Thesis for the Degree of Master of Science

The potential role of gallic acid-*grafted*-chitooligosaccharides in suppression of AGS human gastric cancer cell proliferation

by

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Specialized Graduate School of Science & Technology Convergence

Pukyong National University

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Abstract

Gastric cancer is the second most common cause of cancer-related deaths in the world. In this study, a bioactive derivative of chitooligosaccharides, named gallic acid-*grafted*-chitooligosaccharides (G-COS), was evaluated for its capabilities against the proliferation of AGS human gastric carcinoma cell line. It was found that G-COS treatment caused significant inhibition on gastric cancer cell growth at concentration of 200 and 400 μg/ml. The inhibitory effect of G-COS was evidenced via inducing apoptosis. G-COS-induced cell death was identified by cell viability assay, changes in cell and nuclear morphology, DNA fragmentation, apoptosis analysis and cell cycle analysis. Notably, G-COS-induced apoptosis was related to the increase in the expression of p53, p21, Bax, cytochrome c, caspase (-9 and -3), cleaved PARP, and the decrease in the activation of Bcl-2, p-IκB-α and NF-κB (p50 and p65). These findings indicate that G-COS has a promising potential to be applied in the treatment of gastric cancer as cancer chemopreventive agents.

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List of Abbreviations

^{13}C NMR	^{13}Carbon Nuclear Magnetic Resonance
^{1}H NMR	Proton Nuclear Magnetic Resonance
Apaf-1	Apoptotic protease activating factor-1
Bax	Bcl-2–associated X protein
Bcl-2	B-cell lymphoma-2
cDNA	Combinant deoxyribonucleic acid
Caspase	Cysteine-dependent aspartate-directed proteases
COS	Chitooligosaccharide
DCC	Dicyclohexylcarbodiimide
DIABLO	Direct inhibitor of apoptosis protein (IAP)-binding protein with low PI
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
FACS	Fluorescence-activated cell sorting
FADD	Fas-associated protein with death domain
FBS	Fetal Bovine Serum

FT-IR	Fourier transform infrared
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
G-COS	Gallic acid-*grafted*-chitooligosaccharides
HtrA2	High-temperature requirement protein A2
mRNA	Messenger Ribonucleic Acid
MPT	Mitochondrial permeability transition
MTT	3-(4,5-dimethyl-2-yl)-2,5-diphenyltetrazolium bromide
NF-κB	Nuclear factor-kappaB
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
RT-PCR	Reverse transcription-Polymerase chain reaction
SD	Standard Deviation
SDS	Sodium dodecyl sulfate
Smac	Second mitochondria-derived activator of caspase
TBS-T	Tris-buffered saline and tween 20
TNF	Tumor necrosis factor
TRADD	TNF-receptor associated death domain
TRAIL	TNF-related apoptosis inducing ligand

1. Introduction

1.1. Chitin, chitosan and chitooligosaccharides

Chitin-chitosan is the second most abundant natural biopolymer, next to cellulose, and has been received many attention because of a lot of their applications in various fields.

Chitin, a long chain polymer of *N*-acetylglucosamine ((1→4)-linked 2-acetamido-2-deoxy-β-D-glucan), is a cellulose-like biopolymer exists in the exoskeletons of arthropods and insects, the radulae of molluscs, the cell walls of fungi [1].

Chitosan, a partially deacetylated polymer of *N*-acetylglucosamine, is derived from chitin by deacetylation in the presence of alkali. Chitosan and its derivatives have been applied in many fields (Table 1) including, food, agriculture and cosmetics [1-4]. Especially they have been used in the biomedical and pharmaceutical industries because of their various biological activities [5]. Even though chitosan is known to have many beneficial functional activities, their physicochemical properties such as high molecular size and poor solubility makes them difficult to be absorbed into body. So, sometimes those properties of chitosan have been an obstacle to their wide applications.

Chitooligosaccharides (COS) are chitosan derivatives and can be formed via chemical or enzymatic hydrolysis of chitosan (Figure 1). Compare to chitin-chitosan, COS have lower viscosity, relatively small molecular sizes and short

chain lengths, and are soluble in neutral aqueous solutions. Therefore, they can be easily absorbed *in vivo* system. Because of these property, COS is expected to show better effect than chitosan. Actually, it has been reported that COS have diverse biological functions such as antitumor, antioxidant, antimicrobial, antibacterial, antidiabetic, anti-Alzheimer's and hypocholesterolemic activities [6-15]. Recently, chemically modified COS have attracted a lot of attention because of their enhanced bioactivities [16].

Table 1. Application of chitin and chitosan

Field	Applications
Medicine and pharmaceutics	- Artificial skin / Artificial ear drum - Wound-healing ointment and dressings - Contact lenses - Dentistry - Orthopedic surgery - Drug and gene carriers - Various medicine (antitumor agents, hypocholesterolemic drugs, antibacterial / antifungal drugs, anticoagulant, immunological stimulants…etc)
Food and beverages	- Dietary fiber - Food additive - Food preservation and stabilizing
Cosmetics and toiletries	- Skin care - Oral care - Hair treatment
Biotechnology	- Nanoparticles - Membrane separation - Immobilization of enzyme and cell
Agriculture	- Seed coating - Pollution-free pesticide - Feed ingredients for animal
Other areas	- Dye - Film - Clothes - Waste water treatment

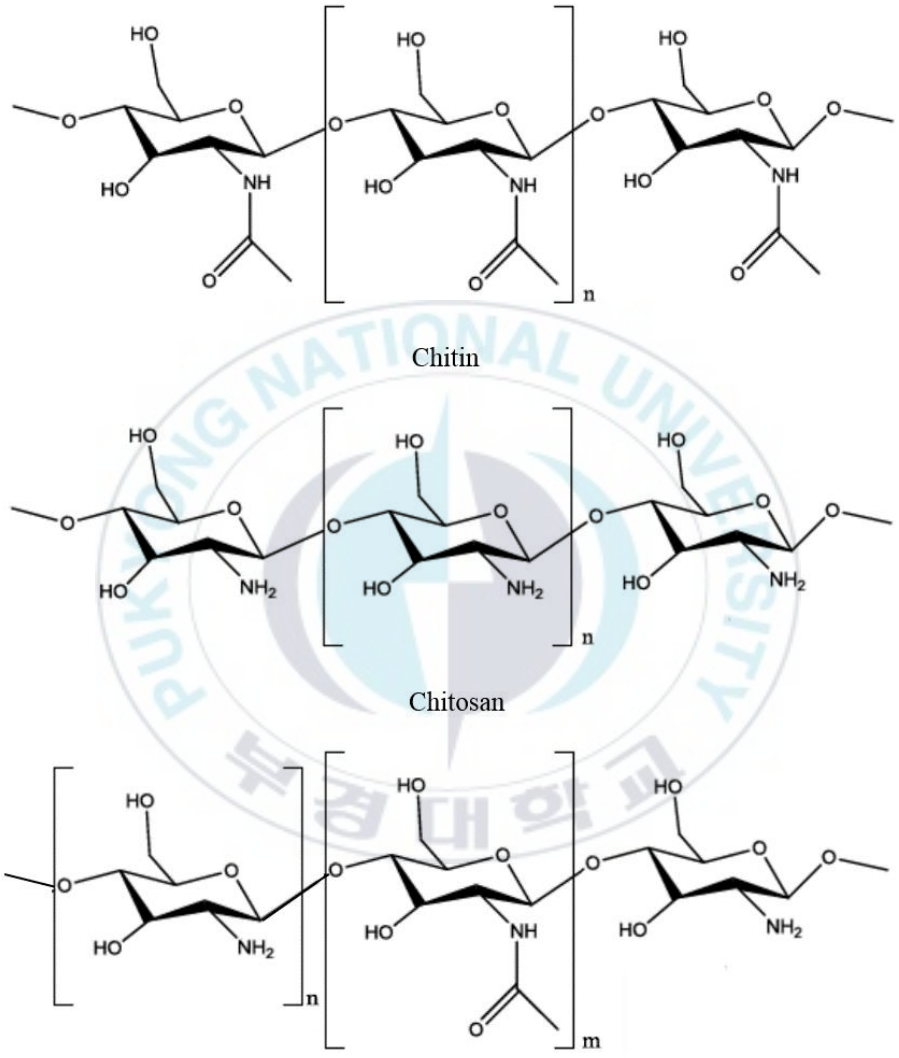


Figure 1. Structures of chitin, chitosan and chitooligosaccharide

1.2. Gallic acid

Polyphenols are structurally various group of compound especially in plants and characterized by the presence of large multiples of phenol structural units [17]. Several polyphenols have some beneficial properties such as antioxidant, anti-inflammatory, and anticarcinogenic effects [18]. Gallic acid (3,4,5-trihydroxybenzoic acid), a well known polyphenol, found abundantly in gallnuts, sumac, tea leaves, oak back, wines, grapes, strawberries and other plants or fruits. Gallic acid also have many biological and pharmacological activities, including antioxidant, antibacterial, anti-inflammatory [19-22]. However, the main interest in gallic acid and its derivatives is related to its anticancer activity about various tumor types. In fact, anticancer activity of gallic acid has been reported in many cancer cells, such as stomach cancer, colon cancer, breast cancer, cervical cancer, prostate cancer, lung cancer, and leukemia [23-26]. Furthermore, gallic acid showed the selective cytotoxicity for multiple cancer cells and has relatively less toxicity to normal cells [27]. Some studies suggest that apoptosis induced by gallic acid and its derivatives is concerned with oxidative metabolism alteration, mitochondrial dysfunction, DNA fragmentation and an increase in intracellular ca^{2+} levels causing caspase activation [28]. Therefore, it can be expected that gallic acid-*grafted*-chitooligosaccharides (Figure 2) may show more enhanced anticancer activity. In the present study, we found that gallic acid-*grafted*-chitooligosaccharides (G-COS) enhance the inhibitory effect against AGS human gastric cancer cells.

