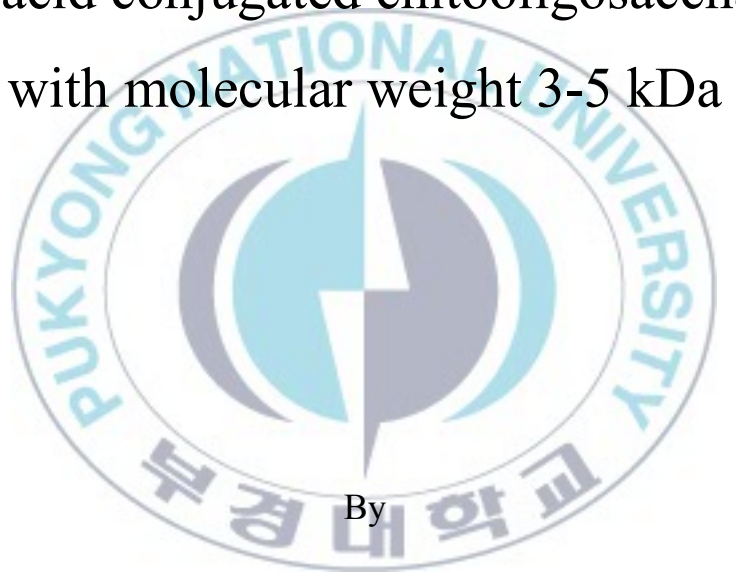
Thesis for the Degree of Master of Science

Inhibition of radical-mediated oxidation of cellular biomolecules and NF-κB expression by gallic acid conjugated chitooligosaccharides with molecular weight 3-5 kDa



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August 2010

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Gallic acid 키토산올리고당(3-5 kDa)의 세포 내 라디칼 저해 및 NF-κB 발현 억제 효과

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by

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Abstract

The aim of this study was to explain the novel possibility of the involvement of gallic acid conjugated chitooligosaccharides with molecular weight 3-5 kDa (G-COS) in improving cellular antioxidant potential and thereby controlling oxidative damage that could be effective for its therapeutic potential in osteoarthritis. The free radicals scavenging G-COS ability was assessed by using electron spin resonance (ESR) technique. In addition, the inhibition of reactive oxygen species (ROS), membrane lipid peroxidation, membrane protein oxidation and DNA oxidation were assessed on mouse macrophages (RAW264.7 cells) and human chondrosarcoma (SW1353 cells). Moreover, the antioxidative mechanisms of G-COS were evaluated by protein expression and gene expression levels of antioxidant enzymes such as superoxide dismutase (SOD) and glutathione (GSH). More importantly, nuclear factor-kappa B (NF-κB) western blot analysis revealed that G-COS inhibits radical mediated expression and activation of NF-κB proteins (transcription factor in the regulation of the expression of a number of genes related to osteoarthritis). This study demonstrated that G-COS can be used as a potential compound-based natural antioxidant in functional foods and pharmaceutical industries.

Key words: Chitooligosaccharides; gallic acid; antioxidant; radical scavenging; mouse macrophages (RAW 264.7), and human chondrosarcoma (SW1353)

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List of Abbreviations

^{13}C NMR	^{13}Carbon Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
AAPH	2,2'-azobis(2-amidinopropane) dihydrochloride
BHA	Butylated hydroxyanisole
BHT	Butylated hydroxytoluene
cDNA	Combinant deoxyribonucleic acid
COS	Chitooligosaccarides
DCC	Dicyclohexylcarbodiimide
DCF	Dichlorofluorescein
DCFH	2′,7′-dichlorodihydrofluorescein
DCFH-DA	2′,7′-dichlorofluorescin diacetate
DMEM	Dulbecco's Modified Eagle's Medium
DMEM-F12	Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12
DMPO	5,5-dimethyl-1-pyrroline N-oxide
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
DPPH	1,1-diphenyl-2-picrylhydrazyl
DPPP	Diphenyl-1-pyrenylphosphine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESR	Electron spin resonance
FBS	Fetal Bovine Serum
FT-IR	Fourier transform infrared

GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-COS	Chitooligosaccharides conjugated gallic acid
GSH	Glutathione
HBSS	Hanks balanced salt solution
IC_{50}	50% Inhibitory concentration
IR	Infrared
mRNA	Messenger Ribonucleic Acid
MTT	3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-kappaB
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POBN	α-(4-pyridyl-1-oxide)-N-tert-butylnitrone
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RT-PCR	Reverse transcription-polymerase chain reaction
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
SOD	Superoxide dismutase
TBARS	Thiobarbituric acid reactive substances
TBST	Tris–buffered saline Teen

Introduction

1. Antioxidant enzymes

It is well known that free radicals play an important role in metabolic and chemical changes in biological systems, which may result in disease, food deterioration, and aging (Halliwell & Gutteridge, 1989). Antioxidants reduce or retard free radical generation and prevent the oxidation of cellular oxidizable substrates. Antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are representative; however, they are synthetic compounds and have to be used under strict regulations (Bran, 1975; Whysner et al., 1994). Vitamins such as vitamin C and vitamin E are well known for the major tissue antioxidants in biological systems; however, we have to be aware of their instability and some adverse effects as prooxidants (Mayo et al., 2003). Recently, much attention has been paid by health professionals and consumers towards natural antioxidants and are searching for alternative ones; especially, those fcf plant extracts, for example, green tea (Nakagawa & Yokozawa, 2002), rosemary (Sebranek et al., 2005), olive oil (Beltran et al., 2005), and marine organisms (Ngo et al., 2010).

To maintain a balance between oxidant and antioxidant, cells possess enzymatic system including superoxide dismutase (SOD), glutathione peroxidase, catalase, glutathione reductase as a part of the cellular defense system. SOD is one of the major antioxidant enzymes and contains redox metals in the catalytic center and catalyzes conversion of superoxide to hydrogen peroxide by up-regulating transcriptional activity. Three different isoforms of SOD have been reported: the cytosolic copper/zinc-containing SOD (SOD-1), the mitochondrial manganese-containing SOD (SOD-2) and the extracellular SOD (SOD-3). The SOD expression level is closely related to oxidative stress.

Glutathione (GSH) is a key component for protecting all cells from oxidative stress and plays a vital role in regulating intracellular redox status. GSH is a very important intracellular antioxidant that directly reacts with reactive oxygen species. GSH also functions as a cofactor of glutathione peroxidase and maintains thiol redox potential in cells for protecting all cells from oxidative stress at all times (Kong et al., 2009).

2. Chitin, chitosan and applications

Many biological compounds including carbohydrates, peptides and some phenolic compounds have been identified as potent radical scavengers. Recently, the antioxidant activity of chitosan and its derivatives attracted a great deal of attention (Chiang et al., 2000).

Chitin-chitosan is the second most naturally abundant copolysaccharide of β-(1-4)-2-acetamido-2-deoxy-β-d-glucose and β-(1-4)-2-amino-2-deoxy-β-d-glucose, next to cellulose, and exists in the shells of crustaceans, insects, and fungi. Polysaccharides, such as sulfated seaweed-based polysaccharide, have been reported as antioxidants. Sulfated polysaccharide from *Porphyra haitanesis* (Zhang et al., 2003), fucoidan from *Fucus vesiculosus*, and fucans from *Padina gymnospora* (Souza et al., 2007) shows the scavenging effect on superoxide radical anion, hydroxyl radical, and lipid peroxide. Similar to the polysaccharide from seaweeds, chitosan has also been reported for its antioxidant ability, in addition to its antibacterial (Kendra & Hadwiger, 1984) and antimutagenetic (Kogan et al., 2004) properties.

Chitosan, a partially deacetylated polymer of N-acetylglucosamine, is prepared by alkaline deacetylation of chitin (Kim et al., 2006). Chitooligosaccharides (COS) are chitosan derivatives (polycationic polymers comprised principally of glucosamine units) and can be generated via either chemical or enzymatic hydrolysis of chitosan (Dou et al., 2007; Jeon & Kim, 2000a,b). Recently, COS have been the subject of increased attention in terms of their pharmaceutical and medicinal applications (Kim & Rajapakse, 2005), due to their missing toxicity and high solubility as well as their positive physiological effects such as ACE enzyme inhibition (Hong et al., 1998), antioxidant (Park et al., 2003) antimicrobial (Park et al., 2004), anticancer (Jeon & Kim, 2002; Shen et al., 2009), immuno-stimulant (Jeon & Kim, 2001), antidiabetic (Liu et al., 2007), hypocholesterolemic (Kim et al., 2005), hypoglycemic (Miura et al., 1995), anti-Alzheimer's (Yoon et al., 2009), anticoagulant (Park et al., 2004) properties and adipogenesis inhibition (Cho et al., 2008).

Chitin and chitosan were applied in broad range of a lot of fields such as agriculture, water-treatment, waste-treatment and food processing. Furthermore, their new

applications are concentrated on producing higher and higher valuable products by means of cosmetics, drug carriers, gene carriers food additives, semi-permeable membranes, and biopharmaceuticals. The applications of chitin and chitosan are summarized in Table 1 (Jeon et al., 2000; Kim et al., 2006; Rinaudo, 2006).

Further, it has been observed that the radical scavenging properties of COS are dependent on their degree of deacetylation and molecular weights. Based on the results obtained from studies carried out using electron spin-trapping techniques, COS with low molecular weight range (1-3 kDa) have been identified to have a higher potential to scavenge different radicals (Park et al., 2003). In contrast, another study reported that low molecular weight chitosans could exhibit more than 80% of superoxide radical anion scavenging activity at 0.5 mg/ml concentration (Yin et al., 2002). In addition, highly deacetylated (90%) COS are more preferable to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide and carbon-centered radicals (Je et al, 2004). Even though the precise mechanism of radical scavenging activity of COS is not clear, it is attributed that amino and hydroxyl groups attached to C-2, C-3 and C-6 positions of the pyranose ring react with unstable free radicals to form stable macromolecule radicals.

Chitosan sulfate and low molecular weight chitosan sulfate were found to have radical scavenging activity on the superoxide and hydroxyl radicals (Xing et al., 2005a,b). Huang et al (2005) proposed hydroxyethyl chitosan sulfate as a radical scavenger as evidenced from the model studies of DPPH, hydroxyl, and carbon-centered free radicals. Xie et al (2001) reported that maleic acid grafted hydroxypropyl chitosan or carboxymethyl chitosan showed potent radical scavenging activity against hydroxyl radicals. It is important to note that for those cases, the approaches are the functionalization with the hydrophilic terminal groups, such as sulfate (Xing et al., 2005a,b), carboxymethyl, hydroxylpropyl (Xie et al., 2001).

As chitosan exhibits strong metal ion chelating ability (Burke et al., 2000) owing to the nitrogen atom, chitosan is a potential preventive antioxidant based on its metal ion deactivation. However, chitosan has some limitations in being a practical antioxidant; especially, its poor solubility due to its inter- and intramolecular hydrogen bond network, and the lack of H-atom donor to serve as a good chain breaking antioxidant. As a result, we need to improve the solubility of chitosan in neutral aqueous solution as well as

introduce the H-atom donor group if we are to consider the development of a chitosan-based antioxidant.

3. Gallic acid

Gallic acid (G, 3,4,5-trihydroxy benzoic acid) (Figure 1) is a natural phenolic antioxidant extractable from plants, especially green tea (Lu et al., 2006). It is widely used in foods, drugs, and cosmetics to prevent rancidity induced by lipid peroxidation and spoilage. From the structural viewpoint, gallic acid is attractive for conjugating onto chitooligosaccharides for a novel green antioxidant because of the high reducing potential and low O-H-bond dissociation enthalpy of the tri-hydroxyl groups on the benzene ring (Ji et al., 2006); the possibility that the bulky group of the benzene ring of gallic acid obstructs the inter- and intramolecular hydrogen bond network of chitooligosaccharides; the multi-functional hydrophilicity based on the hydroxyl and carboxyl groups; the carboxylic acid group for conjugation with chitooligosaccharides; and it being a natural product. It can be expected that gallic acid conjugated chitooligosaccharides may show antioxidant activity, together with the metal chelating ability (Burke et al., 2000; Burke et al., 2002; Guzman et al., 2003; Ngah et al., 2005), biodegradability (Yamamoto & Amaike, 1997), biocompatibility (Richardson et al., 1999), and bioactivity (Dumitriu et al., 1989).

