

Inhibitory effect of a formulated extract from multiple citrus peels on LPS-induced inflammation in RAW 246.7 macrophages

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ABSTRACT:

Background: Formulated Citrus Peel Extract (GL) made from the peels of six citrus fruits available in Japan, namely *navel oranges*, *citrus hassaku*, *citrus limon*, *citrus natsudaidai*, *citrus miyauchi* and *satsuma*, was initially developed as a cosmetic product to protect skin from UV irradiation. Anecdotal evidences of anti-cancer property of GL have been reported by consumers based on the cases such as topical application for melanoma, and oral ingestion for prostate, lung and liver cancers.

Those anecdotal reports stimulated us to investigate anti-tumorigenesis activity of GL. In the previous study, we reported that the topical application of GL inhibited DMBA/TPA-induced skin tumor formation by decreasing inflammatory gene parameters.

Objective: In this study, we mainly investigated the effect of GL on translocation of NF- κ B together with production of nitric-oxide and TNF- α induced by LPS in RAW 264.7 cells.

Results: This investigation showed that GL decreased the release of TNF- α and nitric oxide from macrophage RAW264.7 cells stimulated by LPS in a dose-dependent manner. In addition, GL suppressed the expression of iNOS and nuclear translocation of NF- κ B in RAW264.7 cells, inhibited the degradation of I κ B- α , and scavenged hydroxyl radicals (DMPO/OH adduct) *in vitro*.

Conclusions: Our findings suggest that GL suppresses the inflammation *in vitro*, and exerts chemopreventive activity through the inhibition of production of TNF- α and iNOS proteins due to the inhibition of nuclear translocation of NF- κ B and oxidative stress. GL appears to be a novel functional natural product capable of preventing inflammation and inflammation-associated tumorigenesis.

Keywords: GL, Citrus peel extract, anti-inflammation, Nitric oxide, iNOS, NF- κ B, TNF- α

INTRODUCTION:

Citrus peel has been used traditionally as peptic and bronchial agent in oriental medicine and recently this has been reported to exhibit a broad spectrum of biological activity such as anti-inflammation, suppression of tumorigenesis in experimental animals [1, 2]. The citrus peel is a rich source of flavonoids, as well as many polymethoxylated flavones, which are very rare in other plants [3]. These compounds not only play important roles in biological activities but also in commercial applications in the food and pharmaceutical industries [4].

Mounting evidence suggests that chronic inflammation mediates most chronic diseases, including cancer. Among other transcription factors, nuclear factor-kappaB (NF- κ B) has emerged as major regulators of inflammation, cellular transformation, tumor cell survival, proliferation, invasion, angiogenesis and metastasis [5]. Thus, agents that can inhibit NF- κ B activation pathways have a potential for both prevention and treatment of cancer [6].

Formulated Citrus Peel Extract (GL) is made from the peels of six citrus fruits available in Japan, namely *navel oranges*, *citrus hassaku*, *citrus limon*, *citrus natsudaidai*, *citrus miyauchi* and *Satsuma*. It was initially developed as a cosmetic product to protect skin from UV irradiation. Anecdotal evidences of anti-cancer property of GL have been reported by consumers based on the cases such as topical application for melanoma, and oral ingestion for prostate, lung and liver cancers. These anecdotal reports stimulated us to investigate anti-inflammation and anti-tumorigenesis activities of GL in animal models.

We previously demonstrated that oral feeding of GL suppressed colonic tumorigenesis

induced by azoxymethane [7]. In addition, GL also inhibited DMBA/TPA-induced skin tumor formation [8]. In this study, we investigated the effect of GL on translocation of NF- κ B together with production of nitric-oxide and TNF- α induced by LPS *in vitro*.

MATERIALS AND METHODS:

Citrus Peel Extract: Commercially available formulated citrus peel extract (GL, Miyauchi Citrus Research Center, Gunma, Japan) made from the peels of six citrus fruits was used in this study.