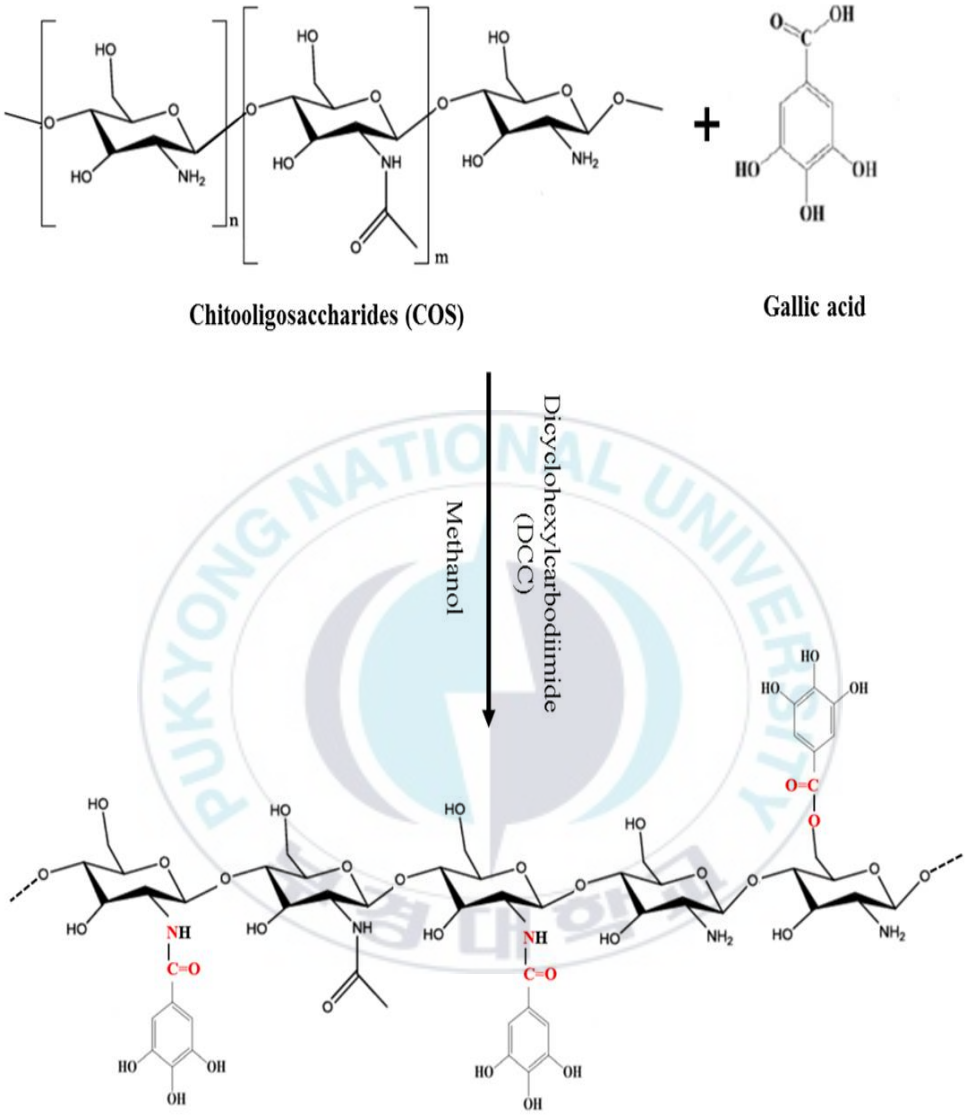


Figure 2. The synthesis of G-COS

1.3. Gastric cancer

Gastric cancer (stomach cancer) is the second leading cause of cancer-related death as one of the most common malignancies with poor prognoses [29]. Especially, the incidence and mortality rates of gastric cancer are highest in Eastern Asia including Korea, China and Japan (Figure 3).

These geographic and ethnic differences in gastric cancer incidence and mortality suggest that gastric cancer is closely related to diet such as ingestion of high salt-preserved food, nitrates, and smoked or pickled food. Besides, nondietary factors such as cigarette smoking and *Helicobacter pylori* infection are also can be a cause of gastric cancer [30].

And this risk could be decreased with a high intake of fresh fruits and vegetables, lower salt diets, the decreased use of cigarette, screening and treatment of *H. pylori* infection [31].

Despite the advanced target therapy and improved understanding of the biology and development of the malignancy, progress in the treatment of gastric cancer has been limited [32]. There are some treatments for gastric cancer such as surgery, endoscopic treatment, chemotherapy and radiotherapy. Except for surgery, most of the cancer therapies are methods to inhibit the growth of cancer cells. Although, the most efficacious remedy for gastric cancer is surgery, many patients have inoperable cancers. Therefore, combination chemotherapy regimens are integral part as primary treatment until now [33].

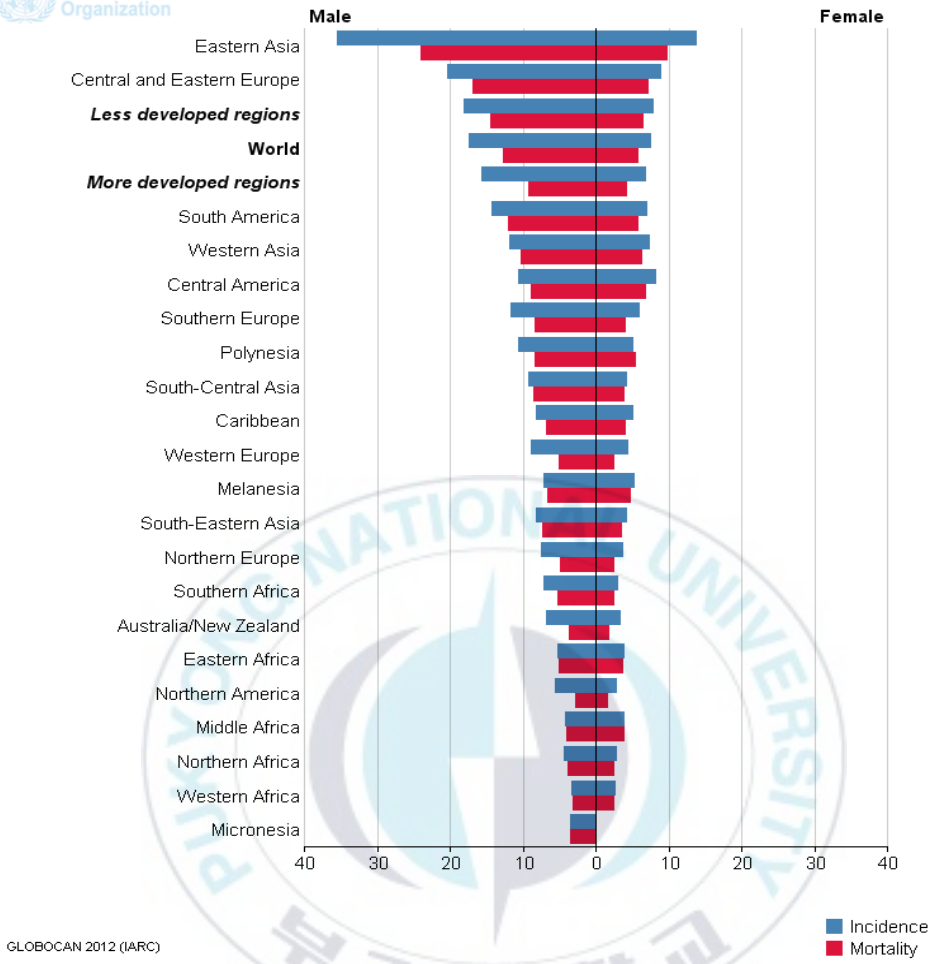


Figure 3. Estimated incidence and mortality for stomach cancer in the world. More than 70% of cases occur in developing countries, and half the world total occurs in Eastern Asia. Age-standardized incidence rates are about twice as high in men as in women, ranging from 3.3 in Western Africa to 35.4 in Eastern Asia for men, and from 2.6 in Western Africa to 13.8 in Eastern Asia for women. The highest estimated mortality rates are in Eastern Asia (24 per 100,000 in men, 9.8 per 100,000 in women), the lowest in Northern America (2.8 and 1.5, respectively).

1.4. Apoptosis

The term “apoptosis” is originated from Greek language meaning “falling of leaves from tree” as compound word of ‘apo- (off)’ and ‘ptosis (falling)’. It was first used by Kerr, Wyllie, and Currie in 1972 to describe a morphologically distinct form of cell death [34, 35]. Apoptosis is often referred to as cell-suicide or programmed cell death and it is known to play an important role in tissue homeostasis, cellular differentiation and development, cancer, immune response by contributing to remove old, superfluous or damaged cells such as virus-infected cells and cancer cells [36].

Apoptosis can be triggered by various external and internal signals, and characterized by typical morphology and biochemical hallmarks, such as chromatin condensation, cell shrinkage, DNA fragmentation, membrane blebbing and apoptotic body formation [37, 38].

Apoptosis play an active role in cells own death, so cell death via induction of apoptosis in cancer cells is considered as one of cancer preventive and therapeutic strategies.

1.4.1. Morphology of apoptosis

Light and electron microscopy have identified the diverse morphological changes that occur during apoptosis. The beginning of apoptosis is characterized by shrinkage of the cell and the nucleus as well as condensation of nuclear chromatin. And later on, the nucleus progressively condense and breaks up (karyorrhexis), also protrusions from the plasma membrane, usually referred to

as ‘blebs’, can be happened (budding phenomenon). After this process, ‘apoptotic body’ was formed, and the apoptotic bodies are phagocytosed into neighbouring cells, including macrophages and parenchymal cells [39, 40].

1.4.2. Pathways of apoptosis

There are two major apoptotic signaling pathways: one is extrinsic pathway (death receptor pathway) and the other is intrinsic pathway (mitochondrial pathway). (Figure 4). The extrinsic pathway is affected by activation of death receptors on the cell surface. In contrast, the intrinsic pathway is regulated by the interplay between pro- and anti-apoptotic members of Bcl-2 family [41].

Two pathways of apoptosis, extrinsic pathway and intrinsic pathway are linked to each other, and that molecules in one pathway can influence the other [42].

These two apoptosis pathways are both related to the activity of protease, caspase. Activation of caspase-3 results in DNA fragmentation, degradation of cytoskeletal and nuclear proteins, crosslinking of proteins, formation of apoptotic bodies, and it leads to cell death [35].

Extrinsic pathway is initiated through activation of death receptors such as Fas, tumor necrosis factor (TNF), and TNF-related apoptosis-inducing ligand (TRAIL) receptors (R). To date, well known combination of ligands and corresponding death receptors involved in cell death are FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4, Apo2L/DR5 [35].

The process of apoptosis by extrinsic pathway is best characterized with the FasL/FasR and TNF-α/TNFR1 models. In these models, the binding of ligand to receptor results in the binding of the adapter proteins such as Fas-associated protein with death domain (FADD) or TNFRSF1A-associated via death domain (TRADD),and transmit apoptotic signals into the cell.

The transmitted signals induce apoptosis via formation of death-inducing signaling complex (DISC) and activation of caspase-8 which leads to the activation of downstream effector caspases such as caspase-3 [35, 43].

Intrinsic pathway is triggered by various stimuli such as DNA damage, hypoxia, viral infections, free radicals, or other cellular stresses caused by chemotherapy or ionizing radiation [44].

These stimuli cause changes in the inner mitochondrial membrane that results in an opening of the mitochondrial permeability transition (MPT) pore, loss of the mitochondrial transmembrane potential and release proapoptotic proteins such as cytochrome *c*, Smac (second mitochondria-derived activator of caspase)/DIABLO (direct inhibitor of apoptosis protein (IAP)-binding protein with low PI), HtrA2/Omi **(**high-temperature requirement protein A2) from the intermembrane space into the cytosol [37,45-47].

