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Peroxynitrite Scavenging Activity of Phloroglucinol from *Ecklonia stolonifera*

Hae Young Chung, Hye Rhi Choi, Hye Jin Park[1], Jae Sue Choi[1] and Won Chul Choi[2]

Department of Pharmacy, Pusan National University, Pusan 609-735, Korea
[1]Department of Food Science and Biotechnology, Pukyong National University, Pusan 608-737, Korea
[2]Department of Biological Science, Pusan National University, Pusan 609-735, Korea

Peroxynitrite ($ONOO^-$), formed by the reaction of superoxide ($^{\cdot}O_2^-$) and nitric oxide ($^{\cdot}NO$), is a cytotoxic species that can oxidize several cellular components such as proteins, lipids, and DNA. It has been implicated in diseases such as cancer, Alzheimer's disease, rheumatoid arthritis, and atherosclerosis. Due to the lack of endogenous enzymes responsible for $ONOO^-$ inactivation, developing a specific $ONOO^-$ scavenger is considerably important. The aim of this study was to evaluate the ability of phloroglucinol isolated from *E. stolonifera* to scavenge $ONOO^-$ and to protect cells against $ONOO^-$. Phloroglucinol was found to be as a potent $ONOO^-$ scavenger. The data demonstrated that phloroglucinol led to decrease $ONOO^-$-mediated nitration of tyrosine through electron donation. Phloroglucinol provided cytoprotection from cell damage induced by $ONOO^-$.

Key Words: Peroxynitrite, scavenging activity, Phloroglucinol, Nitrotyrosine, Cytoprotection, Smooth muscle cells

INTRODUCTION

Peroxynitrite ($ONOO^-$), formed by the reaction of nitric oxide ($^{\cdot}NO$) and superoxide ($^{\cdot}O_2^-$) *in vivo*, is a relatively long-lived cytotoxicant with strong oxidizing properties toward various cellular constituents, including sulfhydryls, lipids, amino acids, and nucleotides.[1)] A number of publications implicate that $ONOO^-$ can induce oxidation of thiol groups on proteins, nitration of tyrosine, and lipid peroxidation.[1~3)] Excessive formation of $ONOO^-$ may also be involved in several human diseases such as cancer, Alzheimer's disease, rheumatoid arthritis, and atherosclerosis.[4)] $ONOO^-$ is considered to be a major reactive species *in vivo* in many forms of cellular and tissue injury with DNA strand breakage and apoptotic cell death.[2,5)] Due to the lack of endogenous enzymes responsible for $ONOO^-$ inactivation, developing specific $ONOO^-$ scavengers is considerably important. There are little described in the medical literature of $ONOO^-$ scavenging activity of natural products and their isolated compounds. Phloroglucinol (1,3,5-trihydroxybenzene) administered intravenously to patients with irritable bowel syndrome at the

Corresponding author : Hae Young Chung, Department of Pharmacy, Pusan National University, Pusan 609-735, Korea. Tel: 051-510-2814, Fax: 051-510-2814, E-mail: hyjung@hyowon.Pusan.ac.kr

end of a test meal was previously reported to reduce rectosigmoid motor response.[6] In the present study, the $ONOO^-$ scavenging mechanism and the protective effect of the active component, phloroglucinol, from *Ecklonia stolonifera* on $ONOO^-$-mediated cell damage were examined.

EXPERIMENTAL PROCEDURES

1) Materials

3-Morpholinosydnonimine (SIN-1) and DL-penicillamine (DL-2-amino-3-mercapto-3-methylbutanoic acid) were obtained from Sigma Chemical Co. Dihydrorhodamine 123 (DHR 123) and $ONOO^-$ were from Molecular Probes (Eugene, OR, USA) and Cayman Chemical Co. (Ann Arbor, MI, USA), respectively. 4,5-Diaminofluorescein (DAF-2) was purchased from Daiichi Pure Chemicals Co. (Tokyo, Japan). All other chemicals were of the highest purity available from either Sigma Chemical Co. (St. Louis, MO, USA) or Junsei Chemical Co. (Tokyo, Japan). Leafy thalli of *Ecklonia stolonifera* were collected at Cungsapo, Pusan in January, 1998 and authenticated by an algologist Dr. K. W. Nam of the Department of Marine Biology, Pukyoung National University. Phloroglucinol was isolated as described previously.[7]

