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Isolation of Phlorotannins from *Eisenia bicyclis* and Their Hepatoprotective Effect against Oxidative Stress Induced by *tert*-Butyl Hyperoxide

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Abstract *Eisenia bicyclis* (Kjellman) Setchell is a common brown alga that inhabits the middle Pacific coast around Korea and Japan. In this study, the ethanol extract and its serial solvent fractions were prepared from fresh *E. bicyclis*, and their hepatoprotective effects were investigated against hepatotoxicity in *tert*-butyl hyperoxide(*t*-BHP)-injured HepG2 cells. When these samples were used at a dose of 10–40 μg/mL^{-1}, they significantly protected the *t*-BHP-induced cell death in HepG2 cells. Among fractions, ethyl acetate fraction (EF) and *n*-butanol extract (BF) exhibited potent hepatoprotective activities (62.60% for EF and 64.86% for BF) in *t*-BHP-injured HepG2 cells at a concentration of 10 μg/mL^{-1}. To find the potential factors for this activity, the samples were characterized on total phenolics, chlorophylls, carotenoids, and radical scavenging activity. Among them, EF showed the highest content of total phenolics and the strongest antioxidant activity both in on- and offline assays. Five phlorotannin compounds, oligomers of phloroglucinol, were isolated chromatographically from this fraction and structurally identified by ^{1}H-NMR and liquid chromatography–electrospray ionization–mass spectrometry analyses as eckol(**1**), 6,6′-bieckol(**2**), 8,8′-bieckol(**3**), dieckol(**4**), and phlorofucofuroeckol A(**5**). Compound **5** among five purified compounds showed the strongest protective activity (45.54%) at a concentration of 10 μM. At the high dose (40 μM), the protective activities of three compounds (compound **2**, **4**, and **5**) were higher than that of quercetin treated with 10 μM concentration. Therefore, we can speculate that they can be developed as potential candidates for natural hepatoprotective agents.

Keywords *Eisenia bicyclis* · Hepatoprotective effect · Oxidative stress · Phlorotannins · Radical scavenging activity

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Introduction

As the largest organ in the human body, liver performs numerous interrelated vital functions. Liver disorders including viral hepatitis, alcohol liver disease, non-alcohol fatty acid disease, autoimmune liver disease, drug-induced liver injury, and metabolic liver disease can lead to acute hepatitis or chronic liver disease [1]. There are three major categories of liver damage according to the original toxic materials [2]. The first is the alcohol-induced liver damage, and the second is the viral hepatitis, mainly responsible for acute and chronic liver diseases. The third is the drug-induced liver damage, which is caused by the use of drugs like paracetamol, pain killers, and antibiotics. Meanwhile, as the drug of liver damage, herbal medicines derived from plant extracts are being increasingly utilized to treat a wide variety of clinical diseases [3]. Until now, there have been a number of herbal medicines whose therapeutic bioactivity has been proven in human and animals. For instance, silymarin, derived from *Silybum marianum* L. is the most well-researched plant extract in the treatment of liver disease [4, 5]. The active fraction is a lipophilic extract from the seeds of the plant and composed of three isomer flavonolignans-silybin, silydianin, and silychristin—collectively known as silymarin. Glycyrrhizin is another well-known herbal medicine, which is an aqueous extract of the licorice root, *Glycyrrhiza glabra* [6, 7]. Its major constituents are glycyrrhizic acid, multiple flavonoids, isoflavonoids, hydroxycoumarins, and sterols. It is believed that the hepatoprotective activity of most herbal-origin agents is due to their antioxidant activity against abovementioned liver disorders [2]. Andrographolide and neoandrographolide, obtained from *Andrographis paniculata* Nees, a well known plant for liver disease, protect liver against hepatotoxins by reducing the levels of the lipid oxidation product, malondialdehyde [8, 9]. Corcumin, a main component of rhizomes of ancient spice, turmeric (*Curcuma* spp.), shows the hepatoprotective activity through a scavenging of a variety of reactive oxygen species, including superoxide anion radicals, hydroxyl radicals, nitrogen radicals, and single oxygen [10, 11]. In addition, silymarin, picroside, kitkoside, and phyllanthin, which are well-known hepatoprotective agents, have been demonstrated that its hepatoprotective effects were due to antioxidant activity [2].

However, the most of herbal materials for liver treatment have been originated from terrestrial plants. Therefore, in the course of searching for hepatoprotective agents from marine materials, the ethanol extract of *Eisenia bicyclis* (Kjellman) Setchell was found to exhibit distinctive hepatoprotective activity at the 10 μg/mL level. *E. bicyclis*, which is common perennial brown alga of Laminariaceae family, inhabits the middle Pacific coast around Korea and Japan. It is one of many species of seaweed used in Japanese dishes and also harvested as the raw material for sodium alginate. A variety of bioactive compounds, including polysaccharides, pyropheophytin, lipids, and tripeptides, have been reported from this alga, and the pharmacological activities such as antioxidant, antiallergic, anticancer, hyperlipidemic, and hypercholesterolemic activities of these compounds have been investigated in associated with those compounds [12–16]. However, in our best knowledge, there have been no reports on the hepatoprotective effect of this species. Therefore, in this study, we performed the profound research to identify main constituents exhibiting the protective effect on liver cells injured by *tert*-butyl hyperoxide(*t*-BHP). In addition to that, the characteristics of the serial solvent extracts of *E. bicyclis* related to antioxidant activity were investigated in order to prove the correlation between hepatoprotective and antioxidant effects.

