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Immunomodulatory Effects of *Aloe saponaria* on Lipopolysaccharide-Activated RAW 264.7 Macrophages

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Abstract

Aloe saponaria (*A. saponaria*) is composed of a high content of polysaccharides; however, few studies exist on this Aloe species. We analyzed the polysaccharide and protein contents of *A. saponaria* and found them to be 90% and 3.6%, respectively. *A. saponaria* reduced nitric oxide production in LPS-stimulated RAW 264.7 macrophages, in addition to decreasing COX-2 mRNA expression. Cytokines, key regulators of the immune response, were specifically regulated in LPS-stimulated RAW 264.7 macrophages treated with *A. saponaria* in a dose-dependent manner, and the expression of co-stimulatory molecules tended to decrease. The concentration of *A. saponaria* crude extract required to exert its immunomodulatory activity is high (500-2000 μg/mL). Further studies will need to purify and optimize the dose required for effective immunomodulation.

Keywords: *Aloe saponaria*; Immunomodulation; Anti-inflammation; Cytokine regulation

Introduction

Aloe species have been used for thousands of years as traditional herbal medicine around the world. *Aloe vera* (*A. vera L.*), *A. arborescens* (*A. arborescens Mill.*), and *A. saponaria* are the primary Aloe species used in nutritional supplement and cosmetics.

Among the Aloe species, numerous studies exist on *A. vera*; however, *A. saponaria* has rarely been studied. *A. saponaria* is phenotypically similar to *A. vera* and is primarily cultivated in South Africa for decorative purposes. It grows well in both tropical and desert areas, and unlike *A. vera,* it can withstand a temperature as low as -7°C.

The *A. saponaria* plant is composed of more than 60% of polysaccharides, which have immunomodulatory activity [1,2]. The immunomodulatory activity of Aloe species, including the promotion of lymphocyte proliferation, complement activation, anti-inflammatory activity, anti-ulcer activity, a protective effect for the ultraviolet light or X- ray, anti-cancer activity, antioxidant activity, and anti-viral activity, is well documented [3-8].

The immunomodulating activity of *A. vera* has been established in several studies [9-14]; however, little is known about the immunomodulatory activity of *A. saponaria* despite its high level of polysaccharides and saponin [15]. In this study, we characterized the immunomodulatory activity of *A. saponaria* by using RAW 264.7 murine macrophages.

Materials and Methods

Chemicals

A. saponaria extract was provided by Friday Meridian (shares) (Seoul, Korea) and analyzed in Carbohydrate Bioproduct Research Center (Seoul, Korea). The sample (SPE_1500_01) is kept in the Immunology Laboratory, College of Pharmacy, Sahmyook University.

Preparation of extracts

Aloe powder (150 g) was boiled in 1500 mL of distilled water for 150 min and the supernatant was collected. One liter of distilled water was added and heated for 100 min; 500 mL of the supernatant was then mixed with ethanol (EtOH) to make 70% EtOH solution and centrifuged for 5 min. The precipitate was dissolved in distilled water and then lyophilized to store as the final polysaccharide product (15 g, yield was 10%) (Figure 1).

Analysis of samples

Total polysaccharide was analyzed using phenol-sulfuric acid method. After mixing 30 μL of sample with 30 μL of 5% phenol, 150 μL of sulfuric acid was added and incubated at 37°C for 20 min. Absorbance was then measured at 490 nm. The protein content was measured using a BCA kit (Thermo Fisher. No. 23225, Pierce BCA protein assay kit, USA).

Cell culture

RAW 264.7 macrophages were obtained from the American Type Culture Collection (ATCC). The cells were cultured using standard cell culture method [16].

Measurement of NO

To activate macrophages, RAW 264.7 cells were treated with lipopolysaccharide (LPS) and co-cultured with *A. saponaria* at 250, 500, 1000, and 2000 μg/mL for 16 h at 37°C in 5% CO_2. After 16 h, the supernatant of each culture condition was transferred to a new plate and 100 μL of 1:1 solution mixed the Griess reagent A (2% sulfanilamide in 5% phosphoric acid) and Griess reagent B (0.2% naphthylethylenediamine dihydrochloride) was added. Absorbance was measured at 570 nm.

