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Anti-inflammatory effect of gallic acid-conjugated chitooligosaccharides in lipopolysaccharide-stimulated RAW 264.7 macrophages

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Abstract:

The aim of the present study is to investigate the anti-inflammatory effect of gallic acid grafted onto COS chains (GA-COS), focusing on the reduction of nitric oxide production, downregulation of inflammatory signals such as inducible nitric oxide synthase (iNOS), gene expression of cytokines such as TNF-α, IL-1β, IL-6, and nuclear factor kappa B (NF-κB) signalling, including the p50 and p65 subunits. The anti-inflammatory effect is mediated through the reduction of nitric oxide production and downregulation of inflammatory proteins such as inducible nitric oxide synthase (iNOS), gene expression of cytokines like TNF-α, IL-1β, IL-6, and nuclear factor kappa B (NF-κB) signalling, including the p50 and p65 subunits. Target proteins were identified by western blot analysis with specific monoclonal antibodies. The levels of gene expression were determined by the RT-PCR method. The results demonstrate that GA-COS effectively reduces nitric oxide generation and downregulates iNOS protein and cytokine expression and NF-κB signalling in lipopolysaccharide (LPS) -induced RAW 264.7 cells. GA-COS exhibits significantly enhanced anti-inflammatory activity compared to the free chitooligosaccharide chain. This study lays the groundwork for future research to demonstrate that GA-COS holds significant potential as a novel compound for the prevention of inflammatory diseases.

Keywords: **anti-inflammatory activity, chitooligosaccharide, chitooligosaccharide conjugated gallic acid, nitric oxide, RAW 264.7 macrophage cells.**

Classification numbers: **3.4, 3.5**

1. Introduction

In response to external agents such as pathogen invasion or exposure to toxic compounds, as well as internal factors such as cellular damage, the body's immune system initiates an inflammatory response [1]. Acute inflammation, characterized by rapid onset over minutes or hours and typically resolving within days, aims to heal intracellular damage. In contrast, chronic inflammation is a prolonged state of acute inflammation resulting from persistent infections that remain unresolved [2, 3]. Chronic inflammation is associated with various diseases, including stroke, chronic respiratory diseases, heart disorders, and cancer [4].

Macrophages, through the secretion of pro-inflammatory cytokines, play a crucial role in the regulation of inflammation [5]. Macrophages can be activated by stimuli such as bacterial LPS from gram-negative bacteria and pro-inflammatory cytokines [6]. In light of these observations, our study focuses on RAW 264.7 mouse macrophages stimulated with LPS as a model to investigate the anti-inflammatory activity of the synthesized compound. However, the regulation of inflammatory modulators involves complex feedback mechanisms [7]. In this study, we investigate the regulation of inflammatory modulators in LPS-stimulated RAW 264.7 cells through the NF-κB signalling pathways.

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In recent years, there has been growing interest in the use of bioactive compounds, particularly phenolic acids, for the treatment of chronic inflammation. Among phenolic acids, GA is a natural compound known for its various biological activities, including anti-inflammatory properties. Nevertheless, GA's limited solubility in water can affect its metabolism and absorption in the human body. Therefore, researchers have explored the grafting of GA onto chitooligosaccharide chains, non-toxic and water-soluble polysaccharides derived from chitin through a deacetylation process, to enhance the solubility of the synthesized compound and improve the bioactivity of the original COS chain [8, 9].

In previous research, GA-COS *via* the free radical-mediated conjugation method demonstrated enhanced antioxidant activities [10, 11]. However, there is currently no information available regarding the anti-inflammatory action of GA-COS against LPS-stimulated macrophages. The aim of the present study is to investigate the anti-inflammatory effect of GA-COS, focusing on the reduction of nitric oxide production, downregulation of inflammatory signals such as inducible iNOS, gene expression of cytokines such as TNF-α, IL-1β, IL-6, and NF-κB signalling, including the p50 and p65 subunits.

2. Materials and methods

2.1. Materials

GA, lactic acid, sodium bicarbonate, ethanol, 3-(4,5-dimethylthiazol2-yl)-2,5-diphenyl tetrazolium bromide (MTT) were purchased from HiMedia (Mumbai, India). The Dimethyl sulfoxide (DMSO), hydrogen peroxide, Folin, TLC silica gel 60 F254, Triton X-100, sodium nitrite, Griess reagent, and agarose were from Merck (Darmstadt, Germany). Dulbecco's Modified Eagle Medium (DMEM), penicillin/streptomycin, foetal bovine serum (FBS), trypsin, 3,3',5,5'-Tetramethylbensidine solution (TMB), Tris hydrochloride (Tris-HCl), 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.