2) Measurement of $ONOO^-$ scavenging activity

$ONOO^-$ scavenging was measured by monitoring the oxidation of dihydrorhodamine 123 by modifying the method of Kooy *et al.*[3] A stock solution of DHR 123 (5 mM) in dimethylformamide was purged with nitrogen and stored at -80°C. Working solution with DHR 123 (f.c. 5 μM) diluted from the stock solution was placed on ice in the dark immediately prior to the study. The buffer of 90 mM sodium chloride, 50 mM sodium phosphate (pH 7.4), and 5 mM potassium chloride with 100 μM (f.c.) diethylenetriaminepentaacetic acid (DTPA) was purged with nitrogen and placed on ice before use. $ONOO^-$ scavenging by the oxidation of DHR 123 was measured with a microplate fluorescence spectrophotometer FL 500 (Bio-Tek Instruments, USA) with excitation and emission wavelengths of 485 nm and 530 nm, respectively, at room temperature. The background and final fluorescent intensities were measured 5 min after treatment with authentic $ONOO^-$ (f.c. 10 μM) in 0.3 N sodium hydroxide. Authentic $ONOO^-$ rapidly oxidized DHR 123 with its final fluorescent intensity being stable over time.

3) Measurement of $^{\cdot}O_2^-$ scavenging activity

2,7-Dichlorodihydrofluorescein diacetate (H_2DCFDA; f.c. 2.5 μM) mixed with esterase (f.c. 1.5 U/ml) was incubated at 22°C for 20 min and placed on ice in the dark until immediately prior to the study. 50 mM Phosphate buffer at pH 7.4 was used. H_2DCFDA was deacetylated to non-fluorescent 2,7-dichlorodihydro-fluorescein (DCFH) by esterase and subsequently oxidized to highly fluorescent 2,7-dichlorofluorescein (DCF) by $^{\cdot}O_2^-$. The conversion of DCFH into DCF was gradually increased by $^{\cdot}O_2^-$. The fluorescence intensity of oxidized DCFH was measured by a microplate fluorescence reader (FL 500, Bio-Tek Instruments) at the excitation and the emission wavelengths of 485 nM and 530 nM, respectively, for 1 hr with or without addition of menadione (f.c., 50 μM) as a $^{\cdot}O_2^-$ source.[8]

4) Measurement of $^{\cdot}NO$ scavenging activity

4,5-Diaminofluorescein (DAF-2) as a specific $^{\cdot}NO$ indicator selectively traps $^{\cdot}NO$ between two amino groups in its molecule, and yields triazolofluorescein, which emits green fluorescence when excited at 490～495 nm.[9] 1 mg DAF-2 in 0.55 ml dimethyl sulfoxide was diluted with 50 mM phosphate buffer (pH 7.4) to 1/400-fold. A $^{\cdot}NO$ donor, sodium nitroprusside (f.c. 2 mM), and DAF-2 (f.c. 3.14 μM) were added to a 96-well microplate. The fluorescence intensity was dependent on the amount of $^{\cdot}NO$ trapped by DAF-2. The fluorescence signal caused by the reaction of DAF-2 with $^{\cdot}NO$ was measured using a fluorescence spectrometer (FL 500, Bio-Tek Instruments) at the excitation and the emission wave-

lengths of 485 nM and 530 nM after 10 min.

5) Interaction of phloroglucinol with $ONOO^-$

To identify the reaction mechanism of phloroglucinol with $ONOO^-$ measured by a spectrophotometric analysis, as described by Pannala *et al.*[10] 500 μM $ONOO^-$ in 0.3 N NaOH was added to a solution containing 100 μM phloroglucinol in 50 mM phosphate buffer (pH 7.4), making a final volume of 1 ml. Each mixed solution was incubated at 37°C with shaking for 1 hr and scanned between 190 nm and 600 nm on a Ultraspec 2000 UV/visible spectrophotometer (Pharmacia-Biotech, England). The spectral changes in the visible region of phloroglucinol in the presence of $ONOO^-$ were monitored at 430 nm to determine the existence of nitration. Samples containing no $ONOO^-$ were also included for comparative purposes.

