice for 40 min. The binding reaction was terminated by rapid filtration onto a Whatman GF/C glass fibre 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 Eq. (1):

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

where DPM, TB, and NSB denote disintegrations per minute, total binding, and non-specific binding, respectively. 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). Values of binding affinity (K_i) were calculated by the following Eq. (2):

Table 1
In vitro binding activities of methanol extracts of green seaweeds to the $GABA_A$–BZD receptor.

Family	Species	Voucher specimen	Displacement (%) of [^{3}H]-flumazenil binding to the $GABA_A$–BZD receptor		
			0.1 mg/ml	1 mg/ml	10 mg/ml
Cladophoraceae	*Cladophora wrightiana* Harvey	AC027	−4.1 ± 3.3	18.6 ± 7.1	70.6 ± 5.0
Codiaceae	*Codium coactum* Okamura	AC012	18.1 ± 2.3	26.3 ± 1.8	46.5 ± 4.5
	Codium contractum Kjellman	AC014	12.3 ± 5.2	23.5 ± 4.5	42.3 ± 2.0
	Codium fragile Hariot	AC023	23.4 ± 3.0	38.3 ± 1.8	55.9 ± 5.6
	Codium latum Suringa	AC013	7.6 ± 5.6	19.0 ± 3.8	48.0 ± 4.0
	Codium minus Silva	AC002	15.4 ± 5.2	17.9 ± 2.0	39.2 ± 1.8
Ulvaceae	*Ulva conglobata* Kjellman	AC003	8.5 ± 4.7	30.8 ± 3.7	86.2 ± 1.9
	Ulva pertusa Kjellman	AC033	−6.1 ± 3.2	32.5 ± 5.8	72.5 ± 3.6

The results were expressed as mean ± SD (n = 3).

$$K_i = \frac{IC_{50}}{1 + [L]/k_d} \quad (2)$$

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

2.6. Pentobarbital-induced sleep test

All experiments were performed between 1:00 and 5:00 PM, and mice were fasted for 24 h prior to the experiment. For oral administration, 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 (sub-hypnotic dose, 30 mg/kg and 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. In the sub-hypnotic dose of the pentobarbital-treatment test, the rate of sleep onset was calculated by the following Eq. (3):

$$\text{Rate of sleep onset}(\%) = \frac{\text{No. of mice falling asleep}}{\text{Total No.}} \times 100 \quad (3)$$

2.7. Determination of total phenol content

The total phenol content (TPC) was determined according to the Folin–Ciocalteu method described by Slinkard and Singleton (1977). 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 (seaweed phenols) (Rodriguez-Bernaldo de Quiros, Frecha-Ferreiro, Vidal-Perez, & Lopez-Hernandez, 2010). TPC was expressed as phloroglucinol equivalents (mg PGE/g).

2.8. HPLC analysis of phlorotannin composition

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 × 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 5–100% methanol. The flow rate was set at 0.8 ml/min with an injection volume of 10 μl and 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.

2.9. Statistical analysis

For multiple comparisons in the pentobarbital-induced sleep test, data were analysed using one-way ANOVA followed by Dunnett's test. Comparisons between two-group data were analysed 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).

3. Results

3.1. Screening of $GABA_A$–BZD receptor-binding activity of seaweed extracts

To explore potential seaweeds for sedative–hypnotic compounds, we screened methanol extracts of 30 seaweeds (eight green seaweeds, 11 red seaweeds, and 11 brown seaweeds) through the $GABA_A$–BZD receptor binding assay. Tables 1 and 2 show displacement (%) of [^{3}H]-flumazenil binding obtained with three concentrations (0.1, 1, and 10 mg/ml) of methanol extracts of green and red seaweeds. *Ulva conglobata* Kjellman (green seaweed) and *Gracilaria verrucosa* Papenfuss (red seaweed) were found to displace over 80% of [^{3}H]-flumazenil binding at a concentration of 10 mg/ml. However, most of the green and red seaweeds did not show any effective binding activity. The most active seaweed was ECK, a brown seaweed (Table 3), and its IC_{50} value was 0.3921 mg/ml (Fig. 1).