Materials and Methods

Materials and Chemicals

The fronds of *E. bicyclis* were harvested from the coast of Ulleung Island, East Sea, Korea in June, 2010. The collected seaweed was washed with fresh water for 12 h and then stored at −20 °C until use. All high-performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific (Pittsburgh, PA, USA). All solvents used for extraction and purification were analytical grade and purchased from Daejung (Gyonggi, Korea). Methanol-d_4 was purchased from Cambridge Isotope Laboratory (Andover, MA, USA).

Characterization of *E. bicyclis* Extract and Fractions

The frozen fronds of *E. bicyclis* (3 kg) were thawed at room temperature and extracted three times with 6 L of 95% ethanol. The extracted solution was then concentrated in a rotary vacuum evaporator to obtain the crude extract (60 g). The serial solvent fractions (*n*-hexane, methylene chloride, ethyl acetate, *n*-butanol, and water) were obtained from 30 g of the crude extract. Total phenolic contents of each fraction were determined according to the Folin–Ciocalteu method using gallic acid as the standard. Gallic acid solutions of different concentration were serially prepared and used for construction of standard curve. Five hundred microliters of each fraction (10 mg/mL in methanol) was mixed with 500 μL of 50% Folin–Ciocalteu reagent. The mixture was incubated for 5 min, with the following addition of 20% aqueous Na_2CO_3 (1 mL). After 10 min of incubation at room temperature, the absorbance of the solution was measured at 730 nm. The total phenolic content was expressed as gallic acid equivalent in milligram per gram sample. Total catotenoid and chlorophyll contents were measured as described in the literature with little modification [17]. In brief, 1 mg of each faction was dissolved in acetone and the absorbances at 470, 644, and 661 nm were measured with a Lambda 3B UV/vis spectrophotometer (Perkin Elmer, Norwalk, CT, USA). The amount of chlorophylls and carotenods were expressed as milligram per gram sample. The radical scavenging activities of each fraction against 2,2′-azino-bis(3-ethylenebenzothiazoline-6-sulfonic acid) (ABTS) and (1,1)-diphenyl-2-picrylhydrazyl (DPPH) radicals were determined as described in the literature with some modifications [18]. For ABTS assay, a 2-mM ABTS stock solution containing 3.5 mM of potassium persulfate was prepared in water and diluted eightfold in HPLC-grade water. This solution was incubated overnight in darkness at room temperature for radical stabilization. Same volume of sample in methanol and ABTS stock solution were allowed to react for 10 min in a dark condition; then, the absorbance was taken at 734 nm using spectrophotometer. For DPPH assay, stock solution was prepared by dissolving 24 mg DPPH with 100 mL methanol and then stored at −20 °C until needed. The working solution was obtained by mixing 100 μL of sample and 400 μL of stock solution, then allowed to react for 30 min in a dark condition. The absorbance was determined at 515 nm using the spectrophotometer. The scavenging activity expressed as (%±SD) for samples working concentration 10 μg/mL is given in Table 1.

HPLC and Online ABTS Radical Scavenging Assay

General HPLC analysis was performed with Agilent HPLC system consisting of binary pump (G1312A), auto sampler (G1367B), diode array detector (G1315D), and degasser (D1379B). The separation was carried out with a reversed-phase Pack Pro-C_{18} [150×

Table 1 Characteristics of the ethanol and solvent fractions from *Eisenia bicyclis*

Samples	Total phenolics[a] (mg/g)	Carotenoids[b] (mg/g)	Chlorophylls[b] (mg/g)	Radical scavenging activity[c]	
				ABTS	DPPH
Ethanol extract (EE)	319.40±14.49	4.79±0.36	23.08±1.41	62.02±1.00	45.95±5.39
n-Hexane fraction (HF)	62.61±1.58	7.90±0.08	58.84±1.35	17.04±1.16	16.29±3.26
Methylene chloride fraction (MF)	165.39±1.98	5.29±0.05	71.90±1.83	44.36±1.18	34.75±2.57
Ethyl acetate fraction (EF)	500.88±9.80	2.27±0.05	0.41±0.03	86.59±2.21	60.23±2.57
n-Butanol fraction (BF)	308.29±4.49	0.69±0.14	0.48±0.03	65.83±2.84	49.62±5.64
Water fraction (WF)	286.27±18.41	0.09±0.02	Not detected	45.99±1.88	38.35±2.05
Ascorbic acid				Over 99	70.79±1.88

All values were determined by three independent experiment and expressed as mean values±SD (n=3)

[a] Values were determined by Folin–Ciocalteu assay, in units of milligram gallic acid equivalent per gram sample

