ANTIOXIDANT EFFECT OF GALLIC ACID-CHITOOLIGOSACHARIDES DERIVATIVES IN RAW264.7 CELL

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SUMMARY

The main aim of this study was to determine the antioxidative effects of gallic acid grafted chitooligosaccharides (COSs) derivatives in the RAW264.7 cell model. The derivatives prepared by a method that uses free radical mediated grafting method with ascorbic acid/hydrogen peroxide catalyst. The antioxidative effects in RAW264.7 cells were determined through its ability to protect biological macromolecules such as DNA and lipid. The results show that the new derivatives have effectively neutralized free radicals to protect the biological macromolecules in RAW264.7 cells. The DNA protection efficacy of the GA-g-COSs is higher than the free COSs at a concentration from 25 to 100 µg/mL. For cell membranes, the protection of the GA-g-COSs is significantly higher than the plain COSs at concentrations from 10 to 100 µg/mL. This study is fundamental for antioxidant activity research in other cell models as well as other activities such as anti-inflammatory activity on the macrophage model.

Keywords: Chitooligosaccharides, antioxidant, gallic acid.

INTRODUCTION

Free radicals are particles which contain unpaired electrons in an atomic orbital. The presence of unpaired electrons makes free radicals to be highly reactive. They can afford to active and accept different unpaired electrons to be a reducing agent and an oxidizing agent. Oxidative stress is the accumulation of excessive of free radical formation that beyonds the control of the antioxidant systems in human. Oxidative stress occurs at the time that the concentration of antioxidants is too low to eliminate free radicals which are formed during the physiological and biochemical processes of the body. Common types of free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS). Many previous reports indicated that oxidative stress is the reason leading to different diseases, such as rheumatoid arthritis disease, Alzheimer's disease, Parkinson disease, amyotrophic lateral sclerosis disease, cardiovascular disease, allergic and respiratory disease, disease relating to immune system, diabetes and cancer disease (Cross *et al.*, 1987; McCord, 2000).

Chitosan is the hydrolyzed product of chitin that is formed by the deacetylation a part of chitin in alkaline medium. The deacetylation process converts the N-acetyl groups to amino group at C2 position in the structure. In this case, the deacetylation is not completely and the product which has the deacetyl degree larger than 50 percentage is called chitosan as well as the deacetyl degree less than 50 percentage is called chitin. Chitosan products have many researchs and diverse applications in different fields. However, chitosan products remain limited like insoluble in water and the human have not enzymes to decompose chitosan to metabolism type in the gastric. Chitooligosaccharides (COSs) is the solution to solve the problem of chitosan products. COSs is the chitosan that has low molecular wieght containing from 3 to 11 glucosamine units and N-acetylglucosamine units which are linked by β-1,4-O-glycoside bond. COSs is the oligomer of chitosan that carries most of the biological activity of chitosan likes anti-oxidant activity, anti-microbial activity, anti-cancer activity, immuno-stimulating activity, anti-inflammatory, and anti-HIV (Lodhi *et al.*, 2014; Hamed *et al.*, 2016).

In the last few years, great interest has been given functional group modifications to improve the bioactivities of COSs, while keeping integrant the background characteristics of COSs. Notably, the coherence of phenolic acids linking functional groups on the COSs chain achieved an interest in functional food, pharmaceutical and medicinal fields. Gallic acid was hydroxybenzoic acids of phenolic acids which has high antioxidative activity and plays an important role in control oxidative stress (Eom *et al.*, 2012; Vo *et al.*, 2017). Research of characterization and biological activity of PVA hydrogel containing chitooligosaccharides conjugated with gallic acid performed by Park et al., 2018. The results of the study showed that the gallic acid grafted COSs derivative has an antioxidative effect. Notably, they have effective against *Propionibacterium acnes* that showed to play an important role in the pathophysiology of common skin diseases such as acne vulgaris. The results suggested that PVA hydrogel can be developed as raw materials for biomedical dressing or a cosmetic product for patients with acne vulgaris (Park *et al.*, 2018). Impacts of aminoethyl-chitooligosaccharide (AE-COS) for anticancer activity due to inhibiting proliferation and inducing apoptosis in human lung A549 cancer cells was evaluated by Ngo et al., 2019. The

results had shown that derivative improved anticancer activity in human lung cancer A549 cells via down regulation of Bcl 2 and up-regulation of caspase 3 and caspase 9. This work showed science evidence of AE-COS material in the application as chemotherapy in cancer treatment in the future (Ngo *et al.*, 2019).