3.2. Hypnotic effects of ECK-E

For evaluation of hypnotic activity of ECK, we prepared ECK-E, which would be safer for human consumption as compared to the methanol extract. In the $GABA_A$–BZD receptor-binding assay, ECK-E was found to have a lower IC_{50} value (0.1269 mg/ml) than that of the ECK methanol extract (Fig. 1). With a hypnotic dose of pentobarbital (45 mg/kg, i.p.), sleep latency and duration of the control group were 59.0 ± 4.6 and 4.5 ± 0.2 min, respectively (Fig. 2). As expected, it was found that a well-known $GABA_A$–BZD receptor agonist, DZP (2 mg/kg, p.o.) significantly potentiated the

Table 2
In vitro binding activities of methanol extracts of red seaweeds to the $GABA_A$–BZD receptor.

Family	Species	Voucher specimen	Displacement (%) of [^{3}H]-flumazenil binding to the $GABA_A$–BZD receptor		
			0.1 mg/ml	1 mg/ml	10 mg/ml
Champiaceae	*Champia parvula* Harvey	AR032	6.1 ± 3.0	7.6 ± 2.6	10.4 ± 3.7
Gelidiaceae	*Gelidium amansii* Lamouroux	AR006	6.4 ± 4.4	25.5 ± 1.4	65.9 ± 1.6
Gigartinaceae	*Chondracanthus tenellus* Hommersand	AR031	11.8 ± 6.4	16.3 ± 5.1	21.7 ± 3.3
	Gracilaria verrucosa Papenfuss	AR001	13.9 ± 3.1	20.7 ± 2.3	80.6 ± 4.8
Grateloupiaceae	*Grateloupia filicina* C.Agardh	AR014	-2.4 ± 1.9	21.8 ± 5.1	16.9 ± 9.2
	Polyopes lancifolius	AR039	10.1 ± 3.6	15.7 ± 2.6	35.3 ± 3.0
Halymeniaceae	*Grateloupia lanceolata* Kawaguchi	AR038	17.8 ± 7.2	24.9 ± 2.3	45.7 ± 1.1
	Polyopes affinis	AR042	8.0 ± 3.9	13.8 ± 6.9	7.4 ± 7.4
Hypneaceae	*Hypnea japonica* Tanaka	AR018	12.5 ± 2.1	10.1 ± 3.8	57.5 ± 2.7
Rhodomelaceae	*Chondria crassicaulis* Harvey	AR041	-6.1 ± 4.0	7.2 ± 2.1	43.6 ± 0.9
	Polysiphonia morrowii Harvey	AR011	15.0 ± 9.1	23.3 ± 4.9	55.0 ± 3.1

The results were expressed as mean ± SD (n = 3).

Table 3
In vitro binding activities of methanol extracts of brown seaweeds to the $GABA_A$–BZD receptor.

Family	Species	Voucher specimen	Displacement (%) of [^{3}H]-flumazenil binding to the $GABA_A$–BZD receptor		
			0.1 mg/ml	1 mg/ml	10 mg/ml
Alariaceae	*Ecklonia cava* Kjellman	AP057	5.2 ± 6.4	43.1 ± 6.2	91.3 ± 2.3
Asperococcaceae	*Myelophycus simplex* Papenfuss	AP032	17.5 ± 3.4	12.6 ± 5.5	25.1 ± 2.2
Corynophloeaceae	*Leathesia difformis* Areschoug	AP046	5.7 ± 7.5	22.0 ± 7.8	21.7 ± 2.3
Dictyotaceae	*Dictyopteris prolifera* Okamura	AP028	18.5 ± 1.0	31.5 ± 4.9	63.8 ± 4.9
Sargassaceae	*Dictyota coriacea*	AP043	16.2 ± 1.7	16.2 ± 5.6	42.1 ± 2.0
	Dictyota dichotoma Lamouroux	AP042	26.3 ± 14.7	25.7 ± 9.3	72.6 ± 1.6
Sargassacea	*Sargassum horneri* C.Agardh	AP052	4.4 ± 3.6	18.8 ± 1.3	70.4 ± 2.4
	Sargassum patens C.Agardh	AP020	3.9 ± 2.8	15.9 ± 4.7	55.7 ± 1.5
Scytosiphonaeae	*Petalonia binghamiae* Vinogradova	AP033	−6.9 ± 2.7	12.6 ± 8.5	22.5 ± 4.4
	Colpomenia sinuosa Derbes et Solie	AP021	16.4 ± 4.7	24.9 ± 2.1	32.6 ± 2.3
Shigeaceae	*Ishige okamurae* Yendo	AP055	−8.3 ± 4.1	20.0 ± 2.3	73.8 ± 1.8

The results were expressed as mean ± SD (n = 3).