The GA-COS powder was prepared according to our previous research [10]. In brief, COSs (0.5 g) were dissolved in approximately 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, gallic acid (0.25 g) was introduced into the mixture. The reaction was conducted at pH 5 and room temperature for 6 hours. After the reaction, the mixture was centrifuged through a 1 kDa filter membrane in a 50 ml centrifuge tube to remove any unreacted ingredients. Finally, the resulting solution was lyophilized using a freeze-dry system (FDU 2110, Eyela, Japan) to obtain solid GA-COS samples.

To confirm the successful grafting of gallic acid onto COS backbones, TLC analysis was performed. Gallic acid, ascorbic acid, and GA-COS were developed on a silica gel plate (TLC silica gel 60 F254, Merck, Germany) with a mobile phase consisting of chloroform - ethyl acetate - acetic acid (50:50:1) and heated at 100°C for 5 minutes. The developed TLC plate was observed under UV light.

The GA-COSs were characterised by proton nuclear magnetic resonance (1H-NMR) spectra. Samples were recorded using a NMR-500 MHz spectrometer (Bruker, Germany) under a static magnetic field of 500 MHz, and the samples were dissolved in D_2O.

2.2. Cell culture

The RAW 264.7 mouse macrophage cells procured from ATCC were cultivated in DMEM medium supplemented with 10% FBS, 100 µg/ml penicillin-streptomycin, and maintained in a 5% CO_2 atmosphere at 37°C. Subsequently, RAW 264.7 cells were detached using a scraper [12].

2.3. Cytotoxicity determination of GA-COS

The cytotoxicity of COS and GA-COS on RAW 264.7 cells was evaluated using the MTT method, as previously described [13]. Briefly, RAW 264.7 cells were seeded in 96-well plates at a concentration of $1x10^4$ cells/well and allowed to incubate for 24 hours. Following the 24-hour culture period, the medium in the wells was replaced with fresh medium containing various concentrations of COS and GA-COS. After an additional 24 hours of incubation, 100 µl of MTT solution was added to each well and further incubated for 4 hours at 37°C. Subsequently, 200 µl of DMSO was added to dissolve the formed formazan salt, and the absorbance of the samples was measured at

540 nm using a microplate reader (PerkinElmer, USA). The percentage of cell viability in untreated cells served as a control for calculating relative cell viability.

2.4. Nitric oxide (NO) assay

RAW 264.7 cells were cultured in DMEM medium without phenol red at a density of $5x10^5$ cells per well for 24 hours. Subsequently, the RAW 264.7 cells were exposed to COS or GA-COS (at concentrations ranging from 10 to 100 µg/ ml)) and LPS (1 µg/ml) for 24 hours. The NO content in the culture medium was determined using the Griess method [14]. Specifically, 50 µl of Griess reagent (comprising 1% sulphanilamide and 0.1% naphthyl ethylenediamine dihydrochloride in 2.5% phosphoric acid) was mixed with 50 µl of the culture medium. After a 20-minute incubation period, absorbance was measured at 540 nm using the same microplate reader as previously mentioned. NO concentration was determined using a standard curve prepared with sodium nitrite.

2.5. Western blot assay

RAW 264.7 cells were cultured in DMEM medium at a density of $5x10^5$ cells per well for 24 hours. After pretreatment with GA-COS or COS (at concentrations ranging from 10 to 100 µg/ml) for 1 hour, LPS (1 µg/ml) was introduced for 24 hours. The RAW 264.7 cells were lysed in RIPA buffer (comprising 150 mM NaCl, 0.5 mM EDTA pH 8.0, 50 mM Tris pH 8.0, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS). Protein concentration was determined using the bio-rad protein assay with bovine serum albumin as the standard. Subsequently, 50 µg of protein was denatured in a 4x sample buffer (3:1) at 90°C for 4 minutes, followed by cooling in water for 2 minutes, repeated twice. The protein samples were then subjected to electrophoresis on a 12.5% polyacrylamide gel using the miniVE electrophoresis and electrotransfer system (Amersham Bioscience, USA). Subsequently, the proteins were transferred onto nitrocellulose membranes using the miniVE system and blocked with 5% skim milk for a minimum of 1 hour at room temperature. The membrane was incubated overnight at 4°C with primary antibodies against iNOS, NF-**κ**B p50, and NF-**κ**B p65 (Proteintech, USA) and then washed three times with 1X PBS/0.2% Tween 20. Following this, the membrane was incubated with a peroxidase-conjugated secondary antibody (1:1000) (Proteintech Group, Inc.) for 1 hour at room temperature and washed three times with 1X PBS/0.2% Tween 20. Finally, the membrane was developed using a 3,3',5,5'-Tetramethylbenzidine solution to visualize the western blot bands [12].