4. Reactive oxygen species (ROS)

The generation of highly reactive oxygen species is an integral feature of normal metabolism and these molecules play an important role in the modulation of inflammatory reactions. Major ROS produced within the cell are superoxide, hydrogen peroxide and hydroxyl radicals (Salvemini et al., 2003). Extracellular release of large amounts of superoxide radical, produced as respiratory burst in leukocytes, is an important mechanism of pathogen killing and also leads to endothelial damage resulting in an increased vascular permeability as well as cell death (Tiidus, 1998). However, recent evidences have implicated that intracellular ROS production plays a key role in

modulation of release of other inflammatory mediators. This is related mainly with the constitutive expression of NAD(P)H oxidases in various tissues (Guzik et al., 2002). ROS produced by this family of enzymes can regulate adhesion molecule expression on endothelium and inflammatory cells, thus affecting cell recruitment to the sites of inflammation (Fraticelli et al, 1996). They also increase chemokine and cytokine expression (Brzozowski et al, 2003). At least part of these effects results from the ability of ROS (in particular H_2O_2) to stimulate MAP kinases activity which leads to activation of several transcription factors. It is possible that intracellular ROS may act as second messengers in inflammatory signal transduction. Reactive oxygen-mediated modifications of macromolecules such as proteins, lipids, DNA, and small cellular molecules are associated with a number of pathogenic processes including aging, diabetes, arthritis, inflammation and cardiovascular diseases (Dhalla et al., 2000; Harper et al., 2004; Henrotin et al., 2003; Ma et al., 2001; Mathy-Hartert et al., 2003).

5. Membrane lipid, membrane protein and DNA oxidation

Oxidation of membrane lipids in diverse tissues of human body is one of the most studied areas in this field. Peroxidation, polyunsaturated membrane lipid by oxygen free radicals is stimulated by several redox-cycling compounds and has been implicate in the cellular toxicity of these agents (Huang et al., 2006). In the oxidation sequence, superoxide radical anions do not sufficiently reactive to abstract hydrogen from unsaturated lipids to initiate peroxidation; rather, secondary reactive oxygen species derived from superoxide radical anion (O_2^{*-}), or H_2O_2, such as $^*OH$, are thought to ultimately initiate lipid peroxidation (Wang et al., 2006). Recently, the most general accepted mechanism for reactive oxygen production from superoxide radical anion; is an iron-dependent Habor-Weiss reaction in which H_2O_2, formed by O_2^{*-}; dismutation, reacts with chelated ferrous iron (ferrous iron reduced by O_2^{*-}) to form $^*OH$ (Shi et al., 1994). Fatty acid peroxidation consists of initiation and propagation reactions. Initiation occurs following hydrogen abstraction by hydroxyl radicals to form carbon-centered lipid radicals. Lipid radicals formed during initiation propagate peroxidation by the concerted effects of two reactions. A second initiation pathway involves iron-dependent

decomposition of lipid hydroperoxides formed during propagation. Decomposition of lipid hydroperoxides is facilitated by both ferric and ferrous iron, although more rapid when catalyzed by the ferrous form (Miyamoto et al., 2003). Ferrous iron-promoted hydroperoxide-dependent initiation appears not to involve O_2^{*-} or $^*OH$, but is markedly attenuated by iron chelators such as ethylenediaminetetraacetic acid (EDTA). Hydroperoxide-dependent initiation reactions require the presence of lipid hydroperoxides formed initially via hydroxyl radical-dependent pathways (Reeder & Wilson, 2001). Once a critical concentration of lipid hydroperoxides is achieved, the predominant mechanism of initiation appears to be liquid hydroperoxide dependent rather than hydroxyl radical-dependent.

Protein oxidation is defined as the covalent modification of a protein induced either directly by ROS or indirectly by reaction with secondary by-products of oxidative stress. The two amino acids that are perhaps the most prone to oxidative attack are cysteine (Radi et al., 1991; Lii et al., 1994) and methionine (Vogt, 1995), both of which contain susceptible sulfur atoms. All oxidizing species can induce modification of cysteine and methionine. In the case of cysteine, oxidation leads to the formation of disulfide bonds, mixed disulfides (e.g., with glutathione), and thiyl radicals (Hu, 1994; Kalyanaraman, 1995). With methionine resides, the major product under biological conditions is methionine sulfoxide (Vogt, 1995). Other amino acids may require more stringent conditions for oxidative modification. In addition, other amino acyl moieties, especially lysine, arginine, proline, and threonine (Stadtman, 1990), incur formation of carbonyl groups (aldehydes and ketones) on the side chains. Another major endogenous oxidizing species is myeloperoxidase-derived HOCl. Interaction of this molecule with tyrosine, tryptophan, lysine, and methionine residues leads to formation of chlorotyrosine, chloramines, aldehydes, and methionine sulfoxide (Hazen & Heinecke, 1997). In a recent series of studies, HOCl was also shown to modify free amino acids to reactive products (Hazen et al., 1998). For example, well-characterized product of free tyrosine oxidation by HOCl is p-hydroxyphenylacetaldehyde (p-HA), which has been shown to attach to lysyl residues in proteins to form a covalent adduct (Hazen et al., 1997).

Among reactive oxygen species, the highly reactive hydroxyl radical reacts with DNA by addition to double bonds of DNA bases and by abstraction of an H atom from the

methyl group of thymine and each of the C-H bonds of 2'-deoxyribose (Sonntag, 1987). These chemical reactions may lead conformational changed to the DNA molecules and thereby altering their specific functions. DNA oxidative mechanism has been demonstrated to possess a potential role in the initiation, promotion, and malignant conversion (progression) stages of carcinogenesis and many other disease conditions (Babbar & Casero, 2006). In addition, the neurodegenerative conditions such as Alzheimer's disease, Huntington's disease, and Parkinson's disease have oxidative stress implicated DNA damage in their pathogenesis (Butterfield et al., 2006; Oikawa et al., 2006), although there are evidences that levels of oxidative DNA damage are not specifically elevated (Alam et al., 2000). Beside these major pathological events related to oxidative DNA damage, many reports reveal that aging has direct relationship with the same (Hazra et al., 2007 & Madia et al., 2007). Broadly speaking, theories of aging are grouped under two categories: damage accumulation aging and developmentally programmed aging. It has been clearly explained that, failure of antioxidant defenses to scavenge all radical species, evident from the increasing background levels of DNA damage with age, will result in the insidious accumulation of damage and gradual loss of function. With this point, it is not difficult to account for why elevated ROS/DNA damage has close relationship with number of degenerative diseases. More importantly, it has been identified that cellular ROS is a direct mediator of activating NF-κB that up-regulate number of pathogenesis (Chang et al., 2004).

6. NF-κB signal in antioxidant activity

Osteoarthritis is the most common type of arthritis in the world, and it is rapidly becoming a major public health issue among the aged population. The initiating event of osteoarthritis is still unknown and believed to be multi-factorial. It is characterized by quantitative and qualitative destructive changes in the architecture and composition of all the joint structures.

Among all malignant bone tumors, chondrosarcoma is the second most common, accounting for 10–26% of all osseous malignancies. It is the most frequently diagnosed bone tumor in the population older than 50 years (Dorfman & Czerniak, 1995; Fletcher et

al., 2002; & Schajowicz, 1994). The most common skeletal sites affected by chondrosarcoma are the bones of the pelvis followed by the proximal femur, proximal humerus, distal femur and ribs (Fletcher et al., 2002). Clinical symptoms are very unspecific. Radiographic findings often suggest the diagnosis of chondrosarcoma because of identification of typical “ring-and-arc” chondroid matrix mineralization and aggressive features of deep endosteal scalloping and soft tissue extension (Murphey et al., 2003). Chondrosarcomas range from locally aggressive tumors with almost no metastatic potential to high-grade malignancies with a marked propensity to metastasize (Dorfman & Czerniak, 1995; Evans, 1977).

In rheumatoid arthritis, oxidative stress has been described as an important mechanism underlying development and progression of destructive proliferative synovitis (Ozturk et al., 1999). Several sources of reactive oxygen species in the synovial joint that could lead to the disturbed redox homeostasis have been proposed. These include exposure to free radicals liberated by activated phagocytic cells at the site of inflammation (Babior, 2000). Especially, neutrophils that are accumulated in affected tissues are involved in production of reactive oxygen species such as superoxide radical anion, hydrogen peroxide, HOCl and highly reactive hydroxyl radical by the Fenton reaction (Harris, 1990). Accordingly, reactive oxygen species can contribute to the pathogenesis of rheumatoid arthritis in a variety of ways including induction of membrane oxidation and instability, irreversible damages to proteins and DNA, cartilage damage and induction of bone resorption as well as signal transduction (Ozturk et al., 1999).

One of the major signal transduction pathways that activates in response to oxidative stress is nuclear factor-kappaB (NF-κB) which plays a key role in regulating the immune and inflammatory responses via its target genes (Chen et al., 1999). NF-κB is found in almost all animal cell types and is involved in cellular responses to stimuli such as stress, cytokines, free radicals, ultraviolet irradiation, oxidized low-density lipoprotein, and bacterial or viral antigens (Brasier, 2006; Gilmore, 1999; Gilmore, 2006; Perkins, 2007; Tian, 2003). Further, NF-κB activation is high in osteoarthritis patients and the p50 and p65 NF-κB are abundant in rheumatoid and osteoarthritic synovitis (Roman-Blas and Jimenez, 2006). Results of recent studies have suggested that G-COS modifies NF-κB activation induced by proinflammatory cytokine and actively participate in reducing the

expression of NF-κB associated inflammatory genes (Largo et al., 2003; Hoffer et al., 2001).

Until now, none has demonstrated the ability of gallic acid conjugated chitooligosaccharides to scavenge free radicals in an intracellular environment and to alter subsequent events resulted from oxidative stress. The aim of this work was to investigate whether G-COS is able to scavenge intracellular free radicals liberated during oxidation. Moreover, its contribution in controlling radical mediated oxidative damage to major cellular biomolecules such as membrane lipids, protein and genomic DNA were also investigated. Further, effects of G-COS on reactive oxygen species induced activation and expression of NF-κB were studied.

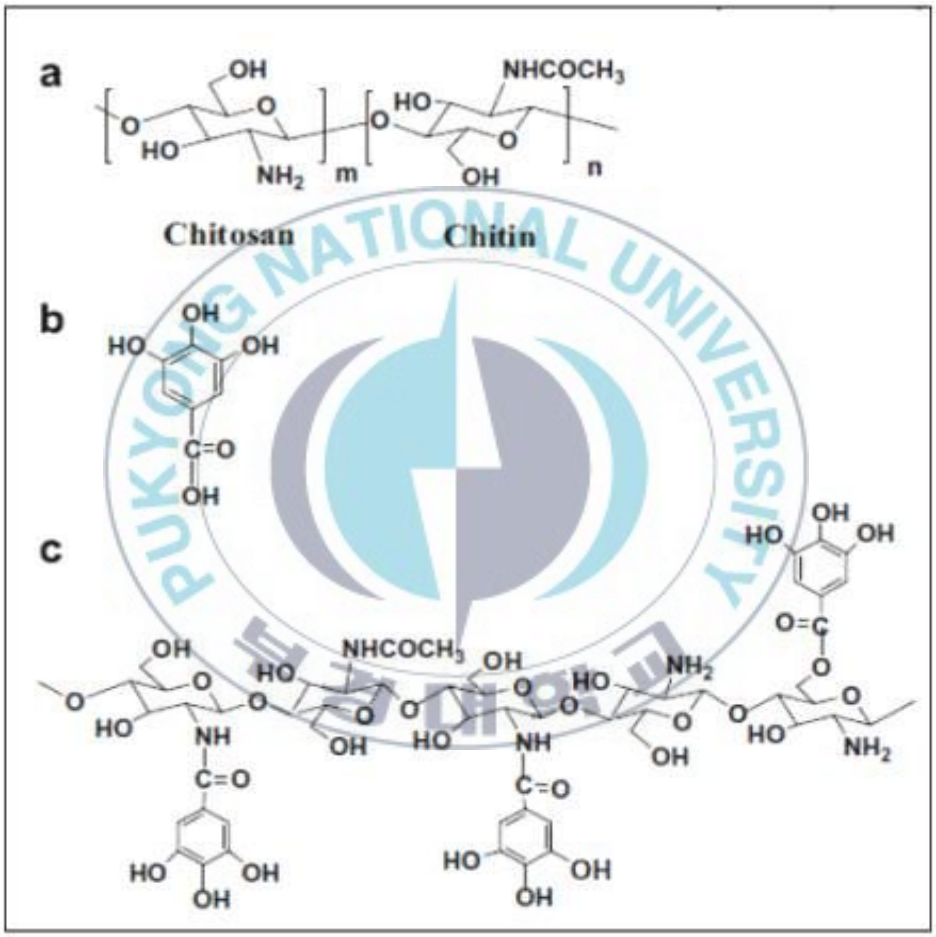


Figure 1. Chemical structures of (a) chitooligosaccharides (COS); (b) gallic acid; and (c) gallic acid conjugated chitooligosaccharides (G-COS)

Table 1. Applications of chitin and chitosan

Field	Applications
Food & beverages	Dietary fiber (reduce cholesterol and lipid- binding agent) Food and fruit preservation (antibacterial and antifungal agents and film) Food stabilizer
Medicine and pharmaceutics	Wound-healing ointments and dressings Contact lenses Orthopedic surgery Drug and gene carriers Antitumor agents Immunological stimulant Dental implants Anticoagulant
Cosmetics and toiletries	Skin care (skin moisture, treat acne) Hair treatment Oral care (toothpaste, chewing gum)
Water and waste treatment	Drinking water and pools Removal of metal ions Ecological polymer
Agriculture	Seed coating Foodstuff for animal
Biotechnology	Enzyme and cell immobilization Nanoparticles

Materials and Methods

1. Materials

Chitooligosaccarides (COS, molecular weight 3.0-5.0 kDa) were kindly donated by Kitto. Life Co. (Seoul, Korea). Gallic acid was purchased from Acros Organics (New Jersey, USA). All chemicals required for synthesis were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

Human chondrosarcoma (SW1353, ATTC HTB94) and mouse macrophages (RAW264.7, ATTC TIB71) cell lines were obtained from American Type Culture Collection (Manassas, VA, USA).

Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12), DMEM, penicillin/streptomycin, and the other materials required for culturing of cells were purchased from Gibco BRL, Life Technologies (Grand Island, NY, USA).

Radical testing chemicals, including 1,1-diphenyl-2-picrylhydrazyl (DPPH), 5,5-dimethyl-1-pyrroline N-oxide (DMPO), $FeSO_4$, H_2O_2, riboflavin, ethylenediaminetetraacetic acid (EDTA), 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH), α-(4-pyridyl-1-oxide)-N-tert-butylnitrone (POBN), Dimethylsulfoxide (DMSO), 2′,7′-dichlorofluorescin diacetate (DCFH-DA), 2,4-dinitrophenyl hydrazine, diphenyl-1-pyrenylphosphine (DPPP), thiobarbituric acid reactive substances (TBARS), guanidine hydrochloride, 3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide (MTT), agarose and fetal bovine serum (FBS) were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

All other reagents were of the highest grade available commercially.

2. Synthesis of gallic acid conjugated chitooligosaccharides (G-COS)

Chitooligosaccharides (COS, 2.4814 g) was dissolved in distilled water (20 ml) and 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 to 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 ×g for 5 h and then filtered. The solution obtained was kept at 2°C overnight and then added diethyl ether (90ml), filtered to obtain the precipitate. The precipitate was dissolved in 20 ml distilled water and then freeze dried to obtain chitooligosaccharides conjugated gallic acid (G-COS).

3. Infrared spectroscopy

The samples prepared in the forms of potassium bromide (KBr) disk, and the films were studied. The KBr disk was prepared according to the method of Sabnis and Block (1997) with slight modifications. The samples (10 mg) were dried overnight at 60°C under reduced pressure. The dried samples were mechanically blended with 100 mg of KBr. The mixture was compacted using an infrared (IR) spectroscopy hydraulic press at a pressure of 8 tons for 60 s. They were dried for 24 h at 60°C under reduced pressure before measuring. The spectra of samples in the forms of KBr disk were obtained using a fourier transform infrared (FT-IR) spectrometer (Perkin Elmer Spectrum GX, Beaconsfield Bucks, England) with a frequency range of 4000-400cm^{-1}.

4. Instrumental analysis

Infrared spectra were recorded as clear signals on KBr plates with a Spectrum 2000 FT-IR spectrophotometer (Perkin Elmer, USA) and proton and carbon NMR (^{1}H and ^{13}C NMR) spectra were recorded in D_2O on a JNM-ECP-400 (400 MHz or 100 MHz) spectrometer (JEOL Japan).

5. Electron spin resonance (ESR) spectrometer

Different radicals were generated and spin adducts were recorded using a JES-FA ESR spectrometer (JEOL Ltd., Tokyo, Japan) at 25°C. Instrument settings were as follows: magnetic field 336 ± 5 mT; sweep time 30 s; sweep width 10 mT; modulation width 0.1 mT and modulation frequency 100 kHz. Radical scavenging activity of sample was

calculated as a scavenging percentage by, $S = (H_o - H)/H_o \times 100\%$; where, H and H_o were ESR signal intensities in the presence and the absence of sample, respectively.

5.1. Hydroxyl radical ($^{*}OH$) assay

Hydroxyl radicals were generated by Fenton reaction and trapped using DMPO nitrone spin trap (Rosen & Rauckman, 1984). The resultant DMPO/$^{*}OH$ adducts was detectable with an ESR spectrometer. A sample solution (15 μl) was mixed with DMPO (0.3 M, 15 μl), $FeSO_4$ (10 mM, 15 μl) and H_2O_2 (10 mM, 15 μl) in a phosphate buffered saline solution (PBS, pH 7.4), and then transferred into a 50 μl quartz capillary tube. After 2.5 min, the ESR spectrum was recorded at 1 mW microwave power and amplitude 200.

5.2. DPPH radical (DPPH*) assay

DPPH radical scavenging activity was measured using the method described by Nanjo et al. (1996). A 30 μl sample solution (or distilled water itself as control) was added to 30 μl of DPPH (60 μM) in methanol solution. After mixing vigorously for 10 s, the solution was then transferred into a 50 μl quartz capillary tube. After 2 min, the ESR spectrum was recorded at 5 mW microwave power and amplitude 1000.

5.3. Superoxide radical anion (O_2^{*-}) assay

Superoxide radical anions were generated by UV irradiated riboflavin/EDTA system (Guo et al., 1999). The reaction mixture containing 0.8 mM riboflavin, 1.6 mM EDTA, 800 mM DMPO and indicated concentration of sample solution was irradiated for 1 min under UV lamp at 365 nm. The reaction mixture was then transferred into a 50 μl quartz capillary tube. The ESR spectrum was recorded at 10 mW microwave power and amplitude 1000.

5.4. Carbon-centered radical (R*) assay

Carbon-centered radicals were generated by AAPH (Hiramoto et al., 1993). 15 μl of PBS (pH 7.4), AAPH (40 mM), POBN (40 mM) and sample were mixed and incubated at 37^{o}C for 30 min. Following incubation, the reaction mixture was transferred into a 50 μl quartz capillary tube and the ESR spectrum was recorded at 5 mW microwave power and amplitude 500.

6. Cell culture and cytotoxicity determination

SW1353 and RAW264.7 cells were grown in DMEM-F12 and DMEM media containing 5% (v/v) FBS, 100 μg/ml penicillin- streptomycin and 5% CO_2 at 37^{o}C, respectively.

Cytotoxicity levels of samples on cells were measured using the MTT method as described by Hansen et al. (1989). The cells were grown in 96-well plates at a density of $5x10^3$ cells/well. After 24 h, cells were washed with fresh medium and treated with different concentrations of samples. After 48 h of incubation, cells were rewashed and 50 μl of MTT (1 mg/ml) were added and incubated for 4 h. Finally, 200 μl of DMSO were added to solubilize the formazan salt formed and the amount of formazan salt was determined by measuring the optical density (OD) at 540 nm using an GENios microplate reader (TECAN Austria GmbH, Grodig/Salzburg, Austria).

7. Determination of intracellular formation of reactive oxygen species (ROS) using fluorescence labeling

Intracellular formation of ROS was assessed according to a method described previously by employing oxidation sensitive dye DCFH-DA as the substrate (Engelmann et al., 2005). The cells were grown in 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 freely 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 sample 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 various 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.

8. Genomic DNA isolation

Genomic high molecular weight DNA was extracted from SW1353 and RAW264.7 cells using standard phenol/proteinase K procedure with slight modifications (Sambrook & Russell, 2001). Briefly, cells cultured in 10 cm culture **dishes** were washed twice with PBS and scraped into 1 ml of PBS containing 10 mM EDTA. After centrifugation cells were dissolved in RNase (0.03 mg/ml), sodium acetate (0.175 M), proteinase K (0.25 mg/ml) and sodium dodecyl sulfate (SDS, 0.6%). The mixture was then incubated for 30 min at 37^{o}C and 1 h at 55^{o}C. Following incubation, phenol/chloroform/isoamyl alcohol was added at a 1:1 (v/v) ratio and the mixture was centrifuged at 6000×g for 5 min at 4^{o}C. Following centrifugation, the supernatant was mixed with 100% cold ethanol at a 1:1.5 (v/v) ratio and kept for 15 min at -20^{o}C. After centrifugation at 12,000×g for 5 min, the pellet was dissolved in 10 mM Tris-EDTA buffer (pH 7.5) and the purity of DNA was spectrophotometrically determined at 260/280 nm. Further, the quality of isolated DNA was evaluated using 1% agarose gel electrophoresis in 0.04 M Tris-acetate, 0.001 M EDTA buffer (pH 7.5).

9.DNA oxidation assay

Hydrogen peroxide mediated DNA oxidation was determined according to Milne et al. (1993). Briefly, 40 μl of DNA reaction mixture were prepared by adding pre-determined concentrations of test sample (or same volume of distilled water as control), and final concentrations of 200 μM of $FeSO_4$, 2 mM H_2O_2 and 5 μl of isolated genomic DNA in

the same order. Then the mixture was incubated at room temperature for 10 min and the reaction was terminated by adding 10 mM final concentration of EDTA. An aliquot (20 μl) of the reaction mixture containing about 1 μg of DNA was electrophoresed on a 1% agarose gel for 20 min at 100 V. Gels were then stained with 1 mg/ml ethidium bromide and visualized by UV light using AlphaEase® gel image analysis software (Alpha Innotech, St. San Leandro, CA, USA).

10. Protein oxidation

The amount of membrane protein carbonyl groups were assessed by a method described elsewhere (Levine et al., 1990) with slight modifications. Cultured cells were collected by centrifugation and washed three times with PBS and lysed with lysis buffer without reducing agents (25 mM Tris-HCl pH 7.8, 2mM EDTA, 180 mM NaCl, 1% Triton X-100). The lysate was aliquoted into microtubes (0.5 ml) and samples were treated with indicated concentrations. After incubation for 30 min at 37°C, 100 μl of 0.1 mM $FeSO_4$ and 100 μl of 2 mM H_2O_2 were added and incubated at 37°C for 1 h. After addition of 400 μl of 20% trichloroacetic acid, solubilized protein (1 mg) was precipitated by centrifugation. The supernatant was discarded, and the remaining pellet was resuspended in 150 μl of 0.2% 2,4-dinitrophenyl hydrazine in 2 N HCl and allowed to stand at room temperature for 40 min. The reaction mixture was vortexed at every 10 min to facilitate the reaction with proteins. The protein was precipitated again with 20% trichloroacetic acid, and the pellet was washed three times with an ethanol/ethyl acetate (1:1 v/v) solution. The pellet was then dissolved in 500 μl of 6 N, guanidine hydrochloride and incubated for 15 min at 37°C. After centrifugation at 6000×g for 5 min, the absorbance of the supernatant was read against a complementary blank at 370 nm. 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 the control group in which samples were not treated.

11. Membrane lipid peroxidation assessment by DPPP fluorescence method

Intracellular lipid hydroperoxide level was determined by the fluorescence probe, DPPP, as described previously with slight modifications to assess the degree of membrane lipid oxidation (Takahashi et al., 2001). For this experiment, the cells growing in culture dishes were washed three times with PBS and labeled with 13 μM DPPP (dissolved in DMSO) for 30 min at 37°C in the dark. The cells were washed three times with PBS and seeded into fluorescence microtiter 96-well plates at a density of 1×10^8 cells/ml using serum-free media. Following complete attachment, the cells were treated with predetermined concentrations of samples and incubated for 1 h. After incubation, 3 mM AAPH in PBS was added and DPPP oxide fluorescence intensity was measured after 6 h at the excitation wavelength of 361 nm and the emission wavelength of 380 nm using a GENios® fluorescence microplate reader (Tecan Austria GmbH, Grodig / Salzburg, Austria).

12. Membrane lipid peroxidation assessment by TBARS method

Cells were analyzed for the generation of lipid peroxidation products by a modification of the thiobarbituric acid-reactive substances (TBARS) method, as previously described (Hino et al., 2001). Briefly, 200 μl of PBS-suspended RAW264.7 and SW1353 cells were added to an eppendorf tube and incubated for 10 min with pre-determined concentrations of test samples or same volume of distilled water as a control. Cellular oxidation was triggered by adding 2 mM H_2O_2 and 0.1 M $FeSO_4$, and incubated for 30 min. Two-fold volume of cold trichloroacetic acid (10% w/v) was added and protein was precipitated by centrifugation. The supernatant was then added to an equal volume of thiobarbituric acid (1% w/v) and heated at 90°C for 30 min. After cooling, reaction mixture was centrifuged and absorbance of the supernatant was determined at 528 nm.

13. RNA isolation

Total RNA was extracted from different cells after treatment with test samples at different concentrations. For that, cells were lyzed with 1ml TRIzol® reagent (Invitrogen Corporation, Faraday Avenue, USA) according to the manufacturer's protocol. Reagent

was added to a 10 cm culture dish, and passing the cell lysate several times through a pipette. The homogenized samples were incubated for 2 min at room temperature. After that, the homogenized samples were transferred into microtube, 0.2 ml chloroform was added into microtube, shook vigorously by hand for 15 seconds and incubated at room temperature for 2 min. The process was followed with centrifugation samples 12,000×g at 4°C for 15 min. The mixture was separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase was transferred to a fresh tube. The RNA was precipitated from aqueous phase by mixing with isopropyl alcohol following ratio 1:1. The mixture was vortexed and kept at 4°C for 10 min, followed by centrifugation 12,000 ×g at 4°C for 15 min. After remove supernatant, RNA pellet was washed once with 1 ml 75% ethyl alcohol. Ethyl alcohol was removed after the sample had centrifuged 12,000 ×g at 4°C for 15 min. At the end of the procedure, RNA pellet was suspended in DEPC-H_2O and stored at -80°C until use. The purity of the RNA was established by reading the optical density of each sample at 260 nm and 280 nm, using GENios microplate reader.