[b] Vaues were determined by spectroscopic measurement with 1 mg/mL solution

[c] Each sample was introduced at 10 μg/mL counting on total volume of reaction mixture

4.6 mm i.d., 3 μm particle size (YMC Co., Kyoto, Japan)] with the mobile phase of acetonitrile (A) and 0.1% TFA in water (B) at a flow rate of 1 mL/min. The gradient program was as follows: 0–10 min, 10% A; 10–50 min, 10–50% A. The chromatogram was detected at 210 nm. For the spontaneous detection of selected compounds and their radical scavenging activity, online ABTS radical scavenging assay was carried out with an Agilent 1200 analysis HPLC system (Agilent Technologies), fitted with an additional pump to supply the ABTS radical stock solution. In brief, a sample in methanol (10 mg/mL concentration, 10 μL) was injected into the online HPLC-ABTS system, and then, individual compounds were separated with an analytical column as described above. After that, the separated compounds were reacted with ABTS radical solution supplied at 0.5 mL/min. The chromatogram for separated compounds was recorded at 210 nm as positive peak, and the chromatogram for radical scavenging activity was recorded at 734 nm to measure the decrease of ABTS radicals as negative peak.

Isolation of Phlorotannins

In order to isolate the major antioxidant compounds, the ethyl acetate fraction (20 g) was subjected to silica gel chromatography (400 g). The sample was fractionated by normal phase silica gel chromatography with the step gradient solvent system as follows: chloroform/methanol/water=80:18:2, *v*/*v*→chloroform:methanol/water=50:49:1, *v*/*v*). Among 13 fractions, fraction 8 (2.73 g) was further fractionated by sephadex LH-20 chromatography with methanol to yield 54 fractions. All fractions were analyzed by HPLC. The fractions showing similar HPLC chromatogram were collected together (G1–G5) and further purified on a Jasco preparative HPLC system (Japan Spectroscopic, Tokyo, Japan) equipped with an ultraviolet detector (MD 2015), pump (PU2089), column oven (CO 2065), and YMC Pack Pro C_{18} RP column (250×20 mm I.D., 5 μm particle size). The mobile phase was same with analytical HPLC with 10 mL/min, and the chromatogram was detected at 210 nm. Eckol (**1**, 35 mg) was purified from G1 (fraction 26–30, 83 mg) with

20% A, and 6,6′-bieckol (**2**, 63 mg) was purified from G2 (fraction 38–44, 133 mg) with 15% A. 8,8′-Bieckol (**3**, 28 mg) and dieckol (**4**, 49 mg) were separated from G4 (fraction 53, 103 mg) in the condition of 20% A for 40 min, then 20–40% A for next 40 min. Phlorofucofuroeckol A (**5**, 12 mg) was purified from G5 in the condition of 20% A for 3 min, then 20–45% A to 80 min. All isolated compounds were identified by ^{1}H-NMR (500-MHz Varian NMR system) and literature comparison.

LC-MS and NMR Analyses

For the structural determination, mass spectrometry (MS) and ^{1}H spectral data were collected and compared with the reported data. Mass data were collected on a model Varian 1,200L LC-MS system (Walnut Creek, CA, USA), with an electrospray ionization (ESI) source (positive mode). The extract of *E. bicyclis* (1 mg min^{-1} in methanol) was injected to LC-MS system, and separation of each compound with YMC Pack Pro-C_{18} [150×4.6 mm i.d., 3 μm particle size] was performed under ambient conditions using a Prostar 230 ternary gradient pump, a Prostar 430 auto sampler, and a Prostar 335 photodiode array detector. The mobile phase acetonitrile–water was used with 0.4 mL/min flow rate. Acetonitrile was gradually increased from 0% to 50% for 50 min. The chromatographic profile was recorded at 210 nm, and mass spectra were acquired in a range of *m/z* 50–1,000. The mass spectrometer conditions were as follows: needle voltage, −4,500 V; nebulizing gas pressure (air), 60 psi; drying gas (N_2) flow rate, 4 L/min^{-1}; and drying gas temperature, 300 °C. The ^{1}H NMR data from the isolated phlorotannins were recorded using a Varian NMR system 500 MHZ (Varian, Palo Alto, CA, USA). All data were obtained in CD_3OD solution, and the chemical shifts are expressed as δ values from tetramethylsilane. The data were processed by the MestReNova program (Mestrelab Research, Santiago de Compostela, Spain).

Cell Culture and Hepatoprotective Activity Assay

The human hepatocellular carcinoma HepG2 cells were obtained from the American Type Culture Collection. HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (Hyclone, Logan, UT, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin. The hepatoprotective effects of the extract, fractions, and compounds from *E. bicyclis* against *t*-BHP were measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell viability assay. In brief, HepG2 cells (1×10^4 cells per well) plated onto 96-well plates were incubated in DMEM at 37 °C for 24 h, then cells were treated with extract (10–40 μg/mL), fractions (10–40 μg/mL), and compounds (10–40 μM) for 24 h. After that, cells were washed with Dulbecco's phosphate-buffered saline and treated with 200 μM *t*-BHP in DMEM without fetal bovine serum for 3 h to induce a hepatotoxicity. Quercetin (10 μM) was used as a positive control. Cell viability was determined using the EZ-Cytox cell viability assay kit (Daeil Lab Service, Seoul) as described in the protocol.

