In Vitro Antioxidant Activity of 5-HMF Isolated from Marine Red Alga Laurencia undulata in Free Radical Mediated Oxidative Systems

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Received: January 4, 2009 / Revised: April 15, 2009 / Accepted: May 21, 2009

Marine red algae of genus Laurencia are becoming the most important resources to produce unique natural metabolites with wide bioactivities. However, reports related to Laurencia undulata, an edible species used as folk herb, are rarely found to date. In this research, 5-hydroxymethyl-2-furfural (5-HMF) was isolated and characterized by nuclear magnetic resonance (NMR) from Laurencia undulata as well as other marine algae. The following characteristics of 5-HMF were systematically evaluated: its antioxidant activities, such as typical free-radicals scavenging in vitro by electron spin resonance spectrometry (ESR) and intracellular reactive oxygen species (ROS) scavenging; membrane protein oxidation; oxidative enzyme myeloperoxidase (MPO) inhibition; as well as expressions of antioxidative enzymes glutathione (GSH) and superoxide dismutase (SOD) on the gene level using the polymerase chain reaction (PCR) method. The results demonstrated that 5-HMF could be developed as a novel marine natural antioxidant or potential precursor for practical applications in the food, cosmetic, and pharmaceutical fields.

Keywords: Marine alga, Laurencia undulata, 5-HMF, antioxidation, PCR technique

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According to the latest publications, the marine red algae of genus Laurencia are well known to produce a wide range of marine biologically active metabolites, mainly including sesquiterpenes, diterpenes, and a C15-acetogenin, etc., which exhibit diverse biological activities, such as antioxidant and anticancer activities [12, 15, 20, 25]. To continue our search for naturally occurring bioactive compounds, Laurencia undulata, one of the edible species with the most potential in genus Laurencia, was selected as our target since reports on this species are rarely found to date. Previous research has revealed only that Laurencia undulata contains polyphenol and [2E]-2-tridecylheptadec-2-enal derivatives [26], and its crude extract showed anti-asthmatic activity [13]. This greatly encouraged us to use it for future investigation in both the chemistry and bioactive fields.

The successful isolation of a methanol extract of Laurencia undulata afforded 5-hydroxymethyl-2-furfural (5-HMF) by column chromatography and HPLC techniques, along with some common components including steroids (cholesterol and fucosterol) and mannitol. Among these isolates, 5-HMF is the most interesting candidate for bioactive screening, not only for the reasons described here below, but also because it is the first time it has been isolated from not only Laurencia undulata but also marine algae in general. Many reports related to 5-HMF have provided significant proof for its potential importance, focusing on its pathways of organic synthesis, quantitative determination by various methods, anticancer activities, and applications as nerve medication, protection of the myocardium, treatments of cardiovascular diseases damage to striated muscles and viscera by combining to protein, and decreasing the accumulation of poisons in the body [7]. The recent research, moreover, showed that 5-HMF can be obtained as the main active component from some traditional medicines, such as Rehmanniae, Cornus, and Schisandra [6, 28]. However, there is no experimental evidence available regarding 5-HMF-mediated inhibition of free-radical damage in cellular oxidizing systems.

