KOREAN J. FOOD SCI. TECHNOL. Vol. 52, No. 6, pp. 622~626 (2020)
https://doi.org/10.9721/KJFST.2020.52.6.622

KOREAN JOURNAL OF
한국식품과학회지
FOOD SCIENCE AND TECHNOLOGY

Canavalia gladiata regulates the immune responses of macrophages differently depending on the extraction method

Ha-Nul Lee[1,#], Young-Min Kim[1,#], Ah-Ra Jang[2], Young Ran Kim[3], and Jong-Hwan Park[2,*]

[1]*Department of Integrative Food, Bioscience and Biotechnology, Chonnam National University*
[2]*Laboratory Animal Medicine, College of Veterinary Medicine and Animal Medical Institute, Chonnam National University*
[3]*College of Pharmacy and Research Institute of Drug Development, Chonnam National University*

Abstract Recent studies have suggested that *Canavalia gladiate*, a dietary food and traditional folk medicine, has promising pharmaceutical potential, but the effects have mostly been demonstrated using its organo-soluble extract. To date, its immunomodulatory effect depending on the extraction method is unclear. Here, the immune responses of macrophages to *C. gladiate* and the underlying mechanisms were studied. *C. gladiate* hot water extract (CGW) induced cytokine production in bone marrow-derived macrophages (BMDMs) in a dose-dependent manner, whereas its ethanolic extract (CGE) did not. Immunoblotting analysis also showed that CGW activated nuclear factor (NF)-κB and mitogen-activated protein kinases (MAPKs). Moreover, an inhibitor assay revealed the involvement of NF-κB, p38, and JNK, but not ERK, in CGW-induced cytokine production. CGE inhibited lipopolysaccharide-stimulated production of pro-inflammatory cytokines and activation of NF-κB and MAPKs in BMDMs. The results suggest that *C. gladiate* regulates the immune responses of macrophages differently depending on the extraction method.

Keywords: *Canavalia gladiate*, cytokine, nuclear factor-κB, mitogen-activated protein kinase, macrophage

Introduction

The immune system is a host defense system that includes many cells and biomolecules with specialized roles in disease protection. The innate immune response provides rapid initial protection and is a dominant system of host defense in most organisms. This response usually involves the phagocytic cells (neutrophils, monocytes, and macrophages) and molecular components (complement, acute-phase proteins, and cytokines) (Delves et al., 2000; Litman et al., 2005). Macrophages are multifunctional cells that play a crucial role in innate and adaptive immune responses (Rooijen et al., 1997). Macrophages protect the host by engulfing and killing pathogens, presenting antigens to lymphocytes, and releasing numerous bioactive molecules that regulate the activity of other cells (Plowden et al., 2004; Porcheray et al., 2005). Therefore, it is important to modify the function of macrophages by selective stimulation or suppression of cell activity.

Canavalia gladiate, commonly known as sword bean, belongs to the legume family (Fabaceae) and is mainly cultivated throughout tropical Asia and Africa. This plant has been used as a dietary food and traditional folk medicine for thousands of years. Recently, many studies have suggested that it has promising pharmaceutical properties. It not only exerts protective effects against bone loss but also has antiangiogenic, hepatoprotective, antioxidant, and anti-inflammatory effects (Byun et al., 2010; Gan et al., 2016; Jeon et al., 2005; Kim et al., 2015; Kumar et al., 2014; Nakatsuka et al., 2014; Pinto et al., 2013). However, most studies have demonstrated the pharmaceutical effects using the organo-soluble extract refluxed with ethanol or methanol solvents. To date, the immunomodulatory effect of *C. gladiate* depending on the extraction method is not clear. In this study, we aimed to compare immune responses of macrophages to *C. gladiate* depending on the extraction method and investigate the underlying mechanisms.

HN Lee and YM Kim contributed equally to this work.