6) Determination of 3-nitrotyrosine

The ability of phloroglucinol to suppress $ONOO^-$-mediated tyrosine nitration was determined by the following procedure as described as previously. 500 μM $ONOO^-$ in 0.3 N NaOH was added to a solution containing 100 μM tyrosine in the presence of 100 μM phloroglucinol in 50 mM phosphate buffer (pH 7.4), making a final volume of 1 ml. The formation of 3-nitrotyrosine by the reaction of tyrosine with $ONOO^-$ was also included for a comparative purpose. The spectrum of the peak displayed at 430 nm indicates the formation of 3-nitrotyrosine.[11]

7) Cell culture

Bovine smooth muscle cells (SMC) were cultured with DMEM medium (Nissui Co., Tokyo, Japan) supplemented with 10% heat-inactivated (56°C for 30 min) fetal serum (Gibco, Grand Island, NY, USA), 233.6 mg/ml glutamine, 0.25 g/ml amphotericin, and 100 units/ml penicilline-streptomycin solution (Gibco), and adjusted to pH 7.4～7.6 by $NaHCO_3$ in an atmosphere of 5% CO_2. The fresh medium was replaced after one day to remove nonadherent cells or cell debris.

8) Cell viability

Trypan blue dye exclusion was assessed as described by Sandoval *et al.*[11] SMCs in a 48-well plate were pre-incubated for overnight. Cells were pretreated either with SIN-1 (f.c. 1 mM) for 6 hrs and then treated with phloroglucinol or penicillamine as the standard for comparison and incubated for approximately 14 hrs. Briefly, cells were detached with trypsin-EDTA (Gibco, BRL, Gaithersburg, MD, USA), and 0.4% trypan blue dye was added to them. Within 10-minute incubation, the number of cells excluding dye was expressed as a percentage of total cells counted from four chambers of the hemocytometer.

9) Statistical analysis

Data are expressed as means±S.E. of 3 determinations. IC_{50} (μM) values (concentration required to inhibit $ONOO^-$ formation by 50%) were calculated using the dose inhibition curve obtained by Microsoft Excel 97.

RESULTS

1) $ONOO^-$ scavenging activity of phloroglucinol from *E. stolonifera*

Table 1 presents IC_{50} values of phloroglucinol for $ONOO^-$, $^{\cdot}O_2^-$, and $^{\cdot}NO$ scavenging activities. Phloroglucinol (0.590±0.440 μM) showed more potent $ONOO^-$ scavenging activity than penicillamine (2.585±0.133 μM). Compared to an antioxidant Trolox (109.950±19.315 μM), phloroglucinol (58.408±4.513 μM) was shown to scavenge $^{\cdot}O_2^-$ much effectively. The $^{\cdot}NO$ scavenging activity of phloroglucinol (56.976±3.137 μM) was lower than carboxy-PTIO (6.433±0.694 μM) as a standard.

2) Interaction of phloroglucinol with $ONOO^-$

To establish whether phloroglucinol can undergo nitration reaction after addition of $ONOO^-$, a spectrophotometric analysis to reveal a change in absorbance at 430 nm was used. The interaction of phloroglucinol with $ONOO^-$ gave no specific

Table 1. IC_{50} (μM) of phloroglucinol on scavenging activities

	$ONOO^-$	$^{\cdot}O_2^-$	$^{\cdot}NO$
Phloroglucinol	0.590±0.440	58.408±4.513	56.976±3.137
Penicillamine*	2.585±0.133		
Trolox*		109.950±19.315	
Carboxy-PTIO*			6.433±0.69

Data are means±S.E. for triplicate measurements.
*Used as a positive control.

