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Chemical components and its antioxidant properties in vitro: An edible marine brown alga, *Ecklonia cava*

Yong Li [b,c,†], Zhong-Ji Qian [b,†], BoMi Ryu [a], Sang-Hoon Lee [a], Moon-Moo Kim [d], Se-Kwon Kim [a,b,*]

[a] Department of Chemistry, Pukyong National University, Busan 608-737, Republic of Korea
[b] Marine Bioprocess Research Center, Pukyong National University, Busan 608-737, Republic of Korea
[c] Resource Institute, Academy of Sciences of Traditional Chinese Medicine of Jilin Province, ChangChun 0431, China
[d] Department of Chemistry, Dong-Eui University, Busan 608-714, Republic of Korea

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ABSTRACT

Seven phlorotannins were isolated and characterized from an edible marine brown alga *Ecklonia cava* (EC), along with three common sterol derivatives (fucosterol, ergosterol, and cholesterol) according to the comprehensive spectral analysis of MS and NMR data. Compounds **5** (7-phloro eckol) and **7** (6,6′-bieckoll) of phlorotannin derivatives were obtained for the first time with the high yields. No reports of compound **3** (Fucodiphloroethol G) was published up to date. The antioxidant properties of all phlorotannins were assessed by total antioxidant activity in a linoleic acid model, free radicals scavenging assay using electron spin resonance spectrometry (ESR) technique, cellular reactive oxygen species (ROS) assay by DCFH-DA, membrane protein oxidation assay; measurement of cellular glutathione (GSH) level in RAW264.7 cell line, and myeloperoxidase (MPO) assay in HL-60 cell line. The results revealed that all phlorotannins had antioxidant properties in vitro, especially, compounds **7** (6,6′-bieckol), **6**, and **3** showed the significant activities compared to the other phlorotannins. Furthermore, the structure–activity relationship (SAR) was discussed based on the structural differences of the tested phlorotannins which have polymeriged phloroglucinal units with diverse skeletons and linkages. It could be suggested that phlorotaninns from this genus would be more potential candidates for the development of unique natural antioxidants for further industrial applications as functional foods, cosmetics and pharmaceuticals. As well as our results makes it clear to understand the reason behind the use of EC as traditional folk herb for a long history.

1. Introduction

The great achievements on the subject area of free radicals are encouraging many leading scientists in this interesting research area continuously.[1,2] Oxygen- and nitrogen-derived free radicals are generated during cellular metabolism and mitochondrial energy production. Reactive oxygen species (ROS), such as hydroxyl ions, superoxide anions, and peroxyl radicals, are involved in oxidative damage to cell components, regulation of signal transduction and gene expression, and activation of receptors and nuclear transcription factors when overproduced.[3] Subsequently it leads to many clinical diseases due to the oxidative stress provided by these kinds of free radicals. Based on this recognition, effective-limited candidates are reported from terraneous resources to solve this problem. At present, marine algae are becoming the focus for targeting effective antioxidants towards oxidative stress in human body due to the presence of diverse natural products with unique structures possibly caused by extreme marine environment. Especially, marine brown and red algae have gain great interest due to their potential ability to produce various bioactive derivatives.[4–7] Among these derivatives, phlorotannins which is a class of compounds with polymerized phloroglucinol units existing in brown algae, have been greatly noteworthy. They have shown broad therapeutic perspectives, such as anti-diabetes,[8] antioxidation,[9] radiation protection,[10] anti-cancer,[11] anti-HIV[12] as well as anti-allergic activities.[13]

In the present study, an edible marine brown alga *Ecklonia cava*, which contains plenty of phloroglucinal derivatives published recently, was taken as our target material to provide unique antioxidants via scavenging and/or reducing the level of free radicals. *E. cava* is widely distributed at the southern coasts of Korea and Japan only. It is abundantly produced in Jeju Island of Korea (30,000 tons per year) for commercial purposes. It is utilized to produce food ingredients, animal feed, fertilizers and folk medicine in gynecopathy and so on.[14] Some related reports already displayed the antioxidant activity using single phlorotaninn from this genus.[15–17] However, this report is systematically investigate the chemical components of EC and antioxidation properties of its phlorotannin

* Corresponding author. Tel.: +82 51 629 7097; fax: +82 51 629 7099.
E-mail address: sknkim@pknu.ac.kr (S.-K. Kim).
† These authors contributed equally to this work.

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derivatives the first time. Chemistry study resulted in two phlorotannins (**5** and **7**) for the first time from this genera besides seven other phlorotannins and three sterols. In addition, no reports can be found regarding the bioactivity of compound (**3**) up to date. First, according to the significant results of total antioxidant activity comparison to tocopherol as positive control in the linoleic acid model system, the tested phlorotannins presented the greatly interesting potential against DPPH, hydroxyl, superoxide, and peroxyl radicals using ESR technique. The subsequent assay on effects of the tested phlorotannins towards cellular ROS undoubtedly supported the above results. In addition, membrane protein oxidation assay, which is effectively used to assess the capacity of antioxidant activity because excessive production and accumulation of ROS can have detrimental effects on membrane proteins, confirmed the potential abilities of these phlorotaninns over again. Finally, the potential inhibition of myeloperoxidase (MPO) and reduction of GSH level strongly suggested the tested phlorotannins can be developed to use as antioxidants in food and pharmaceutical fields. Meanwhile, the SARs were discussed according to the structural features of tested phlorotannins and their natural ratios in the active EC crude extract.

2. Results

2.1. Structural elucidation of phlorotannins and sterols isolated from *Ecklonia cava*

In our continuous investigation in marine edible brown alga-*Ecklonia cava*, ten naturally occurring metabolisms including seven phlorotannins and three sterols (Fucosterol, cholesterol and ergosterol) were isolated from the methanol extract of EC. Their chemical structures were undoubtedly elucidated on the basis of comprehensive spectral analysis of MS and NMR (^{1}H and ^{13}C) data, and also comparing with the data published previously. Two phlorotannins were obtained from this gene for the first time, 7-phloro eckol (**5**),[18] and 6,6′-bieckol (**7**);[19] the other five phlorotannins were assigned as phloroglucinol (**1**),[20] eckol (**2**),[21] fucodiphloroethol G (**3**),[22] phlorofucofuroeckol A (**4**),[19] and dieckol (**6**)[18] as illustrated in Figure 1 and Table 1.

2.2. Determination of total antioxidant activity of phlorotannins

First, determination of total antioxidant activity of phlorotannins, which was screened in a linoleic acid model system by reading the absorbance of ferric thiocyanate values at 500 nm, showed that all of the tested phlorotannins (20 μM, each) exist potential antioxidant activity comparison to the tocophenol used as positive control present in Figure 2 by the following order, compounds **6** > **7** > **3** > **4** > **5** > **2** > **1**. The antioxiditive capacities of compounds **6**, **7**, **3**, **4**, and **5** (in order) were more powerful than the positive control. However, the activity of the other derivatives (**1**, **2**) was obviously lower compared with the positive control from the fifth day on. Simultaneously, the following color development with $FeCl_2$ and thiocyanate at differently incubation intervals yielded in the obvious difference between more active derivatives (**6**, **7**, **3**, **4**, **5**) and less active ones (**1**, **2**) from the fifth day to the seventh day. All these meaningful results encouraged us to investigate the radical scavenging activity on types of free radicals quantitatively and deeply.

2.3. Radical scavenging activities of phlorotannins using ESR technique

The purpose of this study is to examine the most potential antioxidant among the main phloroglucinol derivatives corresponding to the modern antioxidative activity of crude extract of *Ecklonia cava*. The remaining radical concentration after treating with phlorotannins in various radical reactions can be monitored by trapping the short-lived reactive radicals with spin traps and detection of the long-lived spin adducts that is formed with electron spin resonance spectroscopy (ESR). This method consequently provides an excellent way to examine the potential antioxidant ability in the tested phlorotannin derivatives.

2.3.1. DPPH radical scavenging activity

DPPH is the choice of many scientists to evaluate the free radical scavenging activity of natural products. It is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule. Among these tested phlorotannin derivatives, most compounds showed a strong scavenging activity except for compound **1** at various concentrations (from 1 μM to 50 μM) as shown in Figure 3 (A) and Table 2, namely, compounds **6** > **7** > **3** > **4** > **5** > **2** > **1** in order. Especially, compounds **6**, **7**, and **3** showed the strongest scavenging capacities than the others with the IC_{50} values at 8.29 μM, 8.69 μM, and 14.72 μM, respectively. However, compound **1** showed very weak scavenging activity without interesting meaning regarding to the others even the concentration increased up to 50 μM.

