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Depressive Effects on the Central Nervous System and Underlying Mechanism of the Enzymatic Extract and Its Phlorotannin-Rich Fraction from *Ecklonia cava* Edible Brown Seaweed

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Marine plants have been reported to possess various pharmacological properties; however, there have been few reports on their neuropharmacological effects. Terrestrial plants have depressive effects on the central nervous system (CNS) because of their polyphenols which make them effective as anticonvulsants and sleep inducers. We investigated in this study the depressive effects of the polyphenol-rich brown seaweed, *Ecklonia cava* (EC), on CNS. An EC enzymatic extract (ECEE) showed significant anticonvulsive (>500 mg/kg) and sleep-inducing (>500 mg/kg) effects on the respective mice seizure induced by picrotoxin and on the mice sleep induced by pentobarbital. The phlorotannin-rich fraction (PTRF) from ECEE significantly potentiated the pentobarbital-induced sleep at >50 mg/kg. PTRF had binding activity to the gamma aminobutyric acid type A ($GABA_A$)-benzodiazepine (BZD) receptors. The sleep-inducing effects of diazepam (DZP, a well-known $GABA_A$-BZD agonist), ECEE, and PTRF were completely blocked by flumazenil, a well-known antagonist of $GABA_A$-BZD receptors. These results imply that ECEE produced depressive effects on CNS by positive allosteric modulation of its phlorotannins on $GABA_A$-BZD receptors like DZP. Our study proposes EC as a candidate for the effective treatment of neuropsychiatric disorders such as anxiety and insomnia.

Key words: *Ecklonia cava*; phlorotannin; anticonvulsive effect; sleep-inducing effect; gamma aminobutyric acid type A ($GABA_A$)-benzodiazepine receptor

Marine plants, and particularly seaweeds, have been widely used in such applications as foods, nutraceuticals, animal feeds,[1)] and cosmetics.[2)] Marine plants have long been used in traditional medicine, particularly in Asia.[3,4)] The past 3 decades have seen marine plants showing a promising future for the development of new pharmacological agents.[5,6)] Numerous novel compounds isolated from marine plants have been demonstrated to possess various pharmacological properties. These compounds include terpenes, polyphenols, carotenoids, polysaccharides, shikimates, polyketides, alkaloids, and peptides. They have been reported to have antitumorial, antioxidative, antibacterial, antifungal, anticoagulative, antiviral, and anti-inflammatory properties.[3,5,7,8)]

Although a number of biological properties of marine plants have been reported, only limited published data are available on their potential application to treat neuropsychiatric disorders.[9)] For example, an *Ulva reticulata* methanol extract[10)] and phlorotannin compounds from *Ecklonia stolonifera*[11)] have shown a neuroprotective effect by inhibiting acetyl cholinesterase and butyryl cholinesterase activities. Myung *et al.*[12)] have reported that dieckol and phlorofucofuroeckol produced memory-enhancing effects by inhibiting acetylcholinesterase activity in ethanol-treated mice. However, the depressive effects of marine plants on the central nervous system (CNS) have not been widely investigated.

We investigated in the present study the depressive effects on CNS of an *Ecklonia cava* enzymatic extract (ECEE) and its phlorotannin-rich fraction (PTRF). Most neurological activities of marine macroalgae have resulted from phlorotannins (seaweed polyphenols) that are found only in brown marine seaweeds. *Ecklonia cava* (EC) was therefore considered a candidate for the effective treatment of neuropsychiatric disorders because of its rich phlorotannin content. The depressive effects of EC on CNS were evaluated by using pentobarbital-induced sleep and picrotoxin-induced seizure tests on mice. The possible mechanism underlying the depressive effect of EC was elucidated by a gamma aminobutyric acid type A ($GABA_A$)-benzodiazepine (BZD) receptor binding assay and an animal model.

