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Antioxidant activity of gallate-chitooligosaccharides in mouse macrophage RAW264.7 cells

Dai-Hung Ngo[a], Zhong-Ji Qian[b], Thanh-Sang Vo[a], BoMi Ryu[a], Dai-Nghiep Ngo[c], Se-Kwon Kim[a,d,*]

[a] Department of Chemistry, Pukyong National University, Busan 608-737, Republic of Korea
[b] Marine Life Research and Education Center, Chosun University, Gwangju 501-759, Republic of Korea
[c] Department of Biochemistry, Faculty of Biology, University of Science, VNU-HCM, Ho Chi Minh City, Viet Nam
[d] Marine Bioprocess Research Center, Pukyong National University, Busan 608-737, Republic of Korea

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ABSTRACT

In this research, a novel derivative of chitooligosaccharides (COS) was synthesized by covalently linking gallic acid and COS (gallate-COS) via carbodiimide to improve cellular antioxidant activity. The direct intracellular radical scavenging effect of gallate-COS by 2′,7′-dichlorofluorescin diacetate (DCFH-DA) method, and the inhibition of oxidation of cellular macromolecules, such as DNA, protein and lipid in mouse macrophages (RAW264.7 cells) were determined. Furthermore, with the treatment of gallate-COS, the expression levels of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione (GSH) were significantly increased according to reverse transcription polymerase chain reaction (RT-PCR) and Western blot analysis. In addition, gallate-COS decreased reactive oxygen species induced activation of the nuclear transcription factor (NF-κB). Collectively, these results suggest that gallate-COS can be used as a potential natural compound-based antioxidant in the functional food and pharmaceutical industries.

1. Introduction

Antioxidants may have a positive effect on human health since they can protect human body against deterioration by reactive oxygen species (ROS), which attack biomolecules such as proteins, lipids and DNA, lead to many health disorders including aging, cancer, diabetes, neurodegenerative, cardiovascular and inflammatory diseases. Recent studies have shown that nuclear factor-kappa B (NF-κB) is a redox-associated transcription factor and plays a key role in regulating the immune responses, cell survival and cell proliferation via its target genes. Cells possess enzymatic system such as superoxide dismutase (SOD) and glutathione (GSH) to scavenge radicals and protect oxidative damage to major cellular biomolecules (Butterfield et al., 2006; Dhalla, Temsah, & Netticadan, 2000; Seven, Guzel, Aslan, & Hamuryudan, 2008).

Many studies have been carried out to find novel antioxidative compounds from marine resources, such as chitin, chitosan and their derivatives. Chitosan, a partially deacetylated polymer of N-acetyl glucosamine, is prepared by alkaline deacetylation of chitin and chitooligosaccharides (COS) are partially hydrolyzed products of chitosan. COS has important biological properties in medicinal and pharmaceutical applications such as antioxidative (Park, Je, & Kim, 2003), anti-bacterial (Jeon & Kim, 2000), anticoagulant (Park, Je, Jung, & Kim, 2004), immuno-stimulant (Jeon & Kim, 2001), adipogenesis inhibitory (Cho et al., 2008) and anticancer (Shen, Chen, Chan, Jeng, & Wang, 2009) activities.

In recent years, phenolic compounds are of great interest in combining with COS to improve their biological properties. Therefore, the aim of this research is to develop a novel COS derivative by covalently linking gallic acid (3,4,5-trihydroxy benzoic acid) and COS to improve the antioxidant activity of COS due to high antioxidative property of gallic acid (Cho, Kim, Ahn, & Je, 2011; Pasanphan, Buettner, & Chirachanchai, 2010; Pasanphan & Chirachanchai, 2008).

2. Materials and methods

2.1. Materials

COS (molecular weight 3.0–5.0 kDa) was kindly provided by Kitto Life Co. (Seoul, Republic of Korea). Gallic acid was purchased from the Acros Organics (New Jersey, USA). All the chemicals required for synthesis were obtained from the Sigma Chemical Co. (St. Louis, MO, USA). Mouse macrophages (RAW264.7) cell line was obtained from American Type Culture Collection (Manassas, VA, USA). Dulbecco's Modified Eagle Medium (DMEM),

* Corresponding author at: Marine Bioprocess Research Center, Pukyong National University, Busan 608-737, Republic of Korea. Tel.: +82 51 629 7094; fax: +82 51 629 7099.
E-mail addresses: sknkim@pknu.ac.kr, sknkim@mail.pknu.ac.kr (S.-K. Kim).

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penicillin/streptomycin, and the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (Grand Island, NY, USA).