Cytokine assay

LPS-treated RAW 264.7 macrophages were cultured with *A. saponaria* at 250, 500, 1000 and 2000 μg/mL/ for 16 h at 37°C in 5% CO_2. After 16 h, inflammatory cytokines IL-1β, IL-6, and TNF-α were measured in the supernatant of each group by ELISA kits (eBioScience 88-7013, 88-7064 USA, BD 555268 USA).

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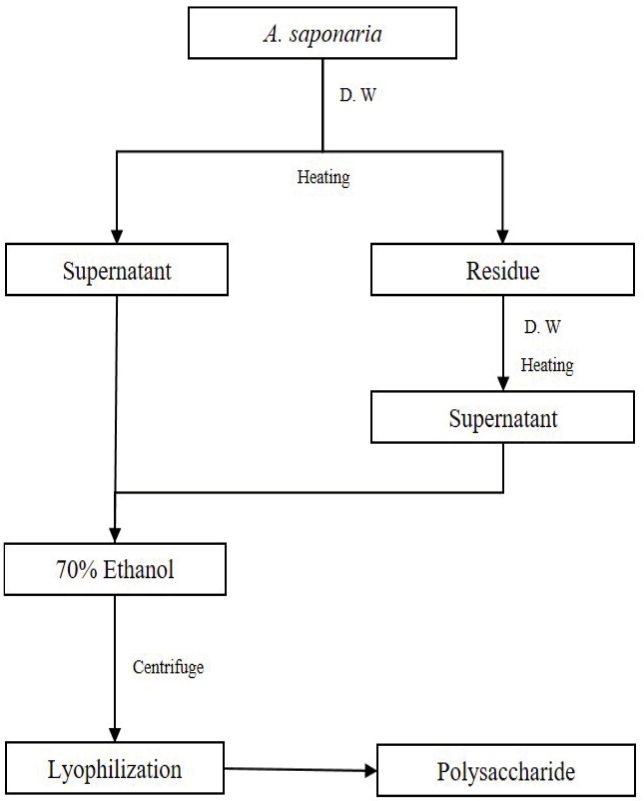

Figure 1: Extraction method from *Aloe saponaria*.

Isolation of total RNA and reverse transcription polymerase chain reaction (RT-PCR)

LPS-treated RAW 264.7 macrophages were cultured with *A. saponaria* at 200, 400, 800, and 1600 μg/mL for 16 h at 37°C in 5% CO_2. After 16 h, the supernatant was removed and the cells remaining on the plate were treated with RiboEx ™ (GeneAll, Korea) to extract total RNA. The extracted RNA was then used for quantitative cDNA synthesis. cDNA synthesis was carried out by using an RNA template, Oligo (dT) primer, and the HyperScript ™ RT premix (GeneAll, Korea) and by incubating at 42°C for 5 min and at 55°C for 60 min. PCR was carried out with Taq polymerase, dNTP, and synthesized cDNA template. PCR incubation conditions were primer-dependent (Table 1). PCR products were analyzed using 0.1% agarose gel electrophoresis and ethidium bromide (EtBr) staining.

Flow cytometry

LPS-treated RAW 264.7 macrophages were cultured with *A. saponaria* at 31.2, 62.5, 125, 250 and 500 μg/mL for 16 h at 37°C in 5% CO_2. The cultured cells were harvested and blocked with purified anti-mouse CD16/32 antibody. Cells were then labeled with anti-ICAM-1, anti-CD80, and anti-CD86 antibody and fixed. The labeled cells were then analyzed using flow cytometry (FC-500, Beckman coulter). All data are expressed as mean ± standard deviation.

Statistics

Statistical significance between groups was determined using Dunnett's test and one-way analysis of variance for repeat measurements. A p value <0.05 was considered statistically significant.

Results and Discussion

Sample analysis

The polysaccharide and protein content in *A. saponaria* was measured using the phenol-sulfuric acid method and BCA protein assay, respectively. The protein and saccharide content were found to be 3.6% and 90%, respectively, and further analysis of these contents yielded the following measurements: fructose (1.76%; peak 2), rhamnose (2.82%; peak 3), arabinose (7.48%; peak 4), galactose (14.73%; peak 6), glucose (43.26%; peak 7), mannose (29.11%), and total sugar content (peak 8) (Table 2 and Figure 2).