*Corresponding author: Joung-Hwan Park, College of Veterianry Medicine and Animal Medical Institute, Chonnam National University, Gwangju 61186, Republic of Korea
Tel: +82-62-530-2834
Fax: +82-62-530-2834
E-mail: jonpark@jnu.ac.kr
Received October 8, 2020; revised October 21, 2020; accepted October 27, 2020

Materials and Methods

Preparation of extract

C. gladiate was supplied from the Gold Farm Food Co. (Jangheung, Korea). The whole parts of the sword bean were refluxed with a 10-fold volume of water (CGW) or 80% ethanol (CGE) at 70°C for 3 h, respectively. After filtration, the extract was concentrated and lyophilized to obtain a powder. The power was dissolved in phosphate-buffered saline for use in subsequent experiments.

Cell culture and treatment

Bone marrow-derived macrophages (BMDMs) were prepared as previously described (Celada et al., 1984). Briefly, BMDMs were cultured in complete Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY, USA) supplemented with 30% L929 cell culture supernatant, 10% fetal bovine serum, 1% penicillin and streptomycin, 1% sodium pyruvate, and 1% MEM non-essential

amino acids in a 5% CO_2 incubator at 37°C for 6 days. The cells were treated with *C. gladiate* extract in the absence or presence of lipopolysaccharide (LPS) sourced from *Escherichia coli* (InvivoGen, San Diego, CA, USA) for 24 h.

Measurement of cytokines

To determine the concentrations of interleukin (IL)-6 and tumor necrosis factor (TNF)-α in the culture supernatants, we used commercial enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's instructions (R&D Systems, Minneapolis, MN, USA).

Immunoblotting

BMDMs were lysed in a lysis buffer containing 1% Nonidet-P40, protease inhibitor cocktail (Roche, Mannheim, Germany), and phosphatase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The lysates were separated by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes. The membranes were immunoblotted with the following primary antibodies: anti-rabbit IκB, anti-rabbit p-65, anti-rabbit p-p38, anti-rabbit p-JNK (Cell Signaling Technology, Beverly, MA, USA), anti-mouse p-ERK, and anti-goat actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA). After immunoblotting with secondary antibodies, proteins were detected using an enhanced chemiluminescence reagent (BioRad, Hercules, CA, USA).

Inhibitor assay

Nuclear factor (NF)-κB and mitogen-activated protein kinase (MAPK) pathway inhibitors were purchased from Calbiochem (San Diego, CA, USA) and Selleck Chemicals (Houston, TX, USA) and included BAY11-7082 (NF-κB inhibitor), SB203580 (p38 inhibitor), SP600125 (JNK inhibitor), and PD98059 (ERK1 inhibitor). The BMDMs were treated with *C. gladiate* extract in the absence or presence of each inhibitor for 24 h.

Statistical analysis

The statistical significance of differences between groups was determined by one-way ANOVA followed by Tukey's post-hoc analysis (GraphPad Prism 5; GraphPad Software Inc., La Jolla, CA, USA). Values of $p<0.05$ were considered significant.

Results and Discussion

CGW stimulated the activity of macrophages

To investigate the immune responses to *C. gladiate*, we prepared CGW and CGE, which were extracted using different solvents. The BMDMs were incubated with various doses of extracts for 24 h, and the cytokines in the culture supernatants were determined by ELISA. CGW induced the production of IL-6 and TNF-α in BMDMs in a dose-dependent manner (Fig. 1A). Particularly, after treatment with 0.3 or 1 mg/mL CGW, both cytokines were released to either the same extent or more than that in the LPS positive control. Endotoxin contamination-stimulated responses were also distinguished (Fig. 1B). The peptide polymyxin B (PMB), an LPS-stimulated TLR4 activation inhibitor, did not affect the production