Reagents: Polyclonal antibodies specific for I κ B- α , NF- κ B and anti-rabbit IgG, HRP-linked antibody were purchased from Cell Signaling Technology (MA, U.S.A.). Biotinylated anti-mouse, goat IgG (H+L) and Vectastain ABC kit were purchased from Vector Laboratories (CA, U.S.A.). The ECL western blot detection reagent was purchased from GE Healthcare (NJ, U.S.A.). Dulbecco's Modified Eagle's Medium (DMEM) and lipopolysaccharides (LPS, form *Escherichia coli*, serotype 026:B6) and protease inhibitor cocktail were obtained from Sigma-Aldrich (MO, U.S.A.). Tumor necrosis Factor alpha (TNF- α) ELISA kit was purchased from Pierce Biotechnology (IL, U.S.A.).

Cell culture: RAW264.7 cells, a murine macrophage-like cell line, were obtained from Riken Cell Bank (Tsukuba, Ibaraki, Japan). Cells cultured in phenol red-free DMEM, supplemented with 10% fetal bovine serum (FBS) (EIDIA, Tokyo, Japan), 100 U/mL penicillin (Meiji seika, Tokyo, Japan), 100 μ g/mL streptomycin (Meiji seika, Tokyo, Japan) and 2mM L-glutamine (Life Technologies Co., NY, U.S.A.). All experiments were performed with cells grown to 80-90% confluence and following no more than 15 passages.

Measurement of cell viability: Cell viability was assessed by MTT assay. RAW264.7 cells plated in 96 well tissue culture dishes (1×10^5 cells) were treated with various concentrations of GL (1, 3, 10%) for 20 hours. Then MTT (Sigma) was added (0.5 mg/mL) and incubated for 4 hours. The culture medium was removed, and cells were dissolved in dimethyl sulfoxide (Wako). The optical densities (OD) at 540 nm and 620 nm were measured using a microplate reader (Model 680, Bio-Rad Laboratories, CA, U.S.A.).

Measurement of NO: Nitrite concentrations in the conditioned medium were determined by using Griess reagent (1% sulfanilamide (Sigma), 0.1% *N*-1-naphthylethylenediamine dihydrochloride (Sigma), and 2.5% phosphoric acid (Kanto chemical, Tokyo, Japan)) (Green

LC et al., 1982). RAW264.7 cells (1×10^5 cells/well) were preincubated at 37°C for 3 hours with or without various concentrations of GL (1, 3, 10%). After pre-incubation, LPS ($10 \mu\text{g/mL}$) was added, and cells were incubated for 20 hours. After incubation, $100 \mu\text{L}$ aliquots of medium were mixed with an equal volume of Griess reagent. Absorbance was measured at 540 nm with a microplate reader after incubating for 5 minutes.

Measurement of TNF- α : RAW264.7 cells (1×10^5 cells/well) were preincubated at 37°C for 3 hours with or without various concentrations of GL (1, 3, 10%). After incubation, LPS ($10 \mu\text{g/mL}$) was added, and cells were incubated for 6 hours. After incubation, the culture media in a 96-well plate were harvested, centrifuged at $370 \times g$ for 10 minutes at 4°C , and the supernatant removed and frozen at -30°C until assay. TNF- α concentration in the supernatants was measured by ELISA.

Western blot analysis: RAW264.7 cells were plated at 2×10^6 cells/well in 6 well plates and preincubated at 37°C for 3 hours with various concentrations of GL. Cells were then treated with LPS ($10 \mu\text{g/mL}$) for the times indicated in the text. The cells were washed in cold PBS and lysed in $75 \mu\text{L}$ lysis buffer (20 mM HEPES pH 7.3) supplemented with 1% Triton-X, 10% glycerol, 1 mM sodium fluoride, 1 mM Na_3VO_4 and protease inhibitor cocktail. After 15 minutes incubation on ice, cells were collected in sample tube by silicon policeman and incubated at 10 minutes on ice. Then cells were centrifuged at $15490 \times g$ for 20 minutes at 4°C . The supernatant fractions were boiled for 5 minutes in sample buffer (50 mM Tris, pH7.4, 4% SDS, 10% glycerol, 4% 2-mercaptoethanol and $50 \mu\text{g/mL}$ bromophenol blue). Preparation of nuclear extracts was performed, as follows. RAW264.7 cells (2×10^7 cells/well) were plated in 10 cm tissue culture dishes. The cells were pre-incubated at 37°C for 3 hours with or without various concentration of GL and treated with LPS for each time. At the end of incubation, the cells were harvested by scraping, followed by centrifugation at $210 \times g$ and 4°C for 5 minutes. After centrifugation, the cell pellet was resuspended in $800 \mu\text{L}$ of Tris-buffered KCl solution (20 mM Tris-HCl, pH 7.8, 50 mM KCl) supplemented with 1 mM dithiothreitol, 50 mM sodium fluoride, 1 mM Na_3VO_4 and protease inhibitor cocktail. Nonidet P-40 substitute (Wako) was added at 0.6% final concentration, and the cell suspension was vortexed for 10 seconds and centrifuged at $15490 \times g$ for 1 minute at 4°C to obtain the nuclei pellet. The nuclei were resuspended in $30 \mu\text{L}$ ice-cold Tris-buffered high-KCl solution (20 mM Tris-HCl, pH 7.8, 500 mM KCl) supplemented with 20% glycerol, 1 mM dithiothreitol, 50 mM sodium fluoride, 1 mM Na_3VO_4 and protease inhibitor cocktail. After 1 hour incubation on ice, the nuclear lysate was centrifuged at $15490 \times g$ for 20 minutes at 4°C ,