A. saponaria regulates excessive nitric oxide production

To determine the state of macrophage activation, we utilized a nitric oxide (NO) assay. Macrophages generate reactive oxygen species and nitrogen species to kill the microorganisms during inflammation; however, if these reactive species are excessively generated, damage to surrounding tissue can occur [17,18].

As shown in Figure 3, NO production from LPS-treated RAW 264.7 macrophages was significantly higher that the control group; however, NO was significantly decreased in RAW 264.7 macrophages treated with *A. saponaria,* in dose dependent manner (p<0.05) (Figure 3). Our data suggest that *A. saponaria* regulates excessive inflammation and subsequent tissue damage by suppressing NO production.

A. saponaria promotes an immune response by increasing cytokine production

LPS-stimulated RAW 264.7 macrophages treated with *A. saponaria* had a dose-dependent increase in IL-1β, IL-6, and TNF-α production by ELISA analysis (Figure 4). Macrophages are a crucial innate immune cell that are activated in response to non-specific stimuli, such as inflammatory cytokines including IL-1β, IL-6, and TNF-α. Additionally, IL-1β, IL-6, and TNF-α mediate important biological processes in the immune system, including proliferation, activation, and inflammation. IL-1β and TNF-α increase expression of adhesion molecules on vascular endothelial cells to recruit immune cells to a specific site, while IL-6 induces a rapid immune response following injury or infection and increases the production of immunoglobulin [19,20]. Our data suggest that *A. saponaria* may enhance the immune response by promoting cytokine production.

A. saponaria suppresses COX-2 mRNA expression

PCR analysis was utilized to measure mRNA expression of inflammation-related genes. As shown in Figure 5, COX-2 mRNA expression, which is associated with prostaglandin (PG) production, was suppressed by *A. saponaria* treatment while mRNA expression of iNOS and IL-1β, IL-6 and TNF-α remained unchanged [21,22].

Although NO and cytokine production was not regulated by *A. saponaria* at the mRNA level, we found that *A. saponaria* suppressed COX-2 mRNA expression (Figure 5), leading to its potential therapeutic use for pain relief via the regulation of an excessive immune response.

A. saponaria alters the expression of co-stimulatory molecules

To determine if *A. saponaria* alters co-stimulatory molecule expression on RAW 264.7 macrophages, we performed flow cytometry. Intercellular cell adhesion molecule-1 (ICAM-1) allows immune cells to move to inflammation sites while CD80/ 86 (B7-1/ B7-2) participate in antigen presenting cell (APC) coupling to T cells. We found that *A.*

Gene	Forward and Reverse	Nucleotide sequences (5'-3')
iNOS	F	ACGCTGAGTACCTCATTGGC
	R	AGCTCCTCCCAGGACCACAC
COX2	F	TGACTGTGGGAGGATACATCTCTC
	R	AAGAAGAAAGTTCATTCCTGATCCC
IL-6	F	TGCTGGTGACAACCACGGCC
	R	GTACTCCAGAAGACCAGAGG
IL-1β	F	CTCTGCAGACTCAAACTCCAC
	R	CAGGATGAGGACATGACACC
TNF-α	F	TCCTCTTCAAGGGCCAAGGC
	R	CTTCTCCAGCTGGAGACCC
β -Actin	F	GGAGGAAGAGGATGCGGCAGT
	R	GTGGGCCGCCCTAGGACCAG

Table 1: Sequence of primers for PCR.

No	Ret.Time min	Peak Name	Height nC	Area nC*min	Rel.Area %	Amount mg/sample 1 mg
1	3.417	-	2.046	0.251	0.31	-
2	3.617	Fructose	8.273	1.420	1.76	0.0011
3	6.134	Rhamnose	6.650	2.275	2.82	0.0021
4	6.600	Arabinose	22.372	6.044	7.48	0.0020
5	7.484	-	1.628	0.435	0.54	-
6	8.150	Galactose	38.245	11.896	14.73	0.0046
7	8.884	Glucose	97.749	34.945	43.26	0.0202
8	9.884	Mannose	51.128	23.516	43.26	0.0180
Total			**228.090**	**80.782**	**100.00**	**0.0481**

Table 2: Component analysis from *Aloe saponaria*.