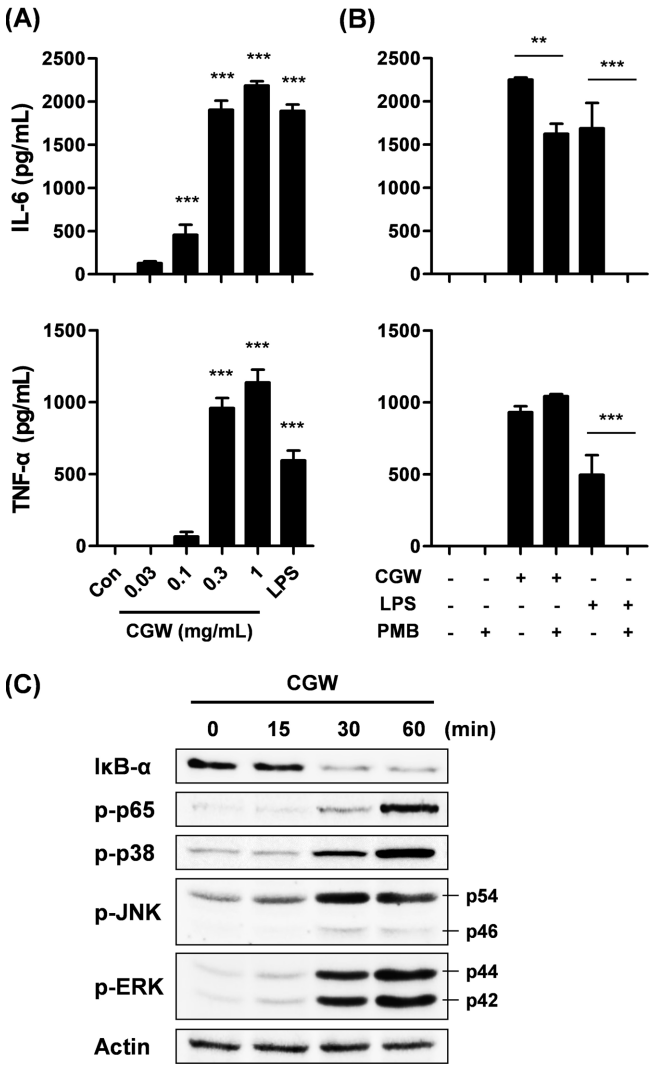

Fig. 1. Effect of *C. gladiate* hot water extract (CGW) on the activation of macrophage immune responses. (A) The levels of IL-6 and TNF-α in the culture supernatants. LPS was used as a positive control. Data are presented as the mean±SD (n=3). ***$p<0.001$ vs. control. (B) Effect of PMB on CGW-induced cytokine production. **$p<0.01$, ***$p<0.001$. (C) Activation of NF-κB and MAPK signaling. Actin was used as a loading control.

of IL-6 or TNF-α induced by CGW, but cytokine production was completely inhibited in LPS-stimulated macrophages. Moreover, there was no significant difference in BMDMs treated with CGE compared with the control group (data not shown). We also investigated the cytotoxicity of the extracts, and neither extract had any effect on the cell viability at the concentrations used in the experiment (data not shown). Based on these results, the concentration of CGW (ranging from 0.03 to 1 mg/mL) and CGE (ranging from 0.1 to 3 mg/mL) were used for subsequent experiments.

The NF-κB and MAPK pathways play an important role as mediators of cellular responses to extracellular signals (Baeuerle et al., 1996; Surh et al., 2001). We determined whether CGW affects the activation of several important components of NF-κB (IκB and p65) and MAPK (p38, JNK, and ERK) signaling. BMDMs were treated with 1 mg/mL CGW for the indicated time periods, and the