Dimethyl sulfoxide (DMSO), 3-(4,5-dimethyl-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), $FeSO_4$, H_2O_2, ethylenediaminetetraacetic acid (EDTA), 2,4-dinitrophenyl hydrazine, diphenyl-1-pyrenylphosphine (DPPP), thiobarbituric acid reactive substances (TBARS), guanidine hydrochloride, agarose and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Primary and secondary antibodies used for Western blot analysis were purchased from Santa Cruz Biotechnology Inc., CA, USA. All other reagents were of the highest grade available commercially.

2.2. Synthesis of gallate-COS

COS (2.4814 g) was dissolved in distilled water (20 ml), methanol (40 ml) and then adjusted to pH 6.8 with triethylamine to obtain a solution A. Gallic acid (0.9404 g) was dissolved in methanol (10 ml) and dicyclohexylcarbodiimide (DCC, 1.0315 g dissolved in 10 ml methanol) reacted with gallic acid to obtain a solution B. The solution B was gradually added to the solution A and stirred in a water bath at 30 °C, 150 rpm for 5 h and then filtered. The solution obtained was kept at 2 °C overnight and thereafter added diethyl ether (90 ml), filtered to obtain a precipitate. The precipitate was dissolved in 20 ml distilled water and then freeze dried to obtain gallate-COS.

2.3. Cell cytotoxicity, membrane lipid, protein, and DNA oxidation determination

RAW264.7 cells were grown in DMEM medium containing 5% (v/v) FBS, 100 μg/ml penicillin-streptomycin and 5% CO_2 at 37 °C. Cytotoxicity levels of samples on cells were measured using the MTT method as described by Hansen, Nielsen, and Berg (1989).

RAW264.7 cells were analyzed for the generation of lipid peroxidation products by modification of the TBARS and DPPP methods (Hino, Morita, Une, Fujimura, & Kuramoto, 2001; Takahashi, Shibata, & Niki, 2001).

The degree of oxidation of the cell membrane proteins was assessed by determining the content of protein by carbonyl groups (Rajapakse, Kim, Mendis, & Kim, 2007).

Genomic DNA was extracted from RAW264.7 cells using standard phenol/proteinase K procedure with slight modifications. Hydrogen peroxide mediated DNA oxidation was determined according to Sambrook and Russell (2001).

2.4. Cellular ROS determination by DCFH-DA

Intracellular formation of ROS was assessed according to a method described by employing oxidation sensitive dye DCFH-DA, as the substrate (Engelmann, Volk, Leyhausen, & Geurtsen, 2005). RAW264.7 cells were grown in a black microtiter 96-well plates and were labeled with 20 μM DCFH-DA in Hanks balanced salt solution (HBSS) and kept for 20 min in the dark. The non-fluorescent DCFH-DA dye which easily penetrates into cells was then hydrolyzed by intracellular esterase to 2′,7′-dichlorodihydrofluorescein (DCFH), and trapped inside the cells. Cells were then treated with different concentrations of test samples and incubated for 1 h. After washing cells for three times with PBS, 500 μM H_2O_2 (in HBSS) were added. The formation of fluorescent dichlorofluorescein (DCF) due to oxidation of DCFH in the presence of several ROS was read after every 30 min at the excitation wavelength of 485 nm and the emission wavelength of 528 nm using a GENios® fluorescence microplate reader (Tecan Austria GmbH, Grodig/Salzburg, Austria). Dose-dependent and time-dependent effects of treatments were plotted and compared with fluorescence intensity of the control group in which samples were not treated.

2.5. RNA isolation and reverse transcription polymerase chain reaction (RT-PCR) analysis

Total RNA was extracted from RAW264.7 cells after treatment with test samples at different concentrations. For that, cells were lyzed with 1 ml TRIzol® reagent (Invitrogen Corporation, Faraday Avenue, USA) according to the manufacturer's protocol. Changes in the steady-state concentration of antioxidant enzyme expression were assessed by RT-PCR. Briefly, total RNA (2 μg) was converted to cDNA using a Reverse transcription System (Promega, Madison, WI, USA). The target cDNA was amplified using the following primer: for SOD, sense 5′-AGGGCATCATCAATTTCGAG-3′ and antisense 5′-TGC-CTC-TCT-TCA-TCC-TTT-GG-3′; for GSH, sense 5′-AGC-ATT-TGG-CAA-AGG-AGA-AA-3′ and antisense 5′-ATC-CGT-GCT-CCG-ACA-AAT-AG-3′; glyceraldehydes 3-phosphate dehydrogenase (GAPDH), sense 5′-GCC-ACC-CAG-AAG-ACT-GTG-GAT-3′ and antisense 5′-TGG-TCC-AGG-GTT-TCT-TAC-TCC-3′. The amplification cycles were carried out at 95 °C for 45 s, 60 °C for 1 min, and 72 °C for 45 s. After 27 cycles, the PCR products were separated by electrophoresis on 1% agarose gel for 20 min at 100 V. Gels were then stained with 1 mg/ml ethidium bromide and observed by UV light using AlphaEase® gel image analysis software (Alpha Innotech, CA, USA).