2.3.2. Hydroxyl radical scavenging activity

Hydroxyl radicals generated in the Fenton system (Fe^{2+}/H_2O_2) were trapped by a stable radical 5,5-dimethyl-pyrrolidine-1-oxyl (DMPO), forming a spin adduct as detected by ESR spectrometer. All phlorotannin derivatives showed wild scavenging activities at different concentrations from 1 μM to 50 μM as presented in Figure 3B and Table 2. The order of IC_{50} values for those derivatives was revealed and arranged as 28.6 μM (**6**) > 29.7 μM (**7**) > 33.5 μM (**3**) > 39.2 μM (**4**) > 39.6 μM (**5**) > 51.8 μM (**2**). Compound **1** showed the weakest scavenging activity with the IC_{50} value at 392.5 μM. In compounds **3**, **4**, and **5**, the obvious differences were not observed due to the similar numbers of hydroxyl groups in the individual structures.

2.3.3. Superoxide anion radical scavenging activity

Superoxide anion is one of the precursors of the single oxygen and hydroxyl radicals, even though it is a weak oxidant, it indirectly initiates lipid peroxidation, and also the presence of superoxide anion can magnify the cellular damage because it consequentially increased other free radicals and oxidizing agents. In the present study, the superoxide anion was induced by irradiation of a riboflavin/EDTA system used as the positive control. Superoxide anion scavenging activity in the presence of the phlorotannins and commercial antioxidant are showed in Figure 3C and Table 2. Comparison to the positive control-EGCG, the results of those phlorotannins revealed the possessed potential scavenging activity in a dose-dependent manner ($p < 0.05$). The order of scavenging ability could be arranged as compounds **7** > **6** > **3** > **4** > **5** > **2** > **1** > EGCG with the IC_{50} values at 15.9 μM (**7**); 14.5 μM (**6**), 18.6 μM (**3**), 21.6 μM (**4**), 21.9 μM (**5**), 26.5 μM (**2**), and 115.2 μM (**1**), respectively. Compounds **7** and **6** showed the highest activity regarding to the lowest compound **1**. The other tested phlorotannin derivatives revealed the similar scavenging activity.

2.3.4. Peroxyl radical scavenging activity

Peroxyl radical is one of alkyl radicals which has been found to be a primary intermediate in many hydrocarbon reactions. These radicals were easily detected by trapping spin adduct using ESR technique. Result showed that all tested phlorotannins possess considerable scavenging activities as shown in Figure 3D and Table

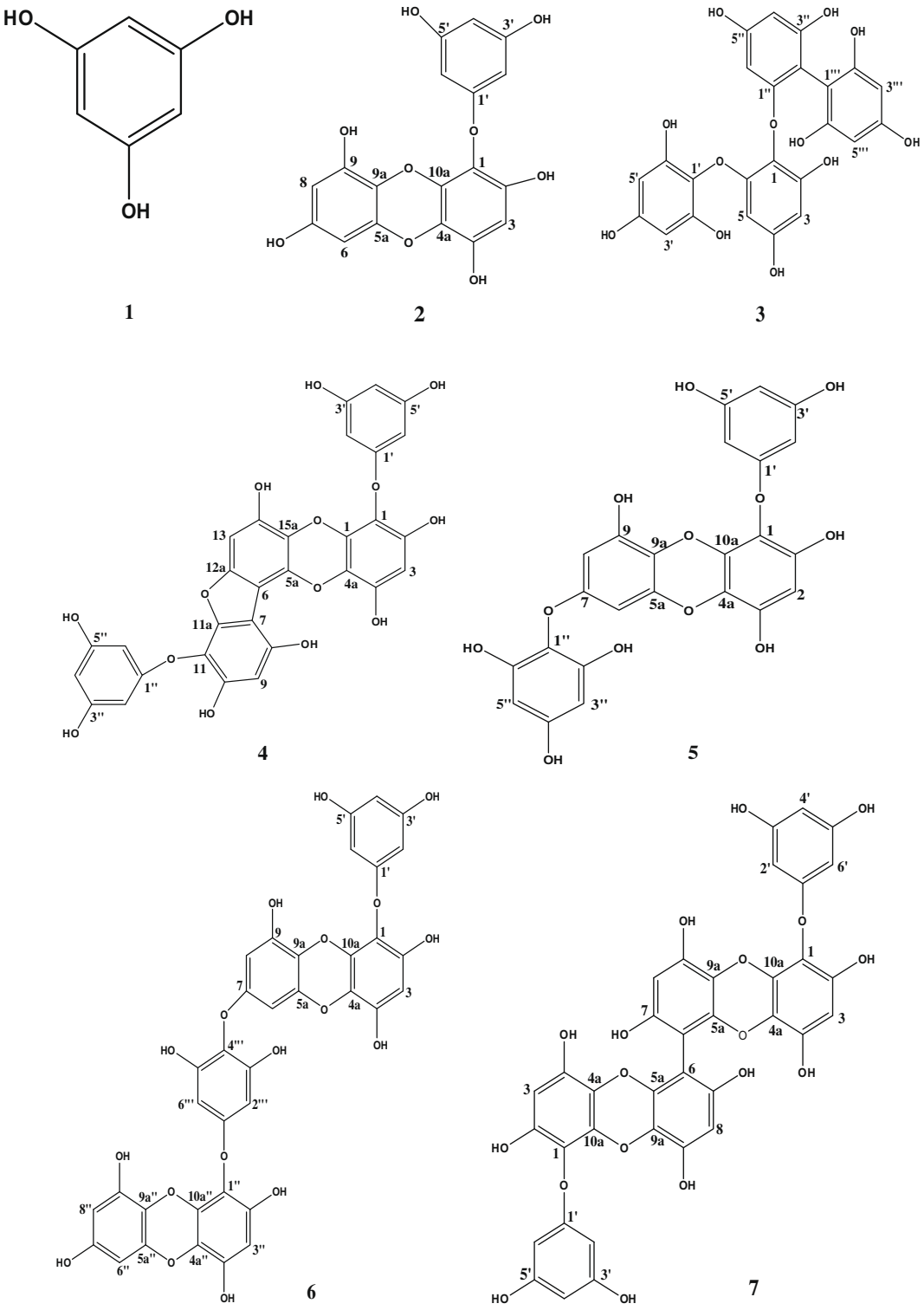

Figure 1. Chemical structures of phlorotannins (**1**–**7**) from *Ecklonia cava*.

2. Compound **6** showed a strongest property than the other compounds with IC_{50} value at 14.5 μM, and compound **7** also showed the similar scavenging capacity with IC_{50} of 17.1 μM, the relatively lowest capacity was observed in compound **1** with IC_{50} value at 128.9 μM. Based on the statistical significance, the abilities of tested phlorotannins were presented in the following sequence, compounds **6** > **7** > **3**> **4** > **5** > **2** > **1** ($p < 0.05$).

2.4. Viability of Phlorotannins on MRC-5, RAW264.7, and HL-60 cell lines

The viabilities of the tested phlorotannin derivatives were carried out on MRC-5, RAW264.7, and HL-60 cultured human cell lines for evaluating the endocellular action of antioxidation. The results showed that the tested phlorotannin derivatives performed no cyto-

Table 1
^{13}C and ^{13}C DEPT NMR (100 MHz) data for phlorotannins (**2**–**7**) in DMSO-d_6