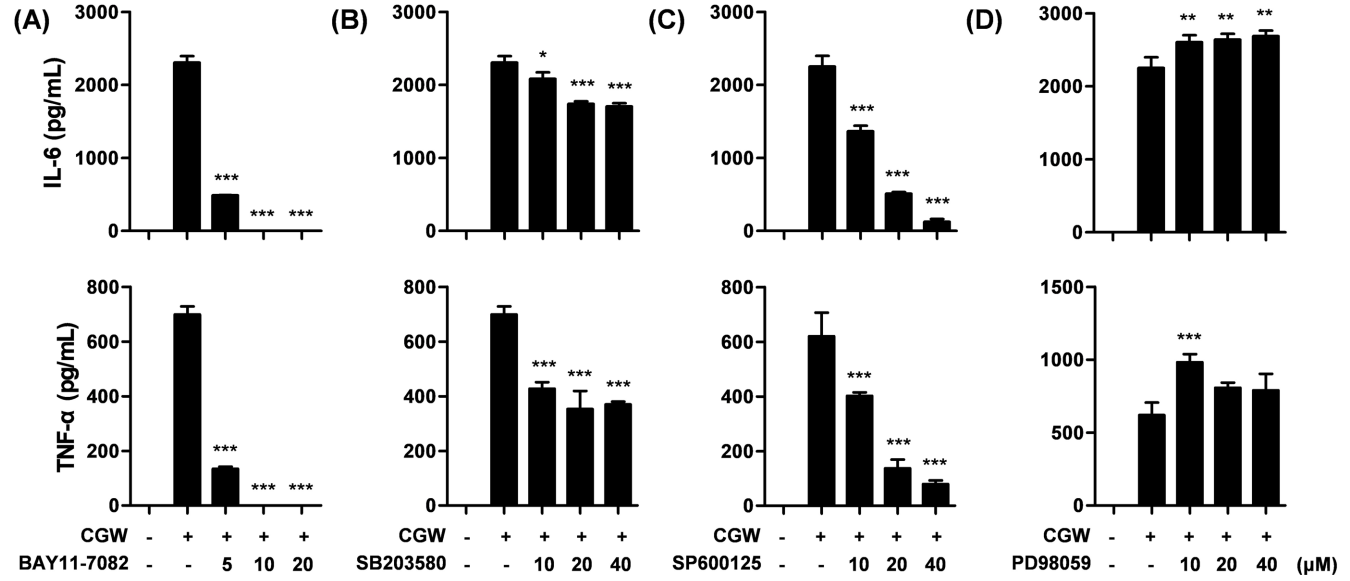

Fig. 2. Effect of the NF-κB and MAPK pathway on CGW-induced cytokine production in macrophages. BMDMs were pretreated with BAY11-7082 (A), SB203580 (B), SP600125 (C), and PD98059 (D) at the indicated concentrations. BAY11-7082, NF-κB inhibitor; SB203580, p38 inhibitor; SP600125, JNK inhibitor; and PD98059, ERK inhibitor. Data are presented as the mean±SD (n=3). *p<0.05 vs. CGW-treated group, **p<0.01 vs. CGW-treated group, ***p<0.001 vs. CGW-treated group.

activation of the components was determined by immunoblotting. CGW reduced the stability of IκB-α and increased the abundance of the phosphorylated form of p65 in BMDMs in a time-dependent manner (Fig. 1C). Thus, CGW activated NF-κB signaling by regulating the phosphorylation of p65, which was dependent on IκB-α. Furthermore, the phosphorylation of p38, JNK, and ERK was strongly activated by CGW treatment in BMDMs.