2.6. Western blot analysis

For separate extraction of nuclear and cytoplasm proteins, CelLytic™ NuCLEAR™ Extraction kit (S26-36-23, Sigma–Aldrich Co., MO, USA) was used following manufacturer's instructions. Protein concentrations in the supernatants were determined with the Bio-Rad protein assay using bovine serum albumin as the standard. Proteins (20 μg) were diluted in 5× sample buffer (10% SDS and 100 mM each DTT, glycerol, bromophenol blue, and Tris–HCl), and resolved in 4–20% Novex gradient gel (Invitrogen, USA), electrotransferred onto a nitrocellulose membrane. Then proteins were transferred onto nitrocellulose membranes, and the blots were blocked with 5% (w/v) bovine serum albumin in Tris–buffered saline and 0.1% Tween 20 (TBST) for at least 1 h at room temperature. Membranes were incubated for 1 h at room temperature with primary antibodies (1:1000) of NF-κB p50, NF-κB p65, SOD and GSH. After washing with TBST, the blots were incubated with the corresponding peroxidase-conjugated secondary antibody (1:5000 dilutions) for 1 h at room temperature. They were then washed again three times with TBST, and developed with enhanced chemiluminescence reagents (ECL, Amersham Biosciences, UK). Western blot bands were visualized using LAS3000® Luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan). Detection of actin (1:5000 antibody dilutions) was used as control for equal loading of protein.

2.7. Statistical analysis

All statistical analysis was performed with independent experiments and data were represented as mean ± standard deviation (SD). The statistical significance was achieved when $p < 0.05$.

3. Results and discussion

3.1. Characterization and antioxidant properties of gallate-COS

According to the Fourier transformed infrared (FT-IR) spectra, gallate-COS show significant bands in 1520 and 1955 cm^{-1}, imply-