C#	2	3	4	C#	5	6	7
1	122.9 (s)	123.3 (s)	122.5 (s)	**1**	122.1 (s)	122.1 (s)	123.5 (s)
2	145.6 (s)	154.5 (s)	147.2 (s)	**2**	145.9 (s)	146.09 (s)	145.4 (s)
3	97.9 (d)	95.7 (d)	98.3 (d)	**3**	98.2 (d)	98.3 (d)	97.7 (d)
4	141.5 (s)	153.0 (s)	142.1 (s)	**4**	141.9 (s)	141.8 (s)	141.4 (s)
4a	122.9 (s)		122.7 (s)	**4a**	123.1 (s)	123.19 (s)	121.9 (s)
5		92.3 (d)		**5a**	142.3 (s)	142.4 (s)	141.3 (s)
5a	142.2 (s)		134.1 (s)	**6**	93.6 (d)	93.5 (d)	99.7 (d)
6	93.6 (d)	150.3 (s)	103.3 (s)	**7**	154.5 (s)	154.2 (s)	151.3 (s)
7	152.6 (s)		103.5 (s)	**8**	98.1 (d)	97.9 (d)	97.8 (d)
8	98.3 (d)		146.6 (s)	**9**	146.0 (s)	145.96 (s)	144.5 (s)
9			99.2 (d)	**9a**	123.9 (s)	123.9 (s)	122.7 (s)
9a	122.3 (s)			**10a**	137.1 (s)	137.2 (s)	137.2 (s)
10			150.5 (s)	**1′**	160.3 (s)	160.3 (s)	160.4 (s)
10a	136.8 (s)			**2′**	93.6 (d)	93.6 (d)	93.7 (d)
11			120.0 (s)	**3′**	158.8 (s)	158.8 (s)	158.8 (s)
11a			149.6 (s)	**4′**	96.2 (d)	96.2 (d)	96.1 (d)
12a			151.0 (s)	**5′**	158.8 (s)	158.8 (s)	158.8 (s)
13			94.8 (d)	**6′**	93.6 (d)	93.6 (d)	93.7 (d)
14			144.9 (s)	**1″**	122.5 (s)	122.2 (s)	
14a			126.4 (s)	**2″**	151.2 (s)	145.92 (s)	
15a			137.0 (s)	**3″**	94.8 (d)	98.2 (d)	
1′	160.0 (s)	121.9 (s)	160.2 (s)	**4″**	154.8 (s)	141.9 (s)	
2′	93.5 (d)	151.1 (s)	93.7 (d)	**4a″**		123.11 (s)	
3′	158.4 (s)	94.7 (d)	158.8 (s)	**5″**	94.8 (d)		
4′	96.0 (d)	154.6 (s)	96.2 (d)	**5a″**		142.6 (s)	
5′	158.4 (s)	94.7 (d)	158.8 (s)	**6″**	151.2 (s)	93.9 (d)	
6′	93.5 (d)	151.1 (s)	93.7 (d)	**7″**		153.1 (s)	
1″		156.5 (s)	159.9 (s)	**8″**		98.5 (d)	
2″		101.6 (s)	93.4 (d)	**9″**		146.06 (s)	
3″		157.2 (s)	159.0 (s)	**9a″**		122.6 (s)	
4″		96.8 (d)	96.7 (d)	**10a″**		137.1 (s)	
5″		157.8 (s)	159.0 (s)	**1‴**		155.9 (s)	
6″		93.3 (d)	93.4 (d)	**2‴**		94.5 (d)	
1‴		101.0 (s)		**3‴**		151.2 (s)	
2‴		157.1 (s)		**4‴**		124.2 (s)	
3‴		94.7 (d)		**5‴**		151.2 (s)	
4‴		157.2 (s)		**6‴**		94.5 (d)	
5‴		94.7 (d)					
6‴		157.1 (s)					

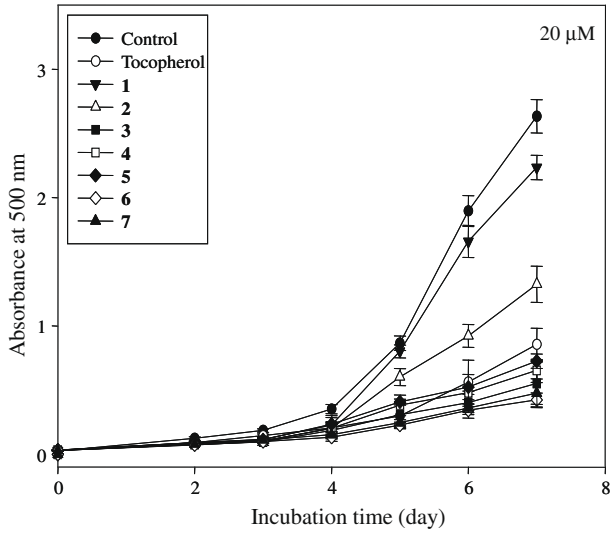

Figure 2. Antioxidative activities of different phlorotannin derivatives (**1**–**7**) from *Ecklonia cava* and α-tocopherol in the linoleic acid emulsion were determined by the ferric thiocyanate method. The control was defined where no antioxidant is added in the antioxidative activity test.

toxic effects even at the highest concentration of 100 μM, and a remarkable difference could not be found between tested phlorotannin derivatives and control as described in Figure 4 ($p < 0.05$).

2.5. Cellular reactive oxygen species (ROS) determination by DCFH-DA

Dichlorofluorescin diacetate (DCFH-DA) has been used as a substrate for measuring intracellular oxidant production in neutrophils. DCFH-DA is hydrolyzed by esterases to dichlorofluorescin (DCFH), which is trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) by action of cellular oxidants. DCFH-DA cannot be appreciably oxidized to a fluorescent state without prior hydrolysis. Due to absence cytotoxic effect of tested phlorotannin derivatives at the required concentration of 100 μM, human cultured cell line RAW264.7 was meaningfully selected for investigating how the tested phlorotannin derivatives affect the intracellular production of ROS. As shown in Figure 5, fluorescence emitted by DCF following ROS-mediated oxidation of DCFH followed a time course increment up to 210 min it was found that pre-incubation with compounds **3**–**7** decreased the DCF fluorescence dose- and time-dependently. Compounds **6** and **7** exerted considerable radical scavenging activities at the concentration of 1 μM. At the concentration of 50 μM, compounds **6**, **7** and **3** could scavenge ROS significantly throughout the incubation time. After 210 min incubation, these three compounds reduced the production of ROS by 84.27%, 83.58% and 75.6% at 50 μM, respectively. Compound **1**, however, reduced the production of ROS with the lowest percentage (%) at the highest concentration 50 μM.

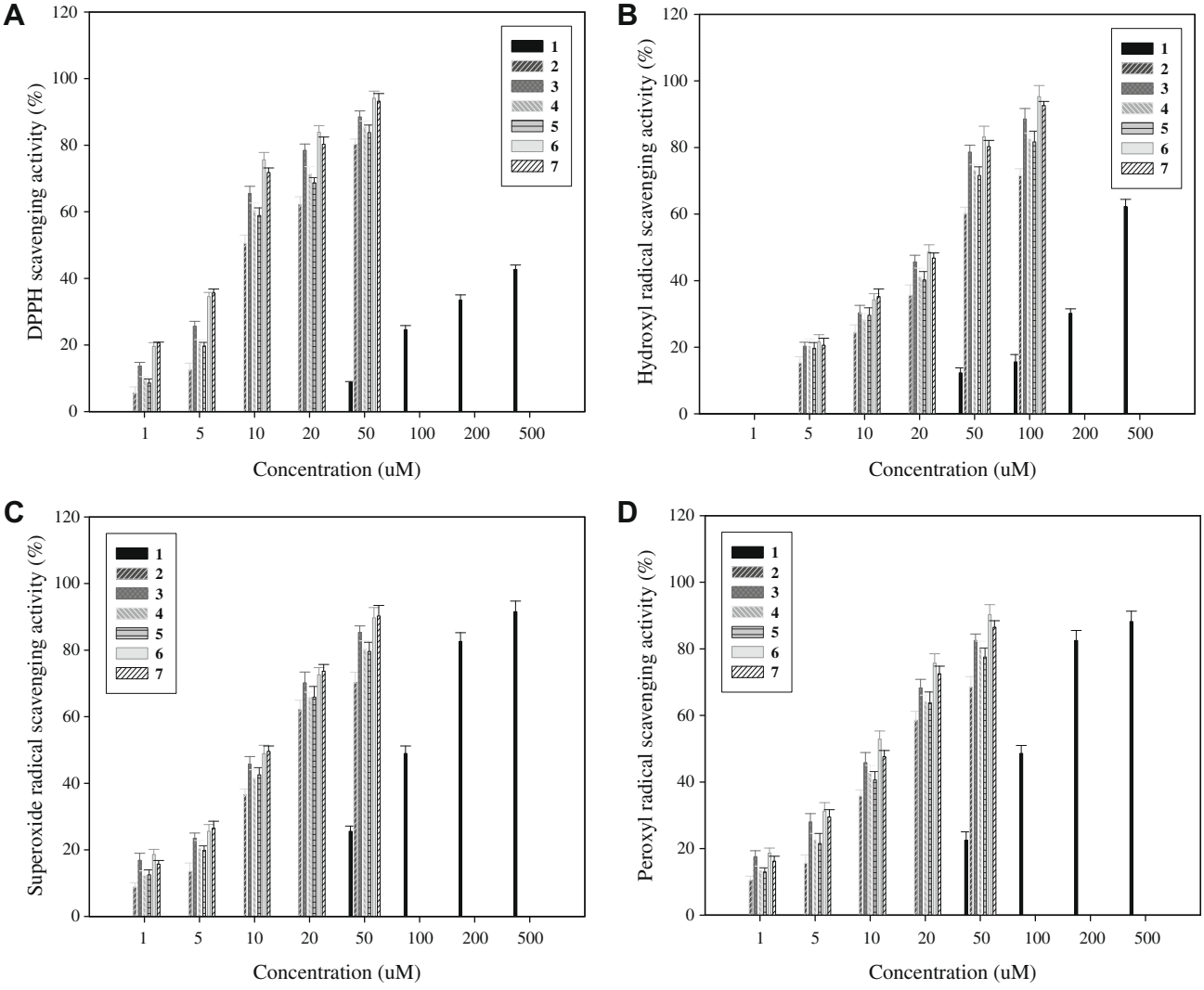

Figure 3. Free radical scavenging activities of phlorotannin derivatives (**1**–**7**) from *Ecklonia cava*, measured with ESR spectrometer. (A) DPPH radical scavenging activity (radical generated by DPPH). (B) Hydroxyl radical scavenging activity (radical generated by Fenton reaction and trapped with DMPO). (C) Superoxide radical scavenging activity (radical trapped with DMPO). (D) Peroxyl radical scavenging activity (radical generated by AAPH and trapped with 4-POPN).