2.6. RNA isolation and reverse transcription-polymerase chain reaction (RT-PCR) assay

RAW 264.7 cells were cultured in a culture medium at a density of 5x105 cells in each dish. Subsequently, the RAW 264.7 cells were incubated with GA-COS or COS (at concentrations ranging from 10 to 100 µg/ml) for 1 hour and LPS (1 µg/ml) for 24 hours. Total RNA was then extracted using the All-In-One DNA/RNA Miniprep Kit (Bio Basic, USA) following the manufacturer's protocol. The factors of interest were determined using the RT-PCR method [15]. To initiate cDNA synthesis, 2 µg of total RNA was converted to cDNA using the primer reverse transcription system (Cole-Parmer, UK). The target cDNA was amplified using specific primers (Phusa Biochem, Vietnam) as follows: for TNF-α, forward 5'-AGC-CCC-CAG-TCT-GTA-TCC-TT-3' and reverse 5'-CAT-TCG-AGG-TCT-CAG-TGA-AT-3'; for IL-1β, forward 5'-GGG-CCT-CAA-AGG-AAA-GAA-TC-3' and reverse 5'-TCA-CAG-TTG-GGG-AAT-TCT-GC-3'; for IL-6, forward 5'-TTC-CAG-AAT-CCC-TGG-ACA-AG-3' and reverse 5'-TGG-TCA-AAC-TCT-TGG-GGT-TC-3'; for glyceraldehyde 3-phosphate dehydrogenase (GADPH), forward 5'-GTC-AAC-GGA-TTT-GGT-CGT-ATT-3' and reverse 5'-AGT-CTT-CTG-GGT-GGC-AGT-GAT-3'. The RT-PCR conditions, suitable for the MyTaq One-Step RT-PCR Kit (Meridian Bioscience, USA), included amplification cycles carried out at 45°C for 20 minutes (1 cycle), 95°C for 1 minute (1 cycle), and 95°C for 10 seconds - 60°C for 10 seconds - 72°C for 30 seconds (40 cycles). A 10 µl mixture was combined with 2 µl of GelRed and subjected to electrophoresis on a 1% agarose gel for 20 minutes at 100 V. Finally, the gels were visualized under UV light using gel documentation image analysis software, VisionCapt.

2.7. Statistical analysis

The data are presented as the mean±standard deviation (SD) of triplicate measurements. Multiple comparisons were conducted using the least significant difference (LSD), Duncan's multiple range test, and one-way analysis of variance (ANOVA) with Statgraphics Centurion software. Statistical significance was determined at $p<0.05$.

3. Results and discussion

3.1. Cell viability

In this study, RAW 264.7 cells were exposed to various concentrations of COS and GA-COS (ranging from 1 to 250 µg/ml) to assess non-cytotoxic concentrations for subsequent experiments. As illustrated in Fig. 1, the cell viability data unequivocally indicate that both COS and GA-COS did not exhibit any significant toxicity towards RAW 264.7 cells at the tested concentrations. GA-COS, in particular, demonstrated a cell viability exceeding 90% [9]. These results substantiate the non-toxic nature of COS and GA-COS at the concentrations assessed. It is noteworthy that previous reports have also established the non-toxicity of GA-conjugated COS in RAW 264.7 cells across all tested concentrations [9, 15].

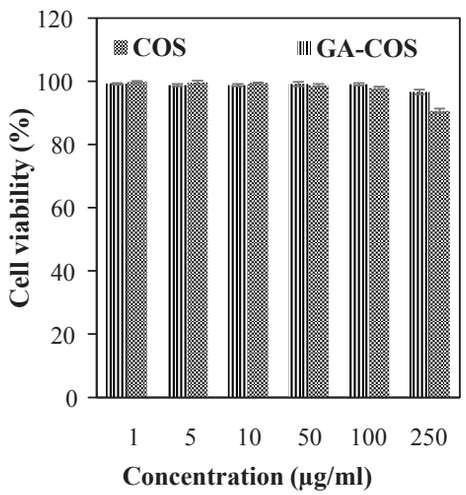

Fig. 1. RAW 264.7 cell viability was determined by the MTT assay.