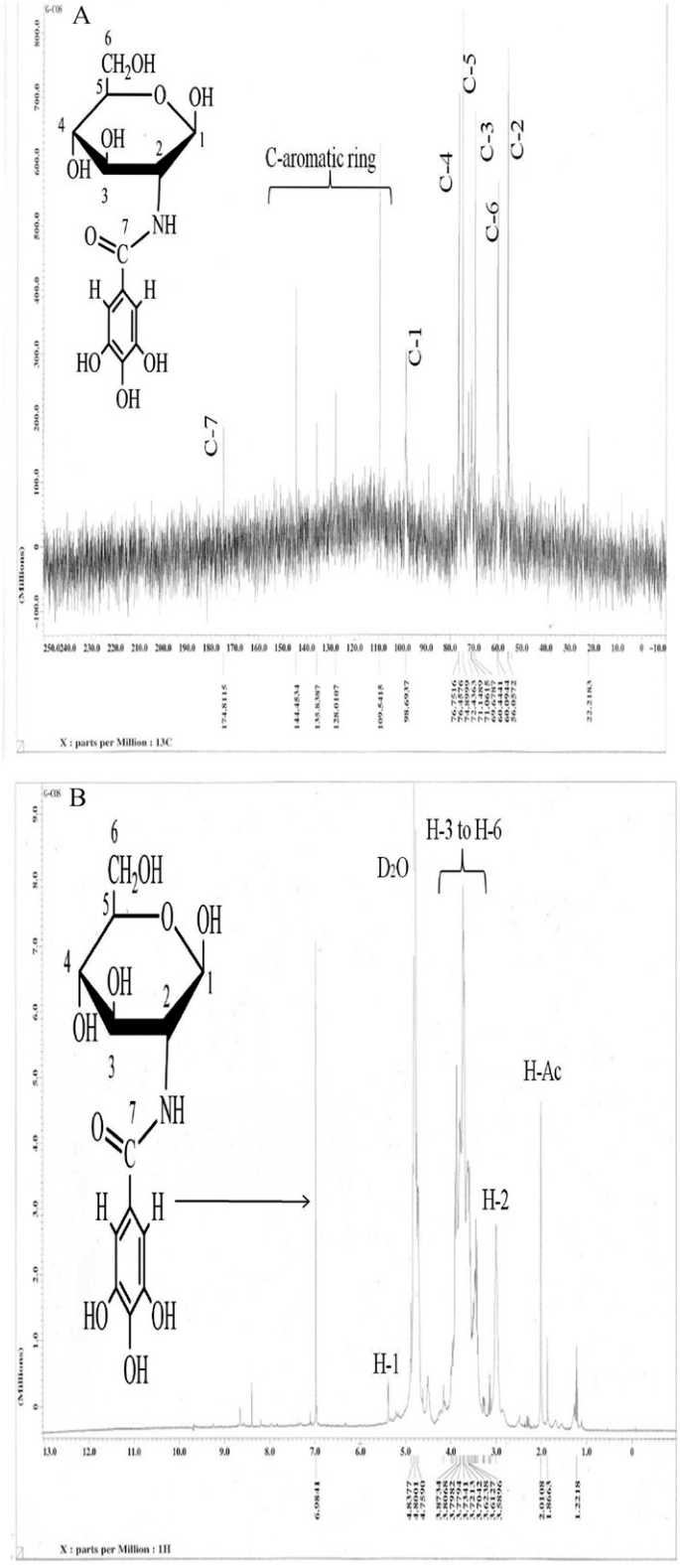

Fig. 1. ^{13}C NMR (100 MHz, D_2O) spectrum of gallate-COS (A) and ^{1}H NMR (400 MHz, D_2O) spectrum of gallate-COS (B).

ing that the amide and ester linkages between gallic acid and COS (data not shown). This shows that the gallyl group of gallic acid was covalently linked with COS via amide and ester linkages. Moreover, ^{13}carbon (^{13}C) NMR spectra, gallate-COS shows the aromatic carbon of the gallyl group at 109.54, 128.01, 135.84, and 144.45 ppm

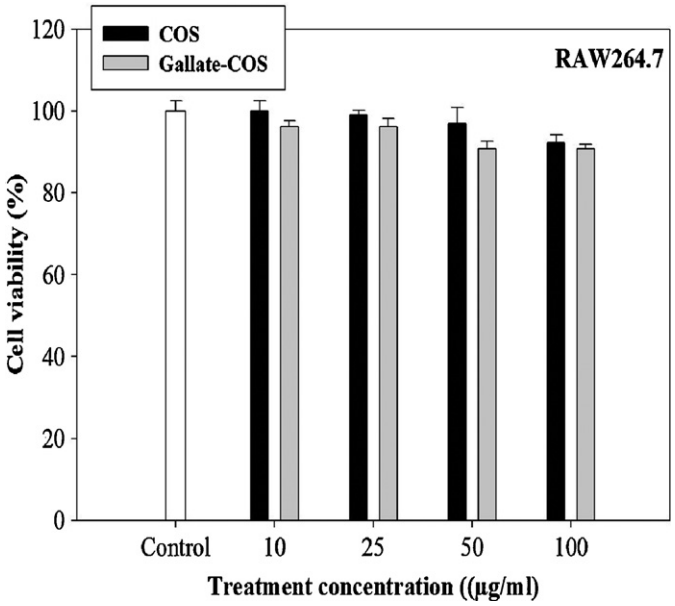

Fig. 2. Cell viability assessed by the MTT assay. The results shown are representative of separate experiments performed in triplicate. Error bars represent the standard error (SD).

(C=C) and 174.8 ppm (C=O) (Fig. 1(A)). From the proton nuclear magnetic resonance (^{1}H NMR) spectra, gallate-COS show a new peak at 6.98 ppm belonging to the phenyl protons as compared with COS (Fig. 1(B)). These results confirmed the successful link gallic acid and COS.

In this research, RAW264.7 cells were treated with varying concentrations of COS and gallate-COS in order to determine the cytotoxic effect of COS and gallate-COS by the MTT assay. The results showed that both COS and gallate-COS exert no cytotoxic effects on RAW264.7 cells at all tested concentrations (Fig. 2). Therefore, those concentrations of COS and gallate-COS were used for further experiments.

The most frequently used method to study membrane lipid peroxidation is the TBARS method. As described in the materials and methods, membrane lipid peroxidation was caused by hydroxyl radicals generated from the Fenton reaction. The results indicated that gallate-COS could decrease TBARS in the cells about 80% at the highest concentration (100 μg/ml) compared with oxidative stress induced control group (Fig. 3(A)). Moreover, gallate-COS had higher antioxidant activity compared to COS at all tested concentrations. The results of this experiment showed that gallate-COS could inhibit cellular lipid peroxidation against scavenging ROS because of the improvement of proton transfer by the gallyl group.