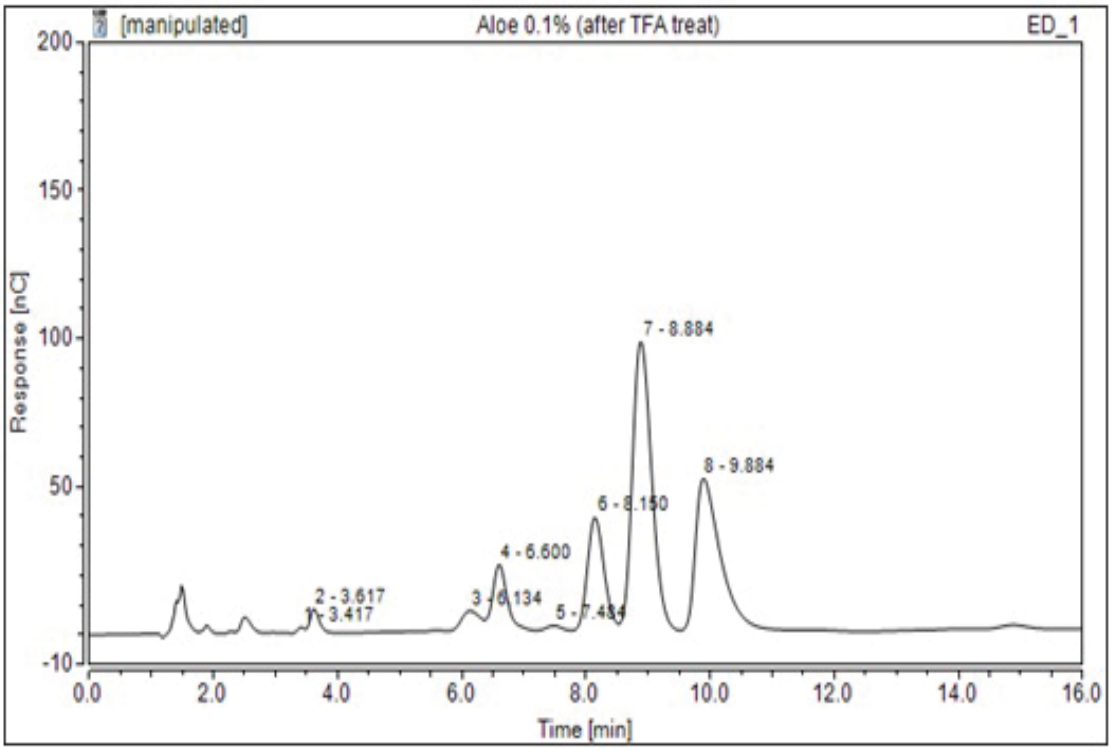

Figure 2: Component analysis from *Aloe saponaria*. Fucose (peak 2), rhamnose (peak 3), arabinose (peak 4), galactose (peak 6), glucose (peak 7) and mannose (peak 8).

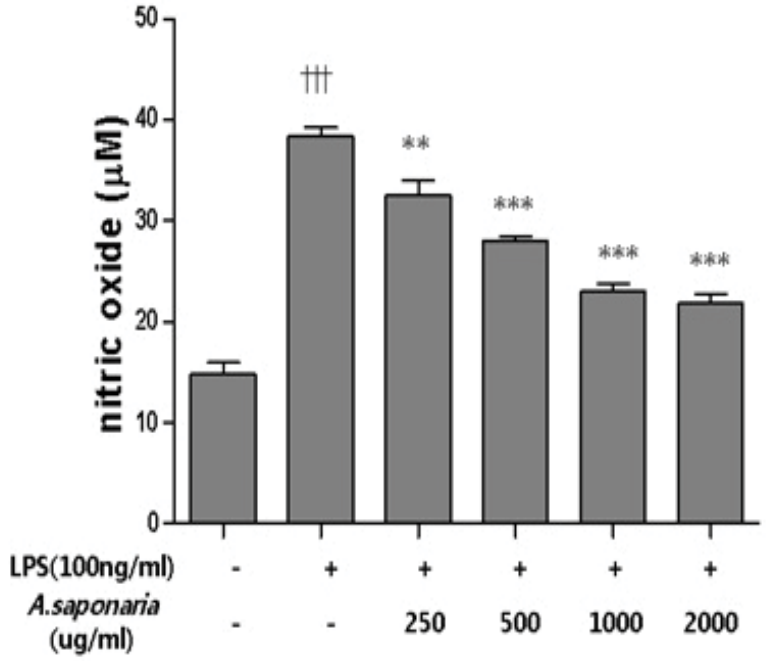

Figure 3: Anti-inflammatory effect of *Aloe saponaria*. RAW 264.7 cells was treated with lipopolysaccharide and *A. saponaira*. Values are means ± SD. †††p<0.0001 compared with cells only levels, **p<0.01, ***p<0.0001 compared with LPS only levels.