The results of Fig. 1 shown are representative of separate experiments performed in triplicate. Error bars represent the standard error (SD).

3.2. Effects of GA-COS on nitric oxide production and protein expressions of iNOS in the LPS-induced RAW 264.7 cells

NO is a crucial mediator produced via the activity of the iNOS enzyme and plays a pivotal role in inflammation [6]. To elucidate the anti-inflammatory potential of GA-COS, an investigation was conducted to ascertain whether GA-COS influenced NO production in LPS-stimulated RAW 264.7 cells. The level of NO in the culture medium following LPS induction was assessed using the Griess reagent. As depicted in Fig. 2, GA-COS exhibited a significantly enhanced inhibitory effect on NO production, whereas COS demonstrated only a slight increase in inhibition at a concentration of 100 µg/ml. Specifically, GA-COS demonstrated an inhibition of NO production at 46.23±1.02%, whereas COS achieved an inhibition of 10.83±0.25%. This outcome underscores the capacity of GA grafted onto COS to improve the inhibition of NO production compared to the unmodified COS chain, thereby substantiating its potential for mitigating inflammation.

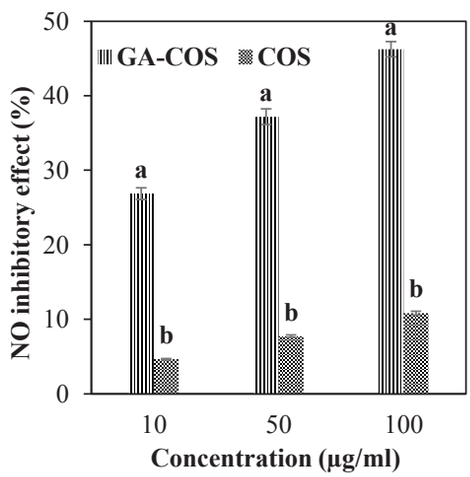

Fig. 2. Effects of GA-COS on NO production inhibition in the LPS-stimulated RAW 264.7 cells.

The results of Fig. 2 shown are representative of separate experiments performed in triplicate. Error bars represent the standard error (SD). Letters a, b indicate significant difference, $p<0.05$.

iNOS is an enzyme that plays a pivotal role in the inflammatory response. Increased expression levels of iNOS result in heightened NO production within cells, thereby contributing to inflammatory diseases. Western

blot analysis was conducted to assess the inhibitory effect of GA-COS on iNOS protein expression. Following a 1-hour pretreatment with GA-COS, RAW 264.7 macrophages were subsequently stimulated with LPS for 24 hours. GA-COS exhibited a concentration-dependent reduction in the expression of iNOS at the translational level (Fig. 3). Notably, a significant difference was observed compared to cells treated with COS, indicating the superior activity of GA-COS in suppressing iNOS expression in LPS-stimulated RAW 264.7 cells.

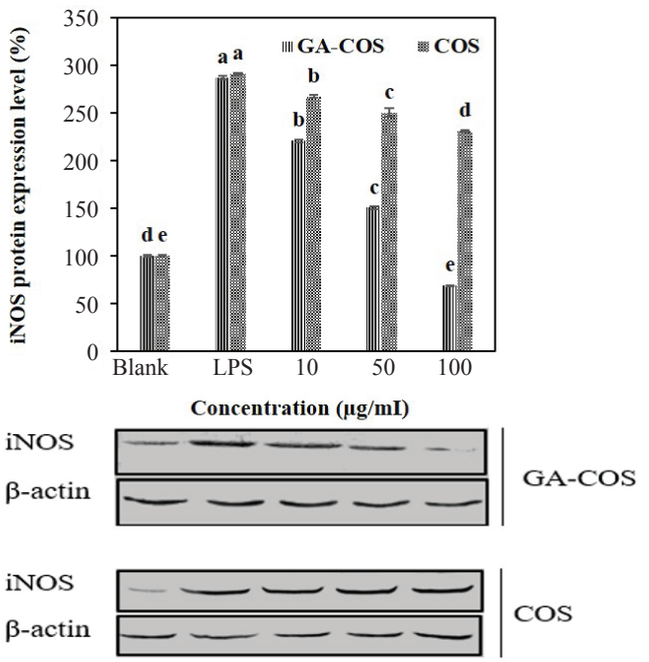

Fig. 3. Effects of GA-COS and COS on protein expression of iNOS in LPS-induced RAW 264.7 cells.