In addition, a sensitive fluorescence probe (DPPP) was used to assess the lipid hydroperoxide level of RAW264.7 cells generated by strong carbon-centered radical generating agent (AAPH). DPPP molecules are diffused into the cellular membranes and DPPP-oxide fluorescence was generated by oxidation products of lipids (hydroperoxides) react to DPPP. DPPP-oxide is fluorescent with high fluorescence although DPPP itself is not fluorescent. The DPPP-oxide fluorescent intensity increased more than threefold due to carbon-centered radical-mediated membrane lipid peroxidation after 6 h of incubation with AAPH (Fig. 3(B)). The results indicated that the DPPP-oxide fluorescence intensity was decreased with the treatment of COS and gallate-COS. A low reduction in fluorescence intensity was studied in the presence of COS about 50% at 100 μg/ml. In contrast, gallate-COS could inhibit membrane lipid peroxidation around 62% at 100 μg/ml. and in a dose-dependent manner.

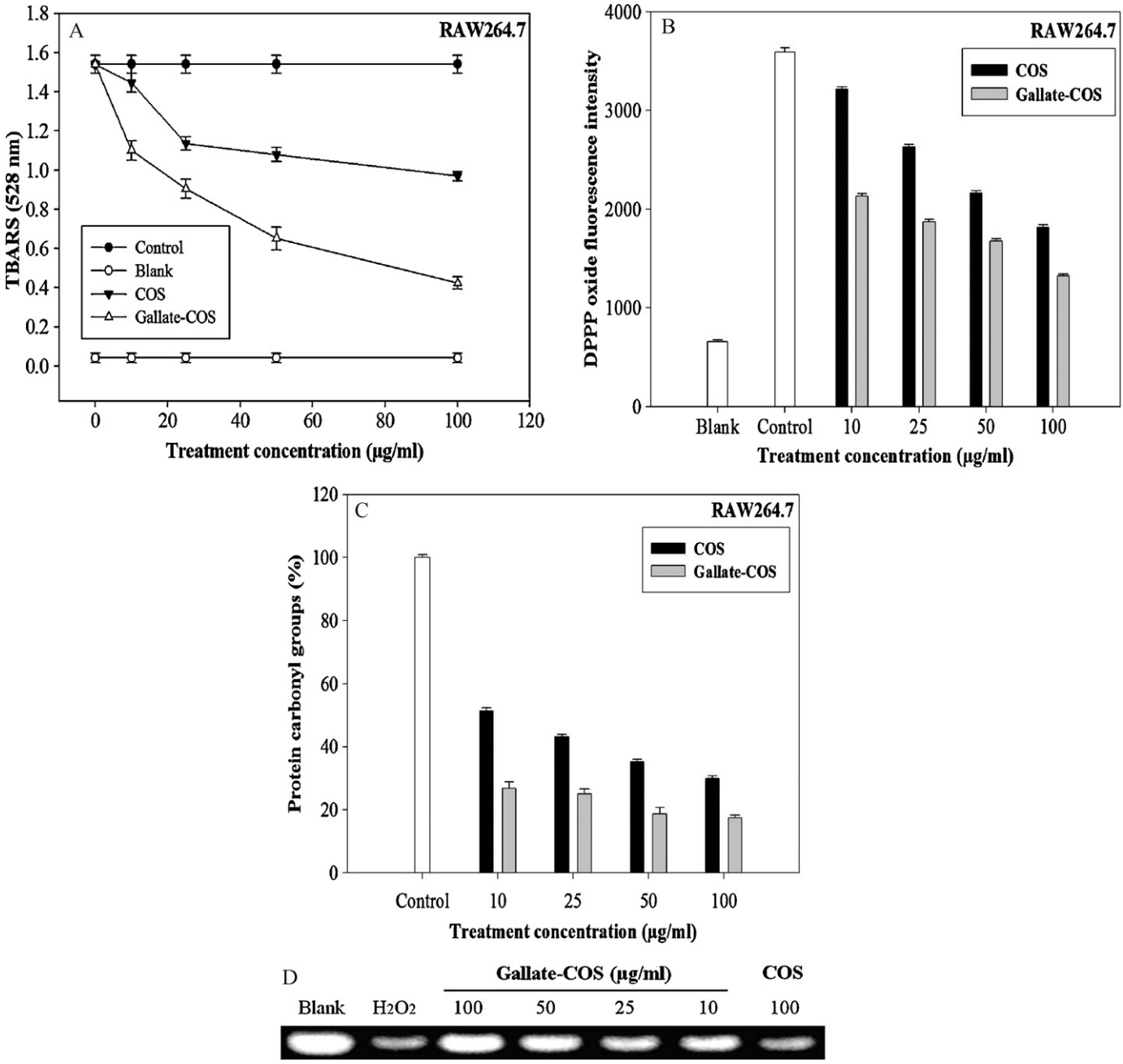

Fig. 3. Membrane lipid peroxidation protection by TBARS method (A), membrane lipid peroxidation protection by DPPP fluorescence method (B), membrane protein oxidative protection (C) and DNA oxidative protection (D) by COS and gallate-COS in RAW264.7 cells. Results are mean ± SD of three independent experiments.