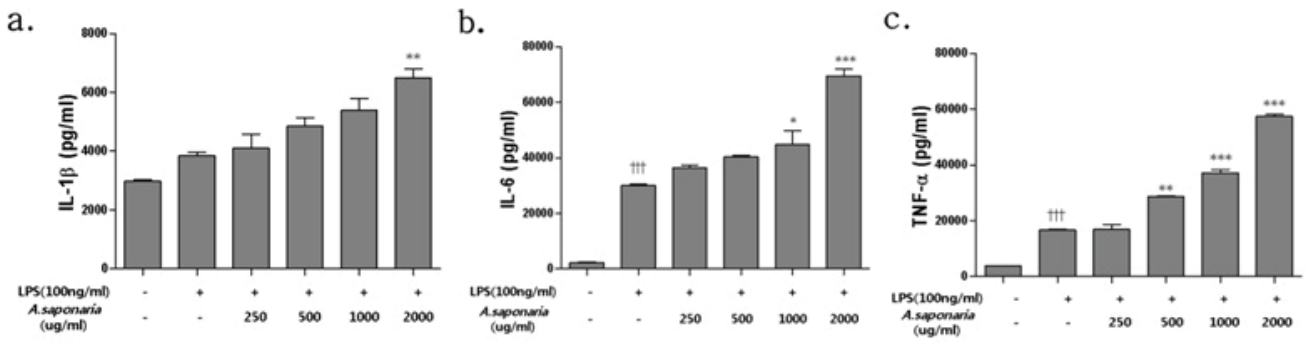

Figure 4: Effect of *Aloe saponaria* on pro-inflammatory cytokine production. RAW 264.7 cells was treated with lipopolysaccharide and *A. saponaira*. IL-1β (a), IL-6 (b), TNF-α (c) were examined using a commercial ELISA kit. Values are means ± SD. †††p<0.0001 compared with cells only levels, *p<0.05, **p<0.01, ***p<0.0001 compared with LPS only levels.

saponaria tended to decrease expression of both ICAM-1 and CD80/86 when treated with LPS (Figures 6 and 7) [23,24].

Macrophages are a major APC that initiates an adaptive immune response by presenting antigen to T and B cells. Macrophage upregulation of co-stimulatory molecules is required for T and B cell interaction. An unregulated immune response may result from excessive co-stimulatory molecule signal cascades and blocking this initial cell interaction event by downregulating co-stimulatory molecule expression may lead to an appropriate immune response. Currently, there are both anti-inflammatory and immunomodulatory therapies that utilize co-stimulatory molecule downregulation.

Conclusion

In this study, we characterized the immunomodulatory activities of *A. saponaria*. *A. saponaria* suppressed excessive inflammation by decreasing NO production and regulating co-stimulatory molecule expression. Further, *A. saponaria* induced cytokine production in a dose-dependent manner, leading to an appropriate immune response. These results suggest that *A. saponaria* could potentially be utilized