The reactivity of antioxidants with free radicals and the relationship with oxidative stress have been the subject of a plethora of scientific researches and consistently top the
list of current topics in health and medicine. In general, free radicals are usually classified into oxygen-, nitrogen-, and carbon-derived reactive species, which can be produced during cellular metabolism and mitochondrial energy production and involved in oxidative damage to cell components, regulation of signal transduction and gene expression, and activation of receptors and nuclear transcription factors. 1,1-Diphenyl-2-pircylhydrazyl (DPPH), hydroxyl, alkyl, and superoxide anion reactive groups are commonly used as effective checking points in vitro for the response of these kinds of radicals described above, and their magnetic signals and levels can be quantitatively monitored and determined by the ESR technique, which provided the first direct proof in 1982 [5, 10]. In addition, reactive oxygen species (ROS) have been demonstrated to be mediators of cellular anomalies, not limited to protein damage, deactivation of enzymatic activity, alteration of DNA, and lipid peroxidation of membranes [3, 8]. Normally, ROS and free radicals can be effectively eliminated by the antioxidant defense systems such as antioxidant enzymes and non-enzymatic factors. However, under pathological conditions, the balance between the generation and elimination of ROS is broken, and as a result of these events, biomacromolecules including DNA, membrane lipids, and proteins are injured by ROS-mediated oxidative stress. Uncontrolled generation of free radicals that attack membrane lipids, protein, and DNA is believed to be involved in many health disorders such as diabetes mellitus, cancer, and neurodegenerative and inflammatory diseases, especially aging caused by the oxidation factor [19]. Therefore, to scavenge overgenerated free radicals, the inhibition of the activities of oxidant enzymes (myeloperoxidase (MPO)), and the increase and/or activation of antioxidant enzymes (glutathione [GSH] and superoxide dismutase [SOD]) should be more effective pathways to protect cells, tissues, and organs from damage caused by direct and indirect acts of reactive free radicals.

Because synthetic antioxidants can generate side effects such as carcinogenicity, the search for natural products is attracting increasing attention from scientists nowadays, in particular, secondary metabolites from marine resources with unique structures resulted from being in an extreme marine environment (high salt, high pressure, etc.). Even though the synthetic antioxidants are effective and cheap compared with natural ones, their applications are restricted because of the potential risks related to health.

In this present work, we describe in detail the extraction, isolation, and structure elucidation of 5-HMF, where HPLC and NMR (1D and 2D) techniques were mainly employed. Its antioxidant activities were also evaluated via a systematical analysis using various assays at the molecular and cellular levels, including free-radical scavenging activities using the ESR technique, cellular ROS scavenging by the DCFH–DA method, membrane protein oxidation level, MPO inhibition level, and GSH and SOD levels via the PCR technique. The results showed that 5-HMF possesses potential antioxidant activity compared with the positive controls. It is suggested that 5-HMF could be developed as an antioxidant in the food-stuffs, cosmetics, and pharmaceutical industries.

**Materials and Methods**

### Plant Material

*Laurencia undulata* was collected along the Coast of Cheju Island in South Korea in October 2007. Prior to experiments, the fresh sample was washed thoroughly with tap water to remove salt, sand, and epiphytes, and then dried to constant weight in the air followed by grinding into powder with a laboratory beater. The sample powder was stored at −20°C until use.

### Materials and Chemicals

1H NMR (400 MHz) and 13C NMR (100 MHz) spectra were recorded on a JEOL JNM-ECP 400 NMR spectrometer (JEOL, Japan), using the DMSO-d₆ solvent peak (2.50 ppm in H and 39.5 ppm in C NMR) as an internal reference standard. For some signals, the chemical shifts approximated the third decimal place. This was 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 *Laurencia undulata* was performed using the Extraction Unit (Donggwon Scientific Co., Korea). The ESR spectra were recorded on a JES-TE100 ESR spectrometer (JEOL, Tokyo, Japan). Silica gel 60 (230–400 mesh; Merck, Germany), Sephadex LH-20 (Sigma, St. Louis, MO, U.S.A.), and thin-layer chromatography (TLC) plates (Kieselgel 60 F 254 grade available commercially). All other reagents were of analytical grade available commercially.
gradient solvent system (10:1, 5:1, 1:1 [v/v], each 4 l), dichloromethane (5 l), and mixtures of dichloromethane and methanol (10:1, 5:1, 1:1 [v/v], each 4 l) to give 12 fractions based on TLC analysis. Fraction 4 yielded the two common derivatives (cholerete and fucosterol). Fraction 6 was further subjected to Sephadex LH-20 chromatography and purified by HPLC to afford compound I (250 mg) and mannotill (25 mg).

