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Hepatoprotective Constituents of the Edible Brown Alga *Ecklonia stolonifera* on Tacrine-induced Cytotoxicity in Hep G2 Cells

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In this study, ethanolic extracts from 18 seaweed variants were assessed for hepatoprotective activity against tacrine-induced cytotoxicity in Hep G2 cells. Only one of these, *Ecklonia stolonifera* Okamura (Laminariaceae), a member of the brown algae, exhibited promising hepatoprotective activity. Bioassay-guided fractionation of the active ethyl acetate (EtOAc) soluble fraction obtained from the ethanolic extract of *E. stolonifera*, resulted in the isolation of several phlorotannins [phloroglucinol (**1**), eckstolonol (**2**), eckol (**3**), phlorofucofuroeckol A (**4**), and dieckol (**5**)]. Compounds **2** and **4** were determined to protect Hep G2 cells against the cytotoxic effects of tacrine, with EC_{50} values of 62.0 and 79.2 μg/mL, respectively. Silybin, a well characterized hepatoprotective agent, was used as a positive control, and exhibited an EC_{50} value of 50.0 μg/mL. It has been suggested that the phlorotannins derived from marine brown algae might prove useful sources in the development of novel hepatoprotective agents.

Key words: *Ecklonia stolonifera*, Hepatoprotective, Phlorotannins, Marine algae, Tacrine, Hep G2 cells

INTRODUCTION

In the bioassay-directed search for hepatoprotective agents obtained from natural sources, employing a model system which mimics the properties of human liver toxicosis is generally considered to be an effective method for the identification of therapeutically applicable agents. Thus, we considered using hepatotoxic agents which are relevant to human liver toxicosis in our assay protocols. Tacrine (1, 2, 3, 4-tetrahydro-9-aminoacridine hydrochloride) is an acetylcholinesterase inhibitor, which has been approved for the treatment of Alzheimer's disease. However, tacrine treatment for Alzheimer's disease also results in reversible hepatotoxicity in 30-50% of patients, which substantially limits its clinical use (Watkins *et al.*, 1994). Therefore, in recent years, a great deal of research has been conducted in order to locate natural products which confer protective effects on tacrine-induced cytotoxicity (An *et al.*, 2005; Jung *et al.*, 2004; Park *et al.*, 2004). In these studies, an immortalized human hepatoma cell line, Hep G2 has frequently been employed for the screening of hepatoprotective agents against tacrine-induced cytotoxicity, because this cell line is known to retain many of the relevant cellular functions (Grant *et al.*, 1988), and is also known to be comparable with rat primary hepatocytes in terms of tacrine-induced cytotoxicity (Viau *et al.*, 1993).

Ecklonia stolonifera Okamura is a perennial brown alga which belongs to the Laminariaceae and grows at a water depth of 2–10 m, is distributed widely throughout countries such as Korea and Japan, and is frequently used as a foodstuff, along with *Laminaria japonica* and *Undaria pinnatifida*. Phloroglucinol, phlorotannins (Taniguchi *et al.*, 1991), and ecklonialactones (Kurata *et al.*, 1989, 1993) have all been previously isolated from *E. stolonifera*. This alga has been associated with antioxidant (Choi *et al.*, 1993; Lee *et al.*, 1996), antimutagenic activity (Lee *et al.*, 1996, 1998; Han *et al.*, 2000), and feeding-deterrent effects (Taniguchi *et al.*, 1991) as well. In the course of our search for hepatoprotective agents that could be obtained from marine natural products, the ethanolic extract of *E. stolonifera* was determined to exhibit a distinct hepatoprotective activity, with a measured EC_{50} of 198.1 μg/mL.

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Further bioassay-directed purification of this extract, using a variety of chromatographic techniques, resulted in the isolation of two active compounds, along with three inactive compounds. Here, we will discuss both the isolation and the biological activities of these compounds.