The degree of oxidation of cellular membrane proteins was determined by the content of carbonyl groups; those have been identified as oxidized protein markers and involved in many different kinds of diseases (Mendis, Kim, Rajapakse, & Kim, 2008). When RAW264.7 cells were exposed to hydroxyl radicals, the formation of carbonyl groups was increased as shown in Fig. 3(C). The carbonyl groups formation was clearly suppressed compared to oxidative stress induced control group when the cells were treated with varying concentrations of COS and gallate-COS. The results show that gallate-COS inhibited oxidation of membrane proteins that was significantly higher than that of COS at all tested concentrations. Furthermore, gallate-COS prevented about 83% of membrane protein oxidation at 100 μg/ml concentration in RAW264.7 cells.

The highly reactive hydroxyl radicals (*OH) react with all components of the DNA molecule, including bases modification (purine and pyrimidine) and also deoxyribose units and damage DNA. This lesion increases with increment of the free radical attacked on cellular DNA, which concerned in cancer, mutagenesis and aging (Hazra et al., 2007; Madia, Gattazzo, Fabrizio, & Longo, 2007; Mello, Hernandez, Marrazza, Mascini, & Kubot, 2006). In this study, oxidation of genomic DNA isolated from RAW264.7 cells was determined by combining effect of 2 mM H_2O_2 and 200 μM Fe(II) on the integrity of DNA. The protective effect of COS and gallate-COS against DNA oxidative damage was assessed by DNA electrophoresis. After 10 min of reaction, almost all DNA was degraded in the control group treated with H_2O_2–Fe(II) alone (Fig. 3(D)). However, gallate-COS showed an inhibitory effect on

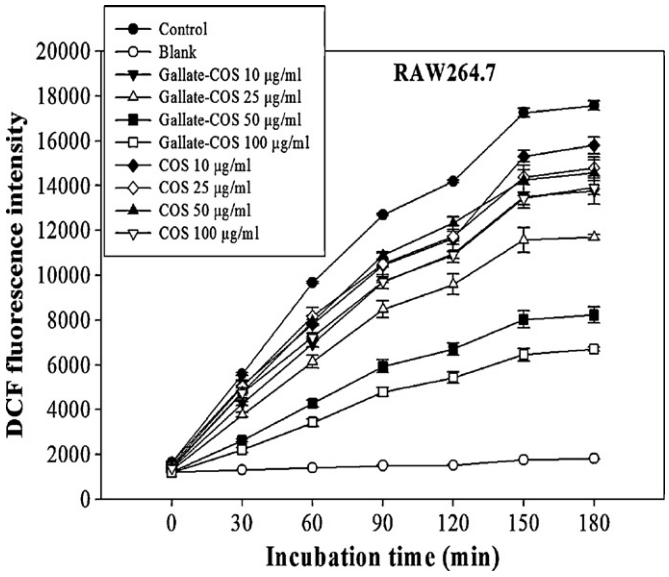

Fig. 4. Intracellular radical scavenging activity of COS and gallate-COS in RAW264.7 cells. The results shown are representative of separate experiments performed in triplicate. Error bars represent the SD.

radical-mediated oxidative DNA damage by dose-dependently. At 100 μg/ml of gallate-COS, DNA damage was inhibited more than 90% determined based on the intensity of DNA bands. In contrast, at 100 μg/ml of COS, DNA damage was inhibited only about 20%.

The cellular direct free radical scavenging effect of COS and gallate-COS were employed on RAW264.7 cells since these cells are able to produce high amount of ROS following stimulation (Arato et al., 2006). For that, the cells were labeled with DCFH-DA as described in the materials and methods section. During labeling, non-fluorescent DCFH-DA dye that easily diffuses through cell membrane gets hydrolyzed by esterase to become DCFH, and this compound is trapped inside the cells. Fluorescence emitted by DCF following H_2O_2-mediated oxidation of DCFH. The monitoring of DCF fluorescence intensities in every 30 min for 3 h duration that radical-mediated oxidation increased with the incubation time. However, the pre-treatment with COS and gallate-COS reduced the DCF fluorescence dose- and time-dependently (Fig. 4). Therefore, we could confirm that gallate-COS scavenged free radicals and inhibited radical-mediated oxidation in RAW264.7 cells.