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Abbreviations: BZD, benzodiazepine; CMC, carboxymethyl cellulose; CNS, central nervous system; CON, control group; DZP, diazepam; EC, *Ecklonia cava*; ECEE, *Ecklonia cava* enzymatic extract; GABA, gamma-aminobutyric acid; IC_{50}, half-maximal inhibitory concentration; i.p., intraperitoneal; PGE, phloroglucinol equivalents; p.o., per-oral; PTRF, phlorotannin-rich fraction; TPC, total phenol content

Materials and Methods

Drugs and chemicals. Pentobarbital was purchased from Hanlim Pharm. Co. (Seoul, Korea). Diazepam (DZP; Myungin Pharm. Co., Seoul, Korea), a $GABA_A$-BZD agonist, was used as a reference sedative-hypnotic drug. Flumazenil and picrotoxin were purchased from Sigma-Aldrich (St. Louis, MO, USA). The radioligand [^{3}H]-flumazenil (Ro 15-1788; PerkinElmer Life and Analytical Sciences, Waltham, MA, USA) was used for the $GABA_A$-BZD receptor-binding assay. All other chemicals and reagents were of the highest grade available.

Preparation of ECEE and PTRF. ECEE and PTRF were dominated from Aquagreentech Co., Ltd. (Jeju, Korea). The samples were prepared according to the process described by Aquagreentech. The EC brown seaweed was collected from the coast of Jeju Island, South Korea. The samples were washed three times in tap water to remove the salt, sand, and epiphytes attached to the surface. The samples were then rinsed carefully with fresh water and freeze-dried. One kilogram of ground dried EC powder was homogenized with 10 L of distilled water and 10 mL of Celluclast was added (Novo Nordisk, Bagsvaerd, Denmark). The reaction with this enzyme was conducted at 50 °C for 24 h. As soon as the enzymatic reaction had been completed, the digest was boiled for 10 min at 100 °C to inactivate the enzyme. The product was clarified by centrifugation (3,000 × *g* for 20 min) to remove any unhydrolyzed residue. The enzymatic digest was subsequently concentrated under vacuum at 50 °C, and the EC enzymatic extract (ECEE) was obtained by freeze-drying. The phlorotannin-rich fraction (PTRF) from ECEE was prepared by mixing concentrated ECEE before drying with 70% ethanol and placing in a shaking incubator for 24 h at room temperature. The ethanol mixture was centrifuged at 3,500 × *g* for 20 min at 4 °C and filtered through No. 1 filter paper (Whatman, Maidstone, England) to remove the residue. The clarified PTRF was evaporated under vacuum at 40 °C and then freeze-dried.

Animals. Male Sprague Dawley (SD) rats of 200–250 g body weight were used to obtain a membrane preparation for the $GABA_A$-BZD receptor-binding assay. Male ICR (imprinting control region) mice weighing 18–22 g were used in the pentobarbital-induced sleep test. All animals were purchased from Koatech Animals (Pyeongtaek, Korea), and were housed at 24 °C with controlled humidity of 55% in a room maintained on a 12 h light/dark cycle (lights on at 9:00 a.m.) with food and water provided *ad libitum*. 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 (permit number KFRI-M-09118).

Picrotoxin-induced seizure test. The picrotoxin-induced seizure test was performed according to the method of Asada *et al.*[13] Mice were treated with a vehicle (10 mL/kg of 0.5% CMC-saline, per oral), ECEE, and DZP. Forty-five minutes after the oral administration, picrotoxin (7 mg/kg) was intraperitoneally (i.p.) administered to induce the seizure response. The mice injected with picrotoxin were immediately placed in individual cages and observed for 90 min. The number of mice with convulsions and latency to the onset of seizure after picrotoxin injection was recorded. A latency of 0 min was recorded if clonic seizure was not apparent within 90 min.

