LPS로 자극된 RAW264.7 대식세포에서 우슬 및 땅두릅 복합 발효추출물의 항염증 효과

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Anti-inflammatory Effects of *Achyranthes japonica* Nakai and *Aralia continentalis* Kitagawa Complex Fermented Extracts on LPS-stimulated RAW264.7 Macrophage

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초 록

본 연구는 RAW264.7 대식세포에 대한 우슬과 땅두릅 복합 열수추출물(1 : 2, 1 : 3, 1 : 5, 2 : 1, 3 : 1 및 5 : 1 비율)의 항염증 효과를 조사했다. 세포 독성은 CCK 분석을 사용하여 확인하였다. 우리는 ELISA 키트를 사용하여 IL-1β, IL-6 및 TNFα를 측정함으로써 우슬 및 땅두릅 복합 열수추출물의 항염증 효과를 평가하였다. 우슬 및 땅두릅 복합 열수추출물은 LPS로 자극된 RAW264.7 대식세포에서 IL-1β 및 TNFα를 유의적으로 억제하였다. 복합 추출물의 다양한 비율을 비교하였을 때, 2 : 1 비율은 LPS로 유도된 RAW264.7 세포에서 훨씬 더 효능이 있었고 TNFα의 생성 또한 억제하였다. 본 연구의 결과는 우슬 및 땅두릅 복합 열수추출물이 RAW264.7 대식세포에 대해 강력한 항염증 효과를 갖는다는 것을 보여주며, 이들 추출물은 염증 질환 예방을 위한 기능성 식품의 좋은 공급원이 될 수 있다고 사료된다.

Abstract

This study investigated the anti-inflammatory effects of mixed extracts of *Achyranthes japonica* Nakai (Aj) and *Aralia continentalis* Kitagawa (Ac) (ratios of 1 : 2, 1 : 3, 1 : 5, 2 : 1, 3 : 1 and 5 : 1) on RAW264.7 macrophages. Cell toxicity was determined using a cell counting kit (CCK) assay. We evaluated anti-inflammatory effects of the mixed extracts of Aj and Ac by measuring interleukin (IL)-1β, IL-6, and tumor necrosis factor (TNF)α using an enzyme-linked immunosorbent assay (ELISA) kit assay. The mixed extracts of Aj and Ac inhibited lipopolysaccharide (LPS)-induced IL-1β and TNFα in LPS-stimulated macrophages. Comparing different ratios of the mixed extracts, the 2 : 1 ratio of Aj and Ac has much more potency and inhibited the production of TNFα in LPS-induced RAW264.7 cells. The results of the present study showed that the mixed extracts of Aj and Ac have potential anti-inflammatory effects on RAW264.7 macrophages. Therefore, these extracts may be used as a good source of functional foods for the protection against inflammatory diseases.

Keywords: *Achyranthes japonica* Nakai (Aj), *Aralia continentalis* Kitagawa (Ac), Anti-inflammatory, Macrophage

1. Introduction

As a series of pathological processes involving various signal transduction properties produced in white blood cells, such as macrophages and mast cells, inflammations involve complex processes run by various immune cells[1,2]. These inflammatory processes include the proc-

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ess of eliminating pathogens through the phagocytosis of immune cells in response to the invasion of pathogens or through their secretion of inflammatory factors, such as cytokines[3]. Among these cells, macrophages are involved in producing nitric oxide (NO), a leading inflammatory mediator, and play a crucial role in inflammatory responses through the release of various factors, like the prostaglandin mediator and pro-inflammatory cytokines (TNF-α (tumor necrosis factor-alpha), IL-1β (interleukin-1 beta), and IL-6)[4-6].

In the East, including in South Korea, various oriental medicines and medicinal herbs have been used since ancient times to treat various diseases. Medicinal herbs have extensive effects through the combined actions of various properties[7,8]. *Achyranthes japonica* Nakai (Aj) is a perennial herb that belongs to the Amaranthaceae family, and it is also termed soimureuip (cow knee) in Korean; this name is apt, its stem nodes are rather pronounced, and, thus, look like cow's knees. In oriental medicine, the dried roots of Aj are used as medicinal herbs to treat various conditions such as labor pains, low blood pressure, joint pain, diuresis, and hepatopathy[9,10]. *Aralia continentalis* Kitagawa (Ac) is a perennial herb that belongs to the Araliaceae family, and it is also referred to as aralias. This herb is wide spread throughout the mountainous areas of East Asia, including Korea, Japan, and China. It is frequently used for nervous breakdowns, kidney diseases, diabetes, muscle pain, and low blood pressure[11,12]. In Korea and other Asian countries, we have been using oriental herbal medicine which is a mixture of oriental medicines and herbal medicines for the treatment of various kinds of diseases from ancient times. These oriental herbal medicines are composed of various herbal medicines and herbal medicines rather than a single medicinal herb, they can exhibit a wide range of effects due to the combined effect of various ingredients[8].

Our previous studies showed that the anti-inflammatory effect after single-fermentation is higher than that before lactic acid fermentation [13,14]. Among lactic acid bacteria, *Lactobacillus* and *Bifidobacterium* produce lactic acid by fermenting sugars as probiotics that facilitate the growth of beneficial bacteria in the body[1,15]. Therefore, they are known to have preventive effects on the development of various diseases and enable physiological control in terms of hindering the absorption of cholesterol in the body, controlling the immune system, and increasing the absorption and utilization of nutrients. It must be noted that using herbs collected from nature as oriental medicines, without any processing, may be inconvenient or cause side effects. Therefore, they are used after inducing changes in their physiochemical and biological activities by processing them from their natural conditions to reduce their toxicity and medicinal properties, thereby changing their efficacy. Further, fermented oriental medicines obtained by culturing lactic acid bacteria in medicinal herbs improve not only the pharmacological functionality of oriental medicines, but also their formulations and processing methods by maximizing both the internal absorption and bioavailability of medicinal properties. Therefore, fermented oriental medicines can create new demand for oriental medicines and encourage the development of high-value oriental medicines and herbal medicinal products[16-18]. We tried to find the ratio of the highest anti-inflammatory activity by mixing Aj and Ac.

In this study, after the combined extraction of certain properties from Aj and Ac, a fermented compound was produced through the fermentation of these extracts using lactic acid bacteria. Thereafter, the anti-inflammatory effects of this compound were identified. When inflammations were induced by lipopolysaccharide (LPS) in RAW264.7 cells, a macrophage cell line, to identify the anti-inflammatory effects of the fermented compound, inflammatory factors (such as nitrite, IL-1β, IL-6, and tumor necrosis factor (TNF-α)) were examined. Based on the results, an investigation was conducted on the possibility of using the compound as an ingredient in functional foods or medicines.

2. Materials and Methods

2.1. Preparation of samples

The Aj and Ac used in this experiment were purchased in the form of powder from J&D (Busan, Korea). The *Lactobacillus plantarum* (KCTC NO. 3108) used in this experiment was purchased from a Korean collection for type cultures (KCTC, Daejeon, Korea). The bacterial strains were incubated in MRS broth (proteose peptone No.3 10 g/L, beef extract 10 g/L, yeast extract 5 g/L, dextrose 20 g/L, polysorbate 80 1 g/L, ammonium citrate 2 g/L, sodium acetate 5 g/L, magnesium sulfate 0.1 g/L, manganese sulfate 0.05 g/L, dipotassium phosphate 2 g/L, Difco) for 7 days in an incubator at 37 ℃ and with 5% CO_2. Aj and Ac powders were sieved with a 850 μm sieve and then added in ratios of 1 : 2, 1 : 3, 1 : 5, 2 : 1, 3 : 1, and 5 : 1 (Aj : Ac); than, 100 g of each was added to a 5 L reactor for 48 h at 37 ℃ and extracted at 70 ℃. After filtration under reduced pressure using filter paper, the solution was concentrated using a rotary evaporator under reduced pressure. Than, *Lactobacillus plantarum*, which is 1% of the weight of the concentrate, was added to the culture solution that had a weight ratio of 10 times of the obtained concentrate, by incubation at 37 ℃ for 1 h. The entire mixture was lyophilized at -70 ℃ for 7 days to obtain the final sample.

2.2. Measurement of the total polyphenol content

The total content of polyphenol was measured using the Folin-Denis method. In a 50 μL sample, the same amount of 1 M Folin reagent was added; and then the mixed sample was kept for 6 min at room temperature to facilitate a reaction. Thereafter, a 100 μL saturated solution of 2% Na_2CO_3 was added to the sample, and the resultant sample was kept for 30 min to facilitate a reaction. The absorbance of this sample was measured using the enzyme-linked immunosorbent assay (ELISA) reader; moreover, the polyphenol content in the sample was measured by drawing the standard calibration curve of galic acid, an indicator substance.

2.3. Measurement of the total flavonoid content

The total flavonoid content was measured by applying the method used by Lin *et al.*[19]. First, 1 mg of each sample was dissolved in 1 mL of distilled water, and then 100 μL of the solution was mixed with 1 mL of diethyleneglycol. After putting 100 μL1N NaOH in the mixture, it was left to react for 1 h at 37 ℃. After the centrifugation

of the mixture for 10 min at 12,000 rpm, its supernatant was collected. Each quantity of 200 µL of the supernatant was put in a 96-well plate, and the absorbance was measured at 420 nm using the ELISA reader. Here, the total flavonoid content was obtained from the standard curve drawn using naringin.

2.4. Cell cultures and the measurement of cytotoxicity

The RAW264.7 macrophages were purchased from the Korean Cell Line Bank (Seoul, Korea). They were cultured under conditions of 37 ℃ and 5% CO_2 using Dulbecco's Modified Eagle Medium in which 10% fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin) were added. All reagents used in the cell experiment were purchased from Welgene. The cytotoxicity of the *Lactobacillus*-fermented combined extracts of Aj and Ac was identified through a cholecystokinin (CCK) assay to determine the maximum concentration for applying the combined extracts to the cells. After the RAW264.7 cells were adjusted to have a concentration of 6×10^3 cells/well in 96-well plates, 100 µL of the cells were dispensed into each well. After these cells were attached through culturing in an incubator 37 ℃ and with, 5% CO_2 for 24 h, the combined extracts were diluted in the medium to yield concentrations of 0, 200, 400, 600, 800, and 1,000 µg/mL and then added to the cells. After a 24-h culture following the addition, the CCK reagent was injected in the cells and they were left to react for 2 h in the incubator at 37 ℃ and with, 5% CO_2. Then, the absorbance was measured at 450 nm. The survival of cells was quantified using the following equation:

$$\text{Cell survival rate (\%)} = \left(1 - \frac{\text{Absorbance of the sample treatment group}}{\text{Absorbance of the control group}}\right) \times 100$$

2.5. Measurement of nitric oxide (NO) concentration

The concentrations of NO produced in the RAW264.7 cell supernatants were measured by quantification using the Griess method to identify the immunity-boosting ability of the combined extracts of Aj and Ac. The RAW264.7 cells were dispensed into 60 mm dishes at a concentration of 1.5×10^5 cells/dish; after 24 h, they were treated with 1 µg/mL LPS and then cultured for 24 h. The combined extracts of Aj and Ac were treated at 400 µg/mL, the maximum concentration without toxicity according to the results of the CCK assay; after 24 h, the experiment was conducted using supernatants. The supernatant of cells without LPS treatment was used for the positive control group, and the supernatant of cells treated with only 1 µg/mL LPS was used for the negative control group. After a 100 µL supernatant mixed with the same amount of the Griess reagent was kept at room temperature for 15 min for a reaction, its absorbance was measured at 540 nm using the ELISA Reader. Here, the standard curve was drawn using sodium nitrite.

2.6. Western blot analysis

Cells (5×10^5 cells/mL) were harvested, washed twice with cold PBS, and lysed in RIPA buffer (150 mM Sodium chloride, 1% Triton X-100, 0.1% SDS, 1% Sodium deoxycholate, 50 mM Tris-HCl pH 7.5, 2 mM EDTA, Volo, Korea) supplemented with a cocktail of protease and phosphatase inhibitors (GenDEPOT, USA). Protein concentrations were measured using a Bradford assay. The protein equivalents of samples were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% bovine serum albumin in TBS, probed with antibodies against cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS) and β-actin overnight at 4, and exposed to horseradish peroxidase (HRP)-conjugated secondary antibodies (Invitrogen) for 1 h. Further, antibody-specific proteins were visualized using an enhanced chemiluminescence detection system (CheBI, NeoScience, Korea).

2.7. Cytokine measurement

In order to measure the effects of the combined extracts of Aj and Ac on the volumes of IL-1β, IL-6, and TNF-α produced by LPS stimulation, RAW264.7 macrophages were dispensed into 60 mm dishes at a concentration of 1.5×10^6 cells/dish and then cultured for 24 h. After inflammations were induced for 24 h by applying LPS (1 µg/mL), each of the 6 different samples of the combined extracts of Aj and Ac was processed. After 24 h, the cell culture fluids were collected and the supernatants that had undergone the process of centrifugation were preserved at -20 ℃ for use as samples. Further, the levels of cytokine production were measured using an ELISA kit (R&D System, Minneapolis, MN, USA) in compliance with its testing guidelines, and the absorbance was measured using the ELISA Reader.

2.8. Reverse transcription-polymerase chain reaction (RT-PCR)

RAW264.7 cells were plated at 5×10^5 cells/mL in a 100 mm dish and incubated overnight. The cells were treated with Aj and Ac extracts for 1 h, followed by treatment with LPS (1 µg/mL) and incubation for an additional 24 h. Total RNA from RAW264.7 cells was extracted using TRIzol reagent (Invitrogen), in accordance with the manufacturer's instructions. The RT-PCR reaction was performed with 1 µg of total RNA, 1 µL of forward primer, 1 µL of reverse primer, and 20 µL of reaction mixture, which was provided by AccuPower RT/PCR PreMix (bioneer). Then PCR was performed in a total mixture volume of 50 µL for 40 cycles at 94 ℃ for 30 s, 56 ℃ for 30 s, and 72 ℃ for 1 min. In addition, amplified cDNA products were separated on 1.2% agarose gel by electrophoresis. The primer sequences of amplified genes are presented in Table 2.

Each sample was analyzed in triplicate, and target genes were normalized to the reference housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Thereafter, fold differences were calculated for each treatment group using normalized CT values for the control.

In order to quantitatively RT-PCR results, the densitometry data for band intensities in different sets of experiments was generated by analyzing the gel images on an Image J program. The density values of the target genes were normalized to an endogenous reference, GAPDH.

2.9. Statistical analysis

All results of this experiment are indicated in terms of means and

Table 1. Total Polyphenol and Flavonoid Contents of Aj and Ac Complex Fermented Water Extracts

Aj : Ac	1 : 2	1 : 3	1 : 5	2 : 1	3 : 1	5 : 1
Total polyphenol contents (μg/mg)	73.79 ± 1.65^a	76.08 ± 2.86^{a,b}	76.86 ± 2.02^b	104.48 ± 1.42^c	102.59 ± 1.28^c	77.98 ± 1.56^b
Total flavonoids contents (μg/mg)	1.40 ± 0.90^a	1.37 ± 0.89^a	1.16 ± 0.20^a	2.98 ± 0.56^c	2.46 ± 0.94^b	2.07 ± 1.08^b

* Values are the means ± SD of three independent experiments (n = 3).
* Values that do not share the same superscript are significantly different by ANOVA ($p < 0.05$).

Table 2. Primers for RT-PCR Analysis

Gene	Forward primer (from 5′ to 3′)	Reverse primer (from 3′ to 5′)	Size
COX-2	TTG AAG ACC AGG AGT ACC GC	GGT ACA GT CCC ATG ACA TCG	324
iNOS	CTG CAG CAC TTG GAT CAG GAA CCT G	GGG AGT AGC CTG TGT GCA CCT GGA A	311
IL-1β	AAG CTC TCC ACC TCA ATG GAC A	GTC TGC TCA TTC ACG AAA ABB GAG	453
IL-6	TCC AGT TGC CTT CTT GGG AC	GTG TAA TTA AGC CTC CGA CTT G	139
TNF-α	GCG ACG TGG AAC TGG CAG AAG	TCC ATG CCG TTG GTT AGG AGG	354
GAPDH	GAA GCT CAT CTC TCC TAT GTG CTG GC	TCC ACC ACC CTG TTG CTG TA	450

standard deviations. The data obtained from this study were first analyzed through a one-way analysis of variance (ANOVA) using SPSS 20 (SPSS Inc., Chicago, IL, USA) and then tested using Duncan's multiple test at $P < 0.05$.

3. Results and Discussion

3.1. Total polyphenol and flavonoid contents

The total contents of polyphenol and flavonoids in the combined extracts of fermented Aj and Ac are presented in Table 1. The content of polyphenols and flavonoids were higher in the group with a high rate of Aj (2 : 1, 3 : 1 and 5 : 1) compared to the group with a high rate of Ac (1 : 2, 1 : 3 and 1 : 5). The total polyphenol content was 104.48 μg/mg at a ratio of 2 : 1 (Aj : Ac) and 102.59 μg/mg at a ratio of 3 : 1 (Aj : Ac), thereby showing statistically significant higher contents than those at other ratios ($p < 0.05$). The total flavonoid content was 2.98 μg/mg at a ratio of 2 : 1 (Aj : Ac), a statistically significant higher content than that at other ratios. Although the contents of the polyphenol and flavonoids of a ratio of 5 : 1 is lower than a ratio of 2 : 1 and 3 : 1, it is still higher than the group with a high rate of Ac. Park *et al.*[20] showed a polyphenol content of 25.38 μg/mg in *Achyranthes japonica* naka, and Han *et al.*[21] showed a content of 58.25 μg/mg in *Aralia continentalis* Kitagawa. These were indeed lower than the polyphenol contents in the combined extracts of Aj and Ac. However, a direct comparison with these former studies is problematic due to the use of different fermentation strains and conditions.

3.2. Cytotoxicity

The cytotoxicity of the combined extracts of Aj and Ac was measured using a CCK assay; the results are presented in Figure 1. When the cell survival rates of RAW264.7 cells were measured using the CCK assay after treating them with the combined extracts of Aj and Ac at 0, 200, 400, 600, 800, and 1,000 μg/mL, all samples showed cell survival rates of 80% or above, up to a concentration of 400 μg/mL.

3.3. Formation potential of NO

NO performs physiological roles, such as controlling blood coagulation, blood pressure, and neurotransmission functions. However, the formation of high-concentration NO is known to play an important role in the formation and progress of cancer, as highly concentrated NO creates harmful properties, such as peroxynitrite and nitrogen dioxide; in addition, a high concentration of NO also causes apoptosis by accumulating harmful antioxidants within cells, causing DNA damage, and releasing apoptosis-inducing factors through the detection of DNA damage in the mitochondria[2,22].

In the NO assay using the combined extracts of Aj and Ac, the Griess method[23] was used for measurement (Figure 2). Inflammatory responses are initiated due to properties such as cytokine secreted in macrophages. When the body is stimulated through the secretion of cytokine, iNOS is expressed through transcriptional control and NO is produced, which performs various physiological and pathological functions[24,25]. In the RAW264.7 cells treated with LPS, the formation of NO was found to decrease in the group treated with the combined extracts of Aj and Ac at all ratios. In particular, at the ratio of 2 : 1 (Aj : Ac), the formation of NO was found to go down to approximately 60% when compared to the group treated with only LPS.

3.4. Levels of cytokine production

The result of the production and expression of IL-1β, IL-6, and TNFα, the inflammatory cytokines produced by RAW264.7 macrophages, and induced by LPS stimulation in combined extracts of Aj and Ac are depicted in Figure 3. The group treated with only LPS showed a higher increase in IL-1β (Figure 3A) at 63.26 pg/mL as compared to the control group (10.12 pg/mL) that was not treated with LPS. However, the levels of IL-1β production were 18.81, 37.35 and 40.14 pg/mL at ratios of 2 : 1, 1 : 5 and 3 : 1 (Aj : Ac), respectively, thereby confirming the suppression of IL-1β production. Notably, the level of IL-1β production decreased to approximately 70% at a ratio of 2 : 1. In the case of IL-6 (Figure 3B), the group treated with only
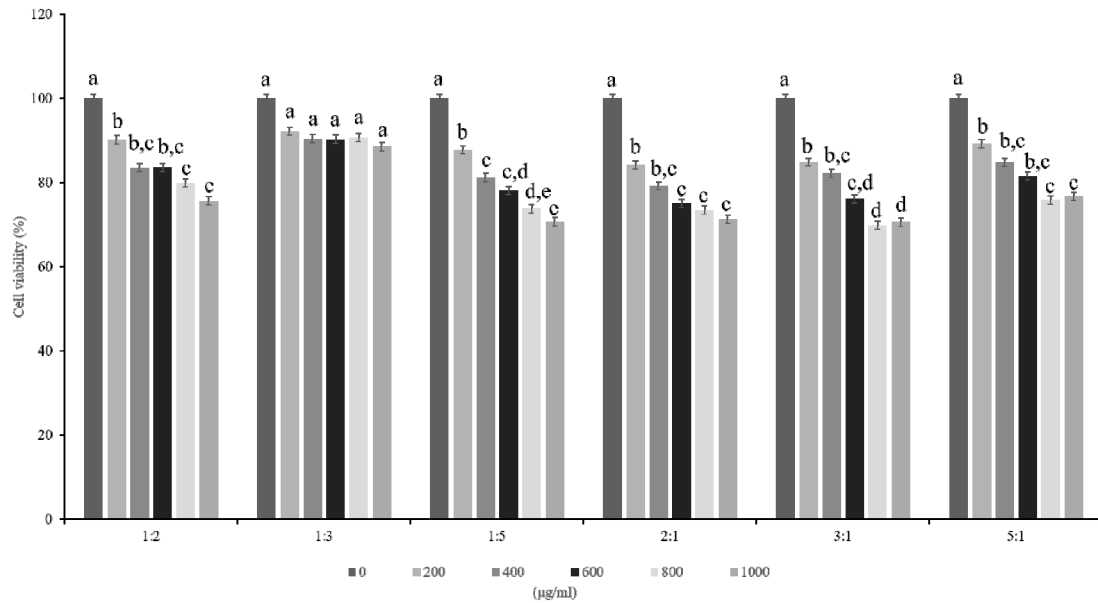
Figure 1. Cell viability was determined by CCK assay. Values are the means ± SD of three independent experiments (n = 3).
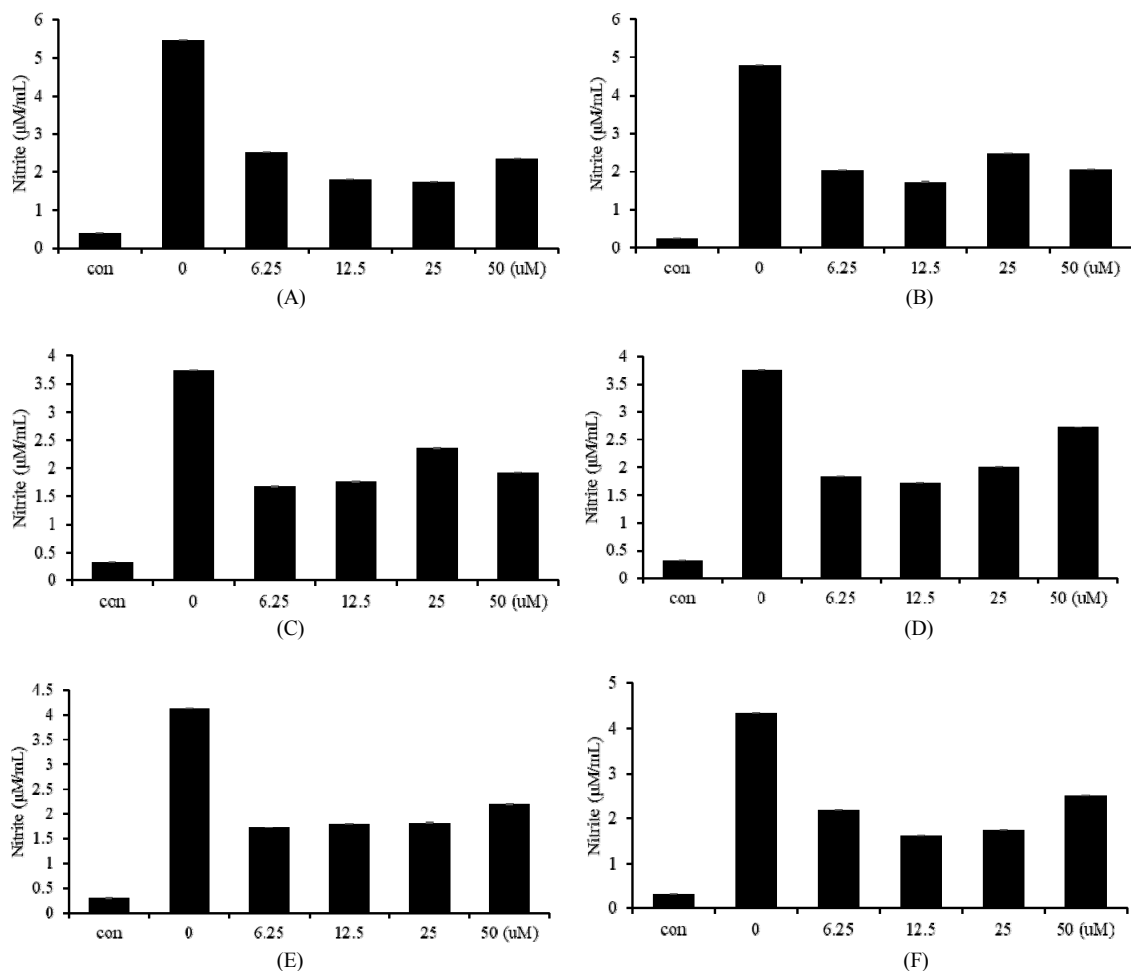

Figure 2. Effects of Aj and Ac complex extracts on LPS-induced NO generation in RAW264.7 macrophage cells. The cells were pre-treated with LPS (1 μg/mL) for 24 h and then treated with various ratios of Aj and Ac complex extracts (400 μg/mL) for 24 h. The NO amounts were determined by Griess assay ((A) 1 : 2, (B) 1 : 3, (C) 1 : 5, (D) 2 : 1 (E) 3 : 1, (F) 5 : 1 (Aj : Ac)). Values are the means ± SD of three independent experiments (n = 3). Values that do not share the same superscript are significantly different by ANOVA ($p < 0.05$).
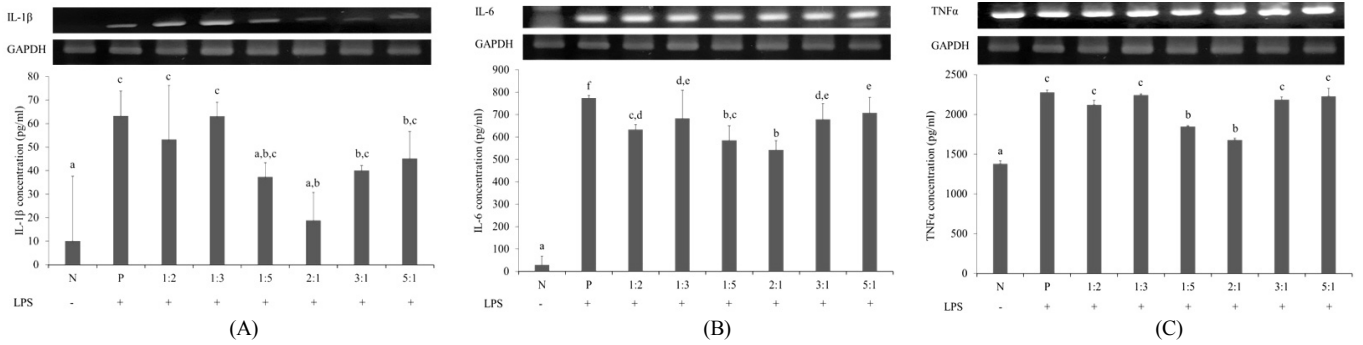
Figure 3. Effects of Aj and Ac complex extracts on IL-1β (A), IL-6 (B), and TNFα (C) generation in LPS-induced in RAW264.7 macrophage cells. The cells were pre-treated with LPS (1 μg/mL) for 24 h and then treated with various ratios of Aj and Ac complex extracts (400 μg/mL) for 24 h. Values are the means ± SD of three independent experiments (n = 3). Values that do not share the same superscript are significantly different by ANOVA ($p < 0.05$).
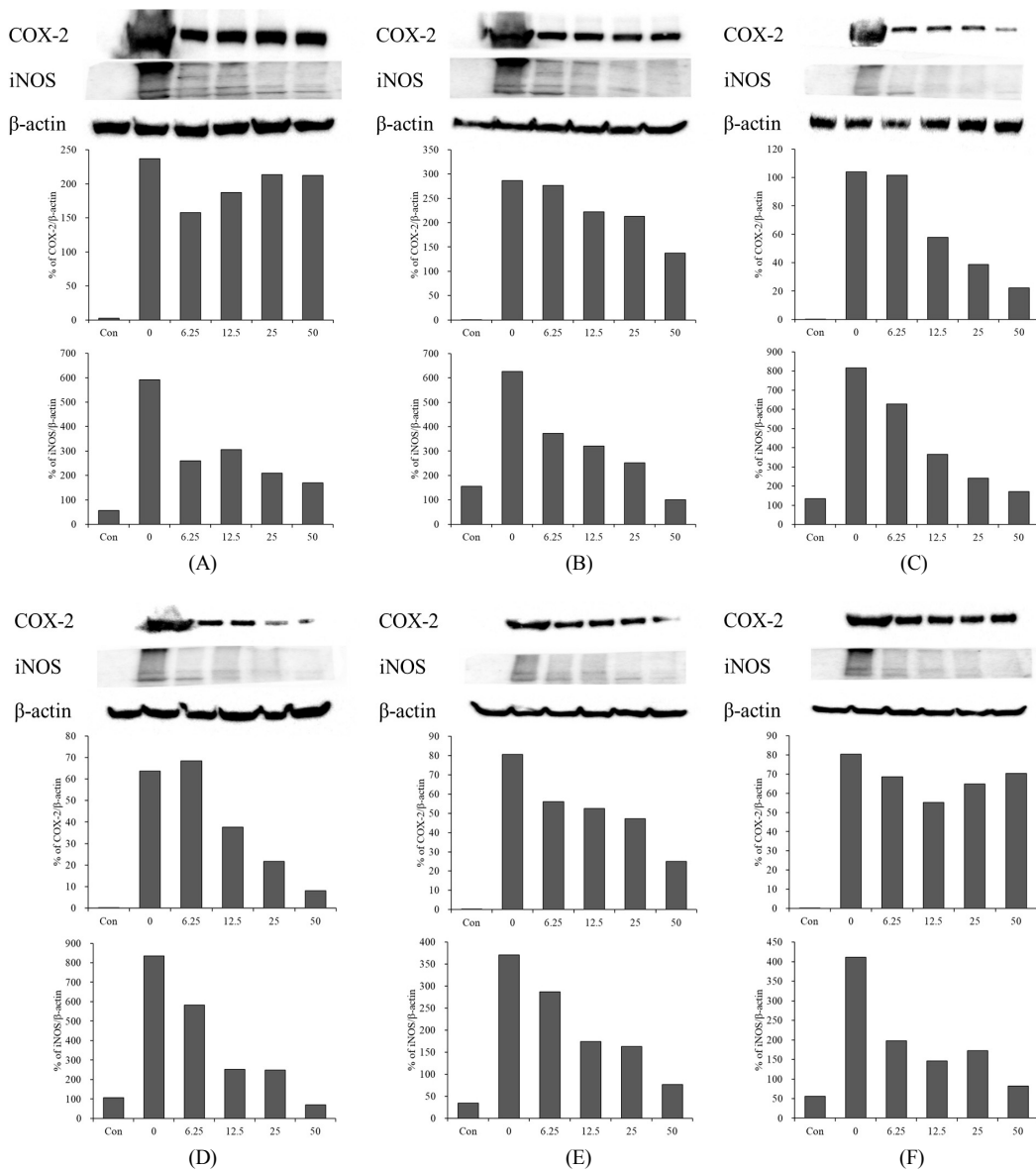

Figure 4. Effects of Aj and Ac complex extracts on COX-2 and iNOS generation in LPS-induced in RAW264.7 macrophage cells. The cells were pre-treated with LPS (1 μg/mL) for 24 h and then treated with various ratios of Aj and Ac complex extracts (400 μg/mL) for 24 h ((A) 1 : 2, (B) 1 : 3, (C) 1 : 5, (D) 2 : 1, (E) 3 : 1, (F) 5 : 1 (Aj : Ac)).

LPS exhibited a high increase in production at 774.49 pg/mL, but its production levels dropped to 678.98, 633.52, and 584.76 pg/mL at ratios of 3 : 1, 1 : 2 and 1 : 5, respectively. In particular, the effect of a 30% decline was observed with 542.68 pg/mL at a ratio of 2 : 1. While the level of TNFα (Figure 3C) production in the group treated with only LPS was 2,275.68 pg/mL, which was 39% higher than 1,379.47 pg/mL in the group without LPS treatment, the levels of TNF α production at ratios of 1 : 5 and 2 : 1 were 1,847.02 pg/mL and 1,679.11 pg/mL, respectively, therby showing a decrease of 19% and 27%, respectively. Vascular inflammatory responses are associated with complex interactions between inflammatory cells (neutrophils, lymphocytes, monocytes, and mocrophages), endothelial cells, and vascular smooth muscle cells. Moreover, cytokines produced by macrophages, T-cells, monocytes, platelets, and endothelial cells have an important role in the onset of inflammatory vascular diseases[26,27].

3.5. Effects on expression of COX-2 and iNOS

The results of the study on the expression of COX-2 and iNOS in RAW264.7 macrophages stimulated by LPS-induced combined extracts of Aj and Ac are depicted in Figure 4. We compared the expression of COX-2 and iNOS by measuring β-actin in a housekeeping gene that does not differ in terms of the degree of cell expression. COX-2 and iNOS are known to be involved in the biosynthesis of a large number of inflammatory mediators by inducing the activity of immune cells. COX-2 is an enzyme that synthesizes prostaglandin from arachidonic acid and is secreted by endothelial cells, macrophages, and osteoblasts in pathological conditions such as inflammation. Inhibition of COX-2 is known to be associated with the treatment of cancer as well as inflammation; currently known selective inhibitors of COX-2 include celecoxib and rofecoxib. NOS is a nitric oxide synthase produced by nitric oxide synthase (NOS). Further, there are three types of NO-endothelial NOS (eNOS), and neuronal NOS (nNOS). Among these, eNOS and nNOS are calcium-dependent and are not always responsive to stimuli; however, they are expressed in small amounts as enzymes that are constantly expressed in cells. Otherwise, iNOS is continuously produced by inflammatory stimulation; when induced by LPS or the like, it