MATERIALS AND METHODS

Plant material

The leafy thalli of *E. stolonifera* were collected at Gijang-gun in Busan, in February 2000, and were authenticated by Prof. H. G. Kim of the Faculty of Marine Bioscience and Technology, at Kangnung National University. The leafy thalli of *Pelvetia siliquosa* were collected from a seashore in Mokpo in February 2003, and were authenticated by Prof. J. A. Shin, at the Yeosu National University. All other leafy algal thalli were collected at Chungsapo, in Busan, Korea in February 2003, and were authenticated by an algalogist, Prof. C. H. Sohn of the Department of Marine Ecology, at the Pukyong National University. A voucher specimens (no. 20000228, 20000328) were deposited in the author's laboratory (J. S. Choi).

Chemicals and reagents

Column chromatography was conducted using silica gel 60 (70~230 mesh, Merck, Germany), RP-18 Lichroprep (Merck, Germany), and Sephadex LH-20 (Sigma, St. Louis, MO). The TLC was performed on a precoated Merck Kieselgel 60 F_{254} plate (0.25 mm), and the spots were detected under UV light, using 50% H_2SO_4 reagent. The RPMI 1640 medium, trypsin-ethylene diaminetetraacetic acid (EDTA), and antibiotics used in this study were purchased from Gibco Laboratories (Grand Island, NY). The fetal bovine serum (FBS) was obtained from Hyclone Laboratories (Logan, UT). Tacrine, silybin, and 3'-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were all purchased from the Sigma Chemical Co. (St. Louis, MO). 96-well tissue culture plates and other tissue culture dishes were obtained from Nunc, Inc. (North Aurora, IL).

Isolation of phlorotannins

The lyophilized powder (3 kg) was refluxed with EtOH (3×9 L) for 3 h. The extract (700 g) was then suspended in water, and partitioned using *n*-hexane (27.9 g), CH_2Cl_2 (25.6 g), EtOAc (25.0 g), *n*-BuOH (99.6 g), in sequence. The EtOAc (25.0 g) fraction was then applied to a silica gel (Merck, 70~230 mesh, 800 g) column (4×80 cm). The column was eluted using EtOAc/MeOH mixtures under stepwise gradient conditions (50:1~5:1), in order to generate the 14 subfractions (F1~F10), *i.e.*, F1~F3; EtOAc/MeOH, 50:1 (5 L), F4~F6; EtOAc/MeOH, 10:1 (5 L), F7~F8; EtOAc /MeOH, 5:1 (5 L), and F9~F10; EtOAc/MeOH, 2:1 (2 L). F1 (3.44 g, IC_{50} = 20 μg/mL) was further subjected to an additional silica gel (70~230 mesh, 250 g) column (3×70 cm) chromatography (*n*-hexane/EtOAc, 1:1) step, yielding 11 subfractions (F1-1~F1-11). Compound **1** (98 mg) was obtained from the RP-18 column chromatography (20% MeOH~100% MeOH, gradient) of F1-4 (257 mg). Compounds **2** (60 mg) and **3** (135 mg) were generated by the RP-18 column chromatography (20% MeOH~100% MeOH, gradient) of F1-5 (1.01 g). Compounds **4** (57 mg) and **5** (87 mg) in F1-6 (945 mg) were obtained *via* the RP-18 column chromatography using a 20% MeOH~100% MeOH gradient, and finally purified *via* Sephadex LH-20 column chromatography, using MeOH as a solvent. The isolated compounds were subsequently identified as phloroglucinol (**1**), eckstolonol (**2**), eckol (**3**), phlorofucofuroeckol A (**4**), and dieckol (**5**), on the basis of the chemical and physicochemical evidence, and were then compared with the previously reported examples in the relevant literature (Fukuyama *et al.*, 1985, 1989a, 1989b, 1990; Nakamura *et al.*, 1996; Kang *et al.*, 2003).

In vitro hepatoprotective activity assay

Our tacrine-induced cytotoxicity assays were conducted using a minor modification of the method developed by Song *et al.* (2001). In brief, human hepatoma Hep G2 cells from the American Type Culture Collection were maintained at a concentration of 2×10^5 cells/well in complete medium consisting of RPMI supplemented with 10% heat-inactivated FBS, penicillin G (100 IU/mL), and streptomycin (100 μg/mL), and then incubated at 37°C in a humidified atmosphere containing 5% CO_2 and 95% air. Cytotoxicity was assessed *via* MTT assay, by incubating cells for 2 h in the corresponding medium, in either the presence or absence of 1.2 mM tacrine. The samples were then tested in triplicate at three different concentrations (100, 200, and 300 μg/mL for extracts or fractions; 10, 50, and 100 μg/mL for compounds). The EC_{50} values for the hepatoprotective effects (defined as percentage viability versus the respective control) were calculated *via* linear regression using the mean values, and are expressed as the means from three independent experiments.