METERIALS AND METHODS

Materials

Lactic acid, sodium bicarbonate, ethanol, thiobarbituric acid (TBA), 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased form HiMedia (Mumbai, India). Gallic acid (GA), Dimethyl sulfoxide (DMSO), sodium hydroxyl, hydrogen peroxide, folin, TLC silica gel 60 F254 and agarose were purchased from Merck (Darmstadt, Germany). Mouse macrophages RAW264.7 (KCLB, Seoul, Korea). Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, fetal bovine serum (FBS), trypsin, and the other materials required for culturing of cells were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ascorbic acid (VWR, Leuven, Belgium) and all other chemicals were of analytical grade or the highest grade available commercially.

Preparation of GA-g-COSs derivatives powder from our previous research. Briefly, the COSs (0.5 g) was dissolved in 25 mL of water and then 1 mL of 1.0 M H_2O_2 containing 0.054 g of ascorbic acid was added. After 30 minutes, the gallic acid (0.25 g) was added to the mixture. The pH value of mixture changed at 5 and the reaction was carried out at room temperature for 6 hours. After reaction, the mixture centrifuged with filter membrane 1 kDa in 50 mL centrifuge tube in order to remove unreacted ingredient. Finally, the resulting solution was lyophilized using freeze dry system (FDU 2110, Eyela, Japan) to obtain GA-g-COSs solid samples. To verify whether gallic acid was successfully grafted onto COSs backbones, TLC analysis was performed. gallic acid, ascorbic acid, and GA-g-COSs were developed on a silica gel plate (TLC silica gel 60 F254, Merck, Darmstadt, Germany) with chloroform - ethyl acetate - acetic acid (50:50:1) as mobile phase, heating at 100°C for 5 minutes. The developed TLC plate was observed under UV light; The GA-g-COSs was characterized by the proton nuclear magnetic resonance (1H-NMR) spectra. Samples were recorded by a NMR-500MHz spectrometer (Bruker, Germany) under a static magnetic field of 500 MHz, samples were dissolved in D_2O.

Methods

Cytotoxicity assay in RAW264.7 Cells

The RAW264.7 cells were grown in DMEM medium containing 5% (v/v) FBS, 100 µg/mL penicillin-streptomycin and 5% CO_2 at 37° C. Cytotoxicity levels of COSs and derivatives were measured by MTT method as the previously described report (Ngo *et al.*, 2012). Briefly, the RAW264.7 cells were grown in 96-well plates at the concentration of $1x10^4$ cells/well. After culture for 24 hours, the cells were treated with the fresh medium and supplemented COSs or derivatives with different concentrations (concentration of samples ranged from 1 to 100 µg/mL, samples were dissolved in deionized water and filtered using a 0.22 µm syringe filter). After incubation for 24 hours, the cells were rewashed. Subsequently, 50 µL of MTT solution (0.5 mg/mL in PBS) was added into every well and incubated for 4 hours. Finally, 100 µL of DMSO was added to solubilize the formazan salt formed and was measured the absorbance at 540 nm by using a microplate reader (PerkinElmer, US). The viable cells were calculated as a percentage of control.

DNA Isolation

Genomic DNA was extracted from RAW264.7 cells using a standard phenol/proteinase K procedure with slight modifications (Ngo *et al.*, 2011). The cells cultured in 10 cm culture dishes were washed twice with PBS and scraped into 1 mL of PBS containing 10 mM EDTA. Then the cells were removed PBS by centrifuge at 12000 rpm for 5 minutes. After, cells were dissolved in sodium acetate (350 µL; 0.2 M), proteinase K (20 µL; 10 mg/mL), SDS (25 µL; 10% w/v), RNase (25 µL; 1 mg/mL). The mixture was incubated at 37° C for 30 minutes and 55° C for an hour. After incubation, the mixture was added a solution of phenol: chloroform: isoamyl alcohol (25:24:1) to 1:1 ratio and centrifuged at 12000 rpm for 5 minutes. Following centrifugation, the supernatant was added cool ethanol to 1:1.5 ratio and incubated for 30 minutes at -20 °C. After that mixture was centrifuged at 12000 rpm for 5 minutes to remove the supernatant. The pellet was dissolved in TE buffer and the purified DNA had an 260/280 nm absorbance ratio of 1.8 - 2.0. Further, the quality of isolated DNA was evaluated using 1% agarose gel electrophoresis.