The results of Fig. 3 shown are representative of separate experiments performed in triplicate. Error bars represent the standard error (SD). Letters a-e indicate significant difference, $p<0.05$.

3.3. Effects of GA-COS on the mRNA expression of TNF-α, IL-1β, and IL-6 in the LPS-induced RAW264.7 cells

To assess the impact of GA-COS on the production of pro-inflammatory cytokines, namely TNF-α, IL-1β, and IL-6, RAW264.7 cells were exposed to GA-COS for 1 hour and subsequently stimulated with LPS (1 µg/ml) for 24 hours. The results, as presented in Fig. 4, demonstrate that GA-COS led to a reduction in mRNA expression levels in LPS-induced RAW264.7 macrophages. At a concentration of 50 µg/ml, GA-COS exhibited inhibitory effects on the secretion of TNF-α, IL-1β, and IL-6 at levels of 92.44±2.99, 94.92±1.22, and 144.37±5.39%, respectively. It is noteworthy that, at equivalent concentrations, the influence of COS in reducing cytokine secretion was significantly lower than that of GA-COS.

The results shown are representative of separate experiments performed in triplicate. Error bars represent the standard error (SD). Letters a-e indicate significant difference, $p<0.05$.

3.4. Effects of GA-COS on NF-κB signalling activation

NF-κB, comprising two subunits, p50 and p65, is a pivotal transcription factor responsible for regulating the expression of numerous proinflammatory mediators, including cytokines (IL-1α, IL-1β, IL-6, and TNF-α) and chemokines (IL-8, CCL2). In normal cells, NF-κB exists in an inactive complex state, sequestered in the cytoplasm by binding to an inhibitor known as I-κB. Stimulation of cells by inflammatory factors, such as UV radiation or bacterial agents, induces NF-κB activation. Following phosphorylation, NF-κB leads to the degradation of I-κB and translocates into the nucleus to initiate the transcription of target genes [16]. In our study, RAW 264.7 cells, stimulated by the extracellular agent LPS for 24 hours, exhibited increased expression of p50 and p65 proteins, with β-actin serving as a control. The inflammatory response was triggered by the interaction between LPS and toll-like receptor 4 (TLR4), culminating in the activation of the myeloid differentiation factor 88 (MyD88) signalling pathway. Subsequently, downstream signalling pathways, such as the NF-κB pathway, were activated, leading to nuclear translocation and the production of inflammatory cytokines and mediators [1]. As depicted in Fig. 5, the GA-COS derivative demonstrated a reduction in the expression levels of p50 and p65 proteins at concentrations ranging from 10 to 100 µg/ml, exhibiting a significant improvement compared to COS. Previous studies have also indicated that gallic acid-conjugated COS downregulates NF-κB