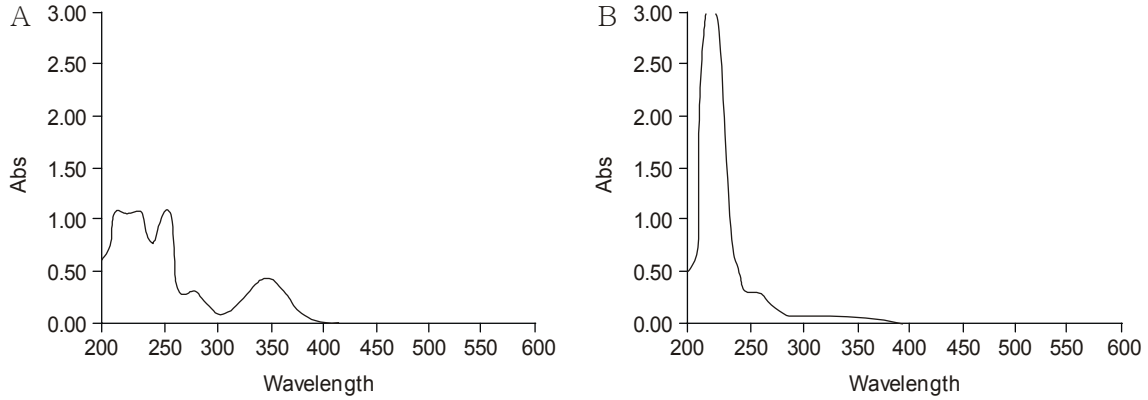

Fig. 1. Interaction of phloroglucinol with $ONOO^-$. Phloroglucinol (100 μM) was incubated without (A) or with $ONOO^-$ (500 μM) (B). Each mixed solution was incubated at 37°C with shaking for 1 hr and scanned between 190 nm and 600 nm with spectrophotometric analysis.

change at 430 nm (Fig. 1), suggesting that no nitration formation occurred. The absence of the peak formation in reaction of phloroglucinol with $ONOO^-$ can be explained by electron donation reaction for its $ONOO^-$ scavenging activity.

3) Effect of phloroglucinol on $ONOO^-$-mediated 3-nitrotyrosine formation

A peak at 430 nm was observed in the reaction of tyrosine with $ONOO^-$, which results in 3-nitrotyrosine (Fig. 2A). Nitration of tyrosine was easily detectable by color change from the colorless to the characteristic yellow color when tyrosine and $ONOO^-$ were mixed. Once nitrotyrosine was formed at pH 7.4, the absorbance was maximal at the 420 nm to 440 nm range. Incubation of phloroglucinol with tyrosine prior to the addition of $ONOO^-$ resulted in the disappearance of the nitrotyrosine peak at 430 nm, implying that phloroglucinol inhibited the formation of 3-nitrotyrosine.

4) Effect of phloroglucinol on cell viability

Cell viability was quantified by trypan blue dye exclusion (Table 2). Bovine smooth muscle cells (SMC) treated with only $ONOO^-$ showed significant decrease (24.7±4.6%) in viability compared to the control group (100.0±4.1%). Incubation of phloroglucinol at three different concentrations (4, 20, and 100 μM) to SIN-1-treated cells led to increase the number of viable cells dose dependently.

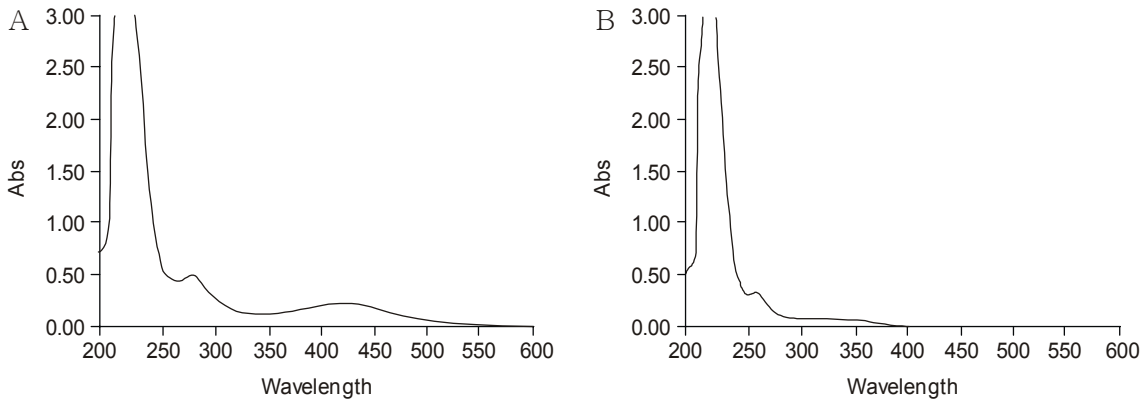

Fig. 2. Effect of phloroglucinol on $ONOO^-$-mediated 3-nitrotyrosine formation. Tyrosine (100 μM) was incubated without (A) or with phloroglucinol (B) prior to the addition of $ONOO^-$. Each mixed solution was incubated at 37°C with shaking for 1 hr and scanned between 190 nm and 600 nm with spectrophotometric analysis. The spectra of the peak displayed at 430 nm reflect the formation of 3-nitrotyrosine.