**Compound I:** 1H NMR (400 MHz, CDCl3): δH ppm 9.60 (1H, s, H-6), 7.22 (1H, d, J=3.6 Hz, H-3), 6.52 (1H, d, J=3.6 Hz, H-4). 13C NMR (100 MHz, CDCl3): δC ppm 177.7 (C-6), 160.4 (C-5), 152.4 (C-2), 122.6 (C-3), 109.9 (C-4), 57.6 (C-7) (see Table 1). LREIMS m/z: 126.05 (52) [M]+, 109.05 (15) [M-OH]+, 97.00 (100) [M-OH-C]+, and 81.00 (12) [M-OH-C-O]+.

**Scavenging Effects of 5-HMF on Free Radicals**

Scavenging effects of 5-HMF on free radicals were assessed using the ESR technique including DPPH, hydroxyl, alkyl, and superoxide anion species. The scavenging capacity was calculated based on the following equation: Radical scavenging activity (%)=(A−A0)/A0×100%

**Scavenging Effect on DPPH Radical**

The scavenging effect of 5-HMF on the DPPH radical was carried out by a method described previously [17]. A 25 µl solution of 5-HMF in 10% DMSO was added to 25 µl of DPPH (60 µM) in methanol solution. After mixing vigorously for 10 s, the solution was then transferred into a 100 µl quartz capillary tube, and then the spin adduct was measured on an ESR spectrometer exactly 2 min later. The instrumental parameters were as follows: magnetic field, 6.5±±5 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000; and sweep time, 30 s.

**Hydroxyl Radicals Scavenging Activity**

Hydroxyl radicals were generated by the iron-catalyzed Fenton Haber–Weiss reaction, and the generated hydroxyl radicals rapidly reacted with nitrone spin trap DMPO [23]. The resultant DMPO–OH adducts were detectable with an ESR spectrometer. The 5-HMF (10% DMSO) solution (15 µl) was mixed with DMPO (0.3 M, 15 µl), FeSO4 (10 mM, 15 µl), and H2O2 (10 mM, 15 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 instrumental parameters were as follows: magnetic field, 336.5±±4 mT; power, 5 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000; and sweep time, 30 s.

**Table 1. NMR (1D & 2D) data for 5-HMF in CDCl3.**

Position δ δ HMBC
1 152.4 (s)
2 122.6 (d) 2, 4, 5, 6
3 109.9 (d) 2, 3, 5
4 160.4 (s)
5 177.6 (d) 2
6 57.6 (t) 4, 5
7 5.50 (1H, d, J=2.1 Hz) 5, 7
7-OH 5.50 (1H, d, J=2.1 Hz)

*Recorded at 400 MHz NMR for 1H and 100 MHz NMR for 13C DEPT; DMSO-d6 was added for HMBC.*

**Alkyl Radical Scavenging Activity**

Alkyl radicals were generated by AAPH according to the method of Hiramoto et al. [11]. Briefly, 15 µl of 40 mM AAPH was mixed with 15 µl of phosphate-buffered saline (PBS), 20 µl of 40 mM 4-POBN, and 15 µl of the indicated concentrations of 5-HMF. The mixture was vortexed and incubated at 37°C for 30 min. Subsequently, the reaction mixture was transferred to a sealed capillary tube and the spin adduct was recorded with controlled spectrometric conditions: modulation frequency, 100 kHz; microwave power, 10 mW; microwave frequency, 9.441 MHz; magnetic field, 336.5±±5 mT; and sweep time, 30 s.

**Superoxide Anion Radical Scavenging Activity**

Superoxide anion radicals were generated by the UV-irradiated riboflavin–EDTA system [9]. The reaction mixture containing 0.8 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO (15 µl), and indicated concentration of 5-HMF fraction was irradiated for 1 min under a UV lamp at 365 nm. The reaction mixture was transferred to the 100 µl quartz capillary tube of the ESR spectrometer for measurement. The instrumental parameters were as follows: magnetic field, 336.5±±5 mT; microwave power, 10 mW; modulation frequency, 9.41 GHz; amplitude, 1×1000; and scan width, 10 mT.