The release of cytochrome c into the cytosol triggers caspase-3 activation through formation of the cytochrome c/Apaf-1(apoptotic protease activating factor 1)/caspase-9-containing apoptosome complex, whereas Smac/DIABLO and HtrA2/Omi promote apoptosis by inhibiting IAP activities [37, 48].

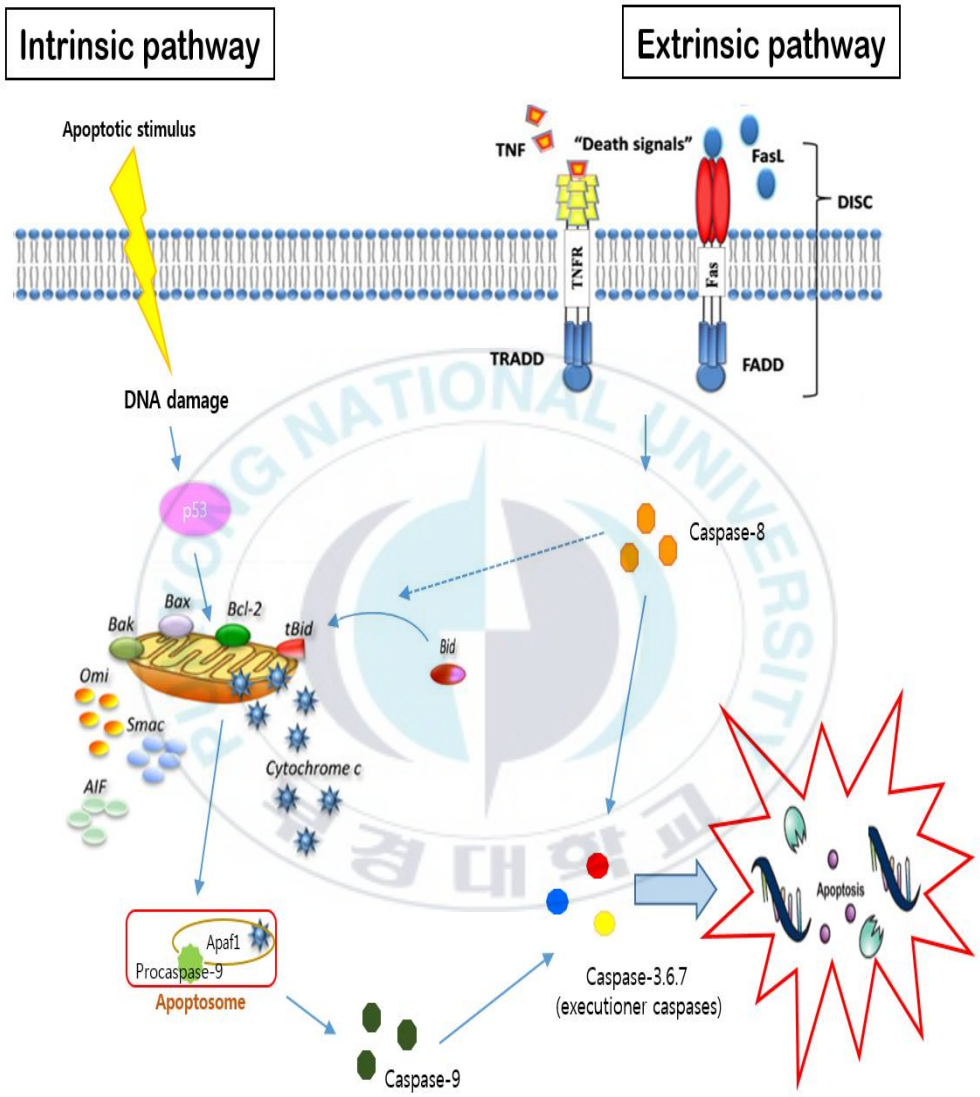


Figure 4. Two kind of apoptosis pathways

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM), 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-diphenyltetrazolium bromide (MTT), agarose and fetal bovine serum (FBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Specific antibodies used for western blot analysis were purchased from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA).

Oligo (dT) 15 primer, M-MLV reverse transcriptase, and GoTaq DNA polymerase were purchased from Promega (Madison, WI, USA). Primers using in this study were obtained from Bioneer (Daedeak-gu, Daejeon, South Korea). All other chemicals were of the highest grade available commercially.

2.2. Synthesis of G-COS

Gallic acid-*grafted*-chitooligosaccharides (G-COS) was produced as described by Ngo et al. (2011) [49]. The synthesis method is as follows : 3-5 kDa COS (2.4814 g) was dissolved in 20 ml distilled water, 40 ml methanol and adjusted to the pH 6.8 with triethylamine to obtain solution A. Gallic acid (0.9404 g) was

dissolved in 10 ml of methanol and mixed with 10 ml of dicyclohexylcarbodiimide (DCC)–methanol mixture (1.0315 g DCC in 10 ml methanol) to obtain solution B. The solution B was gradually added to the solution A while stirring at 30℃and 150 rpm for 5 h, after that, it was filtered to remove dicyclohexyl urea. The reaction mixture was kept at 2℃ overnight and 90 ml diethyl ether was added. Subsequently, the solution was filtered using a filter paper to obtain a precipitate and the precipitate was dissolved in 20 ml of distilled water. The un-reacted gallic acid was removed by using dialysis membrane molecular weight cut off below 1 kDa. Finally, the solution was freeze dried to provide G-COS (1.4882g)

2.3. Cell culture

Human gastric adenocarcinoma cell line AGS was purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured as monolayers in T-75 tissue culture flasks at 5% CO_2 and 37℃ humidified atmosphere using Dulbecco's modified eagle medium (DMEM, Gibco-BRL, Gaithersburg, MD, USA) with 10% fetal bovine serum (FBS) and 100 μg/ml penicillin–streptomycin.

2.4. Cell viability

The effects of G-COS on the viability of AGS were determined using MTT

assay, which is based on the reduction of MTT to MTT-formazan by mitochondrial enzyme. Briefly, 96-well plates were plated with 5 x 10^3 cells/well. After incubation for 24 h, the medium was changed to fresh medium and cells were treated with different concentrations (0, 50, 100, 200, 400 μg/ml) of COS and G-COS for 24 h. 100 μl of MTT solution (1mg/ml) was added to each well, and the cells were incubated for 4h at 37℃.The medium was removed, and 100 μl of DMSO was added to solubilize the formed formazan. Absorbance was determined at 540 nm using a microplate reader (GENios® microlate reader, Tecan Austria GmbH, Austria).The effects of G-COS on cell viability were evaluated by comparing the cell viability of treated cells with that of control cells.

2.5 Morphological analysis and Hoechst staining

AGS cells were grown in 12-well plates, and then cells were treated by COS (400 μg/ml) and G-COS (0, 100, 200, 400 μg/ml) and incubated for 24h. Following, media was discarded and cells were washed twice and fixed in 4% paraformaldehyde (Sigma–Aldrich Corp., St. Louis, MO, USA) in PBS (phosphate buffered saline) for 1h at room temperature. The fixed cells were washed with PBS and their morphological changes were detected by a light microscope (CTR 6000; Leica, Wetzlar, Germany). Furthermore, nuclear deformation was detected by Hoechst 33342 staining.

For Hoechst 33342 assay, AGS cells were treated by various concentrations of

the samples for 24 h. Then, cells were washed twice and fixed in 4% paraformaldehyde in PBS for 1 h at room temperature. After washing with PBS, cells were stained with 1 μg/ml of the fluorescent DNA-binding dye, Bisbenzimide Hoechst 33342 (Sigma–Aldrich Corp., St. Louis, MO, USA) and incubated for 1 h at room temperature to reveal nuclear condensation/aggregation. The Hoechst-stained cells were visualized and photographed under fluorescence microscope (CTR 6000; Leica, Wetzlar, Germany).

2.6. DNA fragmentation

AGS cells were treated with the indicated concentrations of COS and G-COS. After incubation for 24 h, the cells were washed with PBS and lysed on ice in a buffer containing 10 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA and 0.5% Triton X-100 for 20 min. Cell lysates were vortexed and cleared by centrifugation at 1000g for 10 min. After centrifugation, the cells were dissolved in RNase (0.03 mg/ml), NaOAC (0.175 M), proteinase K (0.25 mg/ml) and SDS (0.6%). The mixture was then incubated for 30 min at 37℃ and 1 h at 55℃. Following incubation, fragmented DNA was extracted with an equal volume of neutral phenol:chloroform:isoamylalcohol (25:24:1, v/v/v) and followed by centrifugation at 12,000g for 5 min at 4℃. Then, collected supernatant was mixed with 100% ice cold ethanol at 1:1.5 ratio and kept for 15 min at -20℃. Finally, the mixture was centrifuged at 1200g for 5 min and DNA pellet was

taken. The DNA was dissolved in TE buffer and analyzed electrophoretically on 1.5% agarose gel for 1 h at 100 V. Then, the gels were stained with 1 mg/ml ethidium bromide (EtBr) visualized by UV light using AlphaEase® gel image analysis software (Alpha innotech, CA, USA).

2.7. Cell cycle analysis

AGS cells were cultured in 6-well plates (5 x 10^5 cells/well) at 37℃ in an atmosphere of 5% CO_2 and then the cells were treated with COS (400 μg/ml) and G-COS (0, 100, 200, 400 μg/ml), and incubated for 24 h. The acquired cells were suspended in 300 μl PBS and mixed with 700 μl cold ethanol, and stored at 4℃ for at least 30 min. After centrifugation, the pellet was washed with cold PBS, resuspended in 500 μl PBS and incubated with 50 μl RNase (20 μg/ml) for 30 min. The cells were stained with 50 μg/ml propidium iodide (PI) for 30 min in the dark. The cell cycle was analyzed using a FACSCalibur flow cytometer (Becton – Dickinson Biosciences, San Jose, CA).

2.8. Apoptosis analysis

G-COS-induced apoptosis in AGS cells was determined by flow cytometry using the Annexin V-FITC Apoptosis Detection Kit following the manufacturer's instructions. 5 x 10^5 cells were seeded on 6-well plates and treated with COS (400 μg/ml) and G-COS (0, 100, 200, 400 μg/ml). After 24h,

cells were collected and washed with PBS, and resuspended in 100 μl of binding buffer and then incubated with Annexin V and propidium iodide (PI) in binding buffer at room temperature for 10-15 min in the dark. The stained cell were analyzed using the FACSCalibur flow cytometer (Becton – Dickinson Biosciences, San Jose, CA).

2.9. Western blot analysis

AGS cells were cultured in 6-well plates and treated with COS (400 μg/ml) and G-COS (0, 100, 200, 400 μg/ml) for 24 h. After incubation, collected cells were washed with PBS and lysed in RIPA buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulphate (SDS) and 1% NP-40. Cell debris was removed by centrifugation and supernatant fraction for immunoblotting was collected. Proteins were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto a polyvinylidene fluoride membrane (Amersham Pharmacia Biotech., England, UK) using the TE77 ECL Semi-Dry Transfer Unit (GE Healthcare Life Sciences, Buckinghamshire, UK). The membrane was blocked with 5% BSA (Bovine Serum Albumin) in Tris-buffered saline containing 0.1%Tween20 (TBS, PH 7.6) at room temperature for 4 h, and hybridized with primary antibodies (diluted 1:1000) at 4℃ overnight and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibody at room temperature for 1 h. The immunoreactive protein bands were visualized by chemiluminescent ECL assay

kit (Amersham Pharmacia Biosciences, England, UK) using LAS3000® luminescent image analyzer (Fujifilm Life Science, Tokyo, Japan).