Table 4
Effects of ECK-E 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		2/12	17	7.3 ± 1.1
DZP	2	12/12	100	55.4 ± 4.8***
ECK-E	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 mean ± SEM.
Abbreviations: CON, control group; DZP, diazepam; ECK-E, *Ecklonia cava* Kjellman ethanol extract.
* $p < 0.05$, significant as compared to the control group (Dunnett's test).
*** $p < 0.001$, significant as compared to the control group (Dunnett's test).

pentobarbital-induced sleep in mice ($p < 0.01$) relative to the control group. ECK-E also caused a dose-dependent decrease in sleep latency (Fig. 2A) and an increase in sleep duration (Fig. 2B). In particular, administration of 1000 mg/kg of ECK-E was found to prolong sleep duration up to 142.3 ± 4.0 min, to a level similar to that induced by DZP. With a sub-hypnotic dose of pentobarbital (30 mg/kg, i.p.), ECK-E dose-dependently increased the rate of sleep onset and prolonged sleep duration (Table 4). ECK-E at 1000 mg/kg significantly increased sleep duration (38.7 ± 6.3 min) and the rate of sleep onset (92%). This effect is comparable to that of DZP, which produced 55.4 ± 4.8 min of the sleep duration and a 100% rate of sleep onset.

3.3. Binding activity and hypnotic effects of ECK-E solvent fractions and their correlation with TPC

To identify active compounds with hypnotic activity, ECK-E was further fractionated with different solvents (Fig. 3). *n*-Hexane (9.05 g), EtOAc (2.63 g), *n*-butanol (3.92 g), and H_2O (11.48 g) fractions were obtained from 27 g of ECK-E. The EtOAc fraction was found to have the lowest IC_{50} value (0.0185 mg/ml) for [^{3}H]-flumazenil binding to the $GABA_A$–BZD receptor, and IC_{50} values of *n*-butanol, *n*-hexane, and H_2O fractions was found to be 0.1033, 0.1405, and 0.9607, respectively (Fig. 4A). Hypnotic effects of solvent fractions had a similar tendency with respect to the binding activity. The EtOAc fraction significantly increased the duration of the sleep period at 100 and 200 mg/ml ($p < 0.01$) (Fig. 4B). H_2O fractions did not produce significant hypnotic activity at 200 mg/ml.

According to the previous reports on ECK phlorotannins (Shibata et al., 2004; Kim et al., 2009; Lee, Han, Heo, Hwang, & Jeon, 2010), most phlorotannins were isolated from the EtOAc fraction. Therefore, it was expected that binding activity and hypnotic effects of ECK-E might be due to its phlorotannins, which are known as seaweed polyphenols. The TPC values for EtOAc, *n*-butanol, *n*-hexane and H_2O were found to be 685.7, 262.6, 102.9, and