**Cell Culture**

The MRC-5 and RAW264.7 cell lines used in this study were cultured in DMEM, and the HL-60 cell line, a suspension-dependent cell line, was cultured in Roswell Park Memorial Institute (RPMI) medium, respectively supplemented with 10% heated-inactivated FBS, penicillin (100 U/ml), and streptomycin (100 µg/ml). Cultures were carried out at 37°C in a humidified atmosphere of 95% air and 5% CO2, and the medium was changed every other day.

**MTT Assay**

Cell viability was measured by using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay [2]. The cells were cultured in 96-well microtiter plates (1.5×104 cells/well) with serum-free medium and treated with various concentrations of 5-HMF for 24 h. 5-HMF was dissolved in 10% dimethylsulfoxide (DMSO) and sterilized by filtering through a 0.22 µm filter membrane. The final concentration of DMSO in the culture media never exceeded 0.1%. Sequentially, 20 µl of MTT dye solution was added to each well. After 4 h of incubation, 200 µl of DMSO solution was added for dissolving the formazan crystals and the absorbance was read using a GENios microplate reader (TECAN Austria Gmbh, Austria) at 540 nm by measuring the optical density (OD) of each well, and the cell viability of cells treated with various concentrations of 5-HMF was calculated as follows: Cell viability (%)=OD/ODb×100%, where ODb is the absorbance value of 5-HMF, and OD is the absorbance value of the blank.

**Reactive Oxygen Species Assay by DCFH–DA**

The DCFH–DA method was employed to measure intracellular ROS production, as described previously [21]. RAW264.7 cells growing in 96-well fluorescence microtiter plates were labeled with 20 µM DCFH–DA in Hank’s balanced salt solution (HBSS) and incubated for 20 min in the dark. Cells were then treated with different concentrations of 5-HMF and incubated for another 1 h. After washing the cells three times with PBS, 100 µl of 0.3 mM H2O2 was added. The intensity of the fluorescence signal was detected time-
Cells were then added to the assay mixture containing 0.05 ml of MPO inhibition activity (%)=[1−(A−c)/(A−b)]×100%, where A is the absorbance value of 5-HMF, A c is the absorbance value of the blank, and A b is the absorbance value of the control. Cells in the control and blank were grown in the absence of 5-HMF, and a blank was made without the stimulation with TNF-α.