Pentobarbital-induced sleep test. The pentobarbital-induced sleep test was carried out according to the modified method described by Ma *et al.*[14] All experiments were performed between 1:00 and 5:00 p.m., and the mice were fasted for 24 h prior to the experiment. All samples were suspended in 0.5% (w/v) carboxymethyl cellulose (CMC)-physiological saline for oral administration. A test solution was administered per oral (p.o.) to the mice *via* a sonde needle 45 min prior to the pentobarbital injection. Control mice (0.5% CMC-saline, 10 mL/kg) were tested in parallel with the animals receiving the test sample treatment. Following an intraperitoneal (i.p.) injection of pentobarbital (a sub-hypnotic dose of 30 mg/kg or hypnotic dose of 45 mg/kg), the mice were placed in individual cages and observed for measuring the sleep latency and sleep duration. Individual treatments were unknown to the observers. The sleep latency was recorded from the time of pentobarbital injection to the time of sleep onset, and sleeping duration is defined as the difference in time between the loss and recovery of the righting reflex. The rate of sleep onset with the sub-hypnotic dose of the pentobarbital-treatment test was calculated by the following equation: rate of sleep onset (%) = no. of mice falling asleep/total no. ×100.

$GABA_A$-BZD receptor-binding assay. The $GABA_A$-BZD receptor-binding assay was a modification of the method described by Risa *et al.*[15] The cerebral cortex from 4 male SD rats was homogenized for 10 s in 20 mL of a 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 the Tris–HCl buffer. The washed pellet was homogenized in 20 mL of the Tris–HCl buffer, and the suspension was incubated in a water bath (37 °C) for 30 min to remove endogenous GABA. The suspension was next centrifuged at 27,000 × *g* for 10 min. The final membrane pellet was resuspended in 30 mL of the Tris–HCl buffer and stored in aliquots at −80 °C until being used in the binding assay. The membrane preparation was thawed and washed 3 times with 20 mL of a Tris-citrate buffer (50 mM, pH 7.1, 0–4 °C). The pellet was resuspended at a final concentration of 2.5 μg of protein in a 100-μL binding buffer, and the suspension was used for the binding assay. The membrane suspension (180 μL) was added to 10 μL of a test solution and 10 μL of 1 nM [^{3}H]-flumazenil (final concentration) in a 96-well plate. The solution was mixed and incubated on ice for 40 min. The binding reaction was terminated by rapidly passing through a Whatman GF/C glass fiber filter with an ice-cold 30 mM Tris–HCl buffer to remove any unbound [^{3}H]-flumazenil. The filter was dried at 60 °C for 30 min and suspended in a Wallac microbeta plate scintillation fluid. The amount of filter-bound radioactivity was counted with a Wallac 1450 Microbeta liquid scintillation counter (Perkin Elmer Life and Analytical Sciences, Waltham, MA, USA). Total binding (TB) and non-specific binding (NSB) were respectively determined by using the binding buffer and DZP (1 μM, final concentration). The percentage displacement of radioligand binding was determined by the following equation: binding displacement

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

where DPM denotes disintegrations per minute. Half-maximal inhibitory concentration (IC_{50}) values were calculated from the binding displacement curve which was fitted to a one-site competitive-binding model by using Prism 5.0 (GraphPad Software, San Diego, CA, USA).

Determination of the total phenol content. The total phenol content (TPC) was determined according to the Folin-Ciocalteu method described by Slinkard and Singelton.[16] A dried sample was dissolved in methanol, and 0.5 mL of the sample solution was added to 0.5 mL of the Folin-Ciocalteu reagent and 6.5 mL of distilled water. A 2.5 mL amount of 10% sodium carbonate was then added after 5 min. The mixed sample solution was vortexed for 5 s and then incubated in darkness at room temperature for 60 min. The absorbance of the sample solution was measured at 765 nm. A calibration curve was prepared with phloroglucinol (Sigma-Aldrich, St. Louis, MO, USA), the basic structural unit of phlorotannins (seaweed phenols), TPC being expressed as phloroglucinol equivalents (mg of PGE/g).

Statistical analysis. Data were analyzed by one-way ANOVA and subsequent Dunnett's test for multiple comparisons in the pentobarbital-induced sleep test. Comparisons between two-group data were analyzed by an unpaired Student's *t*-test, differences with $p < 0.05$ being considered statistically significant. The significance analysis was performed by using Prism 5.0.