14. Reverse transcription polymerase chain reaction (RT-PCR) analysis

Total cellular RNA was extracted from treated cells using Trizol® reagent. 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 primers: for superoxide dismutase (SOD), sense 5′-AGGGCATCATCAATTTCGAG-3′ and antisense 5′-TGCCTCTCTTCATCCTTTGG-3′; for glutathione (GSH), sense 5′-AGCATTTGGCAAAGGAGAAA-3′ and antisense 5′-ATCCGTGCTCCGACAAATAG-3′; glyceraldehyde 3-phosphate dehydrogenase (GAPDH), sense 5′-GCCACCCAGAAGACTGTGGAT-3′ and antisense 5′-TGGTCCAGGGTTTCTTACTCC-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).

15. Extraction of nuclear and plasma proteins

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. Briefly, RAW264.7 and SW1353 cells treated with test samples were stimulated with 500 μM H_2O_2 and lysed with 0.5 ml of lysis buffer (500 μl, hypotonic lysis buffer, 5 μl, 0.1 M dithiothreitol (DTT), 5 μl protease inhibitor cocktail) for 15 min on the ice. Igepal CA-630 solution (4 μl) was added and vortexed for 20 s. Nuclei were separated by centrifugation at 10,000×g for 10 min and supernatant (cytoplasm protein) was collected. Precipitated nuclei were lysed with 100 μl of extraction buffer mix (98 μl, extraction buffer, 1 μl of 0.1 M, DTT and 1 μl protease inhibitor cocktail) for 10 min and nuclei protein were collected by centrifugation at 12,000×g for 10 min.

16. Western blot analysis

Protein concentrations in 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, GSH and actin (Santa Cruz Biotechnology Inc. CA, USA). 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.

17. Statistical analysis

All statistical analyses were performed with independent experiments and data are represented as means ± standard deviation (SD). The statistical significance was achieved when $P < 0.05$.

Results

1. Structural characterization of G-COS

The conjugation of gallic acid and COS is possible at either C-2, to obtain the amide linkage, or C-3 and C-6 to obtain the ester linkage. In the FT-IR spectra, G-COS shows significant peaks at 1955 and 1520 cm^{-1}, implying the ester and amide linkages between COS and gallic acid (Figure 2). From these results, it can be concluded that the gallyl group of gallic acid was successfully introduced into COS via amide and ester linkage. Furthermore, the ^{1}H NMR spectra, G-COS shows a new peak at 6.98 ppm belonging to the phenyl protons as compared to COS. This confirms the successful conjugation with gallic acid. For ^{13}C NMR spectra, G-COS showed the aromatic carbon of the gallyl group at 109.54, 128.01, 135.84 and 144.45 ppm (C=C) and 174.8 ppm (C=O) implying successful conjugation.

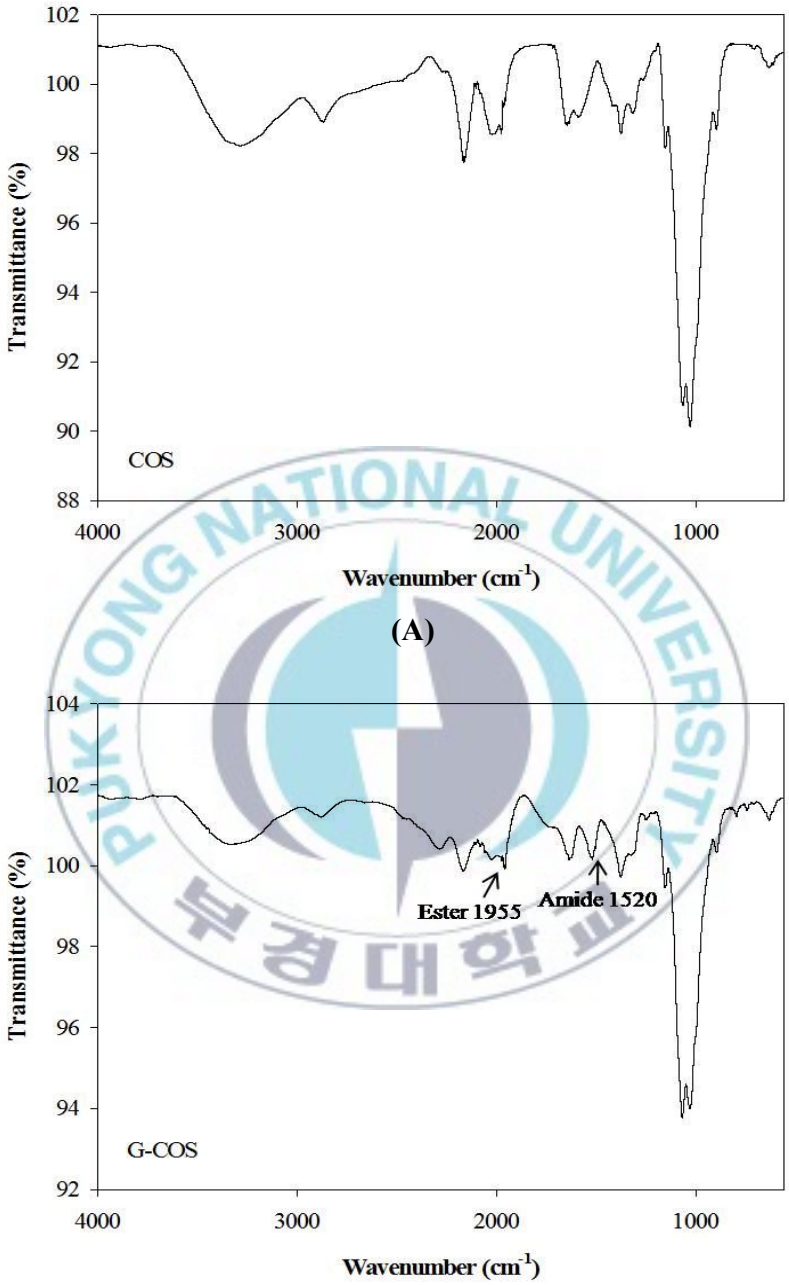


Figure 2. FT-IR (KBr) spectra of COS (A) and G-COS (B)

2. Radical scavenging potency in cell-free systems

Free radical scavenging measured in cell-free systems by ESR with spin-trapping agents followed a similar scavenging trend observed in cell system (scheme 1). ESR spectra obtained with control groups verified that DPPH, DMPO and POBN trapping chemistry is functioning properly to result clear spin adducts, DPPH-H, DMPO/*OH, DMPO/O_2^{*-} and POBN/R^{*} radicals (Mendis et al, 2008). G-COS dose-dependently suppressed the intensity of ESR signal adducts of four radicals at different capacities compared to the control experiments (Table 2). Moreover, G-COS exerted a comparatively lower scavenging effect on carbon-centered radicals and DPPH radical than that of *OH and O_2^{*-}.

2.1. Hydroxyl radical (*OH) scavenging capacity

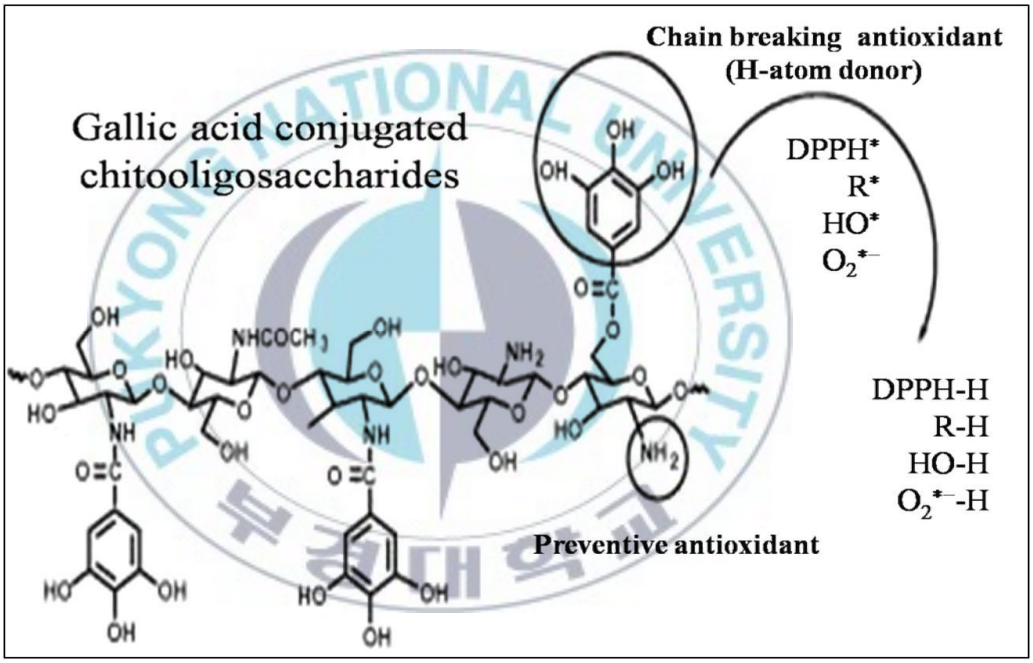
The hydroxyl radical (*OH) is a highly oxidizing product related to the Fenton reaction and Haber-Weiss reaction, which involves the initiation of biological oxidations; for example DNA, protein, and lipid oxidations (Haber-Weiss, 1934). The loosely bound iron is well known to be an important component of biological free radical oxidation as it is a key factor to produce *OH, as seen in equation (Eq.) (1):

$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^{-} + {}^{*}OH$ Eq. (1)

$^{*}OH + \text{G-COS} \rightarrow H_2O + {}^{*}\text{G-COS}$ Eq. (2)

$^{*}OH + \text{DMPO} \rightarrow \text{DMPO}/{}^{*}OH$ Eq. (3)

In this study, hydroxyl radicals were generated via the reaction of Fe^{2+}/H_2O_2 according to the Fenton reaction (Eq. 1) (Pasanphan & Chirachanchai, 2008). The antioxidant capacity of G-COS was determined by the ability of G-COS to stabilize *OH via Eq. (2). The *OH remaining was detected relating to the amount of DMPO/*OH (Eq. 3). The IC_{50} values in scavenging *OH of COS and G-COS are 66.00 ± 0.048 μg/ml and 16.00 ± 0.050 μg/ml, respectively. In other words, G-COS, with only a certain amount of gallate groups, shows antioxidant activity on *OH increase.



Scheme 1. The mechanism antioxidant action of G-COS

Table 2. The IC_{50} values of COS and G-COS to scavenge DPPH, hydroxyl, superoxide and carbon-centered radicals.

	Radical	IC_{50}[a] ± SD (μg/ml)	
		COS	G-COS
1	DPPH	184.00 ± 0.020	31.50 ± 0.050
2	Hydroxyl	66.00 ± 0.048	16.00 ± 0.050
3	Superoxide	78.00 ± 0.050	25.00 ± 0.050
4	Carbon-centered	355.00 ± 0.032	36.25 ± 0.012

[a] Each value is expressed as the mean ± standard deviation (SD, n = 3). The IC_{50} value is defined as the necessary concentration at which the radicals generated by the reaction systems were scavenged by 50%.

2.2. DPPH* scavenging capacity

It is important to clarify whether chitooligosaccharide shows radical scavenging ability via the function of gallic acid or not. The DPPH* is a relatively stable free radical because it can be easily detected by ESR; thus, it is useful and practical for the evaluation of free radical scavenging potential (Williams et al, 1995) The DPPH* radical scavenging activity was investigated from the activity of G-COS to reduce the radicals, as shown in the following Eq. (4):

$$DPPH^* + G\text{-}COS \rightarrow DPPH\text{–}H + {}^*G\text{-}COS \qquad \text{Eq. (4)}$$

The antioxidant capacity was evaluated by plotting the concentrations of antioxidant against the percent of free radical scavenging capacity (Pasanphan & Chirachanchai, 2008). Table 2 shows the comparative studies on the reducing capacity of COS and G-COS. The IC_{50} value, which expresses the antioxidant concentration to reduce the radicals by 50%, is a good indicator to quantify the antioxidant capacity. As shown in Table 2, the IC_{50} values in scavenging the DPPH* of G-COS and COS are 31.50 ± 0.050 μg/ml and 184.00 ± 0.020, respectively.