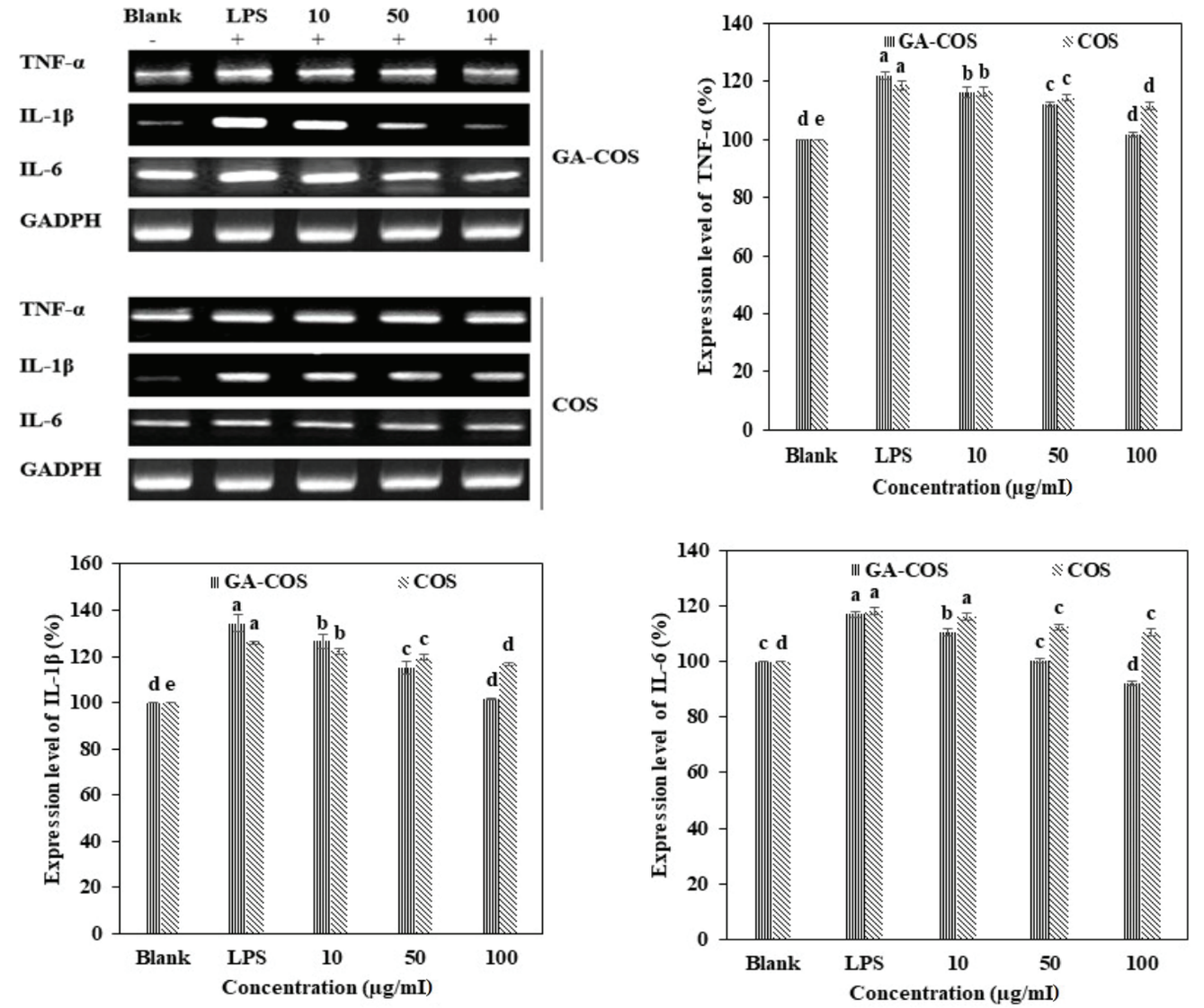

Fig. 4. Effects of GA-COS on the mRNA expression of TNF-α, IL-1β, and IL-6 in the LPS-induced RAW264.7 cells.

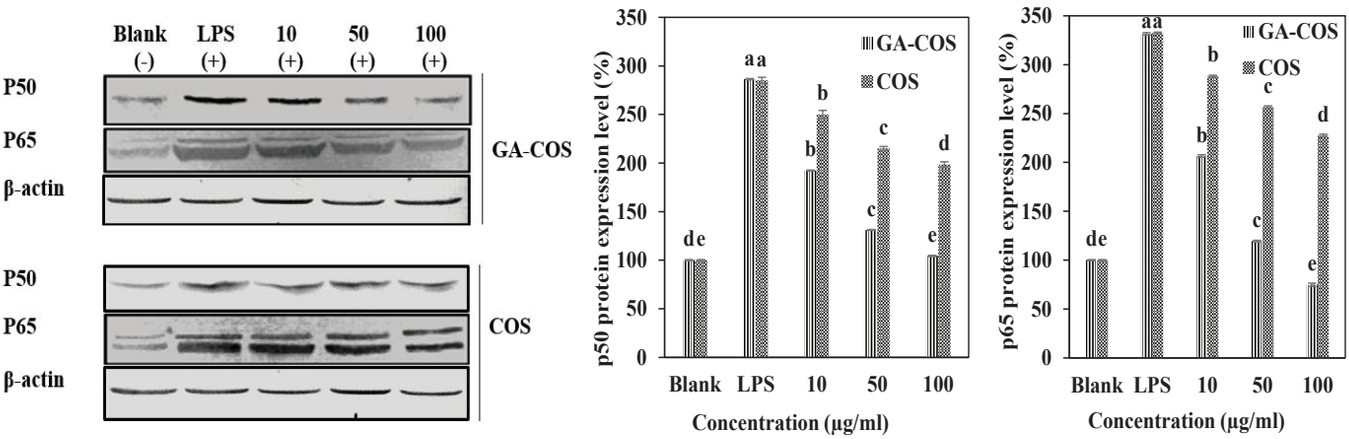

Fig. 5. Effects of GA-COS and COS on p50 and p65 signal in LPS-stimulated RAW 264.7 cells.