Myeloperoxidase Activity Assay

The amount of MPO released by HL-60 human promyelocytic leukemia cells was determined by the o-dianisidine method with modifications [4]. HL-60 cells were suspended in RPMI-1640 without phenol red and FBS and seeded into 96-well plates. Cells were preincubated with various concentrations of samples for 30 min, followed by stimulation with TNF-α (0.05 μg/ml) at 37°C for 30 min. Cells were then added to the assay mixture containing 0.05 ml of 2 mM HCl in 0.1 M phosphate buffer (pH 6.0) and 0.05 ml of 0.2 M o-dianisidine (freshly prepared) in deionized water. The amount of MPO released was measured spectrophotometrically at 460 nm and the inhibition percent of 5-HMF on MPO activity was calculated as follows: MPO inhibition activity (%)=[1−(A−A c)/(A−A b)]×100%, where A is the absorbance value of 5-HMF, A c is the absorbance value of the blank, and A b is the absorbance value of the control. The key correlations from HMBC showed cross bond linkages between protons and carbons were clearly supported by HMQC analysis, except for the hydroxyl proton. The long-range correlations between protons and carbons led to the assignments of three moieties based on HMBC data; that is, a furan skeleton with bisubstituted side chains, hydroxymethyl group, and methyl aldehyde residue. The key correlations from HMBC showed cross peaks between 9.60 (13H) and 152.4 and 122.6 (13C), between 4.72 (1H) and 160.4 and 109.9 (13C), and two quaternary carbon atoms (152.4 and 160.4 ppm). The single-bond linkages between protons and carbons were clearly supported by HMQC analysis, except for the hydroxyl proton. The long-range correlations between protons and carbons led to the assignments of three moieties based on HMBC data; that is, a furan skeleton with bisubstituted side chains, hydroxymethyl group, and methyl aldehyde residue. The key correlations from HMBC showed cross peaks between 9.60 (1H) and 152.4 and 122.6 (13C), between 4.72 (1H) and 160.4 and 109.9 (13C), as well as between 5.50 (1H) and 160.4 and 57.7 (13C). Moreover, the fragment ion peaks in the mass spectrum provided more evidence for the final structure elucidation of compound 1, such as m/z 126.05 (52) [M]+, taking together 1H and 13C NMR spectral data. It contains four unsaturated degrees including three double bonds and one ring. In the 1H NMR spectrum, five signals were observed; two doublets presented at the aromatic region with chemical shift values of 6.52 and 7.22 ppm, respectively. These sp2 proton signals showed the same coupling constant of 3.6 Hz. One sp3 proton doublet appeared at 4.72 ppm with the 2.1 Hz coupling constant being the same as with one triplet signal at 5.50 ppm; in addition, one featured singlet at 9.60 ppm showed the existence of the aldehyde group. The 13C and 13C DEPT spectra revealed six carbon signals consisting of one methane (57.7 ppm), three methines (177.6, 122.6, and 109.9 ppm), and two quaternary carbon atoms (152.4 and 160.4 ppm). The single-bond linkages between protons and carbons were clearly supported by HMQC analysis, except for the hydroxyl proton. The LREIMS of compound 1 suggested the molecular formula C12H10O6, with a molecular ion peak at m/z 262.05 (52) [M]+, taking together 1H and 13C NMR spectral data. It contains four unsaturated degrees including three double bonds and one ring. In the 1H NMR spectrum, five signals were observed; two doublets presented at the aromatic region with chemical shift values of 6.52 and 7.22 ppm, respectively. These sp2 proton signals showed the same coupling constant of 3.6 Hz. One sp3 proton doublet appeared at 4.72 ppm with the 2.1 Hz coupling constant being the same as with one triplet signal at 5.50 ppm; in addition, one featured singlet at 9.60 ppm showed the existence of the aldehyde group. The 13C and 13C DEPT spectra revealed six carbon signals consisting of one methane (57.7 ppm), three methines (177.6, 122.6, and 109.9 ppm), and two quaternary carbon atoms (152.4 and 160.4 ppm). The single-bond linkages between protons and carbons were clearly supported by HMQC analysis, except for the hydroxyl proton. The long-range correlations between protons and carbons led to the assignments of three moieties based on HMBC data; that is, a furan skeleton with bisubstituted side chains, hydroxymethyl group, and methyl aldehyde residue. The key correlations from HMBC showed cross peaks between 9.60 (1H) and 152.4 and 122.6 (13C), between 4.72 (1H) and 160.4 and 109.9 (13C), as well as between 5.50 (1H) and 160.4 and 57.7 (13C). Moreover, the fragment ion peaks in the mass spectrum provided more evidence for the final structure elucidation of compound 1, such as m/z 109.00 (15) [M-OH]+, 97.00 (100) [M-OH-C]+, and 81.00 (12) [M-OH-C-O]+. On the comprehensive spectral analysis, compound 1 was elucidated as a known furan derivative, 5-hydroxymethyl-2-furfural (5-HMF), as shown in Fig. 1A and IB.

Statistical Analysis

The data were expressed as the mean of three replicate determinations and standard deviation (SD), and statistical comparisons were made with the Student’s t-test. P values < 0.05 were considered to be significant.
Effects of 5-HMF on Free Radicals as Determined by ESR

First, the antioxidant activity of 5-HMF was tested using various free-radical species (DPPH, hydroxyl, alkyl, and superoxide anion) by the ESR spin trapping technique. The DPPH radical is a stable nitrogen-derived organic free radical, which can be reduced to a nonradical form (DPPH–H) by accepting an electron or hydrogen in the presence of a hydrogen-donating antioxidant. Because it can accommodate many samples in a short period and is sensitive enough to detect active ingredients at low concentrations, it has been extensively used for screening antiradical activities [1]. The DPPH scavenging activity of 5-HMF, presented in Fig. 2, was shown to be statistically significant at the various concentrations of 100 µM (75.6%), 25 µM (56.3%), 10 µM (35.6%), and 1 µM (10.4%). The IC$_{50}$ value of 27.1 µM compared with control ($p<0.05$) indicated that 5-HMF possessed potential capacity against DPPH radical species in a dose-dependent manner.