2.3. Superoxide radical anion (O_2^{*-}) scavenging capacity

A irradiated riboflavin/EDTA system was applied to test the direct superoxide radical anion scavenging activities of G-COS. Superoxide radical anions generated in the irradiated riboflavin/EDTA system was trapped by DMPO forming a spin adduct which could be detected by ESR. The antioxidant capacity of G-COS was determined by the ability of G-COS to stabilize O_2^{*-} via Eq. (5). The O_2^{*-} remaining was detected relating to the amount of DMPO/O_2^{*-} (Eq. 6).

$$2O_2^{*-} + 2G\text{-}COS \rightarrow H_2O_2 + O_2 + 2{}^*G\text{-}COS \qquad \text{Eq. (5)}$$

$$O_2^{*-} + DMPO \rightarrow DMPO/\ O_2^{*-} \qquad \text{Eq. (6)}$$

The results in Table 2 show that G-COS scavenged superoxide radical anion effectively, the IC_{50} value was 25.00 ± 0.050 μg/ml. Compared with COS 78.00 ± 0.050 μg/ml, G-COS was more effective.

2.4. Carbon-centered radical (R^*) scavenging capacity

Carbon-centered radical (R^*) is one of the representative oxidized products in lipid membranes and lipoproteins (Venkataraman et al., 2004). In this study, we determined the ability of G-COS to scavenge R^* being induced by AAPH (Eq. 7). It is important to point out that the ESR signal of POBN/R^* is related to the antioxidant potential of G-COS (Eq. 8 and Eq. 9):

$R\text{-}N{=}N\text{-}R \rightarrow 2R^* + N_2$ Eq. (7)

$R^* + \text{G-COS} \rightarrow RH + {}^*\text{G-COS}$ Eq. (8)

$R^* + \text{POBN} \rightarrow \text{POBN}/R^*$ Eq. (9)

Table 2 demonstrates the IC_{50} values in scavenging R^* of G-COS is 36.25 ± 0.012 μg/ml, while COS is 355.00 ± 0.032 μg/ml. This indicates that gallic acid on chitooligosaccharide functions in donating an H-atom as well as frees gallic acid.

3. Cell viability

Cytotoxic effects of COS and G-COS were determined by the MTT assay. MTT is a yellow water-soluble tetrazolium salt. Metabolically active cells are able to convert the dye to water-insoluble dark blue formazan by reductive cleavage of the tetrazolium ring. The results showed that any significant toxic effect was not observed on RAW264.7 and SW1353 cells under the tested concentrations (Figure 3 and Figure 4). Therefore, based on the above results, COS and G-COS were used for all experiments.

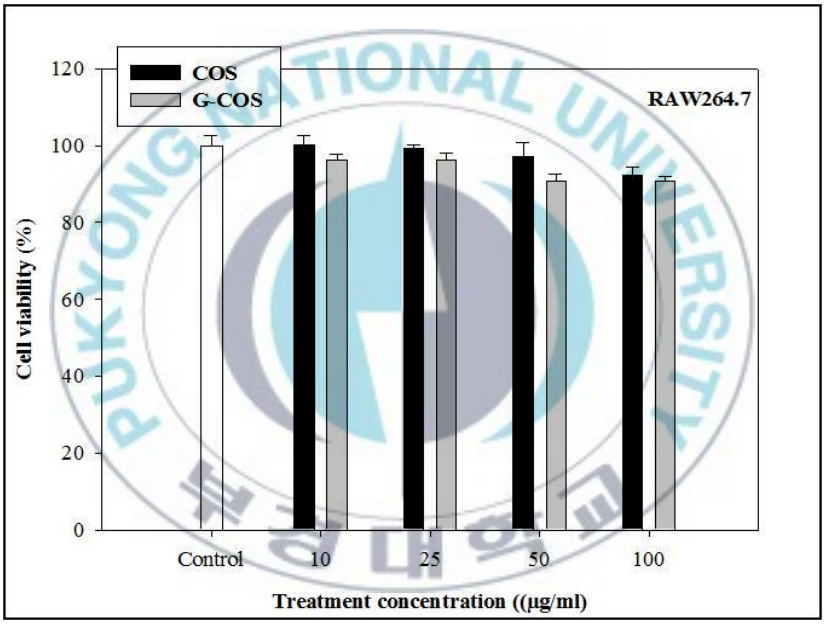


Figure 3. Cell viability determined by the MTT assay. Cytotoxic effects of COS and G-COS on RAW264.7 cells at the different concentrations (10, 25, 50 and 100 μg/ml) and compared with control group was not treated with COS or G-COS. Values are expressed as the means ± SD of three replicate experiments.

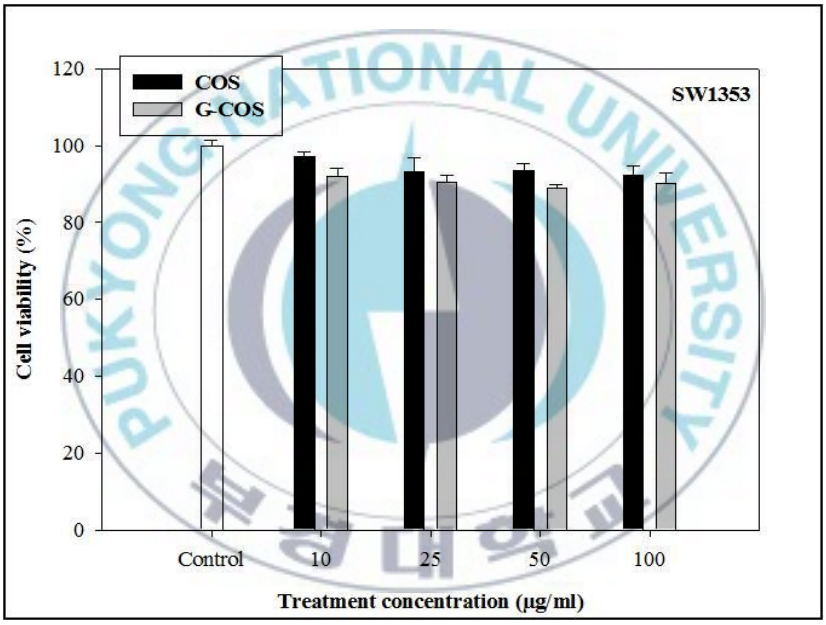


Figure 4. Cell viability determined by the MTT assay. Cytotoxic effects of COS and G-COS on SW1353 cells at the different concentrations (10, 25, 50 and 100 μg/ml) and compared with control group was not treated with COS or G-COS. Values are expressed as the means ± SD of three replicate experiments.

4. Cellular radical scavenging ability

To study the oxidation inhibitory effects of COS and G-COS, mouse macrophage and human chondrosarcoma cell lines (RAW264.7 and SW1353) were employed. These cells are commonly used to study ROS-mediated cellular events since they can produce high amount of ROS following stimulation (Arato et al., 2006). We were interested in studying the direct effects of COS and G-COS to scavenge cellular radicals. For that, RAW264.7 and SW1353 cells were labeled with fluorescence probe 2′,7′-dichlorofluorescein diacetate (DCFH-DA) as described in the materials and methods section. During labeling, non-fluorescent DCFH-DA dye that freely penetrates into cells gets hydrolyzed by intracellular esterases to 2′,7′-dichlorodihydrofluorescein (DCFH), and traps inside the cells (Veerman et al., 2004). As shown in Figure 5 and Figure 6, fluorescence emitted by DCF following ROS-mediated oxidation of DCFH followed a time course increment up to 3 h. Pre-treatment with COS and G-COS decreased the DCF fluorescence dose- and time-dependently.

Even after 30 min of incubation, GCOS exerted a considerable radical scavenging effect at 10 μg/ml concentration. More clearly, at the concentration of 100 μg/ml, G-COS could scavenge radicals significantly throughout the incubation time. This clearly indicated that the oxidation protection effects exerted by G-COS are due to direct scavenging of cellular radicals. These results are in line with our previous observations that G-COS can donate protons under in vitro conditions to scavenge free radicals. Therefore, for the first time here we confirmed the radical scavenging antioxidant effects of a COS derivative (G-COS) in cell systems. Further, we can suggest that G-COS is a potent antioxidant compound that can protect radical mediated oxidation of cellular biomolecules.

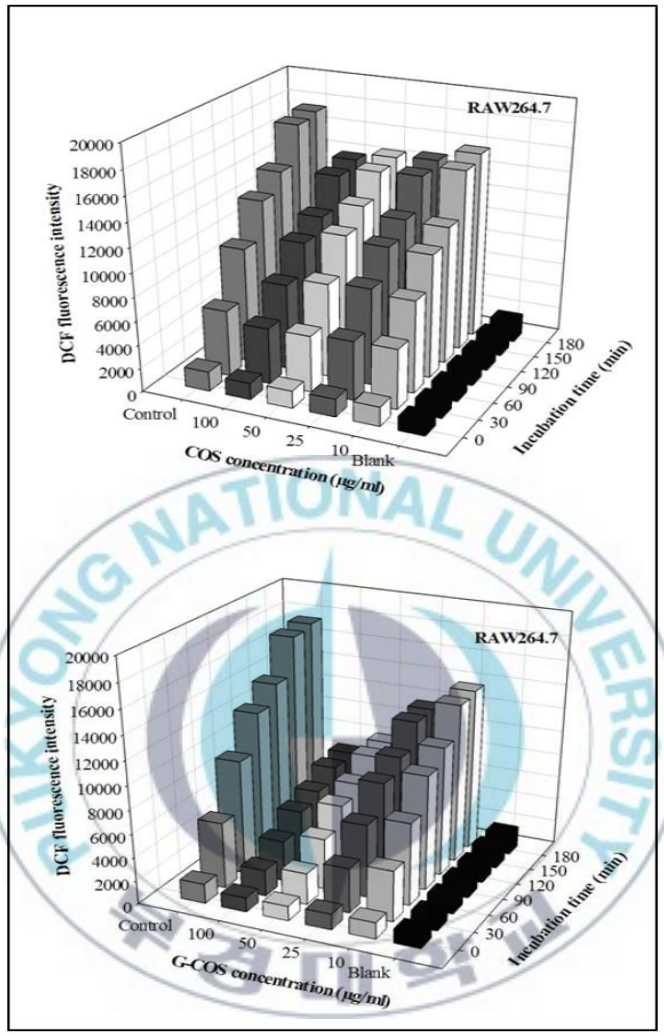


Figure 5. RAW264.7 cells were exposed to serial dilutions of COS and G-COS and then challenged by H_2O_2 to produce ROS. Intracellular radical scavenging activities of COS and G-COS at the different concentrations (10, 25, 50 and 100 μg/ml) and compared with H_2O_2 non-treated blank group and H_2O_2 alone-treated control group. The ROS level is represented as DCF fluorescence. Values are expressed as the means ± SD of three replicate experiments.

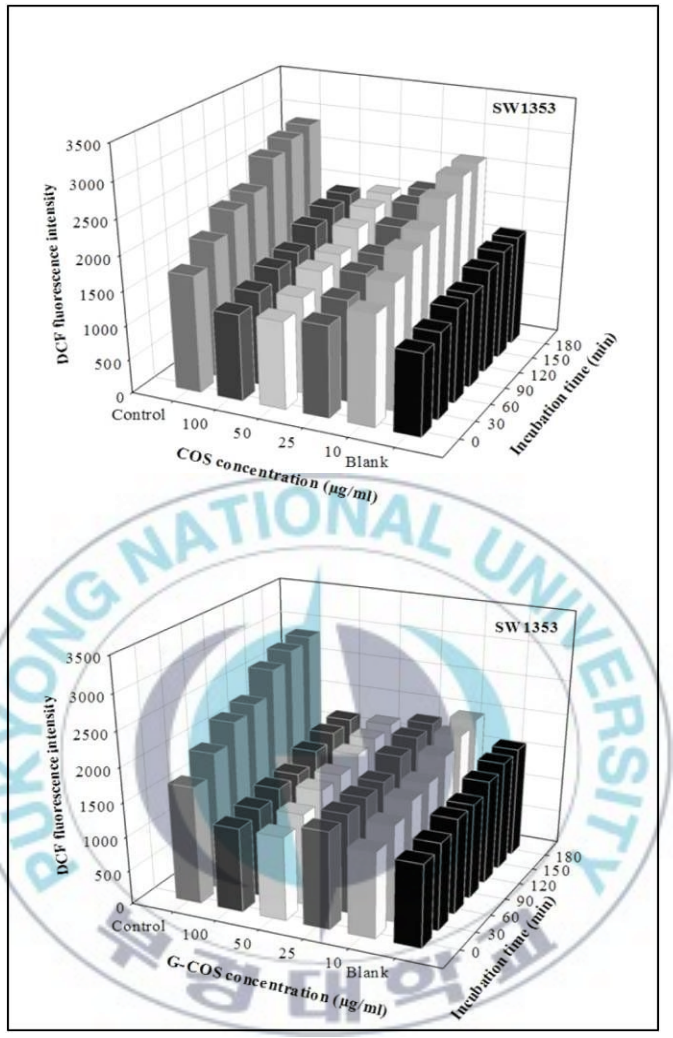


Figure 6. SW1353 cells were exposed to serial dilutions of COS and G-COS and then challenged by H_2O_2 to produce ROS. Intracellular radical scavenging activities of COS and G-COS at the different concentrations (10, 25, 50 and 100 μg/ml) and compared with H_2O_2 non-treated blank group and H_2O_2 alone-treated control group. The ROS level is represented as DCF fluorescence. Values are expressed as the means ± SD of three replicate experiments.