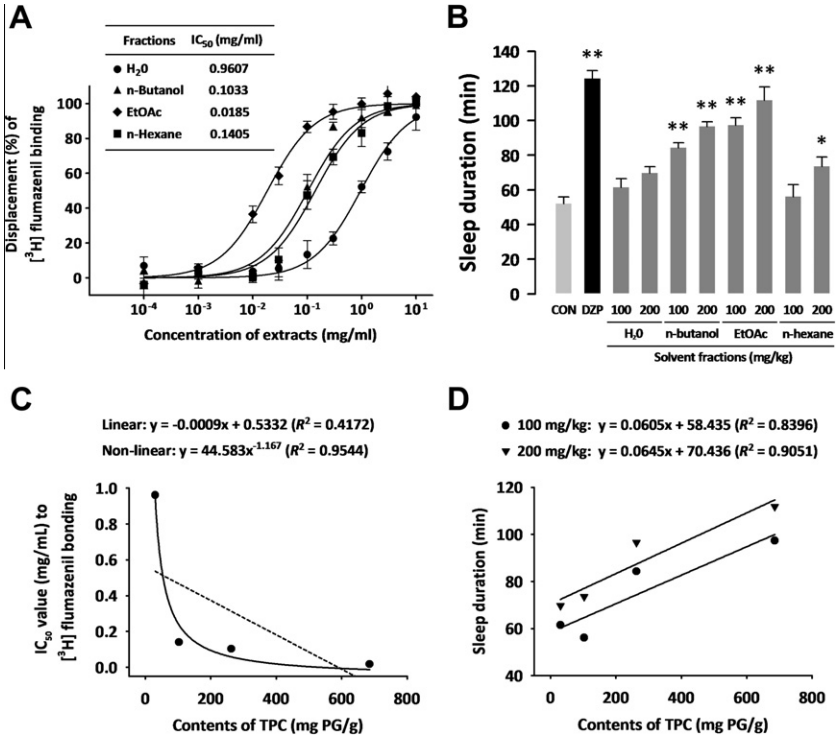


Fig. 4. Correlations of TPC with binding activity and sleep duration in different solvent fractions of ECK-E. (A) IC_{50} values of ECK-E solvent fractions for [^{3}H]-flumazenil binding to the $GABA_A$–BZD receptor. Each data point is expressed as mean ± SD (n = 3). (B) Effects of ECK-E solvent fractions on sleep duration in mice administered a hypnotic dose (45 mg/kg, i.p.) of pentobarbital. Mice received pentobarbital 45 min after oral administration (p.o.) of CON (0.5% CMC-saline, 10 ml/kg), DZP (2 mg/kg), and ECK-E solvent fractions. Each column represents mean ± SEM (n = 10). $^*p < 0.05$, $^{**}p < 0.01$, significant as compared to the control group (Dunnett's test). (C) A correlation between IC_{50} values and TPC. (D) A correlation between sleep duration and TPC. *Abbreviations:* CON, control group; DZP, diazepam; ECK-E, *Ecklonia cava* Kjellman ethanol extract; EtOAc, ethyl acetate; TPC, total phenol content.

Table 5
Total phenol contents (TPC) of ECK-E and its solvent fractions.

Fractions	*n*-Hexane	EtOAc	*n*-Butanol	H_2O	ECK-E
TPC (mg PGE/g)	102.9 ± 0.8	685.7 ± 14.6	262.6 ± 12.2	30.4 ± 2.8	138.5 ± 2.4

Abbreviations: ECK-E, *Ecklonia cava* Kjellman ethanol extract; EtOAc, ethyl acetate; PGE, phloroglucinol equivalents.

30.4 mg PGE/g, respectively (Table 5). The binding activities (Fig. 4C) and hypnotic effects (Fig. 4D) of the solvent fractions were found to be proportional to their TPC values. In the case of the binding activity, the coefficient of determination of the non-linear regression (R^2) was 0.9544. Good correlations were established between TPC content and sleep duration (100 mg/kg, R^2 = 0.8396; 200 mg/kg, R^2 = 0.9051).

3.4. Phlorotannin compositions of ECK-E and its solvent fractions

The hypnotic effect and binding activity of ECK are correlated with TPC. Therefore, the composition of phlorotannins was analysed using HPLC. Major phlorotannin constituents detected in ECK samples were phloroglucinol, eckol, eckstolonol, triphlorethol-A, and dieckol. EtOAc (367.42 mg/g) and *n*-butanol (177.11 mg/g) fractions, which had higher hypnotic effects and binding activities, were found to have higher phlorotannin content than the *n*-hexane (20.49 mg/g) and H_2O (15.24 mg/g) fractions (Table 6). In different solvent fractions, phlorotannin content also had an excellent correlation with sleep duration (100 mg/kg, R^2 = 0.9682; 200 mg/kg, R^2 = 0.9434). Dieckol was the most abundant compound in the EtOAc fraction (274.73 mg/g) and in the *n*-butanol (115.00 mg/g) fraction. Eckol was detected only in the EtOAc and *n*-butanol fractions but not in the *n*-hexane and H_2O fractions. Phloroglucinol and eckstolonol were identified in the EtOAc fraction. ECK-E (92.77 mg/g) included phloroglucinol (7.59 mg/g), eckstolonol (4.06 mg/g), eckol (18.11 mg/g), triphlorethol-A (9.79 mg/g), and dieckol (53.22 mg/g) (Table 6).