and the supernatant (nuclear extract) was mixed with 3×SDS/PAGE sample buffer (50 mM Tris, pH 7.4, 4% SDS, 10% glycerol, 4% 2-mercaptoethanol and 0.05 mg/mL bromophenol blue) at 2:1 ratio, boiled for 5 minutes, and stored at -80°C. The proteins were separated by 8% SDS-PAGE and transferred to a PVDF membrane in transfer buffer (20 mM Tris, pH 8.3, 150 mM glycine, 20% methanol). The PVDF membrane was then blocked by incubation in 20% Block ace (DS Pharma Biomedical., Osaka, Japan). Subsequently, the membrane was incubated with the each antibody for overnight at 4°C and finally incubated for 4 hours at 4°C with a secondary antibody. The protein bands were visualized using an enhanced chemiluminescence system (Amersham Biosciences, NJ, U.S.A.).

Radical scavenging activity: Inhibition of iron-induced hydroxyl radical formation was determined using the electron spin resonance (ESR) trapping technique in combination with DMPO. The DMPO-OH adduct was obtained from the Fenton reaction system containing DMPO (10 µL), H₂O₂ (0.1 mM: 40 µL), and ferrous sulfate (5 mM: 20 µL) with or without GL (5, 10, 20 µL). The mixture was transferred to a flat quartz cell, and the ESR spectrum was measured 4 minutes after the addition of ferrous sulfate. The spin adducts generated in the reaction system were detected using a JEOL RE2X ESR spectrometer (JEOL, Japan). Instrument conditions were as follows: central magnetic field 336 mT (328.5-343.5 mT), 9.425 GHz, microwave power of 1.0 mW, modulation frequency 100 kHz, response rate 0.1s, scanning time 4 minutes, room temperature (23 °C).

Statistical analysis: The data were expressed as a mean ± SEM of the results obtained from a number of experiments. The statistical significance of the results was analyzed by Student's t-test.

RESULTS:

Effect of GL on cell viability of RAW 264.7 cells: To examine cytotoxicity of GL on RAW 264.7 cells, cells were exposed to various concentrations of GL (1, 3, 10%) for 20 hours, and cytotoxicity was monitored using the MTT assay. GL had no influence on cell viability of RAW 264.7 cells (Fig. 1).