Table 2
IC_{50} values of phlorotannins from *Ecklonia cava* against free radicals

Compounds	Radical scavenging activity IC_{50} (μM ± SD)[a] Radicals			
	DPPH	Hydroxyl	Superoxide	Peroxyl
1	Not determined	392.5 ± 2.80	115.2 ± 2.50	128.9 ± 2.25
2	22.89 ± 0.52	51.8 ± 2.50	26.5 ± 1.25	28.4 ± 1.50
3	14.72 ± 1.20	33.5 ± 1.55	18.6 ± 1.50	18.1 ± 1.00
4	17.66 ± 0.80	39.2 ± 1.82	21.6 ± 2.20	21.4 ± 2.10
5	18.64 ± 0.95	39.6 ± 2.10	21.9 ± 1.80	22.7 ± 1.50
6	8.28 ± 0.45	28.6 ± 2.50	16.2 ± 0.95	14.5 ± 1.85
7	8.69 ± 0.35	29.7 ± 1.50	15.9 ± 1.30	17.1 ± 2.20

[a] Radical scavenging activity (%) = [(Blank peak area − Sample peak area)/Blank peak area] × 100 (%). Each value was expressed as the means ± SD (n = 3). IC_{50} value was defined as the necessary concentration at which the radicals generated by reaction systems were scavenge by 50%, respectively.

2.6. Cellular Membrane protein oxidation

Oxidation damage of cellular membranes also results in increased membrane fluidity, compromised integrity, and inactivation of membrane-bound receptors and enzymes. Protein oxidation by ROS plays an important role in the pathomechanism of many diseases. The attack by ROS against proteins results in the modification of amino acid side chain containing amino acids with functional groups such as lysine, arginine, proline, and histidine, and then generates carbonyl moieties (mainly aldehydes and ketones), which has been identified as an early marker for protein oxidation. This is used in measure of protein damage. The inhibitory effects of phlorotannin derivatives on RAW264.7 cells membrane protein oxidation were investigated as described in materials and methods. As shown in Figure 6, when mouse macrophage membranes were exposed to $HO^{\cdot}$ generated by Fe^{2+}–H_2O_2 Fenton reaction, the extent of membrane protein oxidation increased as indicated by the increase of carbonyl groups contents. About 12-times higher amount of carbonyl groups were observed in oxidative stress induced control group compared to those of blank group. Protein carbonyl groups in case of compounds **6** and **7** were lower than those of control group. Pretreatment with compounds **6** and **7** inhibited the oxidation of membrane protein. These compounds can effectively inhibit the protein oxidation by 70.5% and 67.3% of the control, respectively ($p < 0.05$). Compounds **1** and **2** showed very weak inhibitory effects only at the highest tested concentration of 50 μM. The other compounds **4** and **5** showed middle inhibition activity against membrane protein oxidation into carbonyl groups. The results suggested that compounds **6** and **7** could protect membrane proteins in RAW264.7 cells from ROS derived oxidation more effectively.

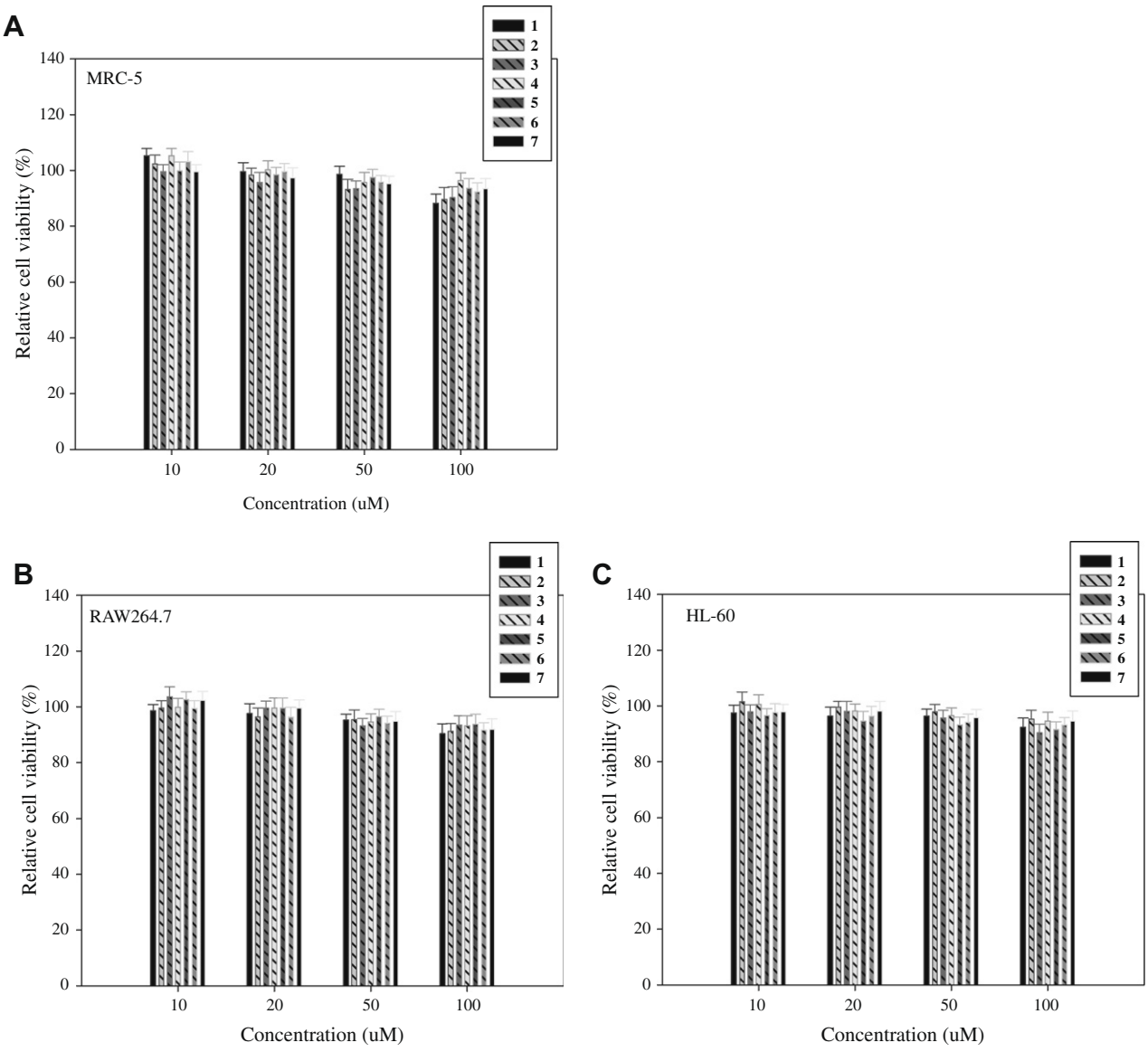

Figure 4. Cytocompatible effects of phlorotannin derivatives (**1**–**7**) on MRC-5 (A), RAW 264.7 (B) and HL-60 cells (C). Different concentrations of phlorotannin derivatives were applied to the cells for 24 h and cell viability was assessed by MTT assay as described in the text. Results are means ± standard error of three independent experiments.

2.7. Measurement of intracellular GSH level

Cellular GSH level was determined using monobromobimane (mBBr) as a thiol-staining reagent. As shown in Figure 7, all tested phlorotannin derivatives showed no remarkably different activity when measured with by mBBr-GSH fluorescence intensity. In these tested derivatives, compounds **6**, **7**, and **3** revealed relatively strong fluorescence intensity than the others at the incubation time of 120 min. the mBBr-GSH fluorescence intensities of these compounds were arranged as compounds **6** > **7** > **3** > **4** > **5** > **2** > **1** ($p < 0.05$).