DNA Oxidation Assay

The DNA oxidation assay indicated the DNA protection ability from RAW264.7 of derivatives and COSs samples. The method was performed according to the previously described report with some modifications (Ngo *et al.*, 2008). The mixture included DNA (5 µL; 1.0 µg), samples (4 µL; concentration ranged from 10 to 100 µg/mL), distilled water (3 µL), $FeSO_4$ (14 µL; 600 µM), H_2O_2 (14 µL; 3 mM) was incubated at room temperature for 10 minutes, added EDTA (4 µL; 130 mM) to stop the reaction. The mixture was stained with GelRed and electrophoresed on a 1% agarose gel for 20 minutes at 100 V. Gels were then visualized by UV light using VisionCapt gel image analysis software (Bio-Print TX4, Vilber, Wembley, WA 6014, Australia).

Membrane Lipid Peroxidation Assay

This method was performed according to the previously described report (Ngo *et al.*, 2011)**.** Briefly, 50 µL sample of COSs and derivatives (concentration ranged 10 to 100 µg/mL) added to Eppendorf tube containing cell solution (50 µL) or the same volume of distilled water as a control and incubated at room temperature for 10 minutes. The mixture was added H_2O_2 (50 µL; 2 mM) and $FeSO_4$ (50 µL; 0.1 M) and incubated at room temperature for 30 minutes. After that, the mixture was added twofold volume of TCA (10% w/v, cooled) to precipitated protein which removed by centrifugation at 12000 rpm for 5 minutes. After centrifuging, the supernatant was collected and added an equal volume of TBA (500 µL; 1%) and incubated at 90^o C for 30 minutes, After cooling (4^o C), the reaction mixture was centrifuged at 12000 rpm for 5 minutes and absorbance of the supernatant was determined at 528 nm.

Statistical Analysis

Data were expressed as the mean ± standard deviation (SD) of triplicates. The least significant difference (LSD), Duncan's multiple range test and one-way analysis of variance (ANOVA) were used for multiple comparisons by statgraphic centurion sofware. The difference was considered to be statistically significant if $p < 0.05$.

RESULTS AND DISCUSSION

Assessment of RAW264.7 Cell Viability

Cytotoxicity levels of the test samples in cells were measured by MTT method. MTT was metabolized to formazan salt by mitochondrial enzymes in living cells. The formazan salt was dissolved by DMSO and measured the absorbance 540 nm. The results in Fig. 1 showed that no significant toxic effects in RAW264.7 cells concentration from 1 to 100 µg/mL. In this case, COSs and GA-g-COSs are the safe materials that are not lead to cytotoxicity in RAW264.7. Therefore, nontoxic concentrations of COSs and GA-g-COSs can apply in further experiments. In previous report, GA-g-COSs was confirmed the nontoxic on RAW264.7 cells at the same concentration (Ngo *et al.*, 2011).

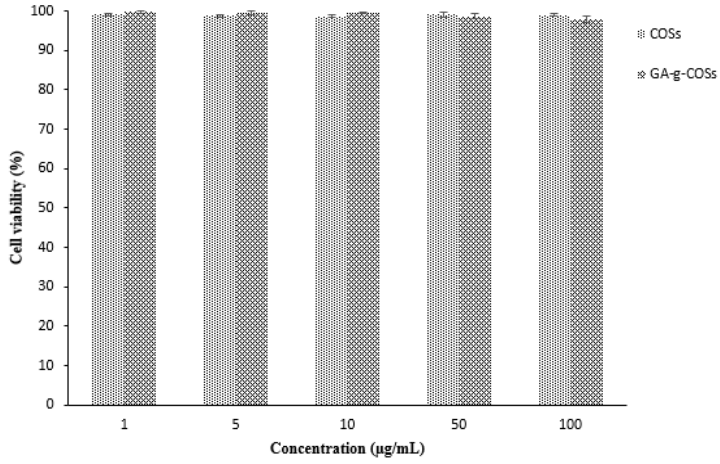

Fig. 1. Cytotoxic effects of COSs and GA-g-COSs in RAW264.7 cells. The cell viability was determined by MTT assay as described in the method. Firstly, the RAW264.7 cells were grown in DMEM medium at a density of 1×10^4 cells/well and treated test samples (1 to 100 µg/mL). After incubation for 24 hours, cells were treated MTT solution for 4 hours. Finally, DMSO solution were added to solubilize formazan crystals and absorbance was recognized at 540 nm. The experiments are run in triplicate and data are presented as means ± SD, $p < 0.05$