3.2. Effect of gallate-COS on antioxidant enzymes

Cells possess enzymatic system such as SOD and GSH as a part of the cellular defense system to maintain the balance between oxidants and antioxidants. SOD which reduces the damaging reactions of superoxide is one of the major antioxidant enzymes. Thus, it protects cells from the toxicity of superoxide radicals. GSH plays an important role in regulating the intracellular redox status. The increase in GSH level protects cells against cell death either by removing free radicals or by conjugating with toxicants (Kong et al., 2009). The antioxidant property of gallate-COS was determined through the activity of antioxidant enzymes, the mRNA and protein expression levels of antioxidant enzymes (SOD and GSH) were studied by RT-PCR and Western blot analysis in RAW264.7 cells. As shown in Fig. 5(A) and (B), all the mRNA and protein expression levels of antioxidant enzymes were up-regulated in the presence of gallate-COS by dose-dependently. The results showed that gallate-COS could increase the expression levels of

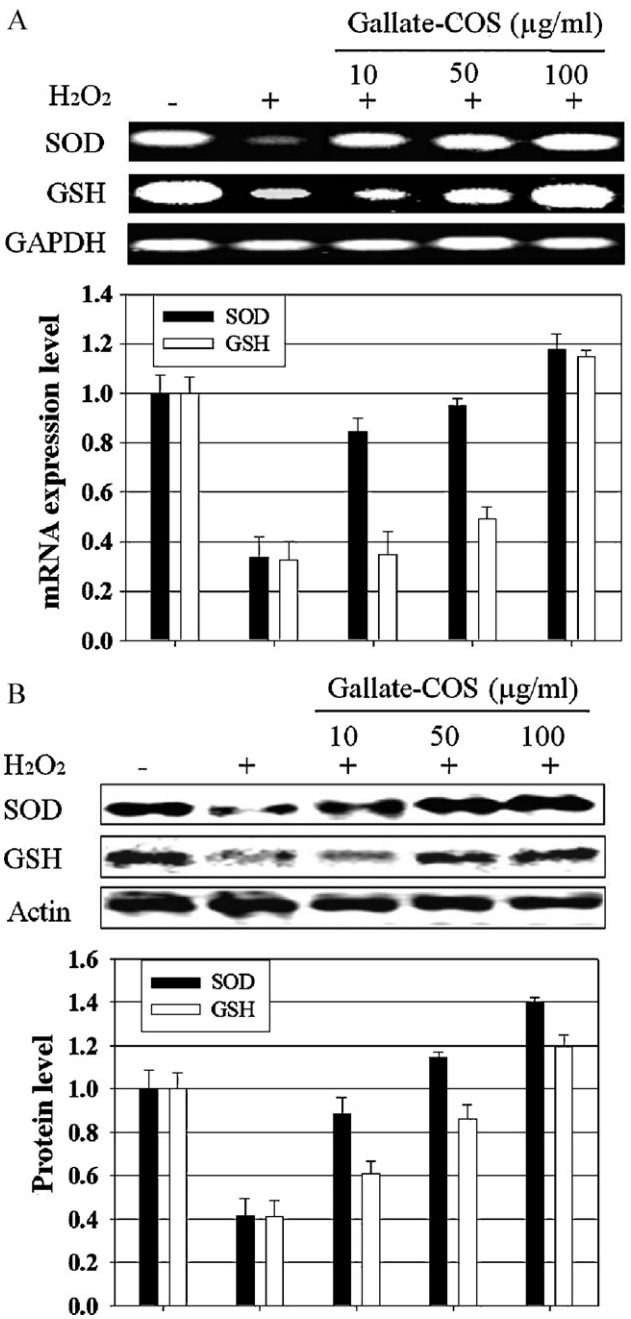

Fig. 5. Effect of gallate-COS on H_2O_2-induced antioxidant enzymes in RAW264.7 cells. The expression levels of mRNA and protein were determined using RT-PCR (A) and Western blot (B) analysis, respectively.

antioxidant enzymes in RAW264.7 cells through scavenging free radicals.