Table 6
Phlorotannin compositions (mg/g) of ECK-E and its solvent fractions.

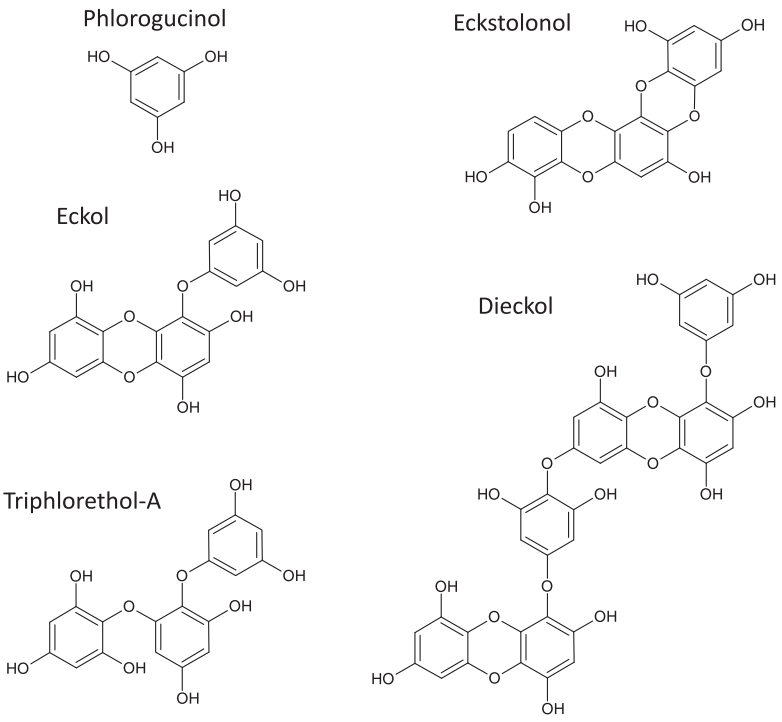
Constituents	*n*-Hexane	EtOAc	*n*-Butanol	H_2O	ECK-E
Phloroglucinol	–	16.14	–	–	7.59
Eckstolonol	–	21.83	–	–	4.06
Eckol	–	33.26	37.55	–	18.11
Triphlorethol-A	11.19	21.46	24.56	9.15	9.79
Dieckol	9.31	274.73	115.00	6.09	53.22
Total	20.49	367.42	177.11	15.24	92.77

Abbreviations: ECK-E, *Ecklonia cava* Kjellman ethanol extract; EtOAc, ethyl acetate.

3.5. Binding affinity of phlorotannin constituents

Fig. 5 shows the structures (Fig. 5A) and binding affinities (K_i, Fig. 5B) of the phlorotannin constituents. The K_i value of DZP, as a reference compound to assess the relative potency of phlorotannin

A



B

Compounds	Molecular weight	IC_{50} (μM)	K_i (μM)
Diazepam (DZP)	284.70	0.019	0.012
Phloroglucinol	126.11	> 1000	> 616
Eckstolonol	370.27	2.422	1.491
Eckol	372.28	1.739	1.070
Triphlorethol-A	374.30	7.180	4.419
Dieckol	742.55	4.991	3.072

Fig. 5. Structures of phlorotannin constituents (A) and their IC_{50} and K_i values for [^{3}H]-flumazenil binding to the $GABA_A$–BZD receptor (B). Diazepam (DZP) was used as a reference $GABA_A$–BZD receptor agonist.

constituents, was 0.012 μM in the $GABA_A$–BZD receptor-binding assay. Among the phlorotannin constituents, eckol (K_i = 1.070 μM) and eckstolonol (K_i = 1.491 μM) had better binding affinity than triphlorethol-A (K_i = 4.419 μM) and dieckol (K_i = 3.072 μM). The K_i value of phloroglucinol, which represents the basic structure of phlorotannins, was not obtained from the concentration range (42.25% inhibition at 1000 μM).