2.8. Myeloperoxidase (MPO) activity

MPO plays an important role in oxidants production by polymorphonuclear neutrophils (PMNs). It is a leukocyte-derived heme peroxidase which has long been considered as a microbial enzyme centrally linked to the unspecific immune defense system. It uses hydrogen peroxide (H_2O_2) and chloride to catalyze the production of hypochlorous acid (HOCl), which is the most powerful oxidant and contributes to both microbial killing and subsequent oxidative injury of host tissue triggering severe inflammatory disorders. Thus, the seeking for compounds towards the inhibition of MPO activity is an important approach to control ROS-mediated oxidation of biomolecules in neutrophils. In the present study, HL-60 cells were used because of its high expression of MPO after stimulation with TNF-α. As shown in Figure 8, the MPO activity was dose-dependently inhibited by pre-treating with compounds **6** and **7** with MPO activity cells of 0.51 IU/10^6 and 0.53 IU/10^6 (10 μM) and 0.45 IU/10^6 and 0.47 IU/10^6 (50 μM), respectively. It could be assumed that this is an indirect way of acting as cellular antioxidant. No significant differences were observed between these two compounds ($p < 0.05$). The tested compounds **3**–**5** showed the middle inhibition activity, and compounds **1** and **2** showed weaker activities compared to others and blank.

3. Discussion

Recently, marine red and brown algae have been well-known as an important source to produce naturally bioactive secondary

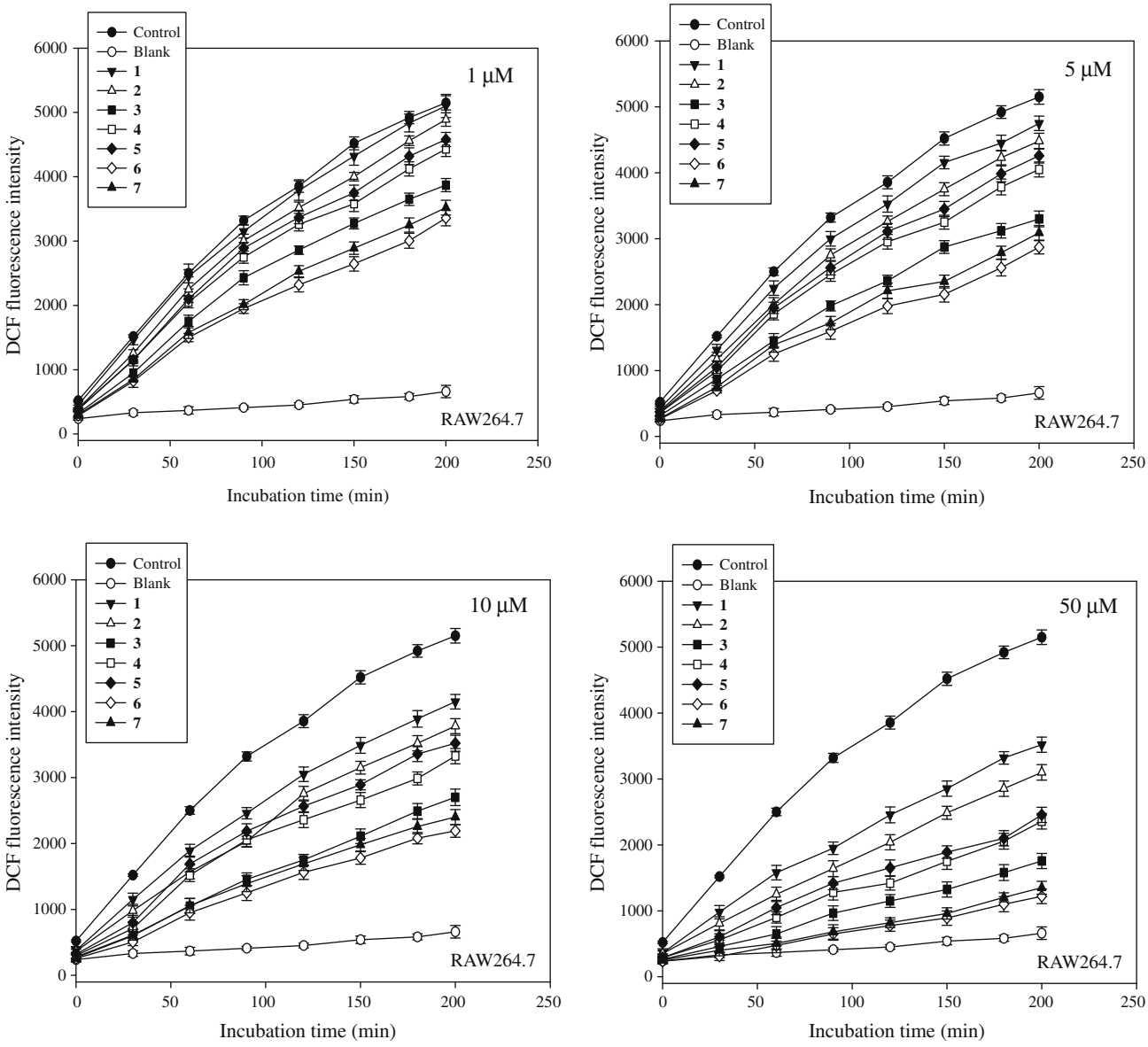

Figure 5. Cellular radical scavenging activities of phlorotannin derivatives (**1**–**7**) at various concentrations (1 μM, 5 μM, 10 μM, and 50 μM). RAW 264.7 cells were labeled with non-toxic fluorescence dye, DCFH-DA, and treated with different concentrations of phlorotannin derivatives. Fluorescence intensities of DCF due to oxidation of DCFH by cellular ROS (generated by H_2O_2) were detected time-dependently ($\lambda_{excitation}$ = 485 nm and $\lambda_{excitation}$ = 528 nm). Effects of phlorotannin derivatives on the scavenging of cellular ROS were compared with H_2O_2 non-stimulated blank and sample non-treated control group in three independent experiments.

metabolites including phenols and polyphenols with unique linkages (ether and/or phenyl).[23,24] *E. cava*, an edible marine brown alga with a long history as folk medicine in Korea, is abundantly distributed only in Korea and Japan according to the latest statistics. The previous reports on *E. cava* have revealed that it contains plenty of phlorotannin derivatives with interesting bioactivities[25] via using crude methanol extract and/or single phlorotannin isolates, such as antioxidation, anti-inflammation, anti-allergy, and anti-HIV activities. Regarding to antioxidation activity, however, it is almost impossible to reflect the antioxidant properties of *E. cava* strictly and completely using one or two single phlorotannins. Hence, this made us take attempt to isolate all phlorotannin derivatives existing in this species for evaluating its antioxidant characteristic properties scientifically and systematically. For the chemistry efforts in this study, the successful isolation and purification of EC methanol extract led to ten single compounds including common sterols (fucosterol, cholesterol, and ergosterols) and phlorotannin derivatives described as phloroglucinol (**1**), eckol (**2**), fucodiphloroethol G (**3**), phlorofucofuroeckol A (**4**), 7-phloroglucinol eckol (**5**), dieckol (**6**), and 6,6′-bieckol (**7**), respectively. All of their structures were undoubtedly established by comprehensive spectroscopic analysis including NMR (^{1}H and ^{13}C) and LREIMS, as well as comparison to previously published data. Among those phlorotannin derivatives, compounds **5** and **7** were isolated for the first time from this genus with the high yields at 1.19% (w/w), and 1.28% (w/w), respectively. The successful purification of all phlorotannins is more meritorious by using Sephadex LH-20 open column chromatography only with eluent instead of HPLC technique. The temperature was maintained <25 °C during the process steps to avoid possible oxidation of hydroxyl groups and over polymerization of phloroglucinal units existing in these phlorotannins. Furthermore, dimethyl sulfoxide (DMSO-d_6) as NMR solvent made proton signals from OH groups appear possibly and clearly in ^{1}H spectrum. It provided more evidence for integral

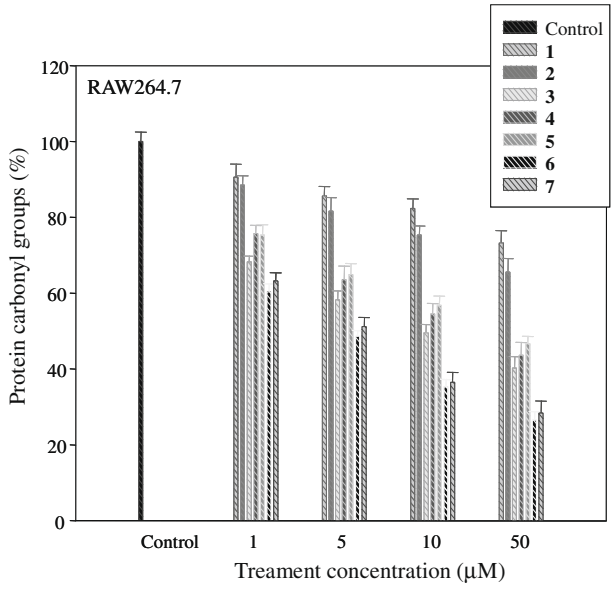

Figure 6. Assessment of cell membrane protein oxidation. Raw 264.7 cell membranes were treated with various concentrations of phlorotannin derivatives (**1**–**7**), and protein oxidation was determined by assessing the amount of carbonylcarbon content as described in the text and compared with –OH non-treated (blank group) and treated (control group). Results are means ± standard error of three independent experiments.