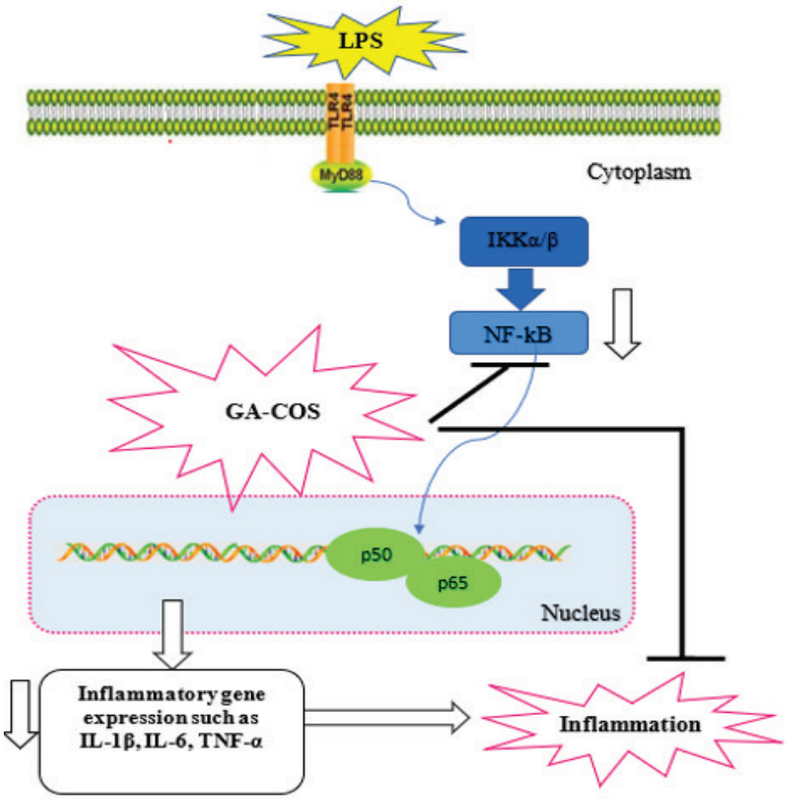

Fig. 6. Diagram displaying the mechanism of NF-κB signal inhibition of GA-COS.

protein signalling expression [12]. Fig. 6 provides an illustrative representation of the mechanism underlying the inhibition of NF-κB signalling by GA-COS.

The results of Fig. 5 shown are representative of separate experiments performed in triplicate. Error bars represent the standard error (SD). Letters a-e indicate significant difference, $p<0.05$.

4. Conclusions

GA-COS exhibited anti-inflammatory properties by suppressing NO formation, inhibiting iNOS protein expression, and downregulating the expression of p50 and p65 protein signals. Furthermore, it regulated the expression of pro-inflammatory cytokines such as TNF-α, IL-1β, and IL-6. This study lays the groundwork for future research to demonstrate that GA-COS holds significant potential as a novel compound for the prevention of inflammatory diseases.

CRediT author statement

Van-Hoai Bui, Hong-Tham N. Vo, Thanh-Tuan Duong, Se-Kwon Kim: Conceptualisation, Methodology, Formal analysis, Software, Data curation, Validation, Visualisation, Investigation; Dai-Nghiep Ngo: Conceptualisation, Methodology, Formal analysis, Supervision, Original draft preparation, Writing - Reviewing and Editing, Project administration.

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COMPETING INTERESTS

The authors declare that there is no conflict of interest regarding the publication of this article.

REFERENCES

[1] L. Chen, H. Deng, H. Cui, et al. (2018), "Inflammatory responses and inflammation-associated diseases in organs", *Oncotarget*, **9(6)**, pp.7204-7218, DOI: 10.18632/oncotarget.23208.

[2] B. Kaminska (2005), "MAPK signalling pathways as molecular targets for anti-inflammatory therapy-from molecular mechanisms to therapeutic benefits", *Biochim. Biophys. Acta*, **1-2**, pp.253-262, DOI: 10.1016/j.bbapap.2005.08.017.