Results

Anticonvulsive activity of ECEE

All the 10 control mice showed tonic seizure induced by an intraperitoneal (i.p.) injection of 7 mg of picrotoxin per kg of body weight. The final mortality rate for the control group was 80% (Table 1). The latency of tonic convulsions was significantly longer (30.4 ± 2.1 min (mean ± SEM), $p < 0.01$), and the final mortality

Table 1. Effects of an *Ecklonia cava* Enzymatic Extract (ECEE) on Picrotoxin-Induced Convulsion in Mice

Groups	Dose (mg/kg)	No. of mice convulsed/Total	Final mortality (%)	Latency of tonic convulsions (min)
CON		10/10	80	8.7 ± 0.2
DZP	2	5/10	20	$30.4 \pm 2.1^{**}$
ECEE	100	10/10	80	9.3 ± 0.3
	250	10/10	70	10.7 ± 0.5
	500	10/10	60	11.8 ± 0.7
	1000	7/10	40	$21.8 \pm 2.7^{**}$

The mice received an intraperitoneal injection of picrotoxin (7 mg/kg) to induce convulsions 30 min after the oral administration of a 0.5% CMC-saline solution (10 mL/kg, control group (CON)), diazepam (DZP), and ECEE. The latency of tonic convulsions is expressed as the mean ± SEM. $^{**}p < 0.01$, significant compared with the value for the CON group (Dunnett's test).

rate (20%) was lower with the positive control group administered with diazepam (DZP, 2 mg/kg) than with the control group. ECEE also increased the latency of tonic convulsions and decreased the final mortality rate in a dose-dependent manner. A significant increase in the latency of tonic convulsions was observed with a dose of 1000 mg/kg ($p < 0.01$).

Sleep-inducing effect of ECEE

The oral administration of ECEE resulted in a dose-dependent decrease in the sleep latency and an increase in the sleep duration of the mice treated with a hypnotic dose (45 mg/kg) of pentobarbital (Fig. 1). A significantly prolonged sleep duration was observed with ECEE doses of 500 (95.9 ± 7.2 min, $p < 0.05$) and 1000 mg/kg (116.3 ± 0.9 min, $p < 0.01$) (Fig. 1B). As expected, the $GABA_A$-BZD receptor agonist DZP (2 mg/kg) significantly potentiated pentobarbital-induced sleep in the mice. The effects of ECEE on the rate of sleep onset and sleep duration induced by a sub-hypnotic dose (30 mg/kg) of pentobarbital are shown in Table 2. None of the 10 mice in the control group that had been treated with the sub-hypnotic dose of pentobarbital fell asleep. The administration of DZP at 2 mg/kg resulted in a rate of sleep onset of 100% and sleep duration of 62.8 ± 4.8 min. ECEE also increased the rate of sleep onset and sleep duration in a dose-dependent manner, the sleep onset rate and sleep duration at 1000 mg/kg were 90% and 42.9 ± 4.7 min, respectively.

Depressive effects and $GABA_A$-BZD receptor-binding activity of PTRF

We prepared PTRF from ECEE to identify the active compounds with anticonvulsive and sleep-inducing activities. PTRF significantly potentiated the pentobarbital-induced sleep in the mice at >50 mg/kg when compared with the control group (Fig. 2). However, the residue fraction did not show any significant sleep-inducing effect at 250 mg/kg. The administration of PTRF in the picrotoxin-induced seizure test significantly delayed the latency of tonic convulsions and decreased the final mortality in a dose-dependent manner, whereas the residue had no effect on the latency of tonic convulsions nor on the final mortality (Table 3). Figure 3 presents the change (%) in the $GABA_A$-BZD receptors by [^{3}H]-flumazenil binding to ECEE, PTRF and the residue. PTRF showed good dose-response binding activity, with a half-maximal inhibitory concentration (IC_{50}) of 0.031 mg/mL. The total phenol content (TPC) of ECEE, PTRF, and the residue was respectively 98.4 ± 1.6, 630.2 ± 11.3, and 26.6 ± 2.8 mg PGE/g (Table 4). The observed binding activity and sleep-inducing effect of each of the three samples tested were found to be proportional to the TPC value.