2.10. RNA extraction and reverse transcription-polymerase chain reaction (RT-PCR) analysis

Total RNA from AGS cell line was isolated using a Trizol reagent (Invitrogen Co., CA, USA) according to the manufacturer's manual. 2 μg of total RNA was converted to single stranded cDNA using a Reverse Transcription System (Promega). The target cDNA was amplified using primers, the sequences of the primer for each gene are shown in Table 2. The amplication cycles were carried out at 95℃ for 45 s, 60℃ for 1 min, and 72℃ for 45 s. After 32 cycles, the amplified PCR products were run in 1% agarose gel for 15-20 min at 100V. Finally, gels were stained with 1mg/ml ethidium bromide (EtBr) and visualized by UV light using AlphaEase® gel image analysis software (Alpha innotech, CA, USA).

2.11. Statistical analysis

The data were presented as mean ± standard deviation (SD) of three independent experiments. The statistical significance was achieved when P-values < 0.05.

Table 2. Sequences of the gene specific primers used for RT-PCR

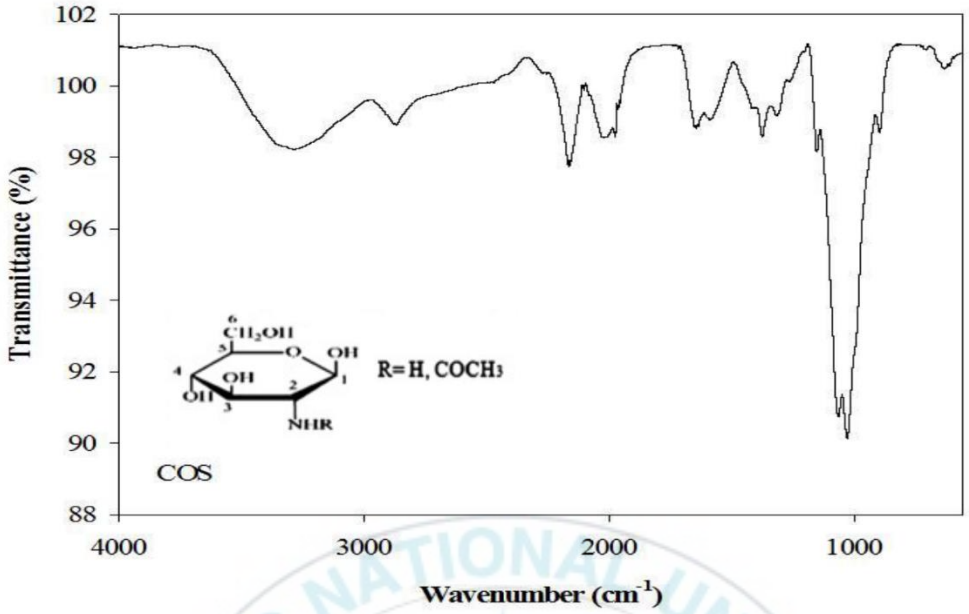
Gene	Direction	Sequence
Bax	Forward	5´-TGC-CAG-CAA-ACT-GGT-GCT-CA-3´
	Reverse	5´-GCA-CTC-CCG-CCA-CAA-AGA-TG-3´
Bcl-2	Forward	5´-CGC-ATC-AGG-AAG-GCT-AGA-GT-3´
	Reverse	5´-AGC-TTC-CAG-ACA-TTC-GGA-GA-3´
Caspase-9	Forward	5´-TGG-ACG-ACA-TCT-TTG-AGC-AG-3´
	Reverse	5´-GCA-AGA-TAA-GGC-AGG-GTG-AG-3´
Caspase-3	Forward	5´-GAA-CTG-GAC-TGT-GGC-ATT-GA-3´
	Reverse	5´-TGT-CGG-CAT-ACT-GTT-TCA-GC-3´
GAPDH	Forward	5´-GAG-TCA-ACG-GAT-TTG-GTC-GT-3´
	Reverse	5´-GAC-AAG-CTT-CCC-GTT-CTC-AG-3´

3. Results and Discussion

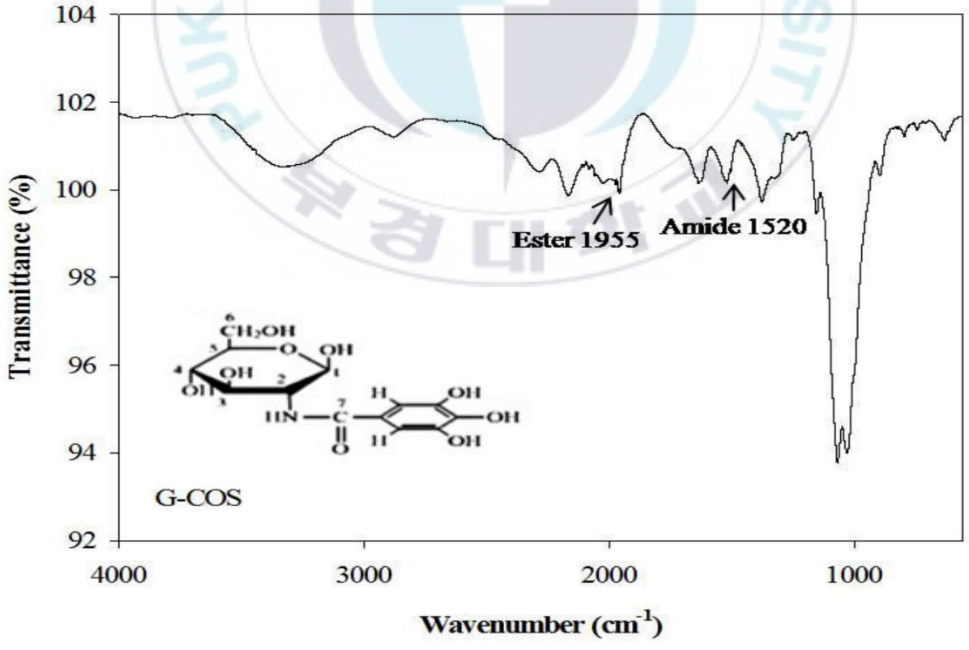
3.1. Structural characterization of G-COS

In the Fourier transform infrared (FT-IR) spectra, G-COS show significant peaks in 1955 and 1520 cm^{-1}, implying the ester and amide linkages between COS and gallic acid (Figure 5). From these results, it can be concluded that the gallyl group of gallic acid was successfully introduced into COS via amide and ester linkages.

Furthermore, the proton nuclear magnetic resonance (^{1}H NMR) spectra, G-COS show a new peak at 6.98 ppm belonging to the phenyl protons as compared with COS (Figure 6A) This confirms the successful conjugation with gallic acid. From ^{13}Carbon (^{13}C) NMR spectra, G-COS show 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 of gallic acid onto COS (Figure 6B).

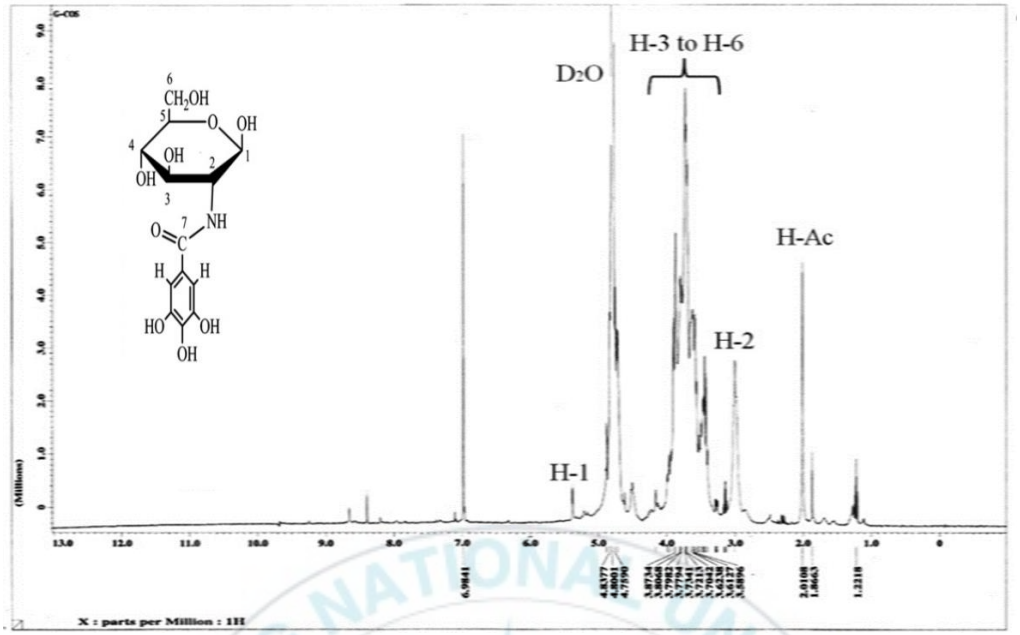


(A)

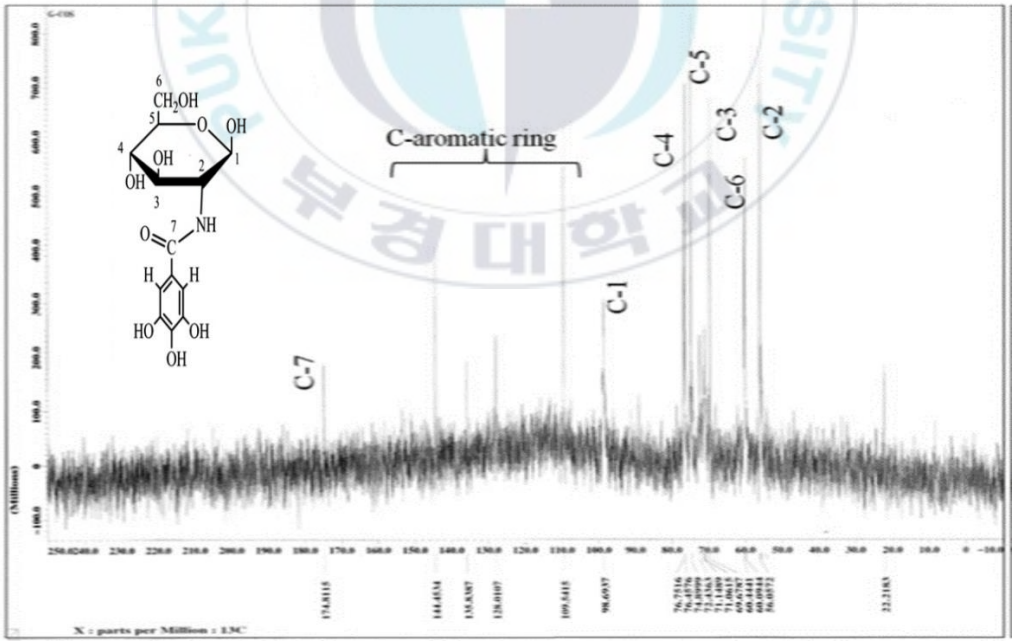


(B)