5. Inhibition of radical mediated DNA damage

In this experiment, combined effect of 200 μM Fe(II) and 2 mM H_2O_2 on the integrity of genomic DNA isolated from RAW 264.7 and SW1353 cells was studied by DNA electrophoresis in the presence or absence of COS and G-COS. After 10 min of reaction, almost all DNA was degraded in the control group treated only with Fe (II)-H_2O_2 (Figure 7 and Figure 8). However, a clear dose-dependent DNA oxidation inhibition effect could be observed when G-COS was treated to the reaction mixture.

For genomic DNA from RAW264.7 and SW1353 cells, at 100 μg/ml of G-COS, DNA damage were inhibited more than 90%. In contrast, treatment of COS did not exert adequate protection towards radical mediated DNA damage. Even at 100 μg/ml of COS only about 20% DNA damage was inhibited.

6. Cell membrane protein oxidation inhibition

Oxidation of membrane protein by Fenton reaction products was determined by assessing the protein carbonyl groups (aldehydes and ketones), those have been identified as the early marker for protein oxidation (Mendis et al., 2008). Interestingly, a clear reduction in carbonyl groups formation was observed following the addition of COS and G-COS to the reaction mixture containing RAW264.7, SW1353 cell membranes (Figure 9 and Figure 10) revealing its higher potency to inhibit protein oxidation. Moreover, even at 10 μg/ml concentration G-COS inhibited about 73% and 80% carbonyl group formation on RAW264.7 and SW1353, respectively. Even though COS inhibited oxidation of membrane protein to some extent, that was significantly lower than that of G-COS at all the tested concentrations.

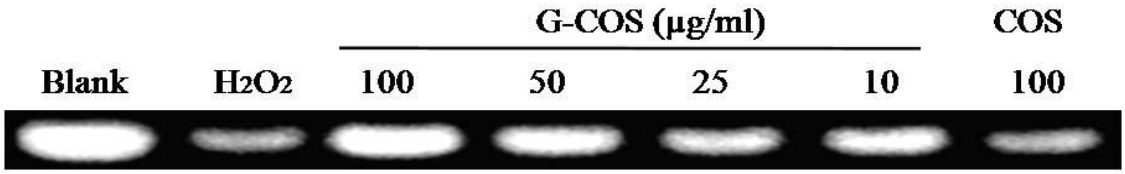


Figure 7. DNA oxidative protection by COS and G-COS. Genomic DNA from RAW264.7 cells was pre-treated with COS or G-COS and exposed to *OH using Fenton chemistry. After 10 min, reaction mixture containing about 1 μg of DNA was electrophorased on a 1% agarose gel for 20 min at 100 V and visualized by UV light after stained with 1 mg/ml ethidium bromide.

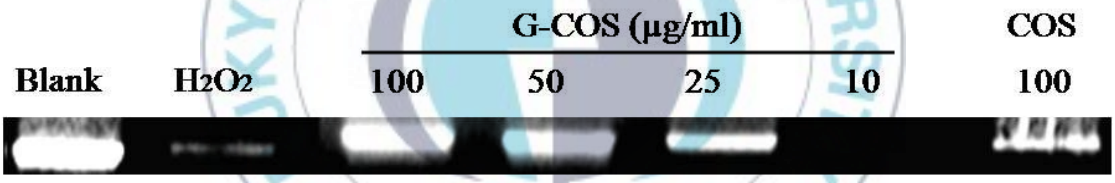


Figure 8. DNA oxidative protection by COS and G-COS. Genomic DNA from SW1353 cells was pre-treated with COS or G-COS and exposed to *OH using Fenton chemistry. After 10 min, reaction mixture containing about 1 μg of DNA was electrophorased on a 1% agarose gel for 20 min at 100 V and visualized by UV light after stained with 1 mg/ml ethidium bromide.

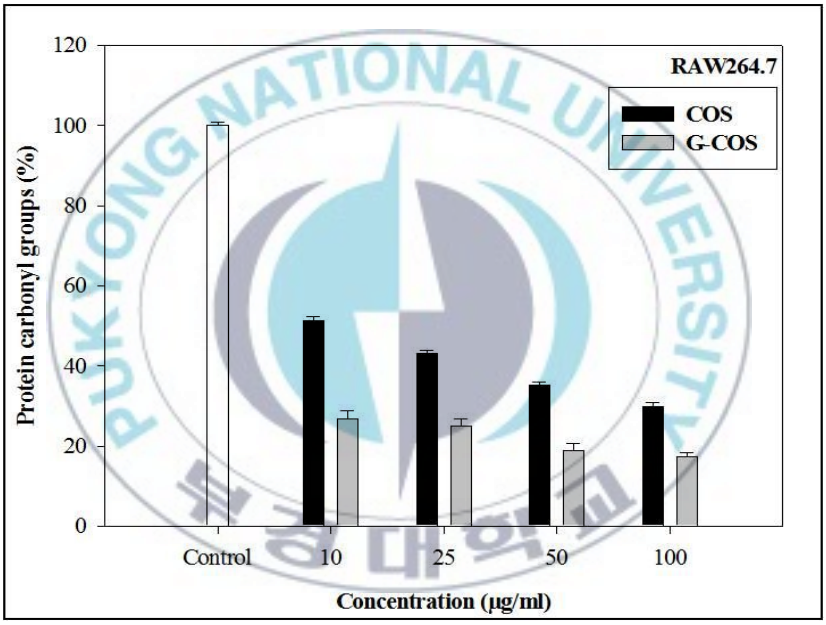


Figure 9. COS and G-COS mediated suppression of radical mediated oxidation in RAW264.7 membrane proteins. Lysates of cells were treated with indicated concentrations of COS and G-COS and protein oxidation was triggered by generating *OH via Fenton reaction. Degree of protein oxidation was determined by assessed the amount of carbonyl groups and compared with *OH non-treated blank group and *OH alone- treated control group. The results shown are representative of separate experiments performed in triplicate. Error bars represent the standard error.

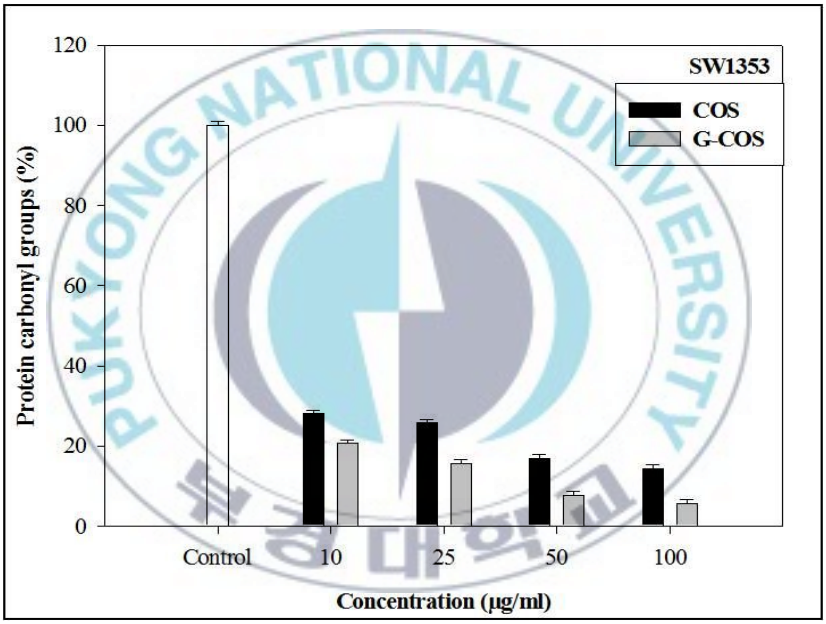


Figure 10. COS and G-COS mediated suppression of radical mediated oxidation in SW1353 membrane proteins. Lysates of cells were treated with indicated concentrations of COS and G-COS and protein oxidation was triggered by generating *OH via Fenton reaction. Degree of protein oxidation was determined by assessed the amount of carbonyl groups and compared with *OH non-treated blank group and *OH alone- treated control group. The results shown are representative of separate experiments performed in triplicate. Error bars represent the standard error.

7. Inhibition of membrane lipid peroxidation

Unsaturated fatty acids in cell membrane lipids are highly susceptible for the free radical attack during oxidation. To study the effects of COS and G-COS on inhibition of cell membrane lipid peroxidation, two different methods were employed. In the first study, a sensitive fluorescence probe, diphenyl-1-pyrenylphosphine (DPPP), was used to determine the lipid hydroperoxide level of RAW264.7 and SW1353 cells exposed to strong carbon-centered radical generator, 2,2′-azobis(2-amidinopropane) dihydrochloride (AAPH). DPPP itself is not fluorescent, but DPPP oxide, resulting products of the reaction with hydroperoxides, is fluorescent with high fluorescence yield. This is possible because, DPPP molecules are incorporated easily into the cell membranes and hydroperoxides (oxidation products of lipids) preferably react with DPPP (Rajapakse et al., 2007). After 6 h of treatment with AAPH, more than threefold increment of DPPP oxide fluorescence was observed in the cells (Figure 11 and Figure 12).

This increment clearly indicated accelerated lipid peroxidation in cell membranes and G-COS could reduce DPPP oxide fluorescence dose-dependently by inhibiting lipid peroxidation. G-COS could inhibit membrane lipid peroxidation dose-dependently even at low concentrations.

In addition to DPPP oxide fluorescence method, thiobarbituric acid reactive substances (TBARS) assay, one of the most popular biochemical analysis methods for the assessment of lipid peroxidation products, was also used to evaluate membrane lipid peroxidation. In this assay, membrane lipid peroxidation was initiated by generating hydroxyl radicals (*OH) via Fenton reaction as described in the section materials and methods (Rajapakse et al., 2007). As we expected, G-COS could reduce TBARS in the cells to a similar pattern observed in the DPPP oxide fluorescence assay (Figure 13 and Figure 14). Moreover, G-COS had higher inhibitory effects compared to COS at all concentrations. Based on the results of present study, we can presume that G-COS inhibit cellular lipid peroxidation by scavenging ROS. In addition, the higher lipid peroxidation inhibitory effect of G-COS can be expected due to improvement of proton donation by the gallyl group.

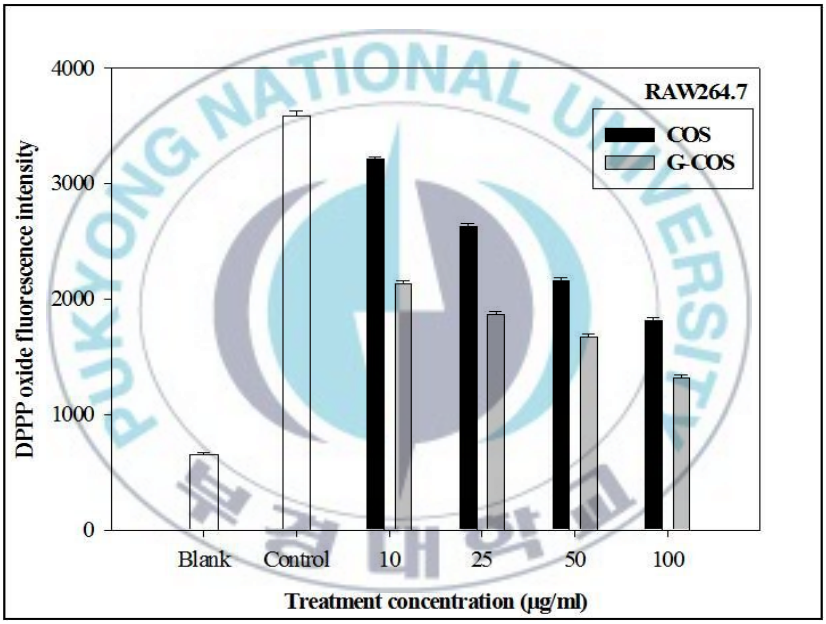


Figure 11. Effect of COS and G-COS on membrane lipid peroxidation inhibition was assessed by DPPP fluorescence assay. RAW264.7 cells treated with different concentrations of COS and G-COS were exposed to carbon-centered radical generating agent, AAPH, to initiate membrane lipid peroxidation. DPPP oxide fluorescence emitted to oxidation of DPPP by lipid hydroperoxides was compared with AAPH non-treated blank group and AAPH alone-treated control group. Results are means ± standard error of three independent experiments.