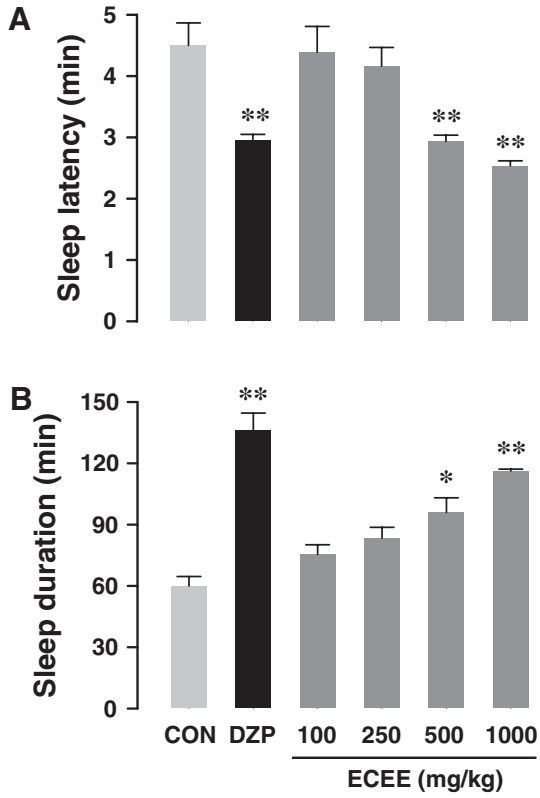

Fig. 1. Effects of an *Ecklonia cava* Enzymatic Extract (ECEE) on the Sleep Latency (A) and Sleep Duration (B) Induced by a Hypnotic Dose (45 mg/kg, i.p.) of Pentobarbital in Mice.

The mice received pentobarbital 45 min after the oral administration of a 0.5% CMC-saline solution (10 mL/kg, control group (CON)), diazepam (DZP, 2 mg/kg), and ECEE. Each column represents the mean ± SEM value (n = 10). $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistically significant values compared with the values for the CON group (Dunnett's test).

Table 2. Effects of an *Ecklonia cava* Enzymatic Extract (ECEE) on the Sleep Onset and Sleep Duration in the Mice Administered with the Sub-Hypnotic Dose (30 mg/kg, i.p.) of Pentobarbital

Groups	Dose (mg/kg)	No. of mice falling asleep/Total	Sleep duration (min)
CON		0/10	0.0 ± 0.0
DZP	2	10/10	$62.7 \pm 4.8^{**}$
ECEE	100	3/10	11.7 ± 1.1
	250	5/10	16.8 ± 4.8
	500	7/10	33.9 ± 2.6
	1000	9/10	$42.9 \pm 4.7^{*}$

The mice received pentobarbital 45 min after the oral administration of a 0.5% CMC-saline solution (10 mL/kg, control group (CON)), diazepam (DZP), and ECEE. 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 compared with the value for the CON group (Dunnett's test).