Inhibition of Radical Mediated DNA Damage in RAW264.7

The hydroxyl radical generated by the Fenton reaction is considered to be the reason for breaking the DNA structure. The hydroxyl radicals are highly when exposed to DNA to destroy purines, pyrimidine and deoxyribose frameworks thereby causing genomic DNA damage. This DNA damage increases with increasing density of free radicals and can be the cause of many diseases such as cancer, mutation, aging, etc (Ngo *et al.*, 2008). In this study, COSs and GA-g-COSs were analyzed for its protective effects against DNA oxidation using genomic DNA isolated from RAW264.7 cells. The isolated DNA was subjected to oxidation by hydroxyl generated via the Fenton reaction. The effects of COSs and GA-g-COSs on the hydroxyl induced DNA damage were observed after electrophoresing the DNA in agarose gel. The results demonstrated GA-g-COSs significantly inhibited oxidative damage of DNA. At 100 µg/mL, the band of GA-g-COSs shows the presence of DNA that is most clearly protected by the test substance and decreases gradually at 50 µg/mL to 10 µg/mL. The results also show that the DNA protection of the derivatives has improved significantly compared to the free COSs and dose dependent

manner. Data were shown in Fig. 2.

The previous report has been shown that gallic acid grafted COSs displayed the protective effect of DNA oxidation more than 90% at 100 µg/mL (Ngo *et al.*, 2011), while this study results showed the protective effect of DNA oxidation more than 88% at the same concentration. In addition, other COSs derivatives also showed the protective ability of DNA damage, such as chitooligosaccharide-4-hydroxybenzyl derivatives that protected DNA damage from Chang liver cells (Trinh *et al.*, 2014). The results of the study clarified that the derivatives formed, which could prevent oxidation from damaging the genomic DNA when the DNA is exposed to the hydroxyl free radical generated in the live cell system.

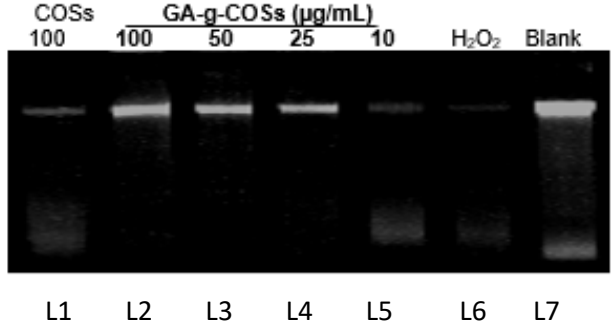

Fig. 2. DNA oxidative protection effects in RAW264.7. Genomic DNA from RAW264.7 cells was treated with COSs or GA-g-COSs and incubated with hydroxyl free radical which generated from Fenton reaction. After 10 minutes, the reaction mixture containing about 1 µg of DNA was electrophoresed on a 1% agarose gel for 20 min at 100 V and visualized by UV light after being stained with GelRed. Lane 1: $FeSO_4$ and H_2O_2 in the presence of COSs at 100 µg/mL; Lanes 2 to 5: $FeSO_4$ and H_2O_2 in the presence of GA-g-COSs; Lane 6: DNA damage control ($FeSO_4$ and H_2O_2); Lane 7: DNA alone (blank). Data are represented by three repeated experiments