3.6. In vivo mechanism of hypnotic effect of ECK-E and EtOAc fraction

In order to verify the *in vivo* mechanism of the hypnotic effect of ECK, the effects of co-administration of ECK-E (1000 mg/kg) and the EtOAc fraction (200 mg/kg) with flumazenil (8 mg/kg), a specific antagonist of the $GABA_A$–BZD receptor were tested. The pretreatment of flumazenil alone did not affect sleep latency (Fig. 6A) and sleep duration (Fig. 6B) induced by pentobarbital (45 mg/kg). As expected, flumazenil was found to significantly inhibit the hypnotic activity of DZP ($p < 0.01$). The hypnotic effect of ECK-E and the EtOAc fraction was also fully antagonised by flumazenil ($p < 0.01$). As a control experiment, the direct hypnotic effect of pentobarbital, DZP, ECK-E, and the EtOAc fraction (data not shown) were assessed. All 15 mice fell asleep upon administration of pentobarbital (45 mg/kg). However, DZP (2 mg/kg), ECK-E (1000 mg/kg), and the EtOAc fraction (200 mg/kg) alone without pentobarbital did not induce sleep.

4. Discussion

During the past two decades, numerous novel compounds isolated from seaweeds have been demonstrated to possess biological activities such as antitumour, antioxidant, antibacterial, anticoagulant, and anti-inflammatory activities (Smit, 2004; Blunt et al., 2007). In addition, neurological activities of extracts and constituents of seaweeds have been reported (Blunt et al., 2007). The methanol extract of *Ulva reticulate* (Suganthy, Karutha Pandian, & Devi, 2010) and phlorotannin compounds from *Ecklonia stolonifera* (Yoon, Chung, Kim, & Choi, 2008) showed the neuroprotective effect based on its inhibitory activity to acetyl and butyryl cholinesterases. Myung et al. (2005) reported that dieckol and phlorofucofuroeckol have memory-enhancing effects by inhibition of acetylcholinesterase in ethanol-treated mice. Neuroprotective effects of seaweeds show potential application as future pharmaceutical candidates to