Figure 5. FT-IR spectra of COS (A) and G-COS (B)



(A)



(B)

Figure 6. ^{1}H NMR spectrum (A), and ^{13}C NMR spectrum (B) of G-COS

3.2. Cell viability

In order to evaluate the effects of G-COS on growth of AGS human gastric cancer cells, the cells were treated with different concentrations (0, 50, 100, 200, 400 μg/ml) of COS and G-COS for 24 h and their viability was determined using the MTT assay (Figure 7). The MTT assay is a colorimetric assay for measuring the activity of cellular enzymes that reduce the tetrazolium dye. MTT is a yellow water-soluble tetrazolium salt, and metabolically active cells can convert the dye to water-insoluble purple formazan by reductive cleavage of tetrazolium ring. Exposure of AGS cells to increasing concentrations of G-COS resulted in a dose-dependent decrease in cell viability relative to control cells. Treatment with COS for 24 h inhibited the proliferation of AGS cells with rates of about 1.4%, 6.3%, 12.4%, 14.6% at concentrations of 50, 100, 200 and 400 μg/ml, respectively. And Treatment with G-COS for 24 h inhibited the proliferation of AGS cells with rates of about 2.3%, 15.7%, 51.2%, 67.3% at concentrations of 50, 100, 200 and 400 μg/ml, respectively. Result of cell viability assay shows that gallic acid significantly enhance the anti-proliferative effect of COS on AGS human gastric cancer cells.

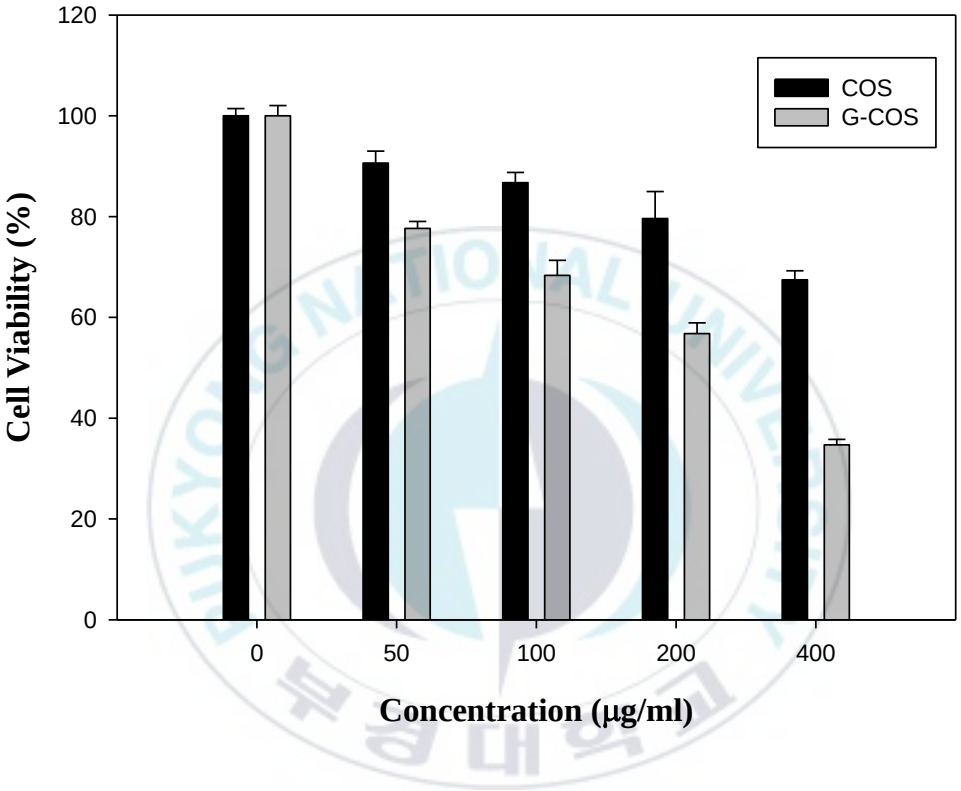


Figure 7. Cell viability of AGS cells treated with different concentration of COS and G-COS. Cells were incubated with testing sample for 24h. The absorbance was measured with 540 nm. Values were expressed as the means ± SD of triplicate experiments.

3.3. Morphological changes and Hoechst 33342 staining

Morphological changes and cell death of AGS cells were characterized using light microscope. Figure 8 shows the morphological changes after 24h exposure to COS (400 μg/ml) and G-COS (0, 100, 200, 400 μg/ml). Also, this method allows us to observe attached cells which are alive. According to the results, the overall cell density were significantly reduced with increasing doses of G-COS. Especially under high concentration of G-COS (200, 400 μg/ml) exposure, irregular shape of cells, and reduction in the number of cells fixed on the bottom of the flask was observed.

Furthermore, to determine the DNA damage induced by the G-COS on AGS cells, DNA staining dye, Hoechst 33342 was used. DNA staining with certain dyes permits the direct observation of the condensation of the cell nucleus. AGS cells were stained with DNA staining dye, Hoechst 33342 dye after 24 h sample treatment and the morphological changes of nuclear were observed under fluorescence microscope in Figure 8. The nuclear degradation of AGS cells significantly observed by fluorescence lighting. Hence the nuclei with chromatin condensation and apoptotic bodies were observed in the cells exposed to G-COS with increasing of concentrations. And also, the results shows that G-COS has enhanced anti-proliferative effect on AGS cells when compare with COS.

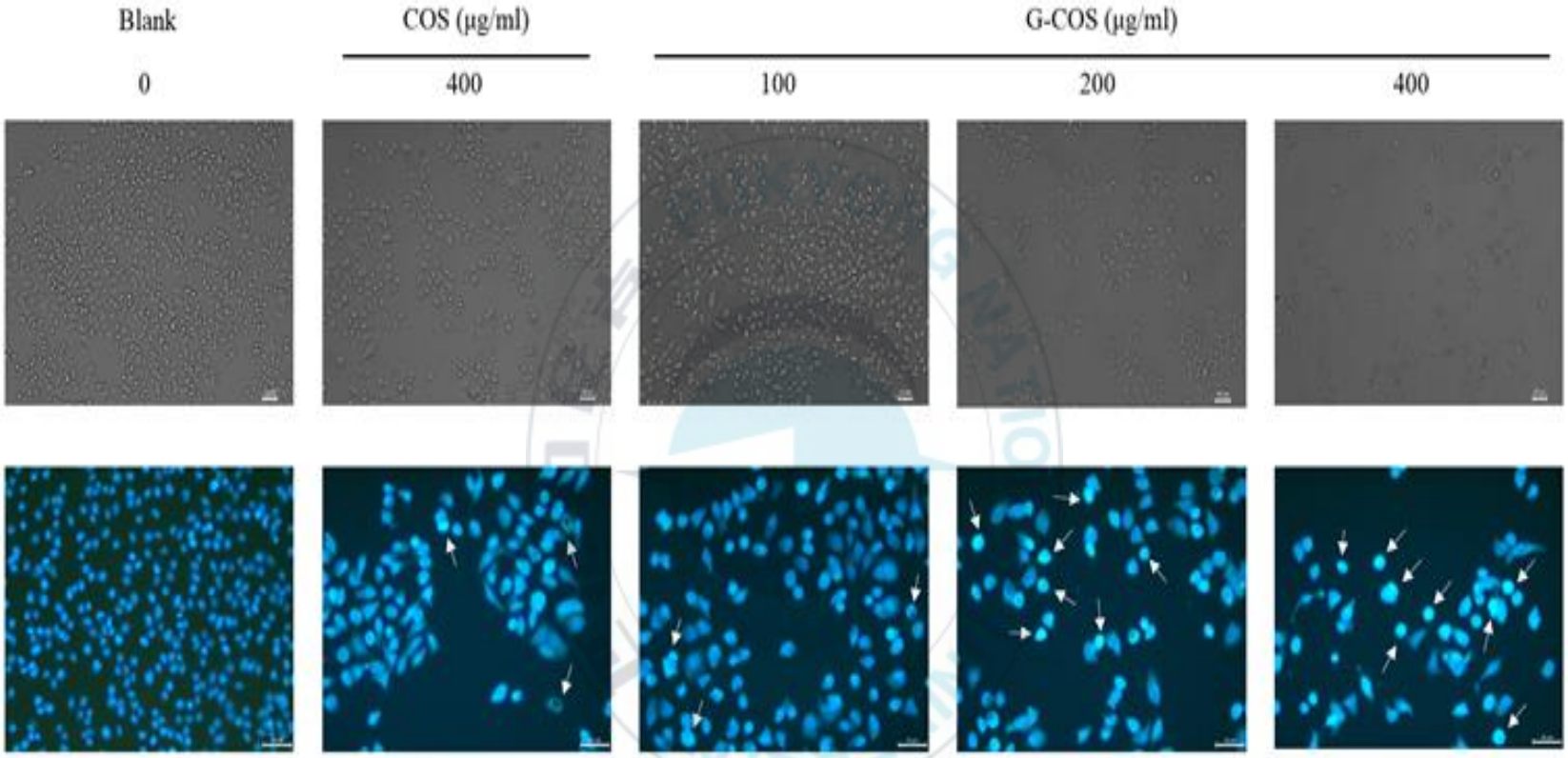


Figure 8. Morphological changes and Hoechst 33342 staining of G-COS treated AGS cells. For observation of morphological changes, cells were grown in 12-well plate and treated with samples for 24h, and then detected by light microscope. For Hoechst 33342 staining, nuclear condition was detected by fluorescence microscope.

3.4. DNA fragmentation

Apoptosis is characterized by chromatin condensation, activation of some caspases and fragmentation of DNA at internucleosomal linker sites, giving rise to discrete bands of multiples of 180-200 base pairs [50]. This form of DNA degradation has been widely observed in apoptosis as one of the biomarkers of apoptosis.

To ascertain that G-COS has potency to induce apoptosis in AGS cells, DNA fragmentation assay was performed. AGS cells were treated with COS (400 μg/ml) and G-COS (0, 100, 200, 400 μg/ml) for 24h, and then DNA fragmentation of AGS cells was detected by an agarose gel electrophoresis. There was a significant DNA fragmentation in G-COS (200 and 400 μg/ml) treated cells compared with the blank as shown in Figure 9. Therefore, this result showed that G-COS induced apoptosis in AGS cells.