The capability of 5-HMF to inhibit hydroxyl radicals generated by the Fenton reaction was measured and is illustrated in Fig. 2. The hydroxyl radical (HO·) is one of the most reactive ROS, which can react rapidly with almost all biological molecules and may be involved in the pathology of many human diseases. The hydroxyl radicals generated by the Fenton system are identified by their ability to form nitrosoxide adducts from the commonly used DMPO spin trap. This spin trap has a greater ability of trapping oxygen-centered radicals than other nitro oxide spin traps [15]. The adduct DMPO–OH radical exhibits a characteristic ESR response that can be detected by an ESR spectrometer. 5-HMF showed scavenging activities of 76.4% (100 µM), 44.4% (25 µM), 28.4% (10 µM), and 9.4% (1 µM). The IC$_{50}$ value of 5-HMF was calculated as 22.8 µM compared with control without the tested sample.

The effect of 5-HMF on alkyl radicals was evaluated using the ESR technique. Alkyl radicals were first generated by the decomposition of AAPH, a water-soluble peroxyl radical initiator, and then trapped by 4-POBN, at 37°C for 30 min, and the decrease of ESR signals with the close increasing. The scavenging activity measured occurred in a dose-dependent manner for all tested samples with different concentrations, as shown in Fig. 2. The 5-HMF showed scavenging activities of 76.4% (100 µM), 44.4% (25 µM), 28.4% (10 µM), and 9.4% (1 µM). The IC$_{50}$ of 5-HMF value was calculated as 45.0 µM compared with control without the tested sample.

The scavenging activity of 5-HMF on the superoxide anion radical was measured by ESR spectrometry (Fig. 2). The superoxide anion radical is generated when oxidation reactions occur in the body; it is a weak oxidant, but it can transform to a powerful and dangerous hydroxyl radical in the presence of metals such as iron and copper, subsequently inducing cell damage and ROS-related diseases. In the present report, superoxide anion radicals were generated in a riboflavin–EDTA system by UV irradiation. With the various concentrations of 5-HMF tested, the results showed strong activity with an IC$_{50}$ value at 33.5 µM.

Free radicals produced from metabolism or environmental sources interact continuously in biological systems, and their uncontrolled generation correlates directly with the molecular level found in many diseases. To measure these
free radicals quantitatively and accurately, the ESR technique, which has been widely used as a powerful method for some typical radicals owing to its convenience, high sensitivity, and short time consumption, can measure radical levels that remain in reactions [24]. In this research, four kinds of typical free radicals were selected to assess the scavenging capacity of 5-HMF, including the nitrogen-derived radical DPPH, oxygen-derived hydroxyl and superoxide anion radicals, and carbon-derived alkyl radical. The results showed that 5-HMF had the strongest scavenging activity on the hydroxyl radical at IC\textsubscript{50} 22.8 µM, compared with the relatively weak alkyl radical with IC\textsubscript{50} 45.0 µM, as shown in Table 2. To our knowledge, this is the first time that the antioxidant activity of 5-HMF has been evaluated by the ESR method.

### Cell Viability of 5-HMF

To assess the antioxidant effects of 5-HMF in cellular systems at the molecular and cellular levels, the cytotoxic effects of 5-HMF were measured on human lung fibroblast (MRC-5), mouse macrophage (RAW264.7), and human promyelocytic leukemia (HL-60) cultured cell lines. The results showed that 5-HMF displayed no cytotoxic effects on these three tested cell lines, as shown in Fig. 3A, and no significant differences could be found among these cell lines. In addition, even at the highest concentration of 100 µM 5-HMF, only about 7% of the cells were decreased among these three cell lines. Thus, this provided scientific evidence that 5-HMF can be used safely and credibly in subsequent researches in cultured cell systems.