[3] T. Liu, L. Zhang, D. Joo, et al. (2017), "NF-κB signalling in inflammation", *Signal Transduction And Targeted Therapy*, **2(1)**, pp.1-9, DOI: 10.1038/sigtrans.2017.23.

[4] D. Furman, J. Campisi, E. Verdin, et al. (2019), "Chronic inflammation in the etiology of disease across the life span", *Nat. Med.*, **12**, pp.1822-1832, DOI: 10.1038/s41591-019-0675-0.

[5] Y.S. Cho, S.H. Lee, S.K. Kim, et al. (2011), "Aminoethyl-chitosan inhibit LPS-induced inflammatory mediator, iNOS and COX-2 expression in RAW264.7 mouse macrophage", *Process Biochemistry*, pp.465-470, DOI: 10.1016/j.procbio.2010.09.019.

[6] A. Cheng, H. Yan, C. Han, et al. (2014), "Polyphenols from blueberries modulate inflammation cytokines in LPS-induced RAW264. 7 macrophages", *International Journal of Biological Macromolecules*, **69**, pp.382-387, DOI: 10.1016/j.ijbiomac.2014.05.071.

[7] N. Fujiwara, K. Kobayashi (2005), "Macrophages in inflammation", *Current Drug Targets-Inflammation & Allergy*, **4(3)**, pp.281-286, DOI: 10.2174/1568010054022024.

[8] J. Liu, J. Lu, J. Kan, et al. (2013), "Synthesis of chitosan-gallic acid conjugate: Structure characterisation and *in vitro* anti-diabetic potential", *Int. J. Biol. Macromol.*, **62**, pp.321-329, DOI: 10.1016/j.ijbiomac.2013.09.032.

[9] H.H. Park, S.C. Ko, G.W. Oh, et al. (2018), "Characterisation and biological activity of PVA hydrogel containing chitooligosaccharides conjugated with gallic acid", *Carbohydr. Polyme*, **198**, pp.97-205, DOI: 10.1016/j.carbpol.2018.06.070.

[10] V.H. Bui, D.N. Ngo (2018), "Synthesis of gallic acid grafted chitooligosaccharides with an ascorbic acid/hydrogen peroxide redox pair to improve chitooligosaccharide activity", *Proceeding of The Biotechnology Conference*, Institute of Biotechnology, Vietnam Academy of Science and Technology, pp.159-164.

[11] V.H. Bui, D.N. Ngo, D.P. Chau, et al. (2020), *Antioxidant Effect Of Gallic Acid-Chitooligosacharides Derivatives In Raw264.7 Cell*, National Biotechnology Conference 2020, pp.960-964.

[12] D.N. Ngo, Z.J. Qian, T.S. Vo, et al. (2011), "Antioxidant activity of gallate-chitooligosaccharides in mouse macrophage RAW264. 7 cells", *Carbohydrate Polymers*, **4**, pp.1282-1288, DOI: 10.1016/j.carbpol.2011.01.022.

[13] D.N. Ngo. M.M. Kim, S.K. Kim (2012), "Protective effects of aminoethyl-chitooligosaccharides against oxidative stress in mouse macrophage RAW 264.7 cells", *International Journal of Biological Macromolecules*, **3**, pp.624-631, DOI: 10.1016/j.ijbiomac.2012.01.036.

[14] M.D.L. Trinh, M.H. Dinh, D.H. Ngo, et al. (2014), "Protection of 4-hydroxybenzyl-chitooligomers against inflammatory responses in Chang liver cells", *International Journal Of Biological Macromolecules*, **66**, pp.1-6, DOI: 10.1016/j.ijbiomac.2014.01.064.

[15] T.S. Vo, D.H. Ngo, L.G. Bach, et al. (2017), "The free radical scavenging and anti-inflammatory activities of gallate-chitooligosaccharides in human lung epithelial A549 cells", *Process Biochemistry*, **54**, pp.188-194, DOI: 10.1016/j.procbio.2017.01.001,

[16] P. Ma, H.T. Liu, P. Wei, et al. (2011), "Chitosan oligosaccharides inhibit LPS-induced over-expression of IL-6 and TNF-α in RAW264.7 macrophage cells through blockade of mitogen-activated protein kinase (MAPK) and PI3K/Akt signalling pathways", *Carbohydrate Polymers*, **4**, pp.1391-1398, DOI: 10.1016/j.carbpol.2011.01.045.