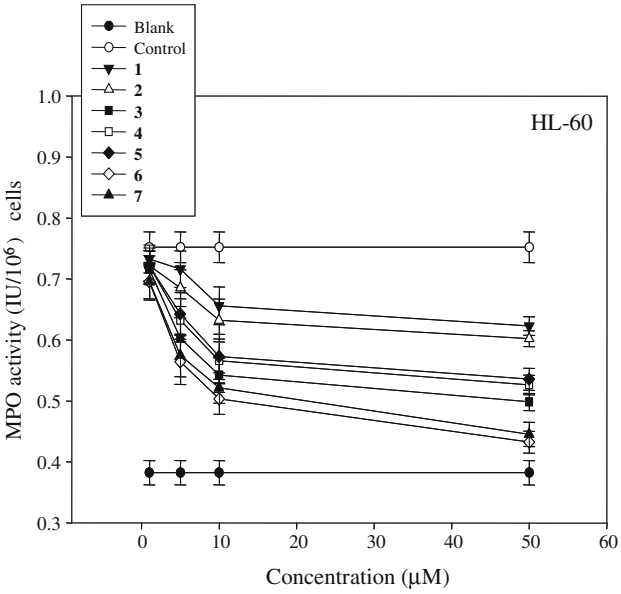

Figure 8. The inhibitory effect of myeloperoxidase activity (MPO) of phlorotannin derivatives (**1**–**7**) in HL-60 cells. Cells were treated with different concentrations of phlorotannin derivatives. An assay mixture containing H_2O_2 and 1.6 mM TMB was added and amount of MPO released was measured spectrophotometrically at 655 nm. MPO activity was compared treated groups with non-treated NA-COS (blank). Results are means ± standard error of three independent experiments.

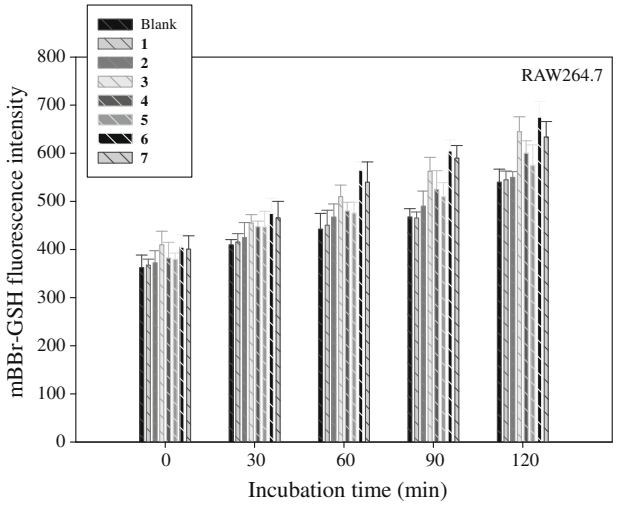

Figure 7. The effect of phlorotannin derivatives (**1**–**7**) on regulation of GSH level in Raw 264.7 cells. Cells were treated with 20 μM of phlorotannin derivatives and incubated for 30 min. Cellular GSH level was determined using mBBr as a thiol-staining reagent according to the method described in the text measuring mBBr-GSH fluorescence intensity at kexcitation = 360 nm and kemission = 465 nm. The average fluorescence values of cell populations were plotted and compared with blank group (non-treated phlorotannin derivatives). Results are means ± standard error of three independent experiments.

structure elucidation than before. Vanillin–H_2SO_4 was employed as the detecting agent instead of UV detector (254 and 365 nm) due to no chemical conjugated systems existing in these phlorotannin derivatives.

For the antioxidation activities testings, all isolated phlorotannins were chosen as our targets except for the isolated sterol derivatives due to the well-known in their bioactivities before. From this study, first, it can make a scientific and entire evaluation for EC containing those phlorotannins with special natural abundance. Second, regarding to the bioactivities of compound **3**, only one paper from our research team can be found in anti-allergy activity field.[25] Therefore, according to our knowledge, it is the first time for the compounds **2**–**6** from EC focusing on antioxidation properties in systematical evaluation mentioned here. In this report, the antioxidant activities were assessed using total antioxidation activity in linoleic acid model, ESR method for four free radicals scavenging, and cellular ROS inhibition assay which has been widely used as a type of reactive molecules that can easily act with some molecules in living organisms and important causative factors which can induce many kinds of diseases such as cancer, atherosclerosis, cardiovascular, aging and so on. Furthermore, membrane protein oxidation, GSH level, and oxidative enzyme (MPO) were also detected, respectively. According to the results described above, it is easy to understand that the most of tested phlorotannin derivatives showed the modern antioxidant properties by the various biological pathways used commonly. In general, this study also revealed the similarly active order as **6** (or **7**) > **7** (or **6**) > **3** > **4**> **5** > **2** > **1**.

The SAR could be described according to the structural differences of these phlorotaninn derivatives. The differences of molecular weights among the tested phlorotannins were not responsible to their antioxidant capacities. However, the number of hydroxyl groups present in these compounds showed to have an important role in antioxidant activity. For an example, compounds **6** (11 OH) and **7** (12 OH) showed the best activities than compounds **1** (3 OH) and **2** (6 OH); The polymers (**5**–**7**) derived from eckol skeleton (compound **2**) showed higher activities than others, demonstrating that the skeletons of phloroglucinal polymers with O-bridge linkages (ether linkage) play a key role in their antioxidation ability, probably due to its unique bonding with corresponding receptors.

In conclusion, *E. cava* is a very interesting resource, not only due to its limited origin (only Korea and Japan) but also due to the presence of unique phlorotannin derivatives with special natural ratio in EC. Based on the above description, it could be suggested that phlorotannins derived from *E. cava*, especially compounds **7**, **6**, and **3**, have noteworthy potential for application as antioxidants

in functional food, cosmetics, and pharmaceutical industries. Meanwhile, additional studies on the mechanisms and in vivo are highly warranted to achieve a better understanding of important antioxidant properties of the isolated phlorotannins from EC.

4. Experimental

4.1. General materials

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Japan), using DMSO-d_6 solvent peak (2.50 ppm in ^{1}H and 39.5 ppm in ^{13}C NMR) as an internal reference standard. For some signals, the chemical shifts approximated the third decimal place. This is to distinguish between signals of very close value but which could nevertheless be clearly differentiated by visual inspection of the spectra. MS spectra were obtained on a JEOL JMS-700 spectrometer (JEOL, Japan). Extraction of EC was performed using Extraction Unit (Dongwon Scientific Co., Korea). Column chromatography was carried out by Silica Gel 60 (230–400 mesh, Merck, Germany), Sephadex LH-20 (Sigma, St. Louis, MO). Thin-layer chromatography (TLC) was run on precoated Merck Kieselgel 60 F_{254} plates (0.25 mm), and the spots on the TLC plate were detected under a UV lamp (254 and 365 nm) using $CHCl_3$/MeOH/H_2O/acetic acid (65:25:4:3, v/v/v/v) as a development solvent system.[26] Vanillin–H_2SO_4 was employed as the detecting agent for phenolic compounds.[27] All the solvent and chemicals used in this study were of a reagent grade from commercial sources.

4.2. Extraction, isolation and purification of phlorotannins

The marine edible brown seaweed, EC was collected from Jeju Island coast of Korea during the period from October 2004 to March 2005. Fresh EC was washed three times with water to remove salt. The lyophilized EC was ground into powder before extraction. The dried EC powder (10 kg) was extracted by stirring extraction unit with MeOH (3 × 5 L) for 10 days. The extract (273 g) was suspended in water and partitioned with *n*-hexane (35.92 g), CH_2Cl_2 (20.49 g), EtOAc (24.87 g), *n*-BuOH (106 g) in sequence. The EtOAc fraction (24.87 g), which exhibited a most potent antioxidation activity, was subjected to a silica gel flash chromatography eluted with hexane/EtOAc/MeOH (gradient) to yield ten subfractions (F1-F10). F5 and F6 (976.235 mg) with the highest activity on antioxidation was further purified by Sephadex LH-20 with MeOH only to afford the phlorotannins, compound **1** (102.85 mg), compound **2** (58.30 mg), compound **3** (47.62 mg), compound **4** (53.71 mg), compound **5** (132.96 mg), compound **6** (118.78 mg), and compound **7** (127.51 mg) as illustrated in Figure 1 and Table 1, respectively.