### Effect of 5-HMF on Intracellular ROS by DCFH–DA

To examine the effect of 5-HMF on intracellular production of ROS, RAW264.7 cells were pre-incubated with the 5-HMF at concentrations of 100 µM, 25 µM, 10 µM, and 1 µM for 1 h and then labeled with the fluorescence probe DCFH–DA, as described in the Materials and Methods section. When DCFH–DA enters viable cells, it can be deacetylated by intracellular esterases to form 2',7'-dichlorodihydrofluorescein (DCF), which can react quantitatively with ROS within the cell to produce 2',7'-dichlorofluorescein (DCF), which is fluorescent [16]. As shown in Fig. 3B, fluorescence emitted by DCF following ROS-mediated oxidation of DCFH followed a time-course increment up to 240 min. It was found that preincubation with 5-HMF decreased the DCF fluorescence in a dose- and time-dependent manner. Even after 30 min of incubation, 5-HMF exerted a considerable scavenging activity at the lowest concentration of 1 µM 5-HMF. At the highest concentration of 100 µM 5-HMF, 5-HMF could scavenge ROS significantly throughout the incubation time. After 210 min of incubation, 5-HMF reduced the production of ROS by 68.8% at 100 µM. The results revealed that the antioxidant activities of 5-HMF is caused by direct scavenging of cellular ROS, and therefore, 5-HMF may be developed as a potent antioxidant candidate to inhibit cellular ROS formation.

### Table 2. IC\textsubscript{50} values of 5-HMF against free radicals by the ESR technique.

<table>
<thead>
<tr>
<th>No.</th>
<th>Radical</th>
<th>IC\textsubscript{50} (µM±SD)\textsuperscript{a}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>DPPH</td>
<td>27.1±1.1</td>
</tr>
<tr>
<td>2</td>
<td>Hydroxyl</td>
<td>22.8±0.8</td>
</tr>
<tr>
<td>3</td>
<td>Alkyl</td>
<td>45.0±1.6</td>
</tr>
<tr>
<td>4</td>
<td>Superoxide</td>
<td>33.5±1.3</td>
</tr>
</tbody>
</table>

\textsuperscript{a}Each value is expressed as the mean±SD (n=3). The IC\textsubscript{50} value was defined as the necessary concentration at which the radicals generated by the reaction systems were scavenged by 50%. The IC\textsubscript{50} value was obtained by interpolation from linear regression analysis.
Effect of 5-HMF on Membrane Protein Oxidation

Study of the effect of 5-HMF on membrane protein oxidation was carried out using a cultured RAW264.7 cell line as described above. As shown in Fig. 4, when mouse macrophage membranes were exposed to HO· generated by the Fe^{2+} - H_{2}O_{2} Fenton reaction, the extent of membrane protein oxidation was increased, as monitored by the increase of carbonyl group contents. The contents of protein carbonyl groups in 5-HMF-treated groups were lower than those of the control group. Pretreatment with 5-HMF for 30 min dose-dependently inhibited the oxidation of membrane protein, indicated as the protein carbonyl group contents at 0.63 (100 µM), 0.96 (25 µM), 1.20 (10 µM), and 1.60 (1 µM) nmol/mg of protein. Membrane protein oxidation arising from intracellular ROS is considered as the main factor to induce many diseases, and subsequently results in the modification of amino acid side chains containing such functional groups as lysine, arginine, proline, and histidine. These then generate carbonyl moieties (mainly aldehydes and ketones) that have been identified as early markers for protein oxidation and used as a measure of protein damage. Oxidative damage of membranes also results in increased membrane fluidity, compromised integrity, and inactivation of membrane-bound receptors and enzymes [18].