Statistical Analysis

Data were expressed as mean±SD. Statistical analysis was carried out by one-way analysis of variance (ANOVA) followed by Dunett's multiple comparison test using the GraphPad Prism 5 software (GraphPad Software Inc., La Jolla, CA, USA). $P<0.05$ were considered statistically significant.

Results and Discussion

Hepatoprotective Effect of the Ethanol Extract and Fractions from *E. bicyclis*

The ethanolic extract (EE) of *E. bicyclis* was found to protect HepG2 cells injured with 200 μM *t*-BHP. Approximately, 70% of HepG2 cells was protected against *t*-BHP injury with 10 μg/mL EE and complete protection was achieved at 40 μg/mL concentration (Fig. 1), implying evident hepatoprotective effect of EE. Therefore, it was necessary to examine the effect of serial solvent fraction from EE, and the result revealed that all fractions possessed the protective effect at 10 μg/mL concentration. The strongest activity was determined from ethyl acetate (EF) and *n*-butanol (BF) fractions, while the weakest activity was found in *n*-hexane fraction (HF). At the 10 μg/mL level, the protection levels were 63% and 65% in EF and BF, respectively while only 35% in HF. However, at the high dose level (40 μg/mL), EF and BF showed almost complete protection (over 90%), and the others also showed over 50% protection against *t*-BHP-injured HepG2 cells (Fig. 1). From these results, it was suggested that the active compounds were not separated completely into certain solvent fraction and possibly distributed in all fractions in different amount. Meanwhile, the cytotoxicity on normal HepG2 cells were determined as over 200 $\mu g/mL^{-1}$ of IC_{50} from EE and fractions of *E. bicyclis*, indicating the safety of the extracts from this species (data not shown). Therefore, *E. bicyclis* was found to be a potential marine biomaterial for liver disease prevention.

Characterization of the Ethanol Extract and Fractions from *E. bicyclis*

In order to characterize EE and all fractions, total phenolics, chlorophylls, and carotenoids were quantified spectrophotometically, and radical scavenging activity was determined against ABTS and DPPH radicals. As a results, the highest (about 500 mg/g) and lowest

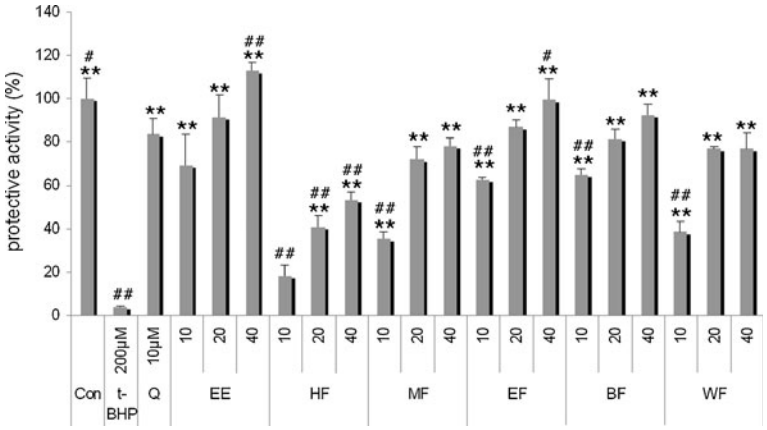

Fig. 1 Protective activities of the extract, fractions from *Eisenia bicyclis* against *tert*-butyl hydroperoxide (*t*-BHP) induced cell death in HepG2 cells. HepG2 cells were pre-treated with ethanol extract (10–40 μg/mL) and fractions (10–40 μg/mL) for 24 h, and then the cells were treated with *t*-BHP (200 μM) for another 3 h. Cell viability was determined by using MTT assay. Quercetin (10 μM) was used as a positive control. The graphs were representatives from three independent experiments. Each bar represents the mean±SD from triplicate experiments. *$P<0.05$ and **$P<0.01$, significant with respect to *t*-BHP treatment. $^{\#}P<0.05$ and $^{\#\#}P<0.01$, significant with respect to quercetin treatment

(about 62 mg/mL) of phenolic compounds were found to be present in EF and in HF, correspondingly (Table 1). Meanwhile, most of carotenoids were distributed from HF to EF, and the HF showed the largest carotenoid content (7.90 mg/g). In the case of chlorophylls, only two solvent fractions, HF and MF, were found to possess chlorophyll compounds. The important thing from these results is to find any correlation between the content of these compounds and the hepatoprotective effect. As mentioned above, high correlation between the hepatoprotective effect and antioxidant activity has been demonstrated from numerous researches. Therefore, radical scavenging activity on ABTS and DPPH radicals was investigated with these fractions (Table 1). At the 10 μg/mL working concentration, both radicals were scavenged in a similar trend with total phenolics. That is, the radical scavenging activities for both radicals were the highest in EF and the lowest in HF. Especially, the radical scavenging activity of EF was comparable with that of ascorbic acid, a well-known antioxidant compound [19]. The R^2 values was 0.919 in the regression equation between total phenolics and ABTS radical scavenging activity and 0.929 in the regression equation between total phenolics and DPPH radical scavenging activity, indicating that the radical scavenging activity of each fraction has high correlation with total phenolics. Therefore, it was easily expected that the hepatoprotective effect in *E. bicyclis* fractions mainly occurred due to the phenolic compounds.