Compound **1** (phloroglucinol): off-white powder; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 8.97 (3H, s, OH-1, 3, 5), 5.66 (3H, s, H-2, 4, 5); ^{13}C NMR (DMSO-d_6, 100 MHz) δ 94.1 (C-2, 4, 6), 158.9 (C-1, 3, 5); LREIMS *m/z* 126.05 $[M]^+$.

Compound **2** (eckol): light brown powder (lyophilized); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.54 (1H, s, OH-9), 9.45 (1H, s, OH-4), 9.21 (2H, s, OH-2, 7), 9.16 (2H, s, OH-3′, 5′), 6.14 (1H, s, H-3), 5.96 (1H, d, *J* = 2.8 Hz, H-8), 5.80 (1H, d, *J* = 1.7 Hz, H-4′), 5.78 (1H, d, *J* = 2.8 Hz, H-6), 5.72 (2H, *J* = 1.7 Hz, H-2′, 6′); ^{13}C NMR (DMSO-d_6, 100 MHz) see Table 1. LREIMS *m/z* 373.00 $[M+H]^+$ ($C_{18}H_{12}O_9$).

Compound **3** (fucodiphloroethol G): off-white powder; ^{1}H NMR (DMSO-d_6, 400 MHz) δ 5.83 (1H, d, *J* = 2.8 Hz, H-3), 5.52 (1H, d, *J* = 2.8 Hz, H-5), 5.84 (2H, br s, H-3′, 5′), 5.98 (1H, d, *J* = 2.3 Hz, H-4″), 5.85 (1H, d, *J* = 2.3 Hz, H-6″), 5.90 (2H, br s, H-3‴, 5‴), 9.11 (3H, s, OH-2, 2′, 6′), 8.99 (1H, s, OH-4), 8.93 (2H, s, OH-4, 6‴), 8.95 (2H, s, OH-3″, 5″), 8.57 (1H, s, OH-2‴), 8.47 (1H, s, OH-4‴); ^{13}C NMR (DMSO-d_6, 100 MHz) see Table 1. LREIMS *m/z* 499.08, $[M+H]^+$ ($C_{24}H_{18}O_{12}$).

Compound **4** (phlorofucofuroeckol A): light brown powder (lyophilized); ^{1}H NMR (DMSO-d_6, 400 MHz) δ 10.17 (1H, s, OH-14), 9.92 (1H, s, OH-4), 9.88 (1H, s, OH-10), 9.48 (1H, s, OH-2), 9.23 (2H, s, OH-3″,5″), 9.21 (2H, s, OH-3′, 5′), 8.23 (1H, s, OH-8), 6.71 (1H, s, H-13), 6.42 (1H, s, H-9), 6.29 (1H, s, H-3), 5.82 (2H, t, *J* = 2.2 Hz, H-4′, 4″), 5.75 (2H, d, *J* = 2.2 Hz, H-2′, 6′), 5.71 (2H, d, *J* = 2.2 Hz, H-2″, 6″); ^{13}C NMR (DMSO-d_6, 100 MHz) see Table 1. LREIMS *m/z* 603.12 $[M+H]^+$ ($C_{30}H_{18}O_{14}$).

Compound **5** (1-(3′,5′-dihydro-xyphenoxy)-7-(2″,4″,6-trihydro-xyphenoxy)-2,4,9-trihydroxydibenzo-1,4-dioxin): light brown powder (lyophilized), ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.65 (1H, s, OH-9), 9.45 (1H, s, OH-4), 9.24 (1H, s, OH-2), 9.16 (2H, s, OH-3′, 5′), 9.15 (2H, s, OH-2″, 6″), 9.03 (1H, s, OH-4″), 6.14 (1H, s, H-3), 5.77 (1H, d, *J* = 2.6 Hz, H-6), 6.00 (1H, d, *J* = 2.6 Hz, H-8), 5.85 (2H, s, H-3″, 5″), 5.71 (2H, *J* = 1.8 Hz, H-2′, 6′), 5.79 (1H, t, *J* = 1.8 Hz, H-4′); ^{13}C NMR data, see Table 1; LREIMS *m/z* 496.13 $[M]^+$.

Compound **6** (dieckol): light brown powder (lyophilized), ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.71 (1H, s, OH-9), 9.61 (1H, s, OH-9″), 9.51 (1H, s, OH-4″), 9.46 (1H, s, OH-4), 9.36 (2H, s, OH-3″, 5″), 9.28 (1H, s, OH-2″), 9.23 (1H, s, OH-2), 9.22 (1H, s, OH-7″), 9.15 (2H, s, OH-3′, 5′), 6.17 (1H, s, H-3″), 6.14 (1H, s, H-3), 6.02 (1H, d, *J* = 2.7 Hz, H-8), 5.98 (1H, d, *J* = 2.7 Hz, H-8″), 5.95 (1H, s, H-2‴, 6‴), 5.82 (1H, d, *J* = 2.7 Hz, H-6), 5.81 (1H, d, *J* = 2.7 Hz, H-6″), 5.80 (1H, t, *J* = 2.0 Hz, H-4′), 5.78 (2H, d, *J* = 2.0 Hz, H-2′, 6′); ^{13}C NMR data, see Table 1, LREIMS *m/z* 743.10 $[M]^+$.

Compound **7** (6,6′-bieckol): light brown powder (lyophilized), ^{1}H NMR (DMSO-d_6, 400 MHz) δ 9.29 (1H, s, OH-9), 9.16 (2H, s, OH-3′, 5′), 9.15 (1H, s, OH-2), 9.09 (1H, s, OH-4), 8.65 (1H, s, OH-7), 6.09 (1H, s, H-3), 6.05 (1H, s, H-8), 5.80 (1H, d, *J* = 2.2 Hz, H-4′), 5.75 (2H, d, *J* = 2.2 Hz, H-2′, 6′), ^{13}C NMR data, see Table 1; LREIMS *m/z* 743.12 $[M]^+$.

4.3. Determination of total antioxidant activity of phlorotannins

The total antioxidant activity was measured in a linoleic acid model system according to the method of Osawa and Namiki.[28] Briefly, the tested phlorotannins (final concentration, 20 μM) was dissolved in 10 ml of 50 mM phosphate buffer (pH 7.0), and added to a solution of 0.13 ml of linoleic acid and 10 ml of 99.5% ethanol. Then the total volume was adjusted to 25 ml with distilled water. The mixture was incubated in a conical flask with a screw cap at 40 ± 1 °C in a dark room and the degree of oxidation was evaluated by measuring the ferric thiocyanate values. The reaction solution (100 μl) incubated in the linoleic acid model system was mixed with 4.7 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate, and 0.1 ml of 2 × 10^{-2} M ferrous chloride solution in 3.5% HCl. After 3 min, the thiocyanate value was measured by reading the absorbance at 500 nm following color development with $FeCl_2$ and thiocyanate at different intervals during the incubation period at 40 ± 1 °C.

4.4. Measurement of free radicals scavenging activity of phlorotannins by ESR

Different radicals tested here were generated according to previously mentioned procedures and spin adducts were recorded using a JES-FA electron spin resonance (ESR) spectrometer (JEOL Lts., Tokyo, Japan).

$$\text{Radical scavenging activity} = \left(\frac{1-H}{H_0}\right) \times 100\%$$

4.4.1. DPPH radical scavenging activity

DPPH radical scavenging activity was carried out by the method described by Nanjo et al.[29] A 30 μl tested phlorotannin sample solution (or ethanol itself as control) was added to 30 μl of DPPH (60 μM) in ethanol solution. After mixing vigorously for 10 s, the solution was then transferred into a 100 μl quartz capillary tube, and the scavenging activity of phlorotannins on DPPH radical was measured using a JESFA-ESR spectrometer (JEOL, Tokyo, Japan). The spin adduct was measured on an ESR spectrometer exactly 2 min later. The running parameters of ESR spectrometer were adjusted as follows: magnetic field 336.5 ± 5 mT; power, 5 mW, modulation frequency, 9.41 GHz, amplitude, 1×1000; sweep time, 30 s. DPPH radical scavenging capacity was calculated according to the above equation, in which H and H_0 were relative peak heights of radical signals with and without sample, respectively. The percentage of scavenging activity was plotted against the sample concentration to obtain the IC_{50} values.