Inhibitory Effect of Membrane Lipid Peroxidation in RAW264.7

Reports of many previous studies had shown that membrane lipid peroxidation is related to many pathologies in the human body (Liu *et al.*, 2017). In this experiment, the hydroxyl free radical was released from the Fenton reaction and the OD value was recorded through the color product of the reaction between malondialdehyde and 2-thiobarbituric acid. The results of the study showed that the cell membrane lipid protection of GA-g-COSs was much better than the original COSs at all concentration. At 100 µg/mL, the lipid protection activity of GA-g-COSs derivatives is 86.35% while that of COSs is 38.63%. According to this result, membrane lipid oxidation inhibition ability of GA-g-COSs in RAW264.7 cells has two-fold more effective than plain COSs. At the other concentrations, activities of derivatives also showed significantly higher than free COSs. The results are detailed in Fig. 3. The previous study showed that gallate grafted COSs have protective effect of membrane lipid in the RAW264.7 cells around 62% at 100 µg/mL (Ngo *et al.*, 2012). Besides, the previously reported result confirmed that gallic acid grafted COSs could decrease membrane lipid peroxidation in the SW1353 cells and indicated that protective effects of membrane lipid were 80% at 100 µg/mL (Ngo *et al.*, 2011).

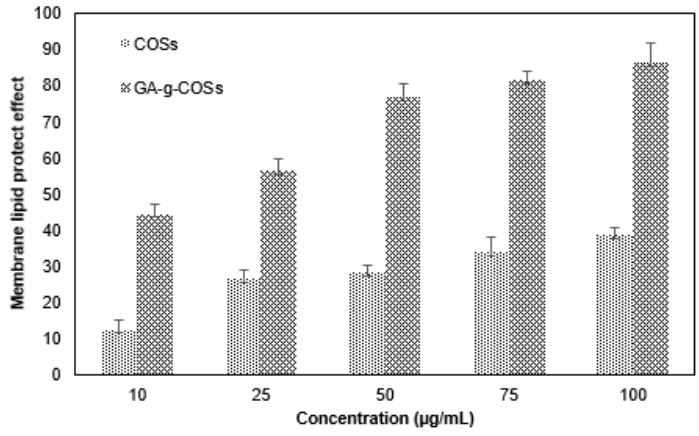

Fig. 3. The protective effects of membrane lipid peroxidation of COSs and GA-g-COSs in RAW264.7. The cells were exposed to hydroxyl radicals generated via Fenton's reaction and peroxidation products of membrane lipids reacted with TBA to form pink color complex which was measured spectroscopically at 528 nm. The experiments are replicated three times and data are shown as means ± SD, $p < 0.05$

CONCLUSION

This study has demonstrated that GA-g-COSs was found to be non-toxic and had protective effects of membrane lipid and DNA injury in RAW264.7 cell stronger than free COSs. GA-g-COSs have potential free radical scavenging effects to inhibit and prevent biological molecular damage of free radicals in live cells system. This study is fundamental for antioxidant activity research in other cell models as well as other activities such as anti-inflammatory activity on the macrophage model.

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HIỆU QUẢ KHÁNG OXI HÓA CỦA DẪN XUẤT GALLIC ACID-CHITOOLIGOSACCHARIDE TRONG TẾ BÀO RAW264.7

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TÓM TẮT

Mục tiêu chính của nghiên cứu là xác định hiệu quả kháng oxi hóa của dẫn xuất chitooligosaccharide (COS) gắn acid gallic trong mô hình tế bào RAW264.7. Dẫn xuất được chuẩn bị theo phương pháp có sử dụng gốc tự do trung gian với xúc tác acid ascorbic/hydrogen peroxide. Hiệu quả kháng oxi hóa trong tế bào RAW264.7 được xác định thông qua khả năng bảo vệ các đại phân tử sinh học như DNA và lipid màng. Kết quả nghiên cứu cho thấy dẫn xuất mới có hiệu quả trung hòa gốc tự do để bảo vệ các đại phân tử sinh học trong tế bào RAW264.7. Hiệu quả bảo vệ DNA của dẫn xuất đều cao hơn COS ban đầu tại nồng độ từ 25 đến 100 µg/mL. Đối với màng tế bào, khả năng bảo vệ của dẫn xuất cao hơn có ý nghĩa so với COS ban đầu tại nồng độ từ 10 đến 100 µg/mL. Nghiên cứu này là nền tảng để nghiên cứu khả năng năng kháng oxi trên mô hình tế bào khác cũng như hoạt tính kháng viêm trên đại thực bào.

Từ khóa: Chitooligosaccharide, kháng oxi hóa, acid gallic.

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