Online HPLC-ABTS Radical Scavenging Assay

Online HPLC system coupled with an additional pump supplying radical reagent enables spontaneous separation of compounds by column and its detection of radical scavenging activity [20, 21]. In order to assess which compounds in the extract of *E. bicyclis* show the radical scavenging activity, online HPLC system supplied with ABTS radical reagent was applied to the EE and fractions of *E. bicyclis* as shown in Fig. 2. The HPLC chromatograms detected at 230 nm showed that the main peaks in the EE (peaks 1–5) demonstrated distinctive ABTS radical scavenging activity as negative peaks and were distributed throughout HF to WF but in a different concentration. Therefore, it was strongly suggested that these compounds were main radical scavengers, and the hepatoprotective effects of *E. bicyclis* occurred from these compounds. In addition, the UV absorption chromatograms

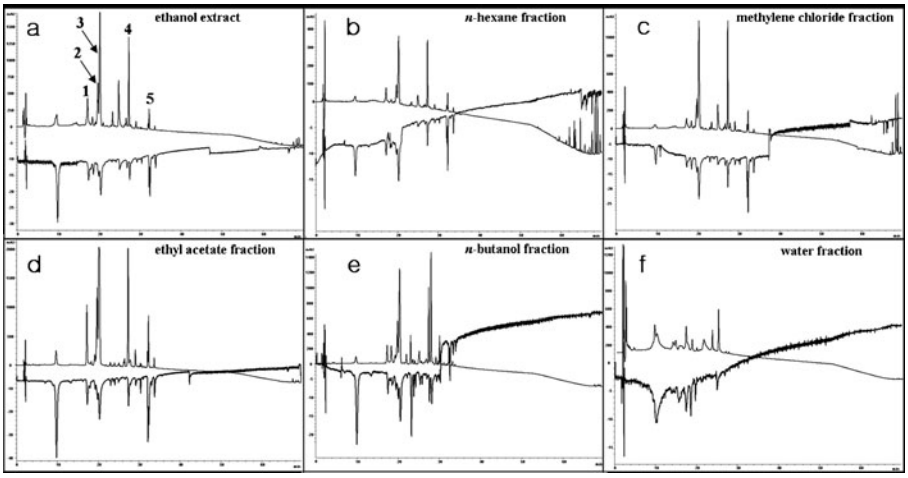

Fig. 2 Online ABTS-HPLC chromatogram of *Eisenia bicyclis* extract and fractions

from these peaks were all similar and showed λ_{max} around 230 and 290 nm, implying that these compounds possessed similar chemical structure. As for the quantification of radical scavenging activity, since same amount of sample was injected to HPLC system, the negative area of each peak could be used as the scale for radical scavenging activity. In the comparison of ABTS radical scavenging activity among serial solvent fractions, EF exhibited the strongest activity, which is identical with offline radical scavenging assay. Therefore, there was a strong chance that main five peaks were target compound for the hepatoprotective activity of *E. bicyclis*.

Isolation and Identification of Antioxidant Compounds from *E. bicyclis*

The silica gel chromatography was carried out to isolate the main antioxidant compounds from EF, and five compounds were successfully obtained and subjected to ^{1}H NMR analysis (Fig. 3). In addition, EE solution in methanol was subjected to LC-ESI-MS analysis in positive mode to obtain the molecular weight information. By combining ^{1}H-NMR, MS, UV spectral data, and literature reports, it was concluded that these compounds belonged to phlorotannins, and the isolated compounds were identified as eckol (**1**), 6,6′-bieckol(**2**), 8,8′-bieckol(**3**), dieckol(**4**), and phlorofucofuroeckol A(**5**) (Fig. 4). Table 2 reports NMR data. In detail, since phlorotannins consist of phloroglucinol units