3.3. Expression and nuclear translocation of NF-κB in the presence of gallate-COS

One of the major signal transduction pathways that activates in response to oxidative stress is NF-κB which plays an important role in regulating the immune and inflammatory responses via its target genes. NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli stress, cytokines, free radicals, ultraviolet irradiation and oxidized low-density lipoprotein (Gilmore, 2006; Perkins, 2007). The results showed that gallate-

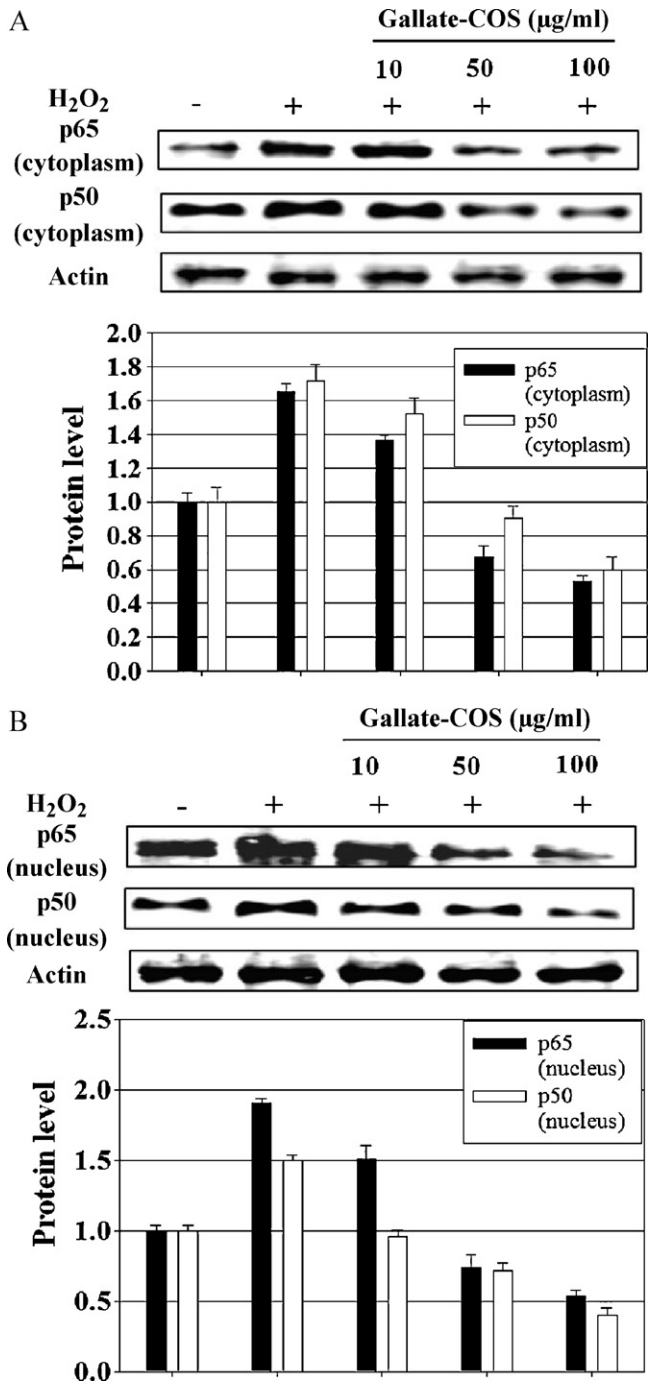

Fig. 6. (A and B) Western blot analysis of p65 and p50 protein expressions in RAW264.7 cells stimulated with H_2O_2.

COS are capable of decreasing activation of NF-κB especially in an environment which induced by oxidative stress. A clear increase of NF-κB (p50 and p65) protein levels was produced by stimulation with H_2O_2 in RAW264.7 cells. Both p50 and p65 protein expression levels were dose-dependently reduced in plasma proteins by gallate-COS treatment compared to H_2O_2 alone-treated group (Fig. 6(A)). Furthermore, when nuclear extracts were performed for both p50 and p65 levels, gallate-COS showed a significant decrease of NF-κB translocation from cytoplasm to nucleus (Fig. 6(B)). These data indicated that gallate-COS can inhibit the activation of NF-κB and nucleus translocation in RAW264.7 cells with the stimulation of oxidative stress.

4. Conclusion

This study has demonstrated a novel antioxidant, obtained by covalently linking gallic acid and amino groups of COS. The chemical structure of gallate-COS has been identified by FT-IR, ^{1}H NMR and ^{13}C NMR. Based on the cytotoxicity assay, COS and gallate-COS were found to be non-toxic and could scavenge cellular radicals in RAW264.7 cells. Furthermore, COS and gallate-COS could inhibit oxidative damage to lipids, proteins and DNA in RAW264.7 cells. In addition, gallate-COS could decrease the activation and expression of NF-κB and increase the level of intracellular antioxidant enzymes (SOD and GSH) in oxidative stress induced RAW264.7 cells. Collectively, gallate-COS could be used as a scavenger to control free radicals that lead to damage to cellular system.

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