Table 2. Effect of phloroglucinol on viability (%) of cultured SMCs exposed to SIN-1 (1 mM) for 6 hours

Samples	Concentration of PH or PA			
	0 μM	4 μM	20 μM	100 μM
Control	100.0±4.1			
SIN-1	24.7±4.6			
SIN-1+PH		46.1±3.1	55.6±1.9	62.9±1.5
SIN-1+PA		46.6±3.0	55.6±1.0	64.6±1.5

Values show the relative % of cell viability by trypan blue assay.
Values are means±S.E. for triplicate measurements.
SIN-1, 3-morpholinosydnonimine; PH, phloroglucinol; PA, DL-penicillamine.

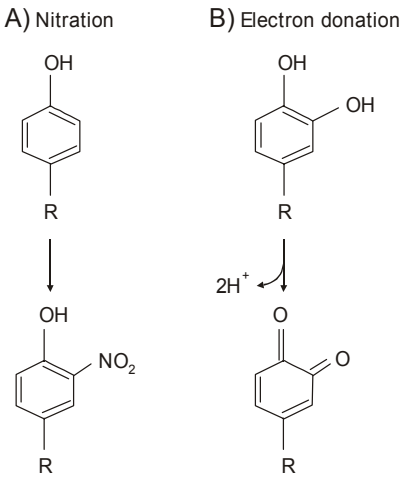

Fig. 3. Possible mechanisms of $ONOO^-$ scavenging.

DISCUSSION

The toxicity of $ONOO^-$ can be attributed to nitration of tyrosine and tryptophan residues and alteration of protein function.[5] In our study, the active component phloroglucinol from *E. stolonifera* scavenged $ONOO^-$ effectively (Table 1). Phloroglucinol not only directly enabled to scavenge $ONOO^-$ but also involved in inhibition of $^{\cdot}O_2^-$ and $^{\cdot}NO$ radical formation (Table 1). Indirectly, the level of $ONOO^-$ can also be controlled by regulation of the levels of the $ONOO^-$ precursors, $^{\cdot}NO$ and $^{\cdot}O_2^-$. The hydroxyl groups in the molecule seemed to be responsible for the strong $ONOO^-$ scavenging activity of phloroglucinol. The detailed mechanism of the $ONOO^-$ scavenging action of each component has not yet clearly known, but the two possible pathways (nitration or electron donation) in the reaction of a phenolic $ONOO^-$ scavenger with $ONOO^-$ were proposed (Fig. 3).[10]

The phenolic compound especially with a

monohydroxyl group like the phenolic amino acid, tyrosine, is preferentially nitrated by $ONOO^-$.[10] The nitration has not clarified whether it is derived from the breakdown of $ONOO^-$ to nitrogen dioxide radical ($^{\cdot}NO_2$) or the nitronium ion (NO_2^+).[11] As seen in Fig. 3A, the conversion of tyrosine into 3-nitrotyrosine by $ONOO^-$ occurred and gave a peak at 430 nm. The increase in absorbance at 430 nm strongly suggests for the $ONOO^-$ scavenger by nitration.[11] However, unlike tyrosine, nitration was not seen with phloroglucinol as the reaction of phloroglucinol with $ONOO^-$ produced no peak at 430 nm. In this respect, electron transfer reaction of the phenolic for its $ONOO^-$ scavenging activity was believed to occur. Phloroglucinol was also examined for its involvement with the reaction of tyrosine and $ONOO^-$. After the addition of phloroglucinol, the peak of 3-nitrotyrosine was no longer observed. This result suggests that phloroglucinol directly blocks the formation of 3-nitrotyrosine by $ONOO^-$ or possibly reduces the availability of $ONOO^-$. $ONOO^-$ plays a role in cytotoxicity that a low dose of $ONOO^-$ has been implicated in apoptosis, whereas its high levels in necrosis.[12] Treatment with phloroglucinol enhanced SMC survival and gave similar cytoprotection against $ONOO^-$ as penicillamine.

In conclusion, the significant $ONOO^-$ scavenging properties of phloroglucinol can be useful for prevention and treatment of the $ONOO^-$-related diseases such as cancer, Alzheimer's disease, rheumatoid arthritis, inflammation, and atherosclerosis.

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