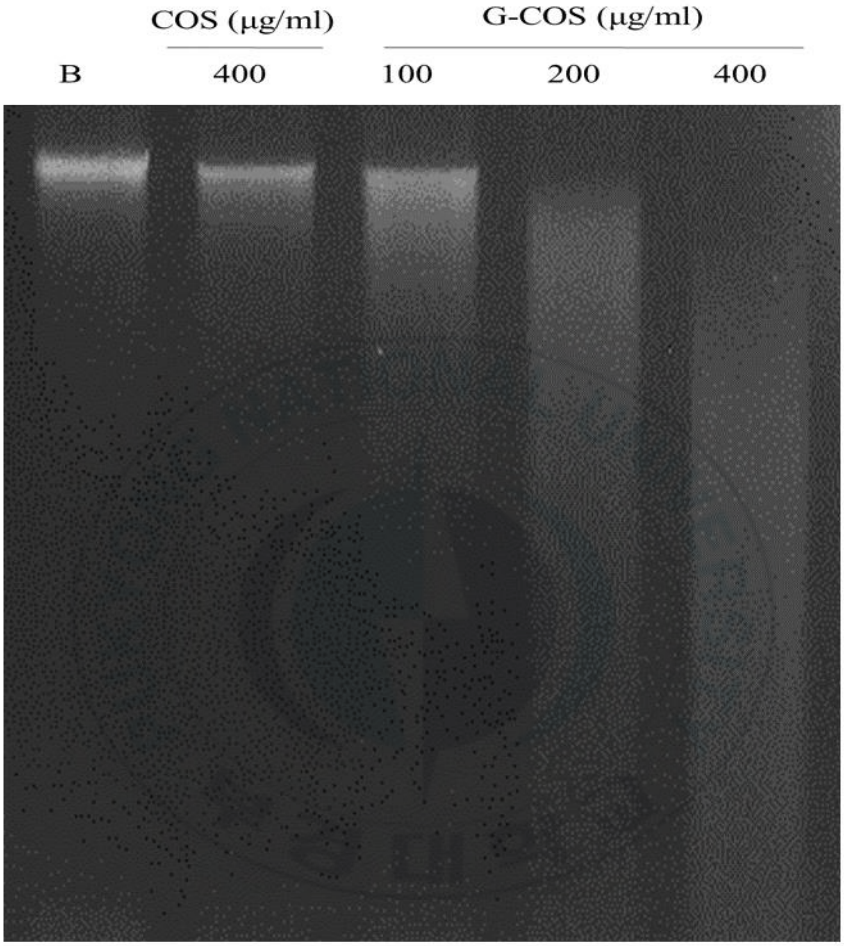


Figure 9. DNA fragmentation of AGS cells which treated with various concentrations of G-COS for 24h. DNA was isolated and separated by 1.5% agarose gel. And then, DNA was stained with ethidium bromide (EtBr) and visualized under UV light.

3.5. Analysis of cell cycle distribution

G-COS inhibit the growth of AGS cells significantly. So, to investigate further the G-COS-mediated death of AGS cells, the cell cycle distribution of AGS cells treated with COS (400 μg/ml) and G-COS (0, 100, 200, 400 μg/ml) was analyzed using flow cytometry. A significant increase in the percentage of cells in Sub-G1 phase was found after treatment with 200 and 400 μg/ml G-COS, compared with control cells (Figure 10). As shown in Figure 10, when cells were exposed to G-COS (200 and 400 μg/ml) for 24 h, the percentage of Sub-G1population showed a noticeable increase (55.64 and 66.64 %) compared with the control (1.50 %), 400 μg/ml COS treatment (5.00 %) and 100 μg/ml G-COS treatment (10.97 %). The cells in Sub-G1 phase refers to the apoptotic cells. In apoptotic cells, the amount of DNA is decreased by DNA fragmentation. Therefore, that cells are less staining by DNA-binding dye, propidium iodide (PI), and get to form Sub-G1 peak on the left side of G0/G1 phase. So it can be deduced that G-COS may induce apoptosis by causing DNA fragmentation.

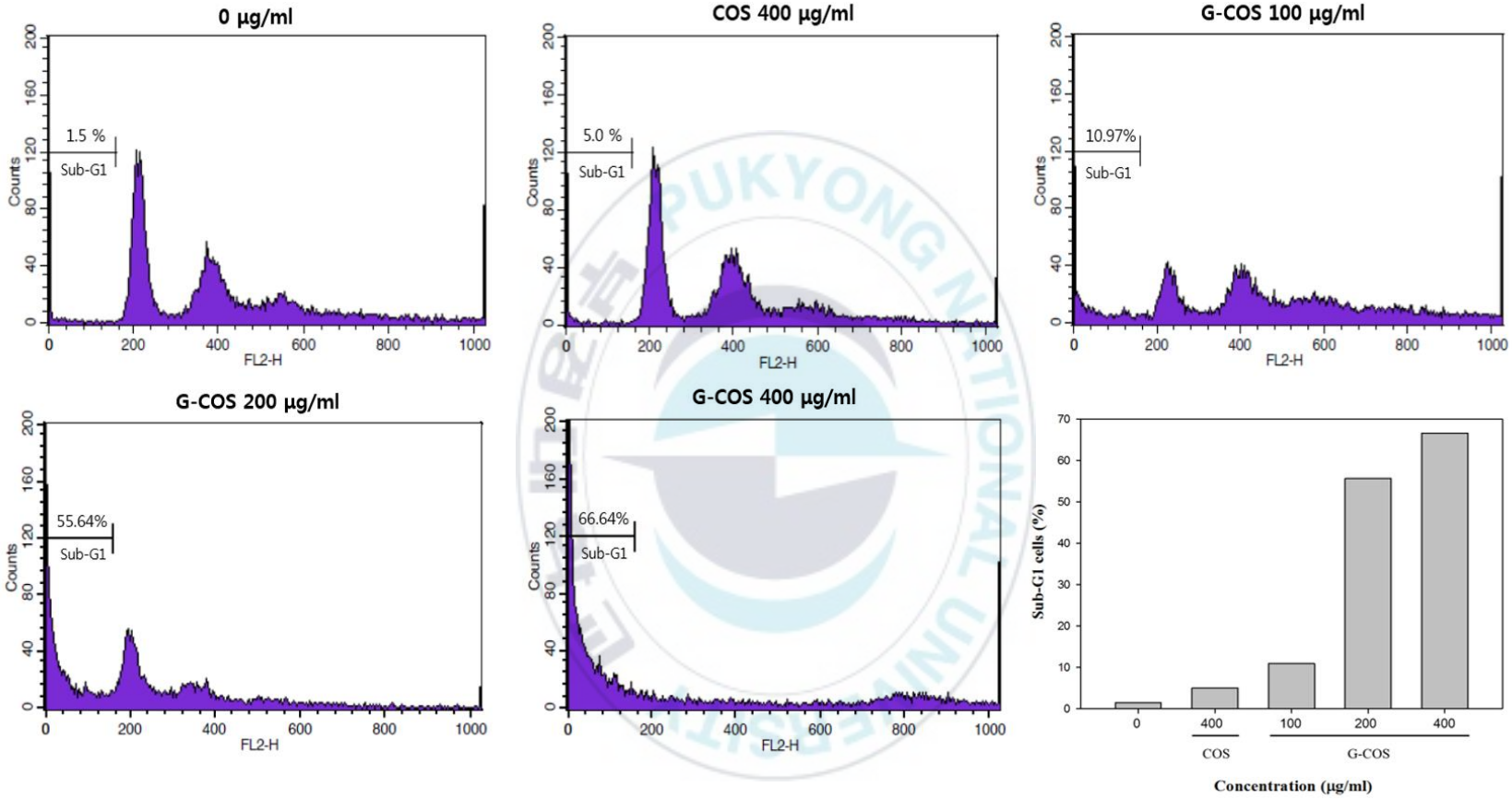


Figure 10. Effects of G-COS on the number of AGS cells in the Sub-G1 phase. Cells were treated with the indicated concentrations of samples for 24h and analyzed by flow cytometry following staining with propidium iodide (PI).

3.6. Analysis of apoptosis

Annexin V has an affinity for negatively charged phospholipids such as phosphotidylserine that is transported to the outer leaflet of the plasma membrane in apoptotic cells. Early apoptotic cells are binding with Annexin V, but not sensitive to propidium iodide (PI) staining. Propidium iodide (PI) intercalates into double-stranded nucleic acids. It is excluded by alive cells but can penetrate cell membranes of dying or dead cells, owing to the loss of plasma membrane integrity. So, PI staining can be visible in late apoptotic cells and dead cells.

To confirm whether G-COS induced apoptosis in AGS cells, flow cytometry analysis was performed. AGS cells were treated with COS (400 μg/ml) and G-COS (0, 100, 200, 400 μg/ml), and the numbers of apoptotic cells were evaluated using the Annexin V-FITC Apoptosis Detection kit. In Figure 11, LL position showed alive cells, whereas LR, UR and UL position indicated early apoptotic cells, late apoptotic cells, and necrotic cells, respectively. As shown in Figure 11, treatment of AGS cells with G-COS for 24 h resulted in significant increase of apoptotic cells (55.6 and 61.9%) in concentration 200 and 400 μg/ml, when compared with other cases (2.14 % in control, 3.36 % in 400 μg/ml COS and 4.08 % in 100 μg/ml G-COS) and the percentage of late apoptotic cell was much higher than early apoptotic cells. These results suggest that G-COS effectively induced apoptosis in AGS cells by decreasing effectiveness time.