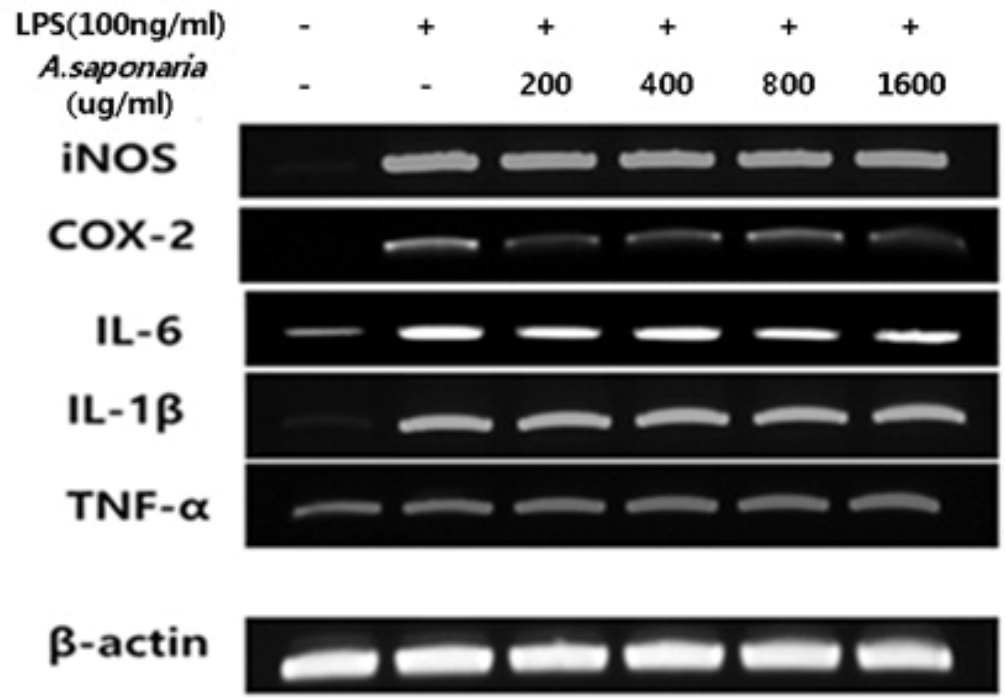

Figure 5: Effect of *Aloe saponaria* on mRNA expression levels of genes related to inflammation. RAW 264.7 cells was treated with lipopolysaccharide and *A. saponaira.* Three experiments were performed and the similar results were observed.

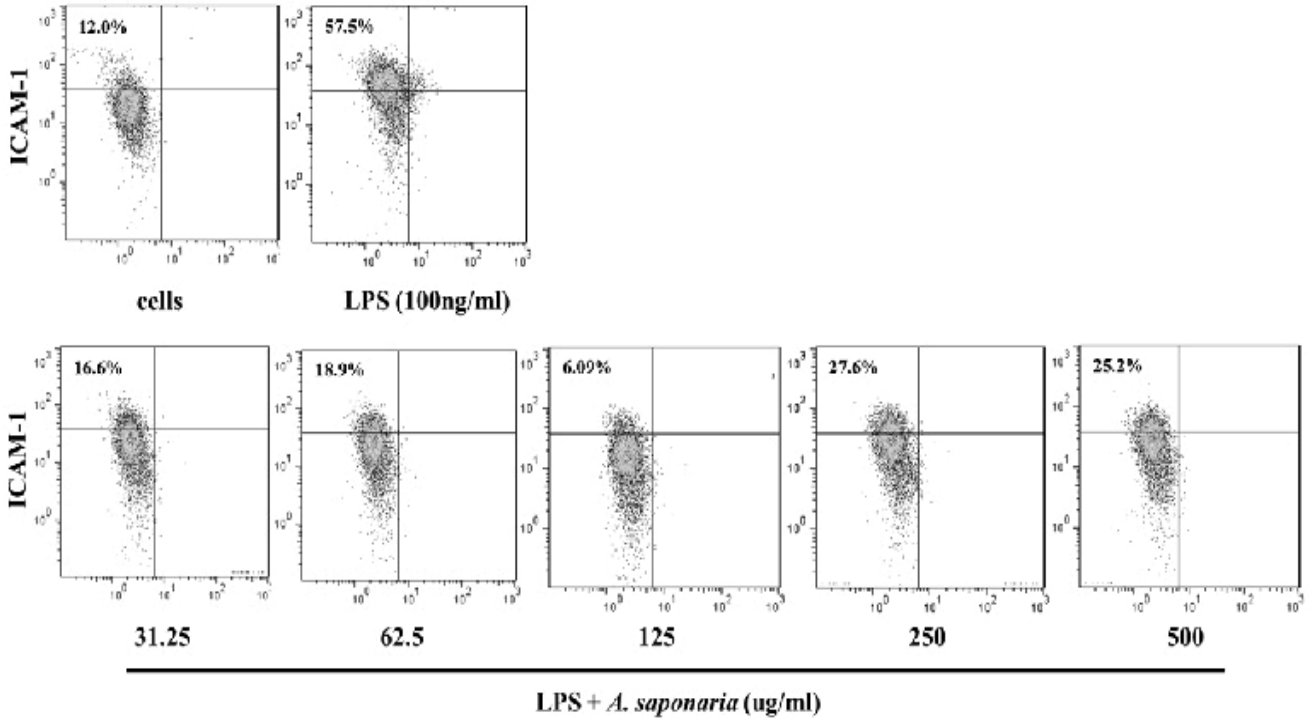

Figure 6: Effects of *Aloe saponaria* on the expression co-stimulatory molecule. RAW 264.7 cells was treated with lipopolysaccharide and *A. saponaira.* The surface ICAM-1 molecules were labeled and examined using a Flow cytometry.