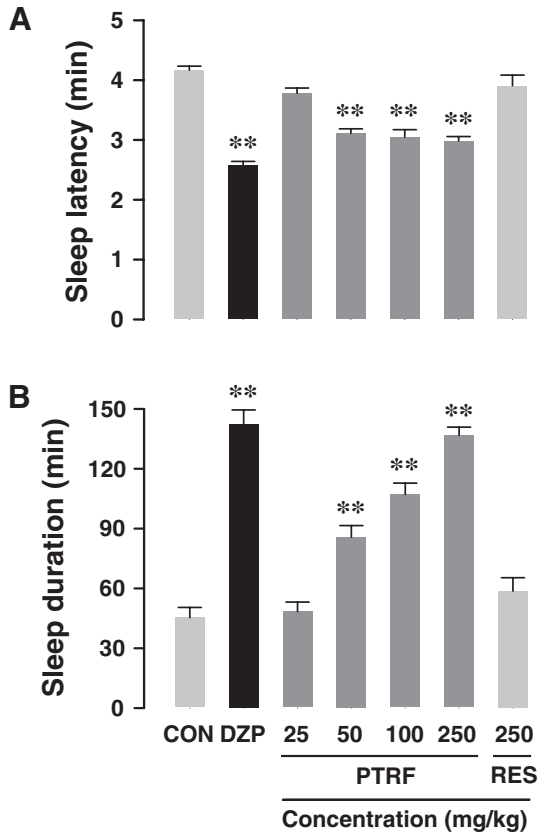

Fig. 2. Effects of the Phlorotannin-Rich Fraction (PTR) from an *Ecklonia cava* Enzymatic Extract (ECEE) on the Sleep Latency (A) and Sleep Duration (B) Induced by a Hypnotic Dose (45 mg/kg, i.p.) of Pentobarbital in Mice.

The mice received pentobarbital 45 min after the oral administration of a 0.5% CMC-saline solution (10 mL/kg, control group (CON)), diazepam (DZP, 2 mg/kg), PTRF, and residue (RES). The residue is the part of ECEE excepting PTRF. Each column represents the mean ± SEM value (n = 10). $^{*}p < 0.05$ and $^{**}p < 0.01$ indicate statistically significant values compared with the values for the CON group (Dunnett's test).

Table 3. Effects of the Phlorotannin-Rich Fraction (PTRF) Separated from an *Ecklonia cava* Enzymatic Extract (ECEE) on Picrotoxin-Induced Convulsion in Mice

Groups	Dose (mg/kg)	No. of mice convulsed/Total	Final mortality (%)	Latency of tonic convulsions (min)
CON		10/10	90	9.0 ± 0.9
DZP	2	3/10	10	28.4 ± 2.1**
PTRF	25	10/10	90	10.7 ± 0.7
	50	7/10	50	14.2 ± 1.9
	100	5/10	40	19.8 ± 2.5*
	250	4/10	20	25.2 ± 3.3**
RES	250	10/10	90	9.2 ± 1.4

The mice received an intraperitoneal injection of picrotoxin (7 mg/kg) to induce convulsions 30 min after the oral administration of a 0.5% CMC-saline solution (10 mL/kg, control group (CON)), diazepam (DZP), and PTRF. Residue (RES) is the part of ECEE excepting PTRF. The latency of tonic convulsions is expressed as the mean ± SEM. $^{**}p < 0.01$, significant compared with the value for the CON group (Dunnett's test).

Possible action mechanism underlying the depressive effect of ECEE and PTRF

To verify the *in vivo* action mechanism underlying the sleep-inducing effect of ECEE and PTRF, we tested the effects of the co-administration of ECEE (1000 mg/kg) and PTRF (250 mg/kg) with flumazenil (8 mg/kg), a specific $GABA_A$-BZD receptor antagonist. Pretreatment with flumazenil alone did not affect the sleep latency (Fig. 4A) or duration (Fig. 4B) induced by a hypnotic dose (45 mg/kg, i.p.) of pentobarbital. However, the inhibitory effect of flumazenil was apparent when co-administered with DZP, a $GABA_A$-BZD receptor agonist. The sleep-inducing effects of ECEE and PTRF were also fully antagonized when co-administered with flumazenil (Fig. 4).

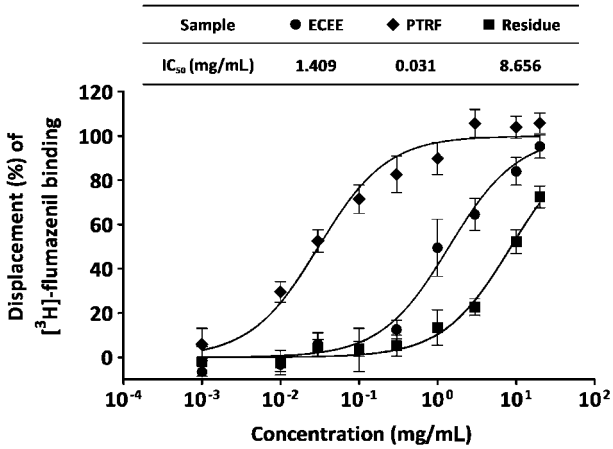

Fig. 3. Dose-Response Curves and Half-Maximal Inhibitory Concentration (IC_{50}) Values for the *Ecklonia cava* Enzymatic Extract (ECEE), Phlorotannin-Rich Fraction (PTRF), and Residue in the $GABA_A$-BZD Receptor-Binding Assay.