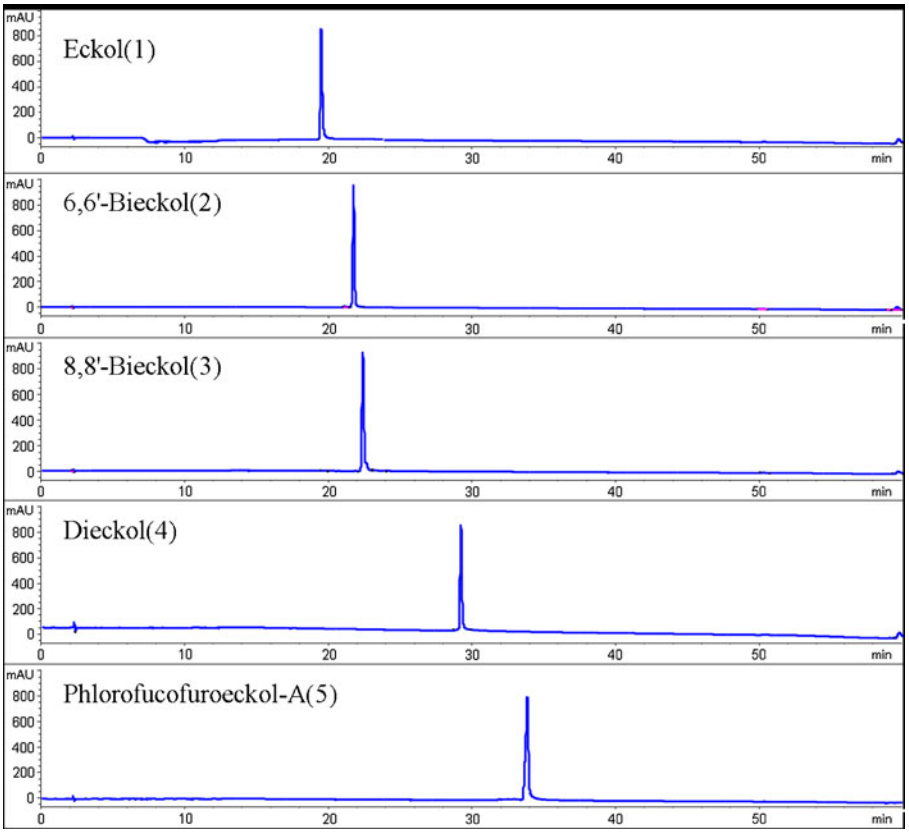

Fig. 3 HPLC chromatograms of isolated phlorotannins from *Eisenia bicyclis*

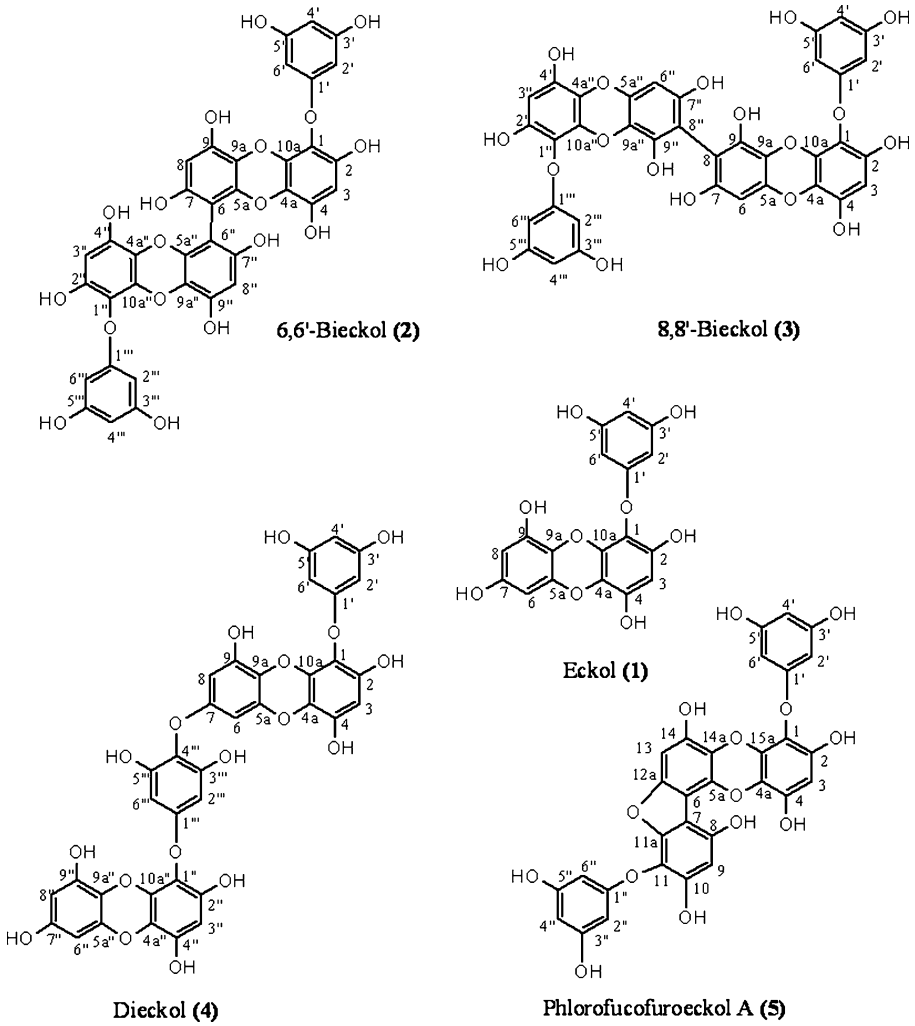

Fig. 4 Chemical structures of phlorotannins isolated from ethyl acetate fraction of *Eisenia bicyclis*