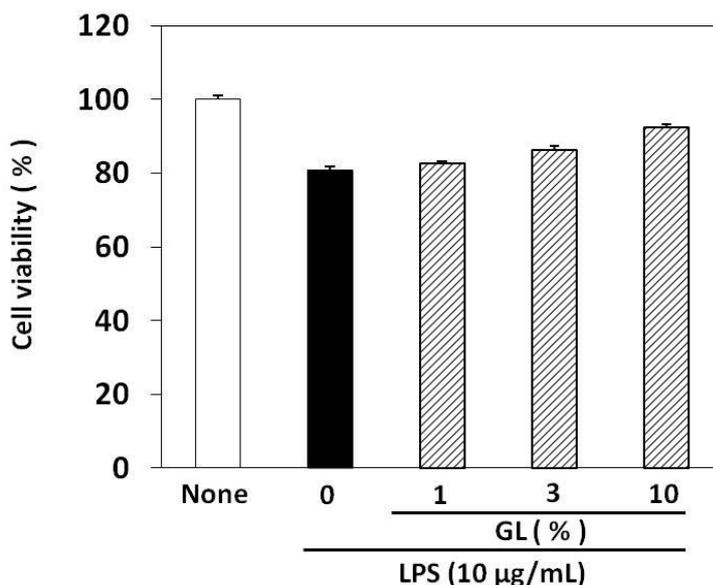


Figure 1. Effect of GL on cell viability of RAW 264.7 cells. RAW264.7 cells (1.0×10^5 cells/well) were pretreated at 37°C for 3h with various concentration of GL (1, 3, 10%) before LPS (10 µg/ml) was added, and cells were incubated for 20h. After incubation, the cell viability were measured by MTT assay. The cell viability was defined as the % of non-stimulated cells. Result shown are representatives of three independent experiments.

Effect of GL on NO and TNF-α production: Incubation with LPS for 20 hours increases the production of nitrite and TNF-α in RAW 264.7 cells. GL reduced the production of nitrite and TNF-α in cell culture supernatants dose-dependently as shown in Fig. 2.

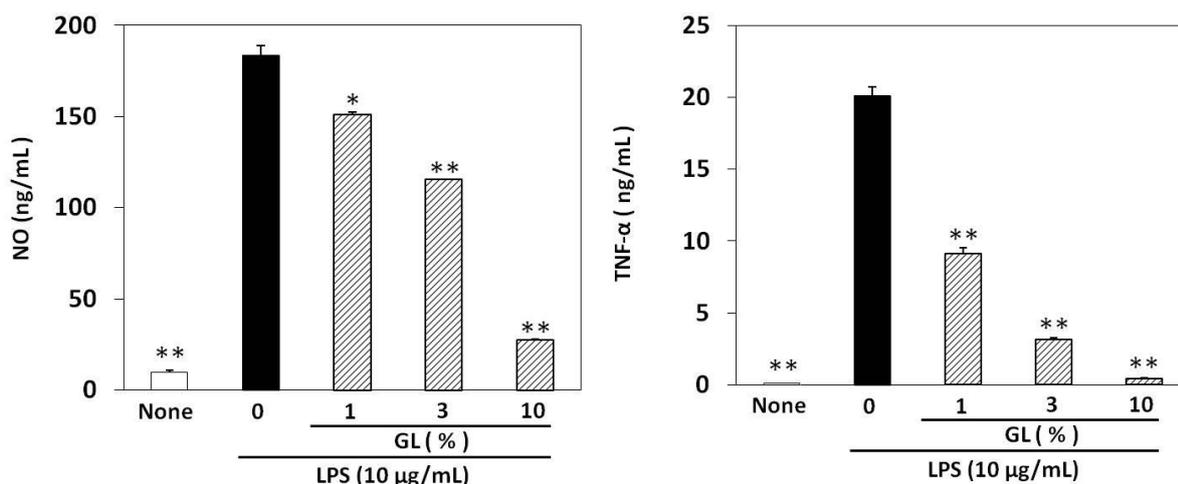


Figure 2. Effect of GL on NO and TNF-α production. RAW 264.5 cells (1.0×10^5 cells/well) were incubated in 96-well plates for 24 hours and GL was added at final concentrations of 1, 3, and 10% and incubated for 3 hours. After the treatment, LPS (10 mg/mL) was added and incubated for further 20 hours, and the nitrite production was measured by Griess reaction. Statistical significance; *P<0.05, **P<0.01 vs. corresponding LPS control.

Effect of GL on iNOS protein expression: To assess whether the reduced production of nitrite in GL-treated RAW 264.7 cells is due to decrease in iNOS protein level, immunoblotting experiments using a specific anti-iNOS antibody were performed. Cells were treated with GL for 3 hours, and stimulated with LPS for 20 hours. As shown in Fig. 3, GL strongly inhibited the expression of iNOS protein dose-dependently.