Effect of 5-HMF on MPO

The effect of 5-HMF on MPO was measured in a cultured HL-60 cell line stimulated by TNF-α, as depicted in Fig. 5. 5-HMF could reduce MPO activity, compared with the TNF-α-stimulated control group. The activity of MPO was dose-dependently inhibited by pretreating with 5-HMF at the different concentrations, with inhibitory activities at 91.4% (100 µM), 78.4% (25 µM), 53.2% (10 µM), and 32.0% (1 µM). MPO is a leukocyte-derived heme peroxidase that has long been considered as a microbial enzyme centrally linked to the unspecific immune defense system. MPO plays an important role in oxidant production by polymorphonuclear neutrophils (PMNs). It uses hydrogen peroxide (H_{2}O_{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 [27]. Accordingly, the results could suggest that 5-HMF may act as a cellular antioxidant in an indirect way in the living cell system.

Effect of 5-HMF on GSH and SOD Expressions on the Gene Level

To examine whether 5-HMF can affect the expressions of GSH and SOD on the gene level, RAW264.7 cells were treated with the tested 5-HMF at different concentrations and the effective trend on cDNA was then measured by PCR, as illustrated in Fig. 6. The tested 5-HMF showed a clear dose-dependent activity to improve the expressions on both GSH and SOD enzymes. At the lowest concentration of 1 µM 5-HMF, an obvious band of cDNA could be observed compared with the nontreated blank group. The increased regulation of GSH and SOD gene expressions was observed in a concentration-dependent manner. Thus, the result demonstrated that the antioxidant effect of 5-HMF was again proven, on the gene level.

Free radicals and reactive oxygen species generated in cells are effectively scavenged by the antioxidant defense system, which consists of antioxidant enzymes such as GSH and SOD. When the activity of the antioxidant defense system decreases or the ROS production increases, oxidative...
stress may occur [11]. GSH is the most abundant low-molecular-weight thiol compound in cells and plays an important role in antioxidant defense and detoxification. GSH provides primary defense against oxidative stress by its ability to scavenge free radicals or participates in the reduction of \( \text{H}_2\text{O}_2 \) catalyzed by GSH peroxidase. A decrease in GSH can compromise the cell’s defenses against oxidative damage and lead to cell death [22]. Treatment of cells with a high concentration (100 \( \mu \text{M} \)) of 5-HMF resulted in a significant increase in GSH gene expression levels. Moreover, superoxide dismutase catalyzes the destruction of superoxide radicals and hence protects oxygen-metabolizing cells from the harmful effect of these free radicals. 5-HMF treatment could lead to decreased oxidative stress and thus could be part of the mechanism for the defensive effect of 5-HMF against oxidative stress.

5-HMF displayed its potential antioxidant character on the molecular, cellular, and gene levels based on the above research. Not only can it effectively scavenge free radicals (DPPH, hydroxyl, alkyl, and superoxide anion) and cellular ROS, but it also protects the cell membrane from oxidation stress, as well as inhibits the activity of the oxidant enzyme MPO. 5-HMF can also significantly increase the expressions of GSH and SOD, which are antioxidant enzymes that play important roles in defense against oxidative damage from reactive free radicals.

Herewith, the structure–activity relationship could be suggested as follows. In a view of the structure of 5-HMF, it contains some interesting functional reactive groups such as double bonds, an aldehyde oxygen atom, and another oxygen existing in the furan ring, which can attract electrons easily; furthermore, one hydroxyl group can also form a bare oxygen after donation of one hydrogen ion. These features should be the main factors for its antioxidant activity, by scavenging overproducing free radicals in the body and decreasing the activity of oxidant enzyme (MPO) or increasing the ability of antioxidant enzymes (GSH and SOD) in certain biological pathways.

In conclusion, Laurencia undulata, one of the most interesting marine algae in the genus Laurencia to produce potential candidates for application in the food and pharmaceutical industries as well as for clinical application, should be given more attention in future study. In addition, 5-HMF will be the next focus for scientists owing to not only its significant antioxidant properties described here, but also for its appearance in some traditional herbs as the main active component. However, its practical application is still a long stretch ahead since further research into its structural modification is still needed because of its unstable property.

Acknowledgment

This research was supported by a grant (M2007-01) from Marine Bioprocess Research Center of the Marine Biotechnology Program funded by the Ministry of Land, Transport and Maritime, Republic of Korea.

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