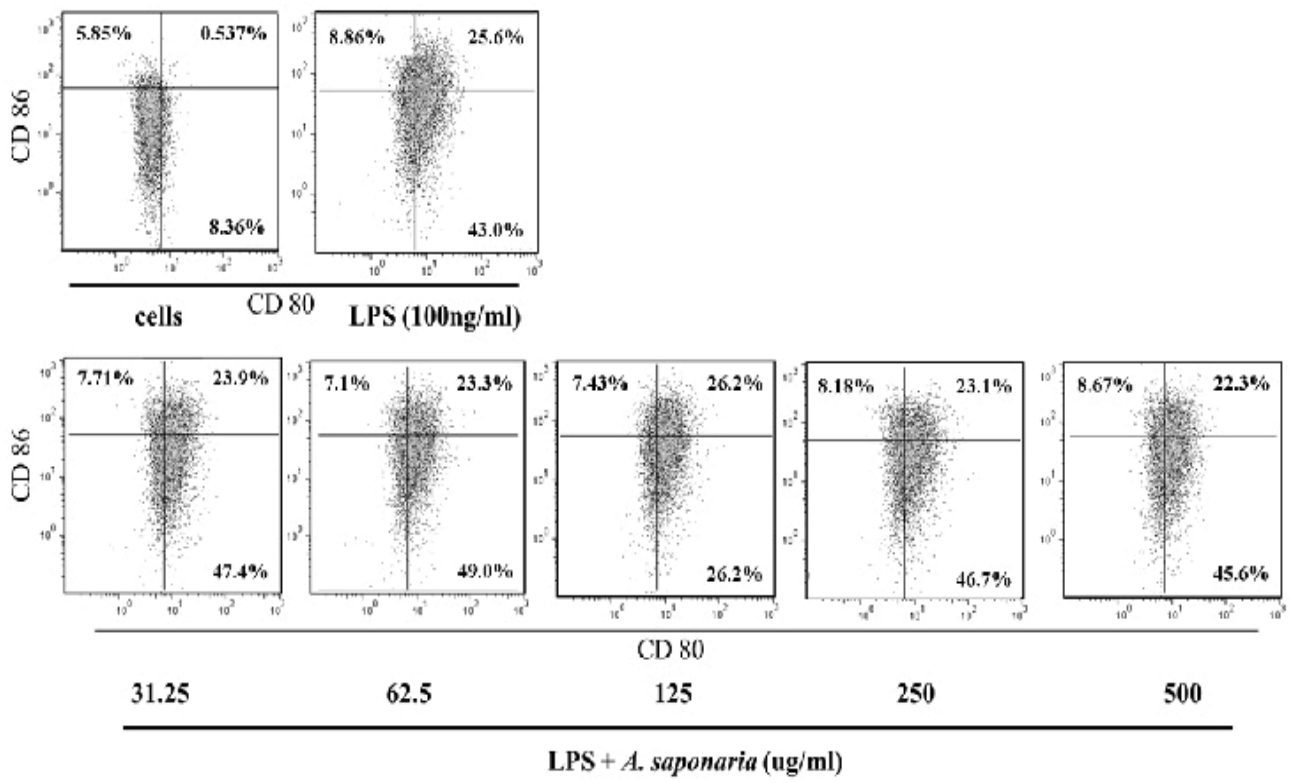

Figure 7: Effects of *Aloe. saponaria* on the expression co-stimulatory molecule. RAW 264.7 cells was treated with lipopolysaccharide and *A. saponaira.* The surface CD86 and CD80 molecules were labeled and examined using a Flow cytometry.

as an immune modulator. In the follow-up study, refinement of A. *saponaria* is of extreme importance and will be performed since the concentration of A. *saponaria* in this study is quite high compared to other herbal medicines [25,26].

Acknowledgements

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