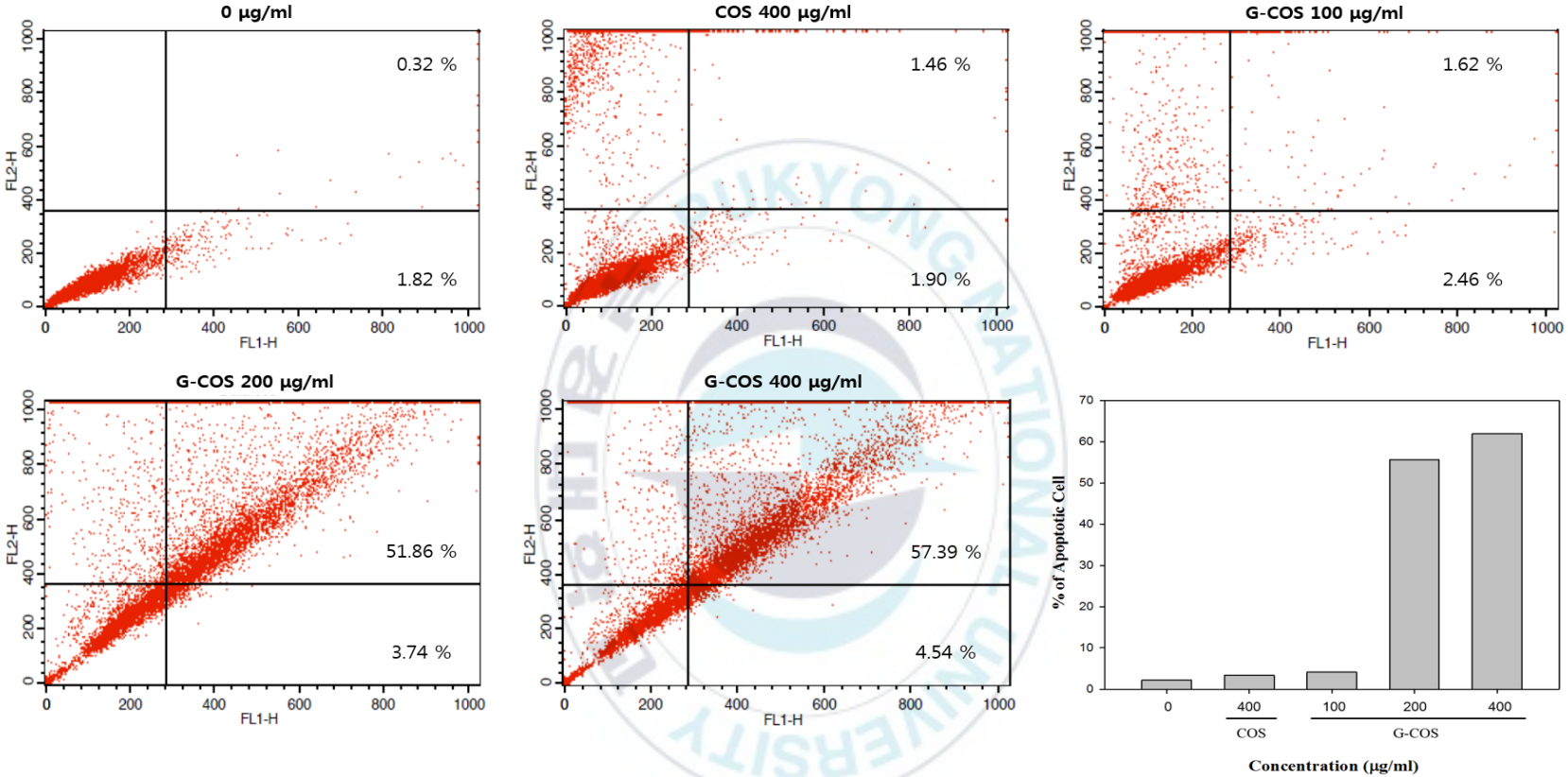


Figure 11. Effect of G-COS on the induction of apoptosis in AGS cells. Cells were exposed to the indicated concentrations of samples for 24h, and double stained with Annexin V-FITC/propidium iodide (PI), and analyzed by flow cytometry. The cells appeared with Annexin V positive/PI negative are considered as early apoptotic cells, while Annexin V /PI double positive as late apoptotic cells. (UL, upper left; UR, upper right; LL, lower left; LR, lower right)

3.7. Effects of G-COS on expression of gene and proteins related to apoptosis

3.7.1. Effect of G-COS on p53 signaling pathway

Although G-COS have been shown to induce apoptosis in AGS cells, the specific mechanism is not fully understood. To know the relevant mechanism, expression level of proteins related to apoptosis was investigated.

A tumor suppressor factor p53 plays a key role in many types of cancer cell death and apoptosis. The p53 is activated by a number of cellular stress, including DNA damage, hypoxia. When DNA are damaged, p53 promotes the transcription of p21, thereby blocking the cell cycle progression and DNA synthesis, and induce apoptosis by regulating members of the Bcl-2 family such as Bax, and Bcl-2 [51-53].

Therefore, to determine whether the p53 and p21 are related to anti-proliferative effect of G-COS, western blot analysis was performed. As shown in Figure 12, treatment of G-COS on AGS cells showed an upregulation effect of p53 and p21 expression levels.

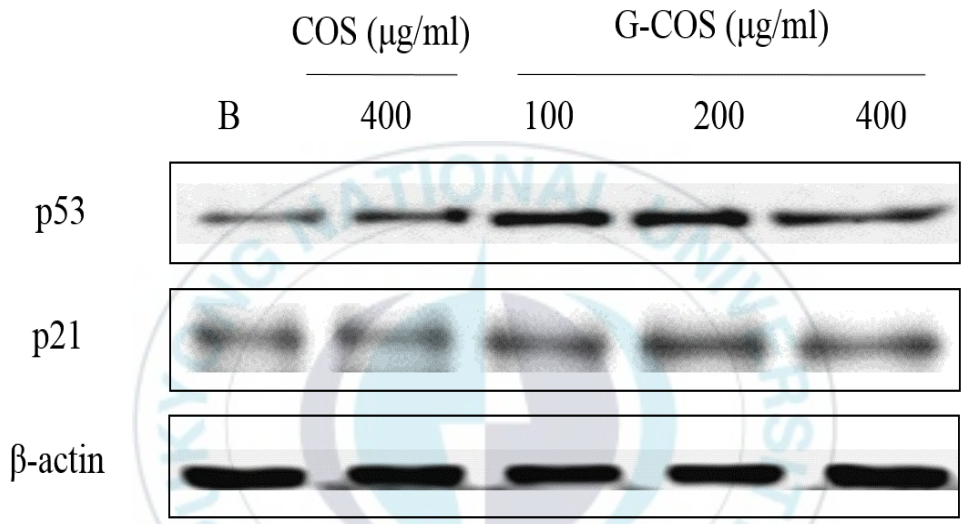


Figure 12. Effect of G-COS on p53 and p21 protein expression. Cells were incubated with G-COS for 24h and the expression levels of p53 and p21 protein were detected by western blot analysis.

3.7.2. Effect of G-COS on Bcl-2 family

It was confirmed that p53 protein expression level was increased by G-COS treatment. Therefore, the expression level of cytochrome c, and Bcl-2 family, which leads to disruption of mitochondrial membrane was investigated.

Members of Bcl-2 family can be subdivided into pro- and anti-apoptotic proteins. Many of these proteins bind each other and form a complex network of homo- and heterodimers. And the relative ratios of pro- and anti-apoptotic Bcl-2 family proteins is a significant factor in determining the reaction degree of apoptosis [54, 55]. The Bcl-2 family proteins can be classified based on function and Bcl-2 homology (BH) domain composition. Bcl-2 family proteins can have up to four Bcl-2 homology domains, BH1, BH2, BH3 and BH4 [55].

To date, about 30 Bcl-2 family members have been identified in mammalian cells and several others in viruses. All members possess at least one of four Bcl-2 homology domains (BH1 to BH4). Among the BH domains, BH3 domain is closely related to inducing apoptosis [56, 57]. All of pro-apoptotic proteins, including Bax, Bak, Bok, Bik, Blk, Bim, Bad, Bid, Hrk, Noxa, PUMA, Bcl-rambo, contain BH3 domain. And some of these proteins (such as Bik, Blk, Bim, Bad and Bid, Hrk, Noxa, PUMA) contain only BH3 domain. In the pro-apoptotic proteins, the BH3 domain has been shown to be required for their killing activity [54-57]. And also, some of the anti-apoptotic proteins include Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A-1, E1B-19K, BHRF1 and CED-9 [56].

The stimuli of intrinsic apoptosis break the balance of these pro- and anti-apoptotic Bcl-2 family, and activate Bax, Bak proteins in mitochondria. When activated, Bax and Bak trigger mitochondrial damage, and after that cytochrome c and other apoptogenic factors are released from the mitochondria into the cytosol. Finally, released cytochrome c activates caspase cascades ultimately leading to cell death [41, 54].

As shown in Figure 13, pro-apoptotic member Bax and cytochrome c level in AGS cells were increased in dose-dependent manner, while anti-apoptotic member Bcl-2 expression was decreased in a dose-dependent manner.

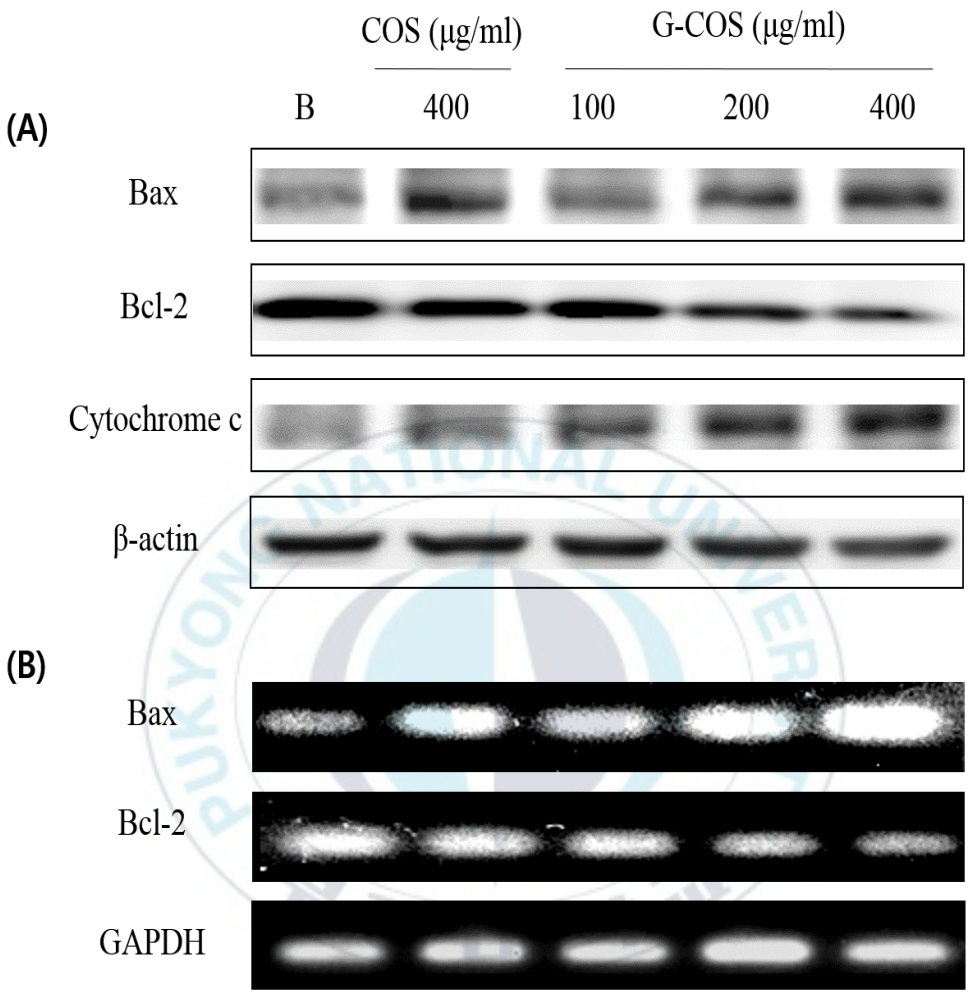


Figure 13. Effect of G-COS on Bax, Bcl-2 and Cytochrome c expression. Cells were incubated with G-COS for 24h and the expression levels of Bax, Bcl-2 and Cytochrome c mRNA and protein were detected by western blot analysis (A) and RT-PCR (B).