Consequently, it was necessary to confirm whether CGW-induced NF-κB and MAPK activation is involved in cytokine production. The BMDMs were pretreated with inhibitors of NF-κB and MAPK signaling at the indicated concentrations for 2 h, followed by incubating with 1 mg/mL CGW for 24 h. Next, the concentrations of IL-6 and TNF-α in the culture supernatants were determined by ELISA. BAY11-7082 (NF-κB inhibitor) and SP600125 (JNK inhibitor) strongly inhibited CGW-induced cytokine production in BMDMs in a dose-dependent manner (Fig. 2A and 2C). In particular, when treated with 5 μM BAY11-7082, the levels of IL-6 and TNF-α were reduced by 21.2 and 19.2%, respectively, as compared with those in BMDMs treated with CGW alone. Moreover, there were no cytokines detected at doses above 10 μM. The release of IL-6 and TNF-α was also inhibited in BMDMs treated with SB203580 (p38 inhibitor) but was not reduced by PD98059 (ERK inhibitor) treatment (Fig. 3B and 3D). This indicates that CGW-induced ERK activation did not affect cytokine production, unlike other components. Collectively, these results indicated that CGW-induced NF-κB and MAPK activation, but not ERK activation, was involved in the production of IL-6 and TNF-α in macrophages.

CGE attenuated LPS-induced pro-inflammatory response

Previous studies have demonstrated that *C. gladiate* can be effective against inflammatory diseases (Kim et al., 2015; Pinto et al., 2013). This prompted us to investigate whether CGE affects LPS-induced pro-inflammatory cytokine production in macrophages. BMDMs were pretreated with various doses of CGE (ranging from 0.1 to 3 mg/mL) for 2 h, and then stimulated with 100 ng/mL LPS for 24 h. Next, the production of the pro-inflammatory cytokines in the culture supernatants was determined by ELISA. Expectedly, CGE significantly inhibited LPS-induced production of IL-6 and TNF-α in a dose-dependent manner (Fig. 3A). Particularly, IL-6 was not detected, and the level of TNF-α was reduced by 18.8% in BMDMs treated with LPS and 3 mg/mL CGE compared with cells treated with LPS alone. This result indicated that CGE was able to inhibit LPS-induced pro-inflammatory cytokine production in macrophages.

Recently, not only pro-inflammatory mediators but also inflammatory signaling pathways have provided various effective targets for the generation of new anti-inflammatory agents (O'neill 2006). Therefore, we needed to confirm whether the previous result corresponds to the inactivation of inflammatory signaling pathways. The BMDMs were pretreated with or without 3 mg/mL CGE for 2 h, followed by LPS stimulation for 1 h. Then, the activation of the components of the inflammatory signaling pathways was determined by immunoblotting. The BMDMs stimulated with LPS induced the degradation of IκB-α and phosphorylated activation of p65, while CGE inhibited LPS-induced NF-κB activation (Fig. 3B). Furthermore, the abundance of the LPS-induced phosphorylated forms of p38, JNK, and ERK was strongly reduced in BMDMs pretreated with 3 mg/mL CGE. These results indicated that CGE was involved in inhibiting LPS-induced activation of NF-κB and MAPK signaling, and it might affect the production of IL-6 and TNF-α in macrophages. Moreover, treatment with CGE alone reduced the phosphorylation of p65 and improved the stability of IκB-α by about 1.2 times compared with the control (Fig. 3B). Similarly, the abundance of the phosphorylated