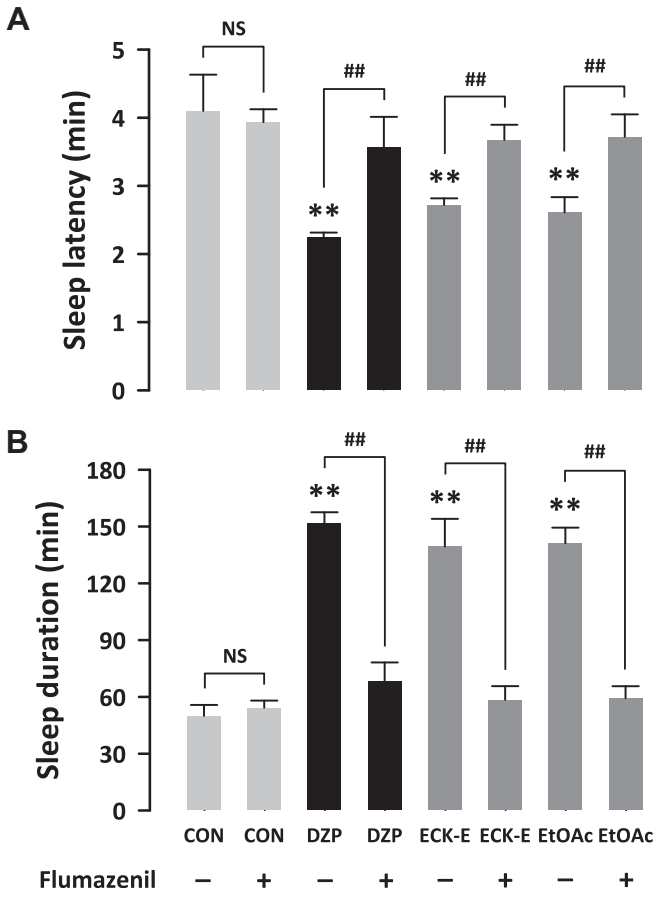


Fig. 6. Effects of flumazenil on the changes in sleep latency (A) and sleep duration (B) in mice treated with DZP, ECK-E, and the EtOAc fraction. Mice received pentobarbital 45 min after oral administration (p.o.) of CON (0.5% CMC-saline, 10 ml/kg), DZP (2 mg/kg), ECK-E (1000 mg/kg), and EtOAc fraction (200 mg/kg). Flumazenil (8 mg/kg, i.p.) was administered 15 min before oral administration. Each column represents mean ± SEM ($n = 10$). $^{**}p < 0.01$, significant as compared to the control group (Dunnett's test). $^{\#\#}p < 0.01$, significant between flumazenil treatment and no flumazenil treatment (unpaired Student's *t*-test). *Abbreviations:* CON, control group; DZP, diazepam; ECK-E, *Ecklonia cava* Kjellman ethanol extract; EtOAc, ethyl acetate; NS, not significant.

prevent neurodegenerative diseases (Pangestuti & Kim, 2011). However, to the best of our knowledge, the sedative–hypnotic activity of seaweeds has not been investigated.

As a result of screening using the $GABA_A$–BZD receptor-binding assay, ECK was found to have the highest binding activity and was selected as a candidate for animal studies. However, the other seaweeds such as *U. conglobata* Kjellman and *G. verrucosa* Papenfuss have the potential to be investigated. ECK is the edible brown seaweed that is distributed in the coastal areas of Korea and Japan (Shibata et al., 2004). The production amount of ECK in Korea is over 30,000 tons per year (Li et al., 2009). In Japan and Korea, ECK is popular as an ingredient of functional foods and traditional medicines. Recently, ECK extract has been used as a commercial functional food in the USA.

The results of the binding assay have indicated the possibility that ECK contains natural ligands that bind to the $GABA_A$–BZD receptor. However, it was not possible to distinguish between agonists and antagonists based on the results of the binding assay (de-Jong, Uges, Franke, & Bischoff, 2005). It is also important that the active compounds are able to pass the BBB to produce the hypnotic activity (Risa et al., 2004). Therefore, it is necessary to confirm the hypnotic activity through *in vivo* animal model assays (Fang et al., 2010). To confirm the hypnotic activity of ECK-E, the pentobarbital-induced sleep test in mice was performed. This method is useful to evaluate the sedative–hypnotic activity (Zhu, Bowery, Greengrass, & Phillipson, 1996). Our results clearly showed that ECK-E potentiates the hypnotic effects induced by both hypnotic (Fig. 2) and sub-hypnotic doses (Table 4) of pentobarbital. These results imply that active compounds in ECK-E are able to pass through the BBB to produce hypnotic activity.

In the different four solvent fractions, hypnotic effects had good correlations with TPC and phlorotannin content (Tables 5 and 6 and Fig. 4). The EtOAc fraction was found to have the highest activities in both the $GABA_A$–BZD receptor-binding assay (Fig. 4A) and the pentobarbital-induced sleep test (Fig. 4B). According to the previous reports for the isolation of ECK compounds, the EtOAc fraction has the characteristics of the phlorotannin-rich fraction (Shibata et al., 2004; Li et al., 2009). Therefore, it was predicted that there is a correlation between phlorotannins and hypnotic activity. Phlorotannins, which are oligomers and polymers of phloroglucinol (1,3,5-tri-hydroxybenzene), are an extremely heterogeneous group of compounds that are structurally different from polyphenols of terrestrial plants based on gallic acids or flavones (Shibata, Fujimoto, Nagayama, Yamaguchi, & Nakayama, 2002). Phlorotannins have only been found to exist within brown seaweeds (Shibata et al., 2004), and ECK contains more phlorotannins than do other brown seaweeds (Heo, Lee, Song, & Jeon, 2003). Thus far, active phlorotannin constituents such as eckol, bieckol, dieckol, phlorofucofuroeckol, triphlorethol-A, and eckstolonol have been isolated from ECK, and among these phlorotannins, dieckol is the main compound (Shibata et al., 2004; Li et al., 2009; Lee et al., 2010). It has been reported that phlorotannins have various biological activities such as antioxidative (Zou et al., 2008), anti-inflammatory (Kim et al., 2009), antibacterial (Nagayama, Iwamura, Shibata, Hirayama, & Nakamura, 2002), and antiallergic (Sugiura et al., 2006) effects.