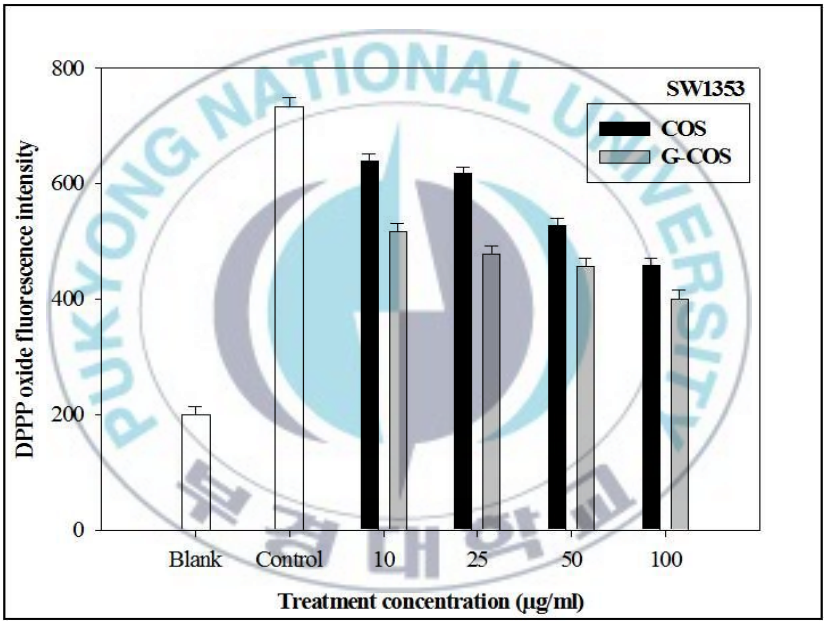


Figure 12. Effect of COS and G-COS on membrane lipid peroxidation inhibition was assessed by DPPP fluorescence assay. SW1353 cells treated with different concentrations of COS and G-COS were exposed to carbon-centered radical generating agent, AAPH, to initiate membrane lipid peroxidation. DPPP oxide fluorescence emitted to oxidation of DPPP by lipid hydroperoxides was compared with AAPH non-treated blank group and AAPH alone-treated control group. Results are means ± standard error of three independent experiments.

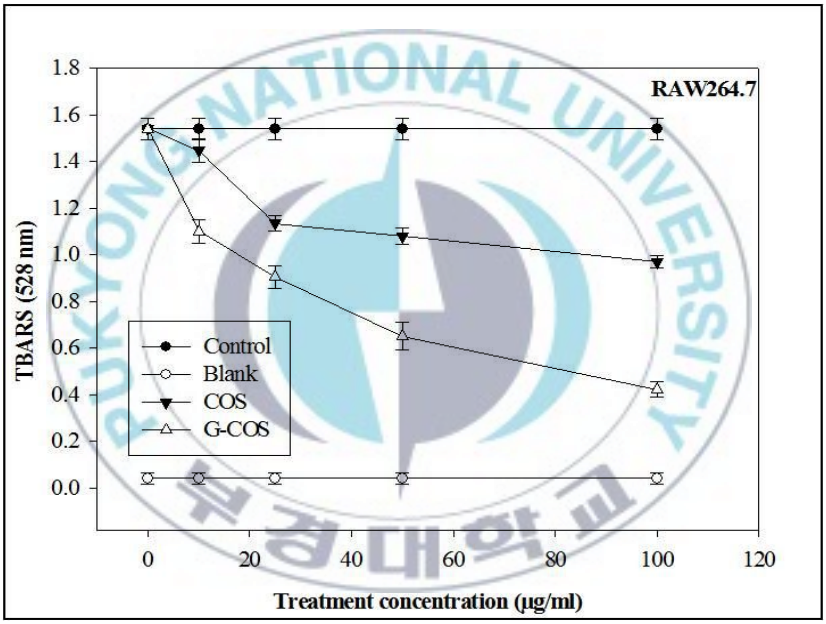


Figure 13. Membrane lipid peroxidation was determined by TBARS method. RAW264.7 cells were exposed to *OH generated via Fenton reaction and oxidation products of membrane lipids which can react with TBA were determined spectroscopically at 528 nm. Effect of COS and G-COS on oxidation inhibition of membrane lipids was compared with *OH non-treated blank group and *OH alone-treated control group in three independent experiments and are presented as means ± SE.

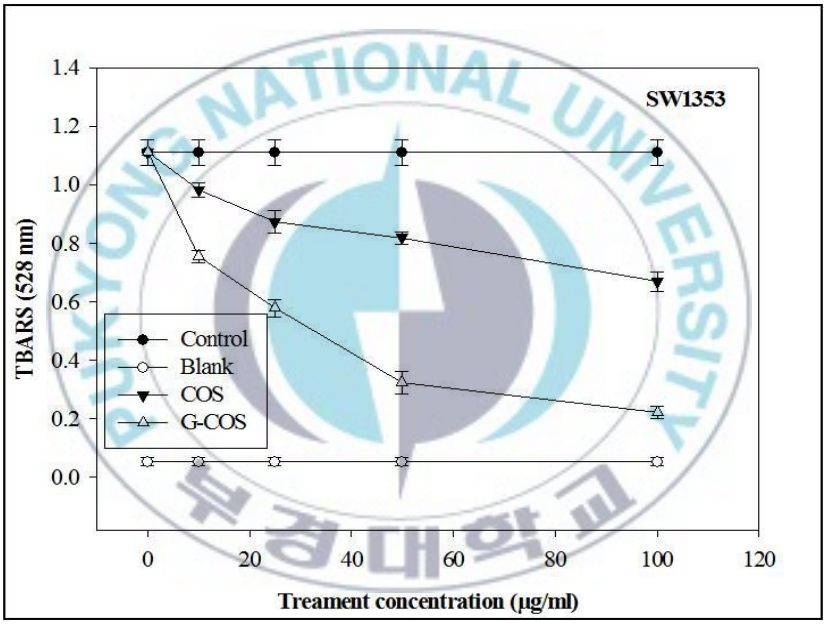


Figure 14. Membrane lipid peroxidation was determined by TBARS method. SW1353 cells were exposed to *OH generated via Fenton reaction and oxidation products of membrane lipids which can react with TBA were determined spectroscopically at 528 nm. Effect of COS and G-COS on oxidation inhibition of membrane lipids was compared with *OH non-treated blank group and *OH alone-treated control group in three independent experiments and are presented as means ± SE.

8. Effect on antioxidant enzymes of G-COS

To evaluate whether the intracellular radical scavenging activities of G-COS are mediated by activity of an antioxidant enzyme, the mRNA and protein expressions of antioxidant enzymes such as SOD and GSH were evaluated using RT-PCR and western blot analysis, respectively in RAW264.7 cells (Figure 15) and SW1353 cells (Figure 16). All the mRNA and protein expressions of antioxidant enzymes were dose-dependently elevated by treatment with G-COS.

9. Expression and nuclear translocation of NF-κB in the presence of G-COS

Stimulation with H_2O_2 caused a clear increase in protein levels of p50 and p65, NF-κB family members in RAW264.7 and SW1353 cells. Lower NF-κB levels were observed in plasma proteins of G-COS treated RAW264.7 and SW1353 cells compared to that of cells treated with H_2O_2 alone (Figure 17A and Figure 18A). The reduction followed a dose dependent pattern and the maximal response in both p50 and p65 protein levels were observed when cells treated with 100 μg/ml concentration of G-COS. Further, a clear reduction in translocation of NF-κB to nucleus in the presence of G-COS was observed when nuclear extracts were analyzed for both p50 and p65 levels (Figure 17B and Figure 18B).

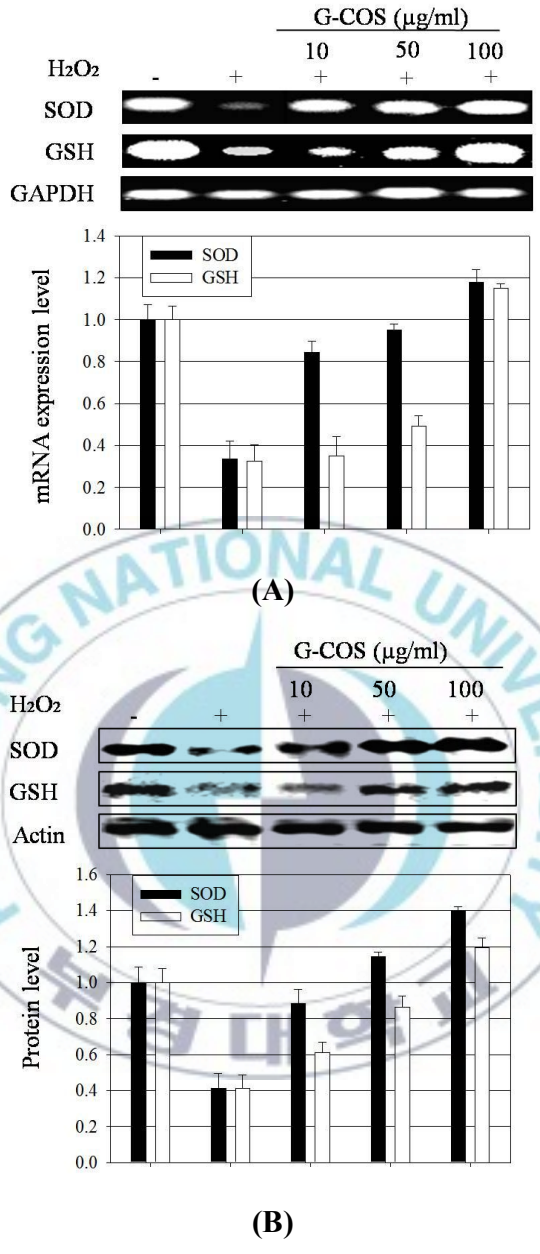
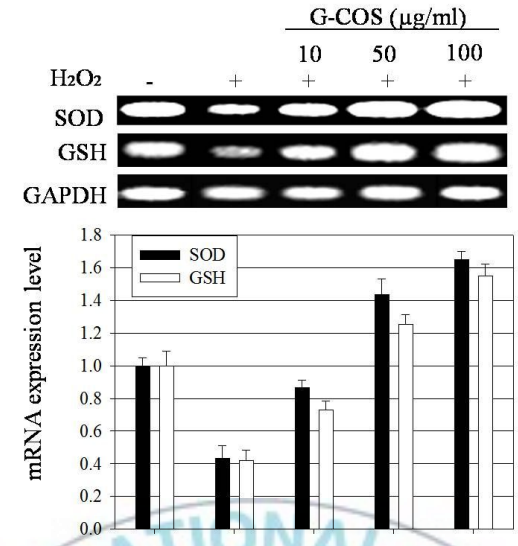
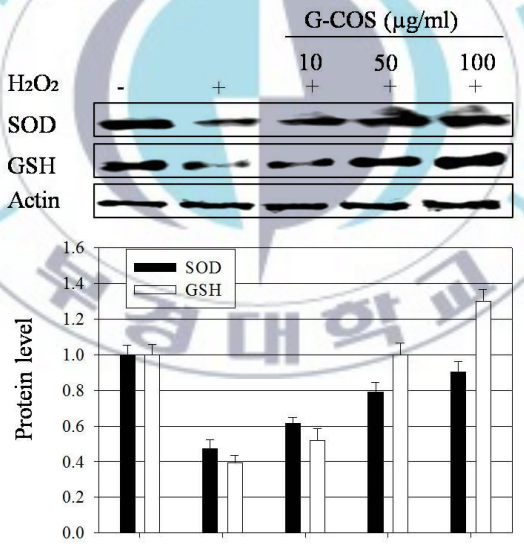


Figure 15. Effect of G-COS on hydrogen peroxide-induced antioxidant enzymes in RAW264.7 cells at the different concentrations (10, 50 and 100 μg/ml) and compared with H_2O_2 non-treated group and H_2O_2 alone-treated group. The expression levels of mRNA and protein were determined using RT-PCR (A) and western blot (B) analysis, respectively.



(A)



(B)

Figure 16. Effect of G-COS on hydrogen peroxide-induced antioxidant enzymes in SW1353 cells at the different concentrations (10, 50 and 100 μg/ml) and compared with H_2O_2 non-treated group and H_2O_2 alone-treated group. The expression levels of mRNA and protein were determined using RT-PCR (A) and western blot (B) analysis, respectively.