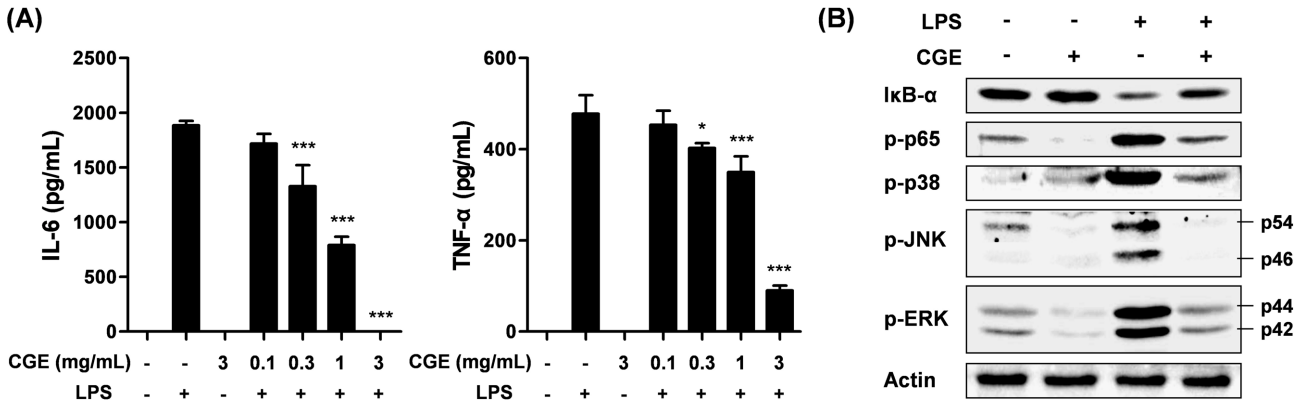

Fig. 3. Effect of *C. gladiate* ethanolic extract (CGE) on the LPS-stimulated inflammatory responses of macrophages. (A) The levels of IL-6 and TNF-α in the culture supernatants. Data are presented as the mean±SD (n=3). *p<0.05 vs. LPS-treated group, ***p<0.001 vs. LPS-treated group. (B) Activation of NF-κB and MAPK signaling. Actin was used as a loading control.

forms of JNK and ERK, which are components of MAPK signaling, was reduced in BMDMs following CGE treatment. This indicated that only CGE was able to inhibit NF-κB and MAPK signaling, mainly the expression of JNK and ERK, in macrophages. This is in contrast to the effect of CGW, which markedly stimulated NF-κB and MAPK activation.

Many previous studies have suggested that polysaccharides possess immunomodulatory activity (Leiro et al., 2007; Liu et al., 2017). We also confirmed that CGW has higher molecular weight polysaccharides than CGE, and this difference might affect CGW-induced immune responses in macrophages (data not shown). Moreover, it has been demonstrated that the ethanolic extract of *C. gladiate* contains more phenolic compounds than its aqueous extract (Kim et al., 2016). Furthermore, many studies have shown that phenolic compounds, especially flavonoids, affect the inflammation induced by macrophages and inhibit the expression of important pro-inflammatory mediators (González et al., 2011; Yahfoufi et al., 2018). Therefore, the abundant phenolic compounds of CGE could probably induce inhibitory effects on LPS-stimulated macrophage-induced inflammation. However, further studies are required to investigate the exact bioactive components of the two *C. gladiate* extracts and elucidate their molecular mechanisms underlying immune responses.

Conclusion

Here, we demonstrated that *C. gladiate* regulates the immune responses of macrophages differently depending on the extraction method. CGW exhibited immunomodulatory activity, while CGE exerted an anti-inflammatory effect. CGW was able to activate the macrophages, inducing cytokine production through the NF-κB and MAPK pathways. In contrast, LPS-stimulated inflammatory responses, such as pro-inflammatory cytokine production and the activation of NF-κB and MAPKs, were inhibited by CGE in macrophages. Hence, the findings of our study may help in better utilizing *C. gladiate* as a functional food and nutraceutical for immune-related diseases such as immunodeficiency and inflammatory diseases.

References

Baeuerle PA, Baltimore D. NF-κB: Ten years after. Cell. 87: 13-20 (1996)

Byun JS, Lee SS. Effect of soybeans and sword beans on bone metabolism in a rat model of osteoporosis. Ann. Nutr. Metab. 56: 106-112 (2010)

Celada A, Gray PW, Rinderknecht E, Schreiber RD. Evidence for a gamma-interferon receptor that regulates macrophage tumoricidal activity. J. Exp. Med. 160: 55-74 (1984)

Delves PJ, Roitt IM. The immune system. N. Engl. J. Med. 343: 37-49 (2000)

Gan RY, Lui WY, Corke H. Sword bean (*Canavalia gladiata*) as a source of antioxidant phenolics. Int. J. Food Sci. Tech. 51: 156-162 (2016)