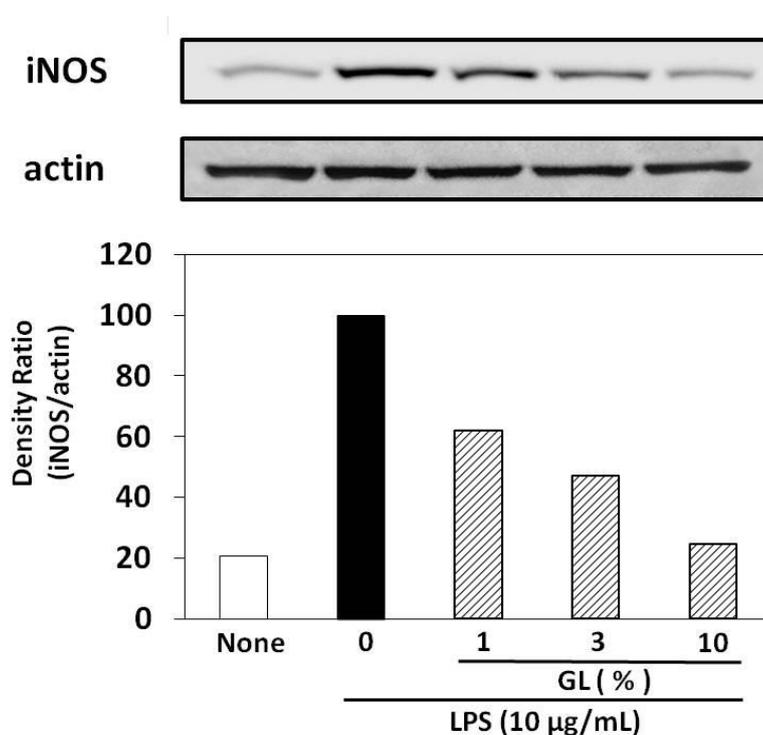


Figure 3. Effect of GL on iNOS protein expression. RAW 264.5 cells were treated with GL at final concentrations of 1, 3, and 10% and incubated for 3 hours. After the treatment, LPS (10 $\mu\text{g}/\text{mL}$) was added and incubated for further 20 hours, and the protein formation was measured by western blot analysis. To facilitate comparisons, the relative density ratios of iNOS protein to actin protein are shown as histogram. The density ratio of LPS control is set to 100. Result shown are representatives of three independent experiments.

Effect of GL on translocation of NF- κ B into nucleus: Activation of NF- κ B is known to be required for iNOS expression and TNF- α production. Therefore, we investigated the translocation of p65, a subunit of NF- κ B, from the cytosol to the nucleus by Western blot analysis. RAW264.7 cells were pretreated for 3 hours with 10% of GL before LPS was added. When RAW264.7 cells were incubated at 37 $^{\circ}\text{C}$ in medium containing LPS for 15 minutes, p65 protein levels were increased. It indicated that NF- κ B translocated into nucleus when treated with LPS. In the presence of GL (10%), translocation of NF- κ B into nucleus was suppressed significantly. On the other hand, GL did not affect on the protein levels of Oct-1 which suggested it did not disturb normal physiological functions in nucleus (Fig. 4).

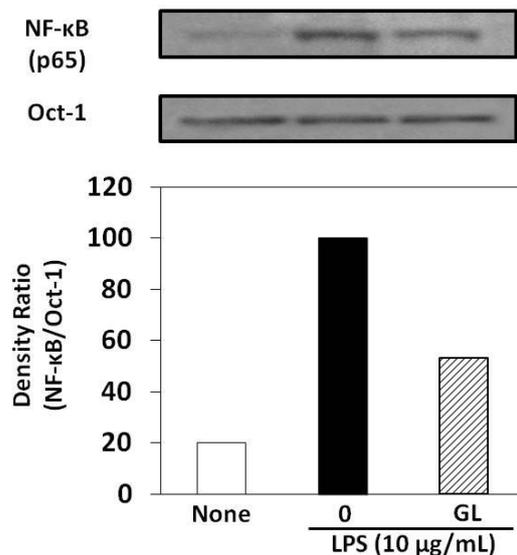


Figure 4. Effect of GL on translocation of NF-κB into nucleus. RAW264.7 cells were pretreated at 37 °C for 3 hours with 10% GL, LPS (10 μg/mL) was added, and cells were incubated for 15 minutes. After incubation, levels of NF-κB (p65) and Oct-1 protein in the nuclear extract samples were determined by Western blot. To facilitate comparisons, the relative density ratios of NF-κB protein to Oct-1 protein are shown as histogram. The density ratio of LPS control is set to 100. Results shown are representatives of three independent experiments.