3.7.3. Effect of G-COS on Caspases

Caspases are a family of cysteine proteases that play a key role in regulating apoptosis by cleaving target proteins at aspartic acid residues.

To date, ten major caspases have been broadly classified into apoptotic initiator caspases (caspase-2,-8,-9,-10), apoptotic effector or executioner caspases (caspase-3,-6,-7) and inflammatory caspases (caspase-1,-4,-5) by their known roles and their mechanism of action [35, 58, 59].

Initiator caspases activate executioner caspases that subsequently coordinate their activities to destroy main structural proteins and activate other enzymes [60]. In apoptosis pathway, the initiator caspase-8,-9 activates other downstream caspases such as caspase-3 ultimately leading to apoptosis.

In order to determine the involvement of caspases in apoptosis by G-COS, the expression level of caspase-9 and -3 was examined using western blot assay and RT-PCR (Figure 14). Treatment with G-COS activated caspase-9 and -3 levels in a dose dependent manner. In addition, the subsequent proteolytic cleavage of DNA repair enzyme PARP, which are down-stream targets of the activated caspase-3, was also investigated [61]. Treatment with G-COS induced the cleavage of PARP known as caspase substrate (Figure 14).

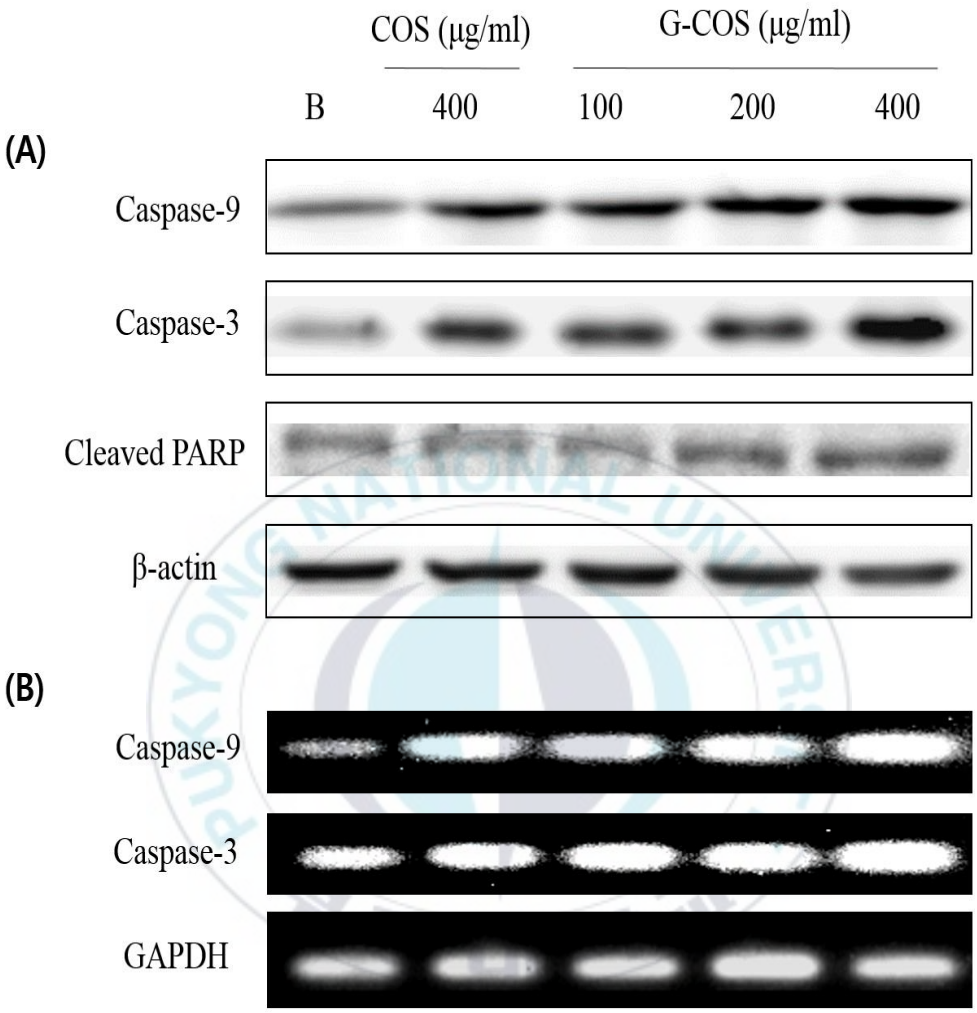


Figure 14. Effect of G-COS on caspase (-9 and -3) and cleaved PARP expression. Cells were incubated with G-COS for 24h and the expression levels of caspase (-9 and -3) and cleaved PARP mRNA and protein were detected by western blot analysis (A) and RT-PCR (B).

3.7.4. Effect of G-COS on NF- κB

NF-κB is a major transcription factor that plays a critical roles in tumor cell proliferation and differentiation, metastasis, inflammation, angiogenesis. And also, NF-κB are known to control apoptosis and cell cycle progression. Many studies on the role of the NF-κB in the regulation of apoptosis in cancer cells have been reported [62, 63].

NF-κB is composed of p50 and p65 subunits. NF-κB p50/ p65 heterodimers in most cells are sequestered in the cytoplasm in an inactive form by an inhibitory proteins, IκB [64]. NF-κB can be activated by various stimuli such as cytokines, growth factors, infectious agents, radiation [65]. In response to these relevant stimuli, IκBs are phosphorylated and degraded. Degradation of IκBs results in liberation of NF-κB allowing translocation into the nucleus where it regulate the expression of numerous genes [63, 66].

Excessively activated NF-κB induces cellular transformation, and results in abnormal cell proliferation. And it has been found that many cytokines that can be controlled by the NF-κB acts as a growth factor for cancer cells. So, when receiving chemotherapy by anti-cancer agents and radiation therapy, NF-κB helps survival of cancer cells in a harsh attack, and it makes the disobedience cancer cells to apoptosis command.

Suppression of NF-κB activity sensitizes a lot of human cell lines to apoptotic stimuli or even directly induces apoptosis. A link between NF-κB and apoptosis was demonstrated by analyzing NF-κB DNA-binding activity. It showed that

inhibition of NF-κB and its downstream pathway is a usual underlying mechanism of apoptosis [67-69]. Therefore, NF-κB has been thought as important target in the treatment of human cancer. So, to examine whether G-COS is related with inhibition of the activation of NF-κB, western blot analysis was performed. Treatment with G-COS induced the decrease in expression level of p-IκB-α, and down-regulations of p50 and p65 were also observed (Figure 15).

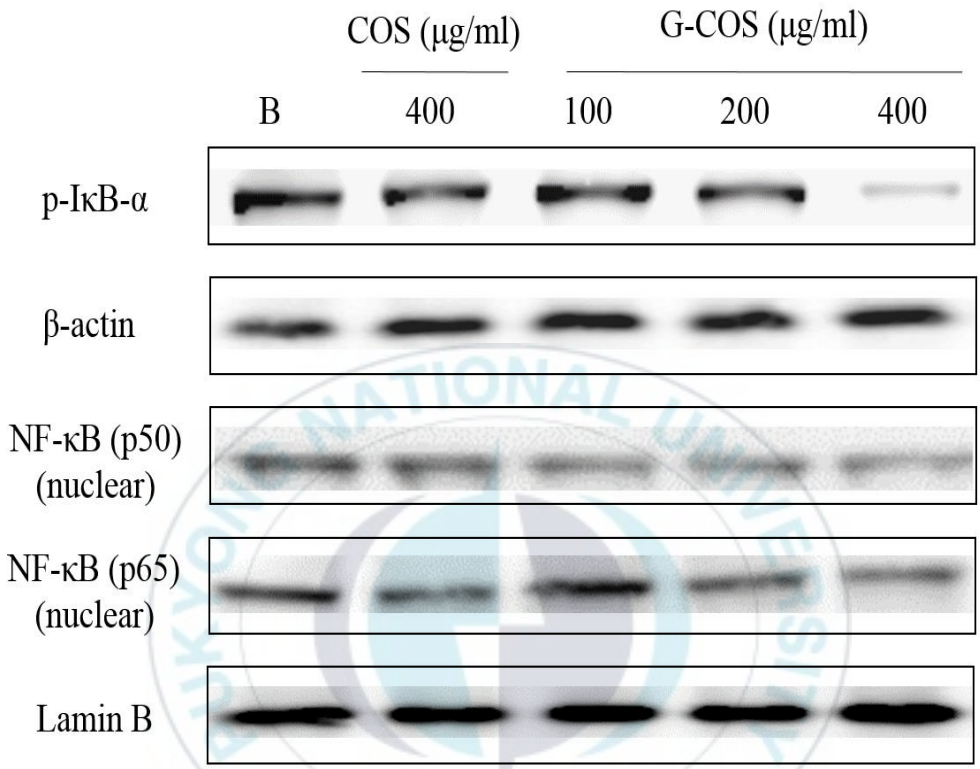


Figure 15. Effect of G-COS on p-IκB-α and NF-κB (p50 and p65) protein expression. Cells were incubated with G-COS for 24h and the expression levels of p-IκB-α and NF-κB (p50 and p65) protein were detected by western blot analysis.

4. Summary and Conclusion

This study conducted to demonstrate the enhanced anti-proliferative effect of COS on AGS human gastric adenocarcinoma cells by conjugating with gallic acid. In the structure of chitosan, there are three hydrogen sources at C-2 (NH_2) and C-3,-6 (OH) positions. But, it is difficult to react with OH of C-3 position because of their steric hindrance. Therefore, the major target of chitosan for modification is introduction on NH_2 or OH of C-2 and -6 positions. G-COS was successfully synthesized by grafting gallic acid with COS at C-2 and C-6. Moreover, the structure of G-COS was identified by FT-IR, ^{1}H NMR and ^{13}C NMR. After synthesis, the samples were treated at the concentrations of 100, 200 and 400 μg/ml for 24h, and this exposure applied for all experiments. Cell viability was confirmed with MTT assay, and morphological changes were observed by using light microscope. Also, Hoechst staining and FACS assay were used for monitored apoptosis with fluorescence technique. The results suggest that the synthesized G-COS have significant apoptotic effect on AGS cells at concentration of 200 and 400 μg/ml. Finally, apoptosis- related gene and protein [p53, p21, Bax, Bcl-2, Cytochrome c, Caspase (-9 and -3), Cleaved PARP, p-IκB-α and NF-κB (p50 and p65)] expressions were investigated with Western blotting and RT-PCR. The results of western blotting and RT-PCR demonstrated that apoptosis caused by G-COS follows the intrinsic pathway (mitochondrial pathway).

In conclusion, G-COS inhibits cell proliferation of AGS human gastric adenocarcinoma cells by inducing apoptosis, and also G-COS showed a significantly higher effect than COS. The present results suggest that G-COS have a promising potential as cancer chemopreventive agents.

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