González R, Ballester I, López-Posadas R, Suárez M, Zarzuelo A, Martinez-Augustin O, Medina FSD. Effects of flavonoids and other polyphenols on inflammation. Crit. Rev. Food Sci. Nutr. 51: 331-362 (2011)

Jeon KS, Na H-J, Kim Y-M, Kwon HJ. Antiangiogenic activity of 4-O-methylgallic acid from *Canavalia gladiata*, a dietary legume. Biochem. Biophys. Res. Commun. 330: 1268-1274 (2005)

Kim O-K, Chang J-Y, Nam D-E, Park YK, Jun W, Lee J. Effect of *Canavalia gladiata* extract fermented with *Aspergillus oryzae* on the development of atopic dermatitis in NC/Nga mice. Int. Arch. Allergy. Immunol. 168: 79-89 (2015)

Kim B, Park J, Yoon H-G, Choi K-C, Kim K, Lee J, Chung JW, Shim S, You Y, Jun W. Effect of ethanol extract of *Canavalia gladiata* on endurance swimming capacity in mice. J. Med. Food. 19: 990-993 (2016)

Kumar P, Reddy. Y N. Protective effect of *Canavalia gladiata* (sword bean) fruit extracts and its flavanoidal contents, against azathioprine-induced toxicity in hepatocytes of albino rats. Toxicol. Environ. Chem. 96: 474-481 (2014)

Leiro JM, Castro R, Arranz JA, Lamas J. Immunomodulating activities of acidic sulphated polysaccharides obtained from the seaweed *Ulva rigida* C. Agardh. Int. Immunopharmacol. 7: 879-888 (2007)

Litman GW, Cannon JP, Dishaw LJ. Reconstructing immune phylogeny: new perspectives. Nature Rev. Immunol. 5: 866-879 (2005)

Liu X, Xie J, Jia S, Huang L, Wang Z, Li C, Xie M. Immunomodulatory effects of an acetylated *Cyclocarya paliurus* polysaccharide on murine macrophages RAW264. 7. Int. J. Biol. Macromol. 98: 576-581 (2017)

Nakatsuka Y, Nagasawa T, Yumoto Y, Nakazawa F, Furuichi Y. Inhibitory effects of sword bean extract on alveolar bone resorption induced in rats by *Porphyromonas gingivalis* infection. J. Periodontal. Res. 49: 801-809 (2014)

O'neill LA. Targeting signal transduction as a strategy to treat inflam-

matory diseases. Nature. Rev. Drug Discov. 5: 549-563 (2006)
Pinto NV, Cavada BS, Brito LF, Pereira RI, Da Silva MTL, Castro RR, De Freitas Pires A, Assreuy AMS. Effects of *Canavalia lectins* on acute inflammation in sensitized and non-sensitized rats. Inflammation. 36: 713-722 (2013)
Plowden J, RenshawHoelscher M, Engleman C, Katz J, Sambhara S. Innate immunity in aging: impact on macrophage function. Aging Cell. 3: 161-167 (2004)
Porcheray F, Viaud S, Rimaniol AC, Leone C, Samah B, Dereuddre Bosquet N, Dormont D, Gras G. Macrophage activation switching: an asset for the resolution of inflammation. Clin. Exp. Immunol. 142: 481-489 (2005)
Rooijen V, Annemarie Sanders N. Elimination, blocking, and activation of macrophages: three of a kind? J. Leukoc. Biol. 62: 702-709 (1997)
Surh Y-J, Chun K-S, Cha H-H, Han SS, Keum Y-S, Park K-K, Lee SS. Molecular mechanisms underlying chemopreventive activities of anti-inflammatory phytochemicals: down-regulation of COX-2 and iNOS through suppression of NF-κB activation. Mutat. Res. 480: 243-268 (2001)
Yahfoufi N, Alsadi N, Jambi M, Matar C. The immunomodulatory and anti-inflammatory role of polyphenols. Nutrients. 10: 1618 (2018)