4.4.2. Hydroxyl radical scavenging activity

Hydroxyl radical scavenging activity was measured using the method described by Rosen et al. Hydroxyl radical was generated by iron-catalyzed Fenton Haber–Weiss reaction and then trapped with DMPO, the resultant DMPO-OH adducts were detected with an ESR spectrometer.[30] A 20 μl tested phlorotannin solution was mixed with DMPO (0.3 M, 20 μl), $FeSO_4$ (10 mM, 20 μl) and H_2O_2 (10 mM, 20 μl) in a phosphate buffer solution (pH 7.4), and then transferred into a 100 μl quartz capillary tube. After 2.5 min, the ESR spectrum was recorded using an ESR spectrometer. The running parameters of ESR spectrometer were adjusted as follow: magnetic field, 336.5 ± 5 mT, power, 1 mW; modulation frequency, 9.41 GHz; amplitude, 1×200; sweep time, 4 min. Hydroxyl radical scavenging capacity was calculated as the above equation, in which H and H_0 were relative peak heights of radical signals with and without sample, respectively.

4.4.3. Superoxide radical scavenging activity

Superoxide anion radicals were generated by UV irradiated riboflavin/EDTA system.[31] The reaction mixture containing 0.3 mM riboflavin (20 μl), 1.6 mM EDTA (20 μl), 800 mM DMPO (20 μl) and indicated concentrations of the tested phlorotannin (20 μl) was irradiated for 1 min under UV lamp at 365 nm. The reaction mixture was then transferred to 100 μl quartz capillary tube of the ESR spectrometer for measurement. The experimental parameters were as follows: magnetic field, 336.5 ± 5 mT; power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000; sweep time, 1 min. Superoxide radical scavenging capacity was calculated as above equation, in which H and H_0 were relative peak heights of radical signals with and without sample, respectively.

4.4.4. Peroxyl radical scavenging activity

Pexoxyl radical scavenging activity was measured using the method described by Hiramoto et al.[32] Briefly, 20 μl of 40 mM 2,2′-azobis (2-amidinopropane) dihydrochloride (AAPH) was mixed with 20 μl of phosphate buffered-saline (PBS), 20 μl of 40 mM α-(4-pyridyl-1-oxide)-*N*-*tert*-butylnitrone (4-POBN) and 20 μl of extracts solution. The mixture was vortexed and incubated at 37 °C for 30 min. Subsequently, reaction mixture was transferred to a sealed capillary tube and spin adduct was recorded with controlled spectrometric conditions; the running parameters of ESR spectrometer was adjusted as follow: modulation frequency, 100 kHz; microwave power, 10 mW; microwave frequency, 9441 MHz; magnetic field, 336.5 ± 5 mT and sweep time, 30 s. Peroxyl radical scavenging capacity was calculated above equation, in which H and H_0 were relative peak heights of radical signals with and without sample, respectively.

4.5. Cells culture and cytocompatible effects of phlorotannins

MRC-5 (Human fetal lung fibroblasts cell line) and RAW264.7 (mouse macrophages cell line) cell lines were cultured in Dulbecco's modified Eagle's medium (DMEM), and HL-60 (human leukemic cell line) cell lines were cultured in Roswell Park Memorial Institute medium (RPMI), supplemented with 10% fetal bovine serum, 2 mM glutamine, and 100 U/ml penicillin–streptomycin at 5% CO_2 and 37 °C humidified atmosphere, respectively. Cytocompatible effects were evaluated using MTT (3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide) assay as described by Hansen et al.[33] The cells were grown in 96-well plates at a density of 5×10^3 cells/well. After 24 h, cells were washed with fresh medium and then treated with different concentrations of phlorotannins (**1**–**7**). After 48 h incubation, cells were rewashed and 100 μl of MTT (1 mg/ml) was added and incubated for 4 h. Finally, DMSO (150 μl) was added to solubilize the formazan salt formed and amount of formazan salt was determined by measuring the OD at 540 nm using an GENios® microplate reader (Tecan Austria GmbH, Austria). Relative cell viability was determined by the amount of MTT converted into formazan salt. Viability of cells was quantified as a percentage compared to the control (OD of treated cells − OD of blank/OD of control − OD of blank × 100%) and dose response curves were developed. The data were expressed as mean from at least three independent experiments and $p < 0.05$ was considered significant.

4.6. Effects of phlorotannins on cellular ROS by DCFH-DA

Intracellular formation of reactive oxygen species (ROS) was assessed as described previously using oxidation sensitive dye 2′,7′-dichlorofluorescin diacetate (DCFH-DA) as the substrate.[34] RAW264.7 cells growing in fluorescence microtiter 96-well plates were labeled with 20 mM DCFH-DA in Hank's balanced salt solution (HBSS) and incubated for 20 min in the dark. Nonfluorescent DCFH-DA dye, that is freely penetrated into cells get hydrolyzed by intracellular esterases to 2′,7′-dichlorofluorescin (DCFH), and tarps inside the cells. Cells were then treated with different concentrations of tested phlortannins and incubated for 1 h. after washing the cells with PBS for three times, 300 μM H_2O_2 dissolved in HBSS was added to cells. The formation of 2′,7′-dichlorofluorescin (DCF) due to oxidation of DCFH in the presence of various ROS was read after every 30 min at the excitation wavelength (Ex) of 485 nm and the emission wavelength (Em) of 535 nm using a GENions® fluorescence microplate reader (Tecan Austria GmbH, Austria). Dose dependant and time dependant effects of treatment groups were plotted and compared with fluorescence intensity of control and blank groups.

4.7. Measurement of intracellular GSH level

Cellular GSH level was determined using monobromobimane (mBBr) as a thiol-staining reagent.[35] For this experiment, Raw 264.7 cells were seeded into fluorescence microtiter 96-well plates at a density of 1×10^7 cells/ml and following attachment, treated with different concentrations of phlorotannins (**1**–**7**) for 30 min. Cells were then labeled with 40 μM mBBr for 30 min at 37 °C in the dark. After staining, mBBr-GSH fluorescence intensity was measured (kexcitation = 360 nm, kemission = 465 nm) using above mentioned fluorescence microplate reader. The average fluorescence values of cell populations were plotted and compared with blank group in which cells were grown without treatment of phlorotannins.

4.8. Myeloperoxidase activity

Amount of myeloperoxidase (MPO) released by HL60, human promyeloblastic cells was determined by *O*-dianisidine method with modification.[36] HL60 cells were suspended in RPMI-1640 without phenol red and FBS and seeded into 96-well plates. Cells were preincubated with various concentrations of phlorotannins (**1**–**7**) for 30 min followed by stimulation with TNF-α (0.05 μg/ml) at 37 °C for 30 min. Cells were then added with the assay mixture containing 0.05 ml of 1 mM H_2O_2 in 0.1 M phosphate buffer (pH 6.0) and 0.05 ml of 0.02 M *O*-dianisidine (freshly prepared) in deionized water. The amount of MPO released was measured spectrophotometrically at 460 nm and MPO activity was plotted as an absorbance value compared to the untreated blank group.

4.9. Membrane protein oxidation

The oxidation degree of cell membrane proteins was assessed by determining the content of protein by carbonyl group.[37] Cultured cells were washed three times with PBS and lysed in lysis buffer without reducing agents. Aliquots of cell lysate were transferred into microtubes and treated with different concentrations of phlorotannins. After incubation for 30 min at 37 °C, 0.1 mM $FeSO_4$ and 2 mM H_2O_2 were added to the mixture and continued to incubate for 1 h. Solubilized protein was precipitated by centrifugation after addition of 400 μl of 20% trichloroacetic acid. The pellet was resuspended in 0.2% of 2,4-dinitro-phenyl hydrazine in 2 N HCl and allowed to stand at 25 °C for 40 min. The protein was precipitated again with 20% trichloroacetic acid and the pellet was washed three times with ethanol/ethyl acetate (1:1 v/v) solution. It was then dissolved in 200 μl 6 N guanidine hydrochloride and incubated for 15 min at 37 °C. After centrifugation at 1500*g* for 5 min, absorbance of the supernatant was recorded against a complementary blank at 370 nm using a UV/visible microplate reader. A blank was prepared with a parallel procedure using 2 N HCl alone instead of 2,4-dinitrophenyl hydrazine reagent. The carbonyl group of protein was expressed by comparing with control group.

4.10. Statistical analysis

The data were expressed as the mean of three replicate determination and standard deviation (SD), statistical comparisons were made with student's test. p values <0.05 were considered to be significant.

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