RESULTS AND DISCUSSION

In the present study, the objective of which was to identify secondary metabolites with hepatoprotective activity from marine natural products, we screened the ethanolic extracts of 18 seaweed variants for their potential protective activity against tacrine-induced cytotoxicity in Hep G2 cells (Table I). Only one of these variants, *Ecklonia stolonifera* Okamura (Laminariaceae), which is a brown algae, exhibited any detectable hepatoprotective activity, evidencing an EC_{50} value of 198.1 μg/mL. In our ongoing

Table I. Hepatoprotective activities of seaweeds on tacrine-induced cytotoxicity

Seaweeds	EC_{50} (μg/mL)[a]
Laminaria japonica Areschoug	>300
Sargassum fulvellum (Turner) C. Agardh	>300
Chondrus ocellatus Holmes	>300
Hizikia fusiforme (Harvey) Okamura	>300
Gigartina tenella Harvey	>300
Gymnogongrus flabelliformis Harvey	>300
Ulva pertusa Kjellman	>300
Pachymeniopsis lanceolata Yamada	>300
Sargassum horneri (Turner) C. Agardh	>300
Ecklonia stolonifera Okamura	198.1
Pelvetia siliquosa Tseng *et* Chang	>300
Codium fragile (Suringar) Hariot	>300
Porphyra tenera Kjellman	>300
Undaria pinnatifida (Harvey) Suringar	>300
Enteromorpha linza J. Agardh	>300
Sargassum species	>300
Chondria crassicaulis Harvey	>300
Sargassum thunbergii (Mertens) O. Kuntze	>300
Silybin	50.0

[a]Hepatoprotective activity was expressed as the mean of 50% effective concentrations of triplicate determinations, obtained by interpolation of concentration-inhibition curve.

study to identify the active components, we also evaluated the solvent-soluble fractions, including *n*-hexane, CH_2Cl_2, EtOAc, and *n*-BuOH, as well as the H_2O layer derived from *E. stolonifera*. Among the partitioned ethanolic extract fractions, the EtOAc-soluble extract exhibited a significant degree of hepatoprotective activity, evidencing an EC_{50} of 195.2 μg/mL (Table II). The subsequent bioassay-guided fractionation of the extract resulted in the isolation of five phlorotannins: phloroglucinol (**1**), eckstolonol (**2**), eckol (**3**), phlorofucofuroeckol A (**4**), and dieckol (**5**) (Fig. 1). Among the compounds isolated, eckstolonol (**2**) and phlorofucofuroeckol A (**4**) exhibited hepatoprotective activity, with EC_{50} values of 62.0 and 79.2 μg/mL, respectively, on tacrine-induced cytotoxicity in the human liver-derived Hep G2 cells (Table III). The hepatoprotective activity exhibited by eckstolonol (**2**) was found to be comparable with that (EC_{50} = 50.0 μg/mL) of a positive control, silybin. The viability of the Hep G2 cells was not altered in the presence (10~100 μg/mL) or absence of compounds **2** and **4**.

Table II. Hepatoprotective activities of various fractions obtained from the EtOH extract of *E. stolonifera* on tacrine-induced cytotoxicity

Samples	EC_{50} (μg/mL)[a]
EtOH ex.	198.1
n-Hexane fr.	>300
CH_2Cl_2 fr.	>300
EtOAc fr.	195.2
n-BuOH fr.	>300
H_2O fr.	>300
Silybin	50.0

[a]Hepatoprotective activity was expressed as the mean of 50% effective concentrations of triplicate determinations, obtained by interpolation of concentration-inhibition curve.