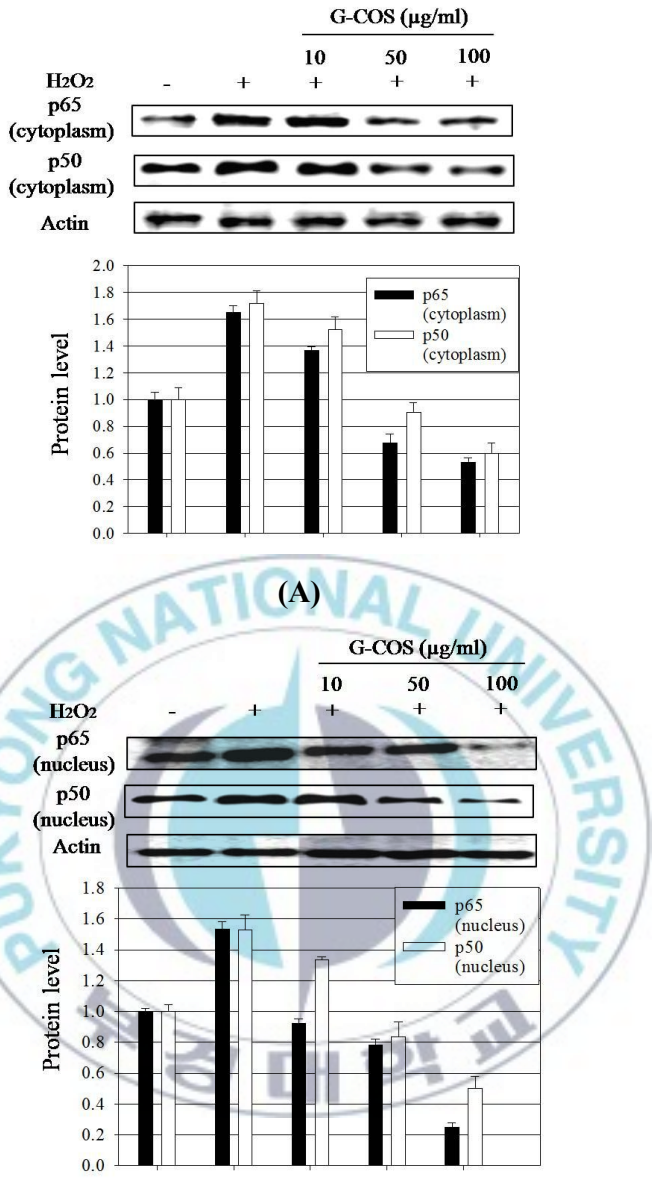
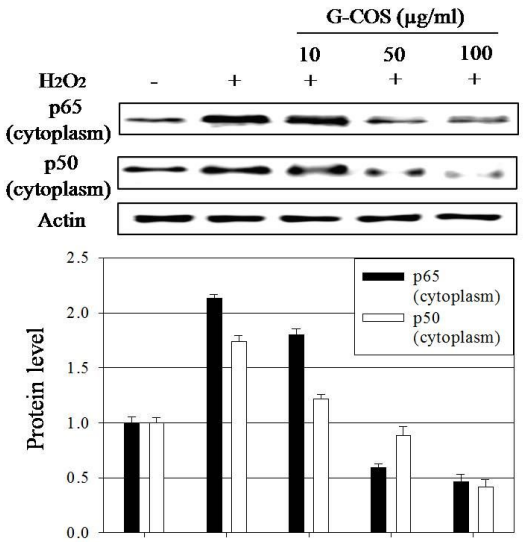
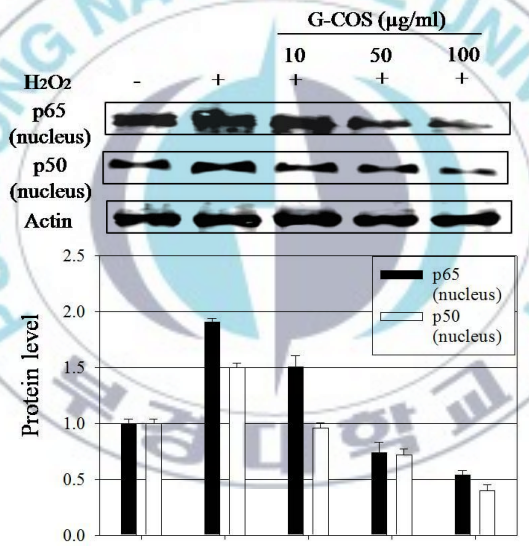


Figure 17. Western blot analysis of p65 and p50 protein expressions in RAW264.7 cells stimulated with 500 μM H_2O_2. After treatment of G-COS, proteins in cytosol and nucleus were extracted and resolved by denaturing SDSPAGE. Proteins (20 μg) were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of actin was used as control for equal loading of protein.



(A)



(B)

Figure 18. Western blot analysis of p65 and p50 protein expressions in SW1353 cells stimulated with 500 μM H_2O_2. After treatment of G-COS, proteins in cytosol and nucleus were extracted and resolved by denaturing SDSPAGE. Proteins (20 μg) were diluted in sample buffer and resolved in 4-20% Novex gradient gel. Proteins were transferred onto nitrocellulose membranes and incubated with primary and secondary antibodies respectively. Western blot bands were developed with enhanced chemiluminescence reagents and visualized using LAS3000® Luminescent image analyzer. Detection of actin was used as control for equal loading of protein.

Discussion

From the results of this study, it was clear that both COS and G-COS act to prevent oxidation of membrane lipids in RAW264.7 and SW1353 cells in different potencies. Clear results were obtained for inhibition of membrane lipid peroxidation in the presence of G-COS when RAW264.7 and SW1353 cells were subjected to diphenyl-1-pyrenylphosphine (DPPP) assay, thiobarbituric acid reactive substances (TBARS) assay. Macrophages are particularly vulnerable to oxidative damage due to the high degree of polyunsaturated fatty acids in their membranes leading to formation of lipid peroxidation products. Further, lipid peroxidation of the cell membrane has been considered to be critically involved in a variety of pathological events including osteoarthritis (De Valk & Marx, 1999; Heller et al., 1998). Therefore, the ability of G-COS to act against lipid oxidation is an interesting future observed in this study. However, the activity difference observed with the treatment of G-COS and COS was suggested due to the occupancy of the gallyl group that offers the unique opportunity to have a substantial affinity to lipids thereby inhibit the lipid peroxidation. The nature and spatial arrangements are causative factors that account for affinity of the residues to lipids that required for successful inhibition of lipid peroxidation.

The reactive oxygen species mediated damage to proteins modifies amino acids (lysine, arginine, proline and histidine) generating carbonyl moieties, which has been identified as an early marker for protein oxidation and are used as a measure of protein damage. Damage to proteins especially proteins in cell membranes is plausible cause to negatively regulate normal cell growth and functions depending on the extent of damage. Moreover, oxidized protein levels have been reported to be higher in osteoarthritis patients. Olszowski et al. (2003) have reported that HOCl/OCl^-, a product of activated neutrophils modify the structure of collagen type II which is the major protein of joint cartilage in osteoarthritis. Our results demonstrated that G-COS as well as COS are capable of protecting proteins in RAW264.7 and SW1353 cells from H_2O_2 derived oxidation. Since the concentration of H_2O_2 used for these experiments were not toxic and did not affect cell proliferation, we could confirm that the oxidation inhibitory effects were purely due to effects exerted by the test compounds.

Interestingly, we found that G-COS involved in protecting DNA damage mediated through *OH produced from the concerted action of H_2O_2 and Fe (II). In this study, genomic DNA was isolated from RAW264.7 and SW1353 cells to study protective effects of G-COS against DNA oxidative damage. In cartilages, chondrocytes synthesize various kinds of proteoglycans which, together with a collagen matrix form a supramolecular complex giving to this tissue a biological spring function able to resist to high compressive forces, in addition of being involved in tissue growth and remodeling. The number and the proper functionality of chondrocytes guarantee the anatomical and biological features of the cartilage. It is important to stress that, even though a high inhibition of DNA oxidation was observed in the presence of G-COS, our results do not show physical association of G-COS to DNA to achieve its protection against oxidative assault. In line with the fact that positively charged nature is required to form a complex with DNA, the net negative charge of G-COS does not confer it's binding to negatively charged DNA backbone. However, even with the comparatively high net positive charge it does not seem that COS make an effective interaction between DNA to protect against *OH mediated DNA oxidation. Moreover, despite the inability to bind with DNA, this novel property of G-COS to protect DNA damage caused by oxidative stress provides a focus on the mechanism of direct scavenging of hydroxyl radicals.

Therefore, direct measurement of scavenging of reactive oxygen species was assessed using 2′,7′-dichlorofluorescin diacetate (DCFH-DA) assay to confirm the radical scavenging effect G-COS and COS. To measure specifically *OH scavenging activity of tested compounds in cell system, mouse macrophages and human chondrosarcoma were labeled with DCFH-DA and Fe(II)/H_2O_2 was added to the system. Esterases in living cells hydrolyze DCFH-DA to dichlorofluorescin (DCFH), which is trapped within the cell. This nonfluorescent molecule is then oxidized to fluorescent dichlorofluorescin (DCF) mainly by action of cellular *OH. Confirming our assumption, in the presence of G-COS high radical scavenging effect was observed. However, a substantial effect was not observed in the presence of COS. These effects were further confirmed by direct scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, superoxide and carbon-centered radicals generated during their interaction using electron spin resonance (ESR) in cell-free systems. Since stable radicals accumulate in the system, spin-trapping is an

integrative method of measuring radical scavenging effects. Also results are accurate than that observed in the presence of instantaneous or steady state generation of free radicals. Exactly similar trend was observed with direct ESR methods. G-COS has exhibited its potency to scavenge different radicals in the order of hydroxyl, DPPH, superoxide and carbon-centered radicals. These results indicated that G-COS directly participated in scavenging some radicals. Moreover, in accordance with other related studies it was probable to conclude that G-COS involves in scavenging of radicals through improved proton donation (Xing et al., 2005).

Moreover, the present study describes the effect of G-COS on NF-κB nuclear translocation in an oxidation induced environment. Further, pre-incubation with G-COS inhibited both p50 and p65 protein migration to the nucleus. These results seem particularly important since NF-κB has been linked to joint cartilage destruction during inflammation. NF-κB activation prior to the onset of clinical manifestations of arthritis has been suggested based on results obtained using animal models of inflammatory arthritis supporting the concept that NF-κB plays a very active role in the development and progression of arthritis in vivo (Makarov, 2001; Mor et al., 2005). Therefore, the results of this study further strengthen the capability of G-COS to alter NF-κB activity particularly in an environment stimulated with reactive oxygen species.

Consistent with a role for oxidant stress in pathogenesis of osteoarthritis, a number of clinical and preclinical studies have correlated increases in markers of oxidative stress and lower levels of body's natural antioxidants such as superoxide dismutase (SOD) and glutathione (GSH) with disease severity (Gambhir et al., 1997). Treatment with G-COS time-dependently and dose-dependently induced the increased GSH level. GSH level was also increased slightly with the treatment of G-COS. The increase in GSH level protects cells against death either by eliminating free radicals or by making conjugation with toxicants (Sanchez-Reus et al., 2005). These results suggested that G-COS could be effective against scavenging radicals by increasing cellular GSH level. SOD works as an antioxidant because it outcompetes the damaging reactions of superoxide. Therefore, it protects cells from the toxicity of superoxide. As such, SOD is one the principal reactive oxygen species in cells, and is therefore a very important antioxidant.

The results of this study demonstrated that SOD and GSH are up-regulated in the presence of G-COS and this ability promotes their potency to act against intracellular oxidative stress. Also induction of SOD and GSH level at high concentrations of G-COS were dynamically parallel to inhibition of radical damage in macrophages and chondrosarcoma.

The data indicate that G-COS acts as a symptom and a structure modifying agent in the treatment of osteoarthritis. However, the mechanisms associated with these ways of action for G-COS are still not totally clarified. One could potentially extrapolate from these data to partially explain the reported comparative beneficial effects of G-COS in arthritis compared to COS. Therefore, these results contribute to clarify the mechanisms of actions of G-COS in cells involved in arthritis. Taken together, these results suggest that oxidation inhibition potential of G-COS is another facet of major biological relevance to control osteoarthritis. Therefore, we can conclude that if these data can be clinically translated, the therapeutic potential of G-COS can be expanded considering its effect as oxidative damage inhibitory agent with a great biocompatibility.

Conclusion

The present work has demonstrated a novel antioxidant (G-COS) by conjugating chitooligosaccharides (COS) with gallic acid. G-COS was successfully synthesized by grafting gallyl groups with COS at C2, or C3 and C6 position. Moreover, G-COS has identified for the chemical structure by FT-IR, ^{1}H NMR and ^{13}C NMR. In this study, a facile way to modify the structure of COS has applied and thereby improved its antioxidant activity.

The findings of this research provide a new understanding on free radical scavenging properties of COS and G-COS on hydroxyl radical, superoxide radical, DPPH radical and carbon-centered radical. COS and G-COS are non cytotoxic in RAW264.7 and SW1353 cells. The emphasis placed on the force for COS and G-COS to prevent damaging effects of free radicals on DNA, membrane proteins and membrane lipids in RAW264.7 and SW1353 cells. In addition, G-COS has increased the level of antioxidant intracellular enzymes (SOD and GSH) and suppressed the NF-κB activation in H_2O_2-induced RAW264.7 and SW1353 cells.

In conclusion, G-COS and COS have potential free radical scavenging effects by both indirect and direct ways to reduce and prevent damage to biological molecular from free radicals in live cells. COS and G-COS have shown to be effective in antioxidant activity, which G-COS exhibited a higher antioxidant activity than COS. They can be used as scavengers for controlling free radicals that lead to damage of cell systems. Collectively, G-COS can be used as a potential compound-based natural antioxidant in functional foods and pharmaceutical industries.

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