The residue is the part of ECEE excepting PTRF. Each data value is expressed as the mean ± SD (n = 3).

Table 4. Total Phenolic Contents of ECEE and Its Fractions

Sample	ECEE	PTRF	Residue
TPC (mg PGE/g)	98.4 ± 1.6	630.2 ± 11.3	26.6 ± 2.8

ECEE, *Ecklonia cava* enzymatic extract; PGE, phloroglucinol equivalents; PTRF, phlorotannin-rich fraction; TPC, total phenolic content. Residue is the part of ECEE excepting PTRF.

Discussion

The depressive effects of such terrestrial plants as valerian (*Valeriana officinalis*) and chamomile (*Matricaria recutita*) on CNS have been widely investigated during the past 4 or 5 decades.[17] Phenols of the terrestrial plants in particular have been considered as major sedative-hypnotic compounds.[18,19] For example, it has been reported that hispidulin, a flavone, had the ability to stimulate a GABA-induced chloride current. Hispidulin had positive allosteric properties and permeability across the blood-brain barrier in an *in situ* rat perfusion model.[20] Chlorogenic acid[21] and epigallocatechin gallate[22] exhibited anxiolytic effects by acting as $GABA_A$-BZD receptor agonists.

Although such marine plants as brown seaweeds are rich in various polyphenols, they have not been considered a potential resource for neuropharmacological applications. We therefore investigated in this study the anticonvulsive and sleep-inducing effects of EC which has greater TPC than other brown seaweeds.[23,24] ECEE exerted significant anticonvulsive (Table 1) and sleep-inducing effects (Fig. 1 and Table 2) in the

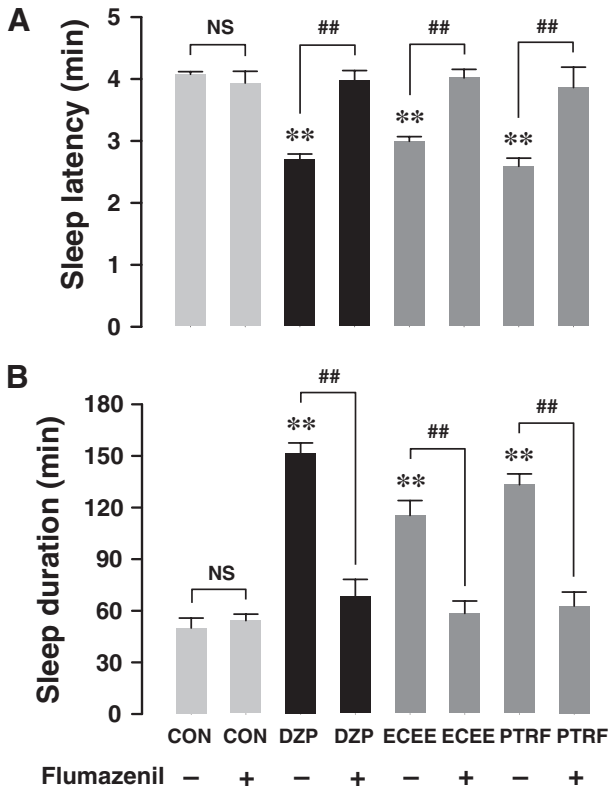

Fig. 4. Effects of Flumazenil on the Changes in Sleep Latency (A) and Sleep Duration (B) in the Mice Treated with Diazepam (DZP), the *Ecklonia cava* Enzymatic Extract (ECEE), and Phlorotannin-Rich Fraction (PTRF).