Effect of GL on degradation of IκB-α: To determine whether GL inhibited degradation of IκB-α, cell lysates of RAW264.7 treated with GL cells were prepared. RAW264.7 cells were pretreated with GL (10%) for 3 hours and then incubated in the presence of LPS for various periods (5, 10, 15, 30, 60 minutes). When RAW264.7 cells were incubated at 37 °C for 10 or 15 minutes in medium containing LPS, the IκB-α was degraded. However, when the cells were pretreated with GL (10%), the IκB-α level did not change. Furthermore, GL (10%) did not affect the actin level (Fig. 5).

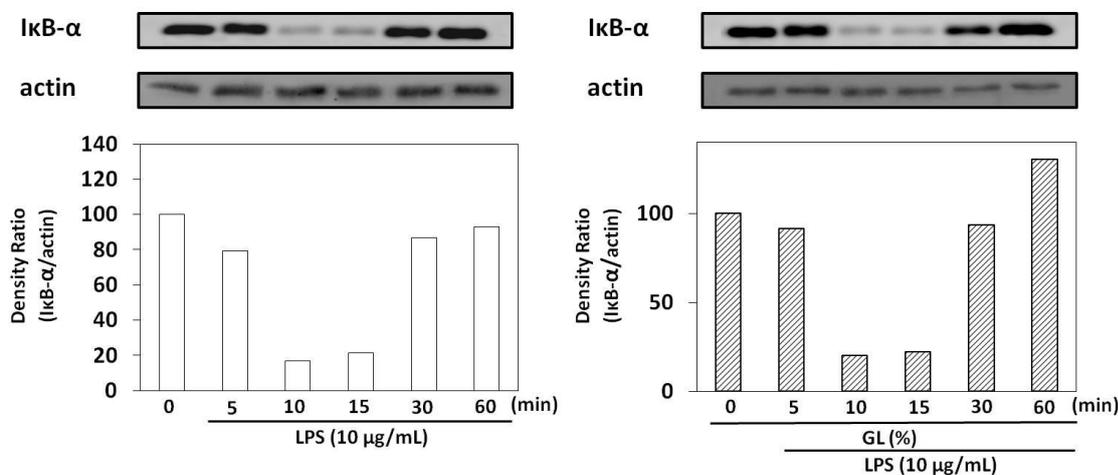


Figure 5. Effect of GL on degradation of IκB-α. RAW264.7 cells (2.0 X 10⁶ cells/well) were incubated at 37 °C for 3 hours in 2 mL medium in the presence or absence of GL (10%) before LPS (10 μg/mL) was

added, and cells were incubated for indicated time (0, 5, 10, 15, 30, 60 minutes). The protein levels of I κ B- α and actin were determined by Western blot. To facilitate comparisons, the relative density ratios of I κ B- α protein to actin protein are shown as histogram. The density ratio of non-stimulated control is set to 100. Results shown are representatives of three independent experiments.

Radical scavenging effect of GL: The antioxidant activity of GL was evaluated by Fenton reaction system with the ESR spin-trapping technique. Figure 6 showed that GL at concentrations of 6.7% (B), 12.5% (C) and 22.2% (D) reduced the ESR signal intensity by 22%, 42%, 70% as compared with that of control (A), respectively.