In the present study, eckol, eckstolonol, dieckol, and triphlorethol-A were for the first time characterised as a gamma-aminobutyric acid type A–benzodiazepine receptor ligand (Fig. 5). Eckol showed the greatest binding affinity ($K_i = 1.070$ μM), and K_i values of dieckol, the main compound of ECK, was found to be 3.072 μM (Fig. 5). A number of flavonoids with affinity to the $GABA_A$–BZD receptors have been isolated from terrestrial plants (Jäger & Saaby, 2011). For example, apigenin, which was isolated from chamomile (*M. recutita*), was found to have a K_i value of 4 μM (Viola et al., 1995). 6-Methylapigenin (*Valeriana wallichii*) (Wasowski, Marder, Viola, Medina, & Paladini, 2002) and hispidulin (*Artemisia herba-alba*) (Salah & Jäger, 2005) were isolated from the sedative plants, and were found to have K_i values of 0.5 and 8 μM, respectively. Wogonin with a K_i value of 0.92 μM was isolated from *Scutellaria baicalensis* Georgi through the $GABA_A$–BZD receptor-binding assay (Hui et al., 2002). Binding affinities of eckol and dieckol were found to be similar to those of flavonoids isolated from terrestrial sedative plants.

In order to elucidate the *in vivo* mechanism of the hypnotic effect of ECK, the effects of co-administration of ECK-E and the EtOAc fraction with flumazenil, a specific BZD antagonist were tested. The binding sites of BZDs and barbiturates are the targets for various therapeutic agents that act as positive allosteric modulators at $GABA_A$ receptors (Johnston, 2005). BZD and barbiturates are known to bind to two different binding sites at the $GABA_A$ receptors (Gottesmann, 2002). Although acting as a modulator, barbiturates such as pentobarbital at a higher dose can directly activate $GABA_A$ receptors and induce sleep (Sigel & Buhr, 1997). Sedative–hypnotic agents acting at the BZD-binding site of the $GABA_A$ receptor are known to potentiate pentobarbital-induced sleep (Cho et al., 2010; Fang et al., 2010). The BZD antagonists such as flumazenil inhibit the sedative–hypnotic activity of BZD agonists such as DZP by blocking the binding of DZP at the BZD site of $GABA_A$ receptors (Johnston, 2005). In the present study, ECK-E and the

EtOAc fraction alone did not induce sleep (data not shown) but potentiated the pentobarbital-induced sleep in a manner similar to DZP (Fig. 6). The hypnotic effects of ECK-E and the EtOAc fraction were completely blocked by flumazenil (Fig. 6), indicating that active compounds in ECK-E and the EtOAc fraction act directly at the BZD-binding site of $GABA_A$ receptors. These findings support the idea that the hypnotic effects of ECK should be attributed to the allosteric modulation of $GABA_A$ receptors at the BZD-binding site, via a mechanism similar to that of DZP.

5. Conclusions

It was demonstrated for the first time, that ECK has hypnotic effects originating from its phlorotannins, which have the characteristics of $GABA_A$–BZD receptor ligands. ECK should prove to be useful for developing natural sleep aids and sedative–hypnotics for insomnia. Future studies are needed to evaluate the effects of individual phlorotannin constituent on inhibitory post synaptic currents of $GABA_A$ receptors and changes in sleep structure. There are over 10,000 species of seaweeds worldwide, and most of these are underutilised or unused (especially in the West) (McHugh, 2003). Therefore, our study proposes that seaweeds have the potential to be applied in the field of functional foods for mental health.

Acknowledgments

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