The mice received pentobarbital 45 min after an oral administration of the CMC-saline solution (10 mL/kg, control group (CON)), DZP (2 mg/kg), ECEE (1000 mg/kg), and PTRF (250 mg/kg). Flumazenil (8 mg/kg, i.p.) was administered 15 min before the oral administration of each test sample. Each column represents the mean ± SEM value (n = 10). $^{**}p < 0.01$ indicates statistically significant values compared with the values for the CON group (Dunnett's test). $^{\#\#}p < 0.01$ indicates a statistically significant difference between the treatment with flumazenil and the treatment without flumazenil (unpaired Student's *t*-test). NS, not significant.

respective picrotoxin-induced seizure and pentobarbital-induced sleep tests.

EC is an edible brown seaweed distributed in coastal areas of Korea and Japan.[25] The amount of EC produced per year in Korea is more than 30000 tons,[26] and EC is popular as an ingredient of functional foods and traditional medicines in Japan and Korea. An EC extract has recently been used in the United States as a commercial dietary supplement because of its antioxidative activity. It has been reported that EC had bioactive properties that were antioxidative,[27] immune-enhancing,[28] and inhibitory of histamine release[26] and human immunodeficiency virus 1 reverse transcriptase.[29]

Previous studies have reported the major constituents of the bioactive properties of EC to be phlorotannins which are found only in brown seaweeds.[25] Phlorotannins, oligomers and polymers of phloroglucinol (1,3,5-tri-hydroxybenzene), are an extremely heterogeneous group of compounds that are structurally different from the polyphenols of terrestrial plants based on gallic acids or flavones.[30] EC phlorotannin contains such phenolic secondary metabolites as eckol, 6,6′-bieckol, dieckol, phlorofucofuroeckol, and triphlorethol-A.[23,24] It has been reported that phlorotannins have such biological properties as antioxidative,[31] anti-inflammatory,[32] antibacterial,[33] antiallergic,[34] and α-glucosidase inhibitory[35] effects. To investigate whether the marine polyphenol, phlorotannin, had the same depressive effect on CNS as terrestrial polyphenols, we evaluated the anticonvulsive and sleep-inducing effects of PTRF fractionated from ECEE. PTRF significantly potentiated pentobarbital-induced sleep and delayed the latency of tonic convulsions induced by picrotoxin at a lower concentration than that of ECEE.

Compounds with anticonvulsive and sleep-inducing properties have the potential to modulate $GABA_A$-BZD receptors in CNS.[36,37] The BZD binding site is the target for therapeutic agents that act as positive allosteric modulators at $GABA_A$ receptors.[19] Such BZD agents as DZP stimulate the ability of GABA to cause hyperpolarization of the membrane by allowing an influx of chloride current. The result is that neurotransmission is inhibited, and these agents subsequently induce anticonvulsive, sleep-inducing, and anxiolytic effects.[38] We performed a $GABA_A$-BZD receptor-binding assay to investigate the relationship between EC and the GABAergic system. ECEE and PTRF showed good dose-response binding activity, with IC_{50} values of 1.409 and 0.031 mg/mL (Fig. 3). In the *in vivo* test of the mechanism underlying the sleep-inducing effects of DZP, ECEE, and PTRF, the effects were fully inhibited by flumazenil, a specific $GABA_A$-BZD receptor antagonist (Fig. 4). Such BZD antagonists as flumazenil inhibit the depressive activity of $GABA_A$-BZD receptor agonists like DZP in CNS by blocking the binding of DZP to the $GABA_A$ receptors at the BZD site.[39] These findings support the notion that the sleep-inducing effects of ECEE and PTRF would be attributable to allosteric modulation of the $GABA_A$ receptors at the BZD-binding site *via* a mechanism similar to that of DZP.

Conclusion

We demonstrated that ECEE and its PTRF have anticonvulsant and sleep-inducing effects by positive allosteric modulation of $GABA_A$ receptors. Future studies are needed to evaluate the depressant effects of individual phlorotannin constituents on the CNS. Our study proposes the potential application of marine plants in treatments of neuropsychiatric disorders such as insomnia and anxiety.

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