Although the mechanism underlying tacrine-induced hepatotoxicity has yet to be precisely elucidated, it has been determined that tacrine alters intracellular glutathione concentrations in cultured hepatocytes, which suggests the involvement of reactive oxygen species (ROS) generation and lipid peroxidation in tacrine-induced cytotoxicity (Osseni *et al.*, 1999). This means that antioxidative compounds may exert protective effects against tacrine-induced hepatotoxicity. We previously investigated the inhibition of total ROS generation on these isolated compounds, and all of the compounds exhibited inhibitory effects against ROS generation, in the order: **4** > **3** > **2** > **5** > **1** (Kang *et al.*, 2004). Antioxidative action is considered to be a rather complex process, which may include the prevention of formation or the scavenging of free radicals. Although some of the isolated compounds did not exhibit hepatoprotective properties, the inhibition of ROS generation is one of the probable hepatoprotective mechanisms underlying the observed effects of compounds **2** and **4**. However, further studies will be required in order to confirm this, and to elucidate the mechanistic bases for the hepatoprotective effects associated with compounds **2** and **4**.

Phlorotannins, the common secondary metabolite constituents of brown algae, are polymers of acetate-malonate derived 1, 3, 5-trihydroxybenzene (phloroglucinol), and are considered to be analogues of terrestrial condensed tannins (Higa, 1981). Eckstolonol (**2**) and phlorofucofuroeckol A (**4**, Kang *et al.*, 2004) have been previously reported to exert tyrosinase-inhibitory effects, and phlorofucofuroeckol A (**4**) has been shown to possess an anti-plasmin inhibitory property (Fukuyama *et al.*, 1990). However, the hepatoprotective activity of compounds **2** and **4** observed in this study has not, to our knowledge, been previously reported, which suggests that these compounds should be evaluated further, for their potential roles as hepatoprotective agents.

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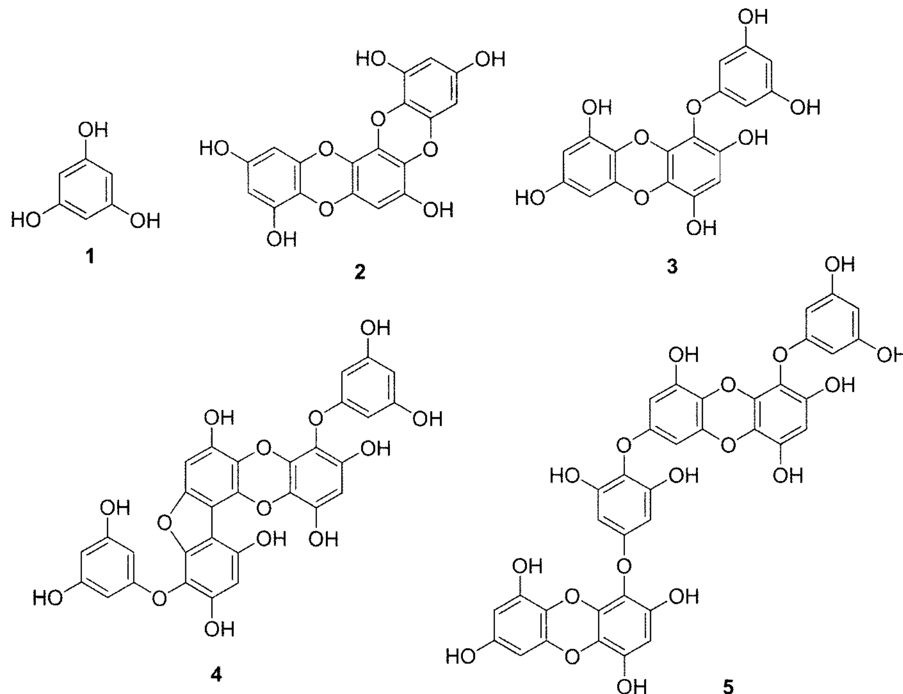

Fig. 1. The structures of the phlorotannins from *E. stolonifera*

Table III. Hepatoprotective activity of isolated compounds **1~5** from the EtOAc fraction of the EtOH extract of *E. stolonifera*

Compounds	EC_{50} (μg/mL)[a]
Phloroglucinol (**1**)	>100
Eckstolonol (**2**)	62.0
Eckol (**3**)	>100
Phlorofucofuroeckol A (**4**)	79.2
Dieckol (**5**)	>100
Silybin	50.0

[a]Hepatoprotective activity was expressed as the mean of 50% effective concentrations of triplicate determinations, obtained by interpolation of concentration-inhibition curve.

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