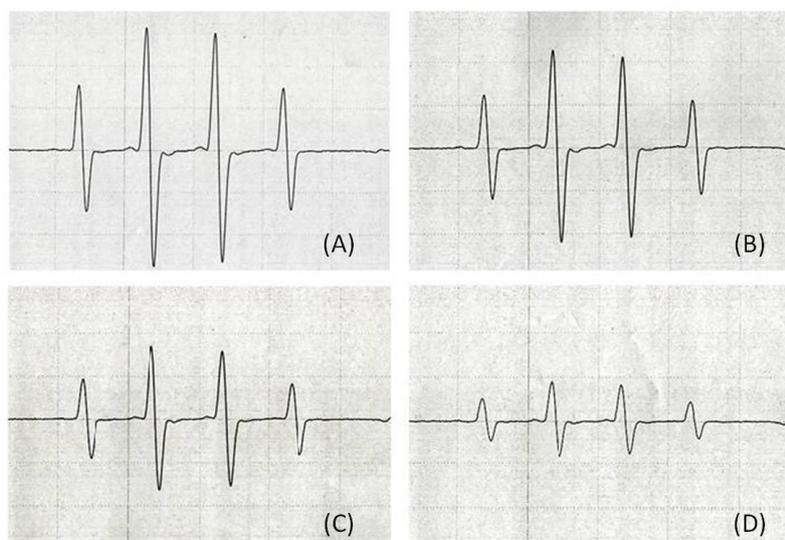


Figure 6. Radical scavenging effect of GL. The DMPO-OH adduct was obtained from the Fenton reaction system containing DMPO (10 μ L), H₂O₂ (0.1 mM: 40 μ L), and ferrous sulfate (5 mM: 20 μ L) with or without GL (5, 10, 20 μ L). ESR spectra of DMPO adduct trapping hydroxyl radical (A) without, (B), (C), (D) with 6.7%, 12.5%, and 22.2% GL, respectively.

DISCUSSION:

In this study, we evaluated the anti-inflammatory role of GL in RAW 264.7 cells and demonstrated that it attenuated LPS-induced inflammatory events by inhibiting iNOS induction to reduce NO production, translocation of NK- κ B into nucleus and generation of oxygen radicals.

Nitric oxide (NO) produced by the inducible forms of iNOS protein is an important cellular signaling molecule involved in many physiological processes [9]. However, excess production of NO plays an important role in various inflammatory conditions [10-13]. LPS was known to induce the expression of this enzyme which leads to the production of abundant amounts of NO. GL dose-dependently inhibited the production of NO caused by the inhibition on the expression of iNOS.

Numerous evidences from preclinical and clinical studies have shown that NF- κ B and NF- κ B-mediated pro-inflammatory cytokines play a central role in the progression of inflammation and cancer-related symptoms. NF- κ B activity is induced by several factors including LPS and ROS. Activation of the NF- κ B is initiated by the degradation of I κ B, and then translocate into nucleus to leads to the given physiological response such as inflammatory response and cellular proliferation [14-17]. This study showed that GL inhibited the nuclear translocation of NF- κ B while it did not inhibit degradation of I κ B- α . Therefore, it is considered that anti-inflammatory activity of GL was due to the inhibition on the translocation of NF- κ B into nucleus and consequently inhibiting the production of iNOS, TNF- α and oxygen radicals.

In the previous study, GL significantly inhibited TPA-induced mouse skin inflammation and DMBA/TPA-induced skin tumor formation and as the results the tumor incidence and tumor multiplicity of papillomas were reduced at 20 weeks [8]. We also reported that chemical analysis on GL revealed its richness of flavonoids including nobiletin, sinisetin, tangeretin, naringin and hesperidin with a total measured content as high as 464 ppm or 0.46 mg/mL [8]. It is assumed that the biological activities of GL are due to synergistic effects among the rich flavonoids, particularly polymethoxyflavones in this formulated extract.

Although ROS such as OH-radicals can help the host defense systems, excessive ROS is capable of inducing DNA damaging and other bio-molecules. Increased formation of radicals can promote the development of malignancy, and the continuous generation of radicals may account for the increased risk of cancer development in the aged [18, 19]. It can be inferred that chemo-preventive activity of GL on the skin tumor development is linked to the radical scavenger activity together with inhibition of NO and iNOS generation.

CONCLUSION:

In conclusion, GL inhibited iNOS induction in LPS-stimulated RAW 264.7 cells in a dose-dependent manner and reduced NO production by inhibition of iNOS activity, translocation of NF- κ B into nucleus and radical scavenging activity. Therefore, these results indicate that GL serves as a valuable protective agent in inflammatory diseases as well as a preventing agent of tumor development.

Authors' Contributions: All authors contributed to this study.

Abbreviations:DMBA/TPA;7,12-dimethylbenz(a)anthracene/12-O-tetradecanoylphorbol-13-acetate ROS; reactive oxygen species

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