linked to each other in various ways [22], what should be examined first is the MS data to analyze the number of phloroglucinol unit. In Table 2, compound **1** was found to have three phloroglucinol units with the molecular ion at *m/z* 373 $[M+H]^+$. In a similar manner, compound **2**, **3**, and **4** were composed of six phloroglucinol units with the parent ion at *m/z* 743 $[M+H]^+$. In the ^{1}H-NMR spectrum of **1**, an AB_2 system at δ 5.71 (2H, *d*, *J*=2.09), δ 5.79 (1H, *t*, *J*=2.08), and an AB system at δ 5.78 (1H, *d*, *J*=2.74), δ 5.95 (1H, *t*, *J*=2.74) were observed. In addition, a singlet at δ 6.13 (1H, *s*) and five phenolic OH protons at δ 9.12~δ 9.48 were also observed. According to above data and values in the literature, **1** was identified as eckol [15, 23]. Compound **4** showed very similar ^{1}H spectrum with that of eckol, except for the absence of proton signals at carbon 7 and 4‴, indicating the connection point between two dieckol units. The above data for **4** were consistent with the literature values for dieckol [15, 23]. The MS data of compounds **2** and **3** showed the presence of six phloroglucinol units and ^{1}H spectrum of those compounds revealed the linkage of two

Table 2 ^{1}H NMR (500 MHz, DMSO-d_6) and ESI/MS data of phlorotannin compounds from *Eisenia bicyclis*

C no.	Eckol (1)		6,6′-Bieckol (2)		8,8′-Bieckol (3)		Dieckol (4)		C no.	Phlorofucofuroeckol A (5)	
	^{1}H (ppm)	*mult*, *J (Hz)*	^{1}H	*mult*, *J*	^{1}H	*mult*, *J*	^{1}H	*mult*, *J*		^{1}H	*mult*, *J*
1									1		
2	9.15[a]	s	9.12[a]	s	9.13[a]	s	9.33[a]	s	2	9.44[a]	s
3	6.13	s	6.08	s	6.14	s	6.14	s	3	6.29	s
4	9.42[a]	s	9.05[a]	s	9.42[a]	s	9.58[a]	s	4	9.87[a]	s
4a									4a		
5a									5a		
6	5.78	*d*, *J*=2.74			5.93	s	5.82	*d*, *J*=2.85	6		
7	9.15[a]	s	8.61[a]	s	7.87[a]	s			6a		
8	5.95	*d*, *J*=2.74	6.04	s			6.02	*d*, *J*=2.86	7a		
9	9.48[a]	s	9.25[a]	s	8.78[a]	s	9.67[a]	s	8	8.22[a]	s
9a									9	6.42	s
10a									10	9.85[a]	s
1′									11		
2′	5.71	*d*, *J*=2.09	5.74	*d*, *J*=2.09	5.72	*d*, *J*=2.09	5.72	*d*, *J*=2.07	12		
3′	9.12[a]	s	9.13[a]	s	9.08[a]	s	9.2[a]	s	13	6.71	s
4′	5.79	*t*, *J*=2.08	5.8	*t*, *J*=2.08	5.77	*t*, *J*=2.07	5.79	*t*, *J*=2.07	14	10.14[a]	s
5′	9.12[a]	s	9.13[a]	s	9.08[a]	s	9.2[a]	s	14a		
6′	5.71	*d*, *J*=2.09	5.74	*d*, *J*=2.09	5.72	*d*, *J*=2.09	5.72	*d*, *J*=2.07	15a		
1″									1′		
2″			9.12[a]	s	9.13[a]	s	9.33[a]	s	2′	5.76	*d*, *J*=2.04
3″			6.08	s	6.14	s	6.16	s	3′	9.17[a]	s
4″			9.05[a]	s	9.42[a]	s	9.58[a]	s	4′	5.83	*t*, *J*=2.19
4a″									5′	9.17[a]	s
5a″									6′	5.76	*d*, *J*=2.04
6″					5.93	s	5.81	*d*, *J*=2.73	1″		
7″			8.61[a]	s	7.87[a]	s	9.25[a]	s	2″	5.72	*d J*=2.03
8″			6.04	s			5.98	*d*, *J*=2.73	3″	9.2[a]	s
9″			9.25[a]	s	8.78[a]	s	9.67[a]	s	4″	5.83	*t*, *J*=2.19
9a″									5″	9.2[a]	s
10a″									6″	5.72	*d J*=2.03
1‴											
2‴			5.74	*d*, *J*=2.09	5.72	*d*, *J*=2.09	5.95	s			
3‴			9.13[a]	s	9.08[a]	s	9.48[a]	s			
4‴			5.8	*t*, *J*=2.08	5.77	*t*, *J*=2.07					
5‴			9.13[a]	s	9.08[a]	s	9.43[a]	s			
6‴			5.74	*d*, *J*=2.09	5.72	*d*, *J*=2.09	5.95	s			
ESI/MS		$[M+H]^+$= *m/z* 373		$[M+H]^+$= *m/z* 743		$[M+H]^+$= *m/z* 743		$[M+H]^+$= *m/z* 743			$[M+H]^+$= *m/z* 603

s singlet, *d* doublet, *t* triplet

[a] Signals for hydroxyl protons

dieckol, with different linkage way. The linkage point was identified to carbon 6 and 6′ in **2** and carbon 8 and 8′ in **3** through the two singlet protons at δ 6.04 (2H, *s*) for **2** and δ 5.93 (2H, *s*) for **3** and the comparison with literature data [23, 24]. Therefore, it was concluded that compound **2** was 6,6′-bieckol and **3** was 8,8′-bieckol. In the case of compound **5**, ^{1}H

spectrum was quite different with above compounds in the respect of eckol unit. From the literature search and MS data (molecular ion at m/z=603 $[M+H]^+$), this compound was concluded to be a phlorofucofuroeckol-A [25]. All phlorotannins from *E. bicyclis* isolated in this study were found to be belonged to phlorotannins with dibenzodioxin linkage among four subclasses of phlorotannins depending on linkages of different types: ether, phenyl, ether+phenyl, and dibenzodioxin linkages [22]. Figure 4 shows the chemical structures of five phlorotannins with numbering system.

Hepatoprotective Effect of Phlorotannins from *E. bicyclis*

The protective activities of phlorotannins from *E. bicyclis* were investigated against *t*-BHP induced cell death in HepG2 cells (Fig. 5). All phlorotannins exhibited the concentration dependent activity. However, compound **1** showed only minor protective activity (7–21% protection) in the tested ranges (10–40 μM). The others exhibited stronger hepatoprotective acitivities than that of **1** and was in the order: **5**>**4**>**2**>**3**>**1** at the working concentration of 20 and 40 μM and **5**>**4**>**2**>**1**>**3** at 10 μM. The hepatoprotective activities exhibited by dieckol(**4**) and phlorofucofuroeckol A(**5**) at 20 μM were comparable with that of a positive control, quercetin at 10 μM. According to the previous study, *t*-BHP induced hepatotoxicity originates from lactate dehydrogenase and alanine transferase leakage in hepatocyte cells [26], and therefore, *t*-BHP widely has been used as a chemical inducer for oxidative stress. In this context, we could speculate that protective effects of phlorotannins isolated from *E. bicyclis* against *t*-BHP induced hepatotoxicity are due to their antioxidant activities illustrated in the present study. This was further supported by the literature report that eckol (**1**), dieckol(**4**), and phlorofucofuroeckol(**5**) isolated from *Ecklonia stolonifera* exhibited hepatoprotective activity [27] and **5** showed the highest protection among them, which is consistent with the result in this study. However, to our knowledge, this is the first report that the hepatoprotective acitivity was demonstrated with the extract, as well as phlorotannins from *E. bicyclis*.

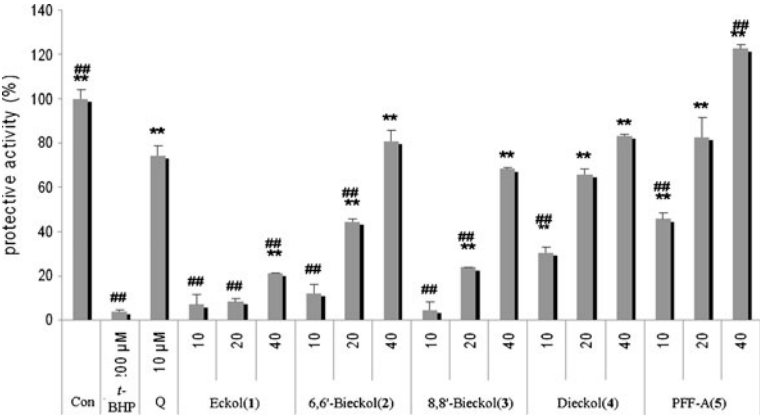

Fig. 5 Protective activities of phlorotannins from *Eisenia bicyclis* against *tert*-butyl hydroperoxide (*t*-BHP) induced cell death in HepG2 cells. HepG2 cells were pre-treated with phlorotannin compounds (10–40 μM) isolated from ethyl acetate fraction of *E. bicyclis* for 24 h, and then the cells were treated with *t*-BHP (200 μM) for another 3 h. Cell viability was determined using MTT assay. Quercetin (10 μM) was used as a positive control. The graphs were representatives from three independent experiments. Each bar represents the mean±SD from triplicate experiments. *P<0.05 and **P<0.01, significant with respect to *t*-BHP treatment. $^{\#}P$<0.05 and $^{\#\#}P$<0.01, significant with respect to quercetin treatment

In conclusion, five phlorotannins isolated from the ethyl acetate fraction of *E. bicyclis* ethanolic extract were demonstrated to be mainly responsible for the antioxidant activity of fraction, showing the highest radical scavenging activity among serial solvent fractions. Therefore, the hepatoprotective activities in EE and fractions were concluded to occur from these phlorotannis, indicating that *E. bicyclis* can be a potential source for marine natural hepatoprotective agents. However, further analyses related in the concise mechanism of protection against *t*-BHP induced cytotoxicity and the combinational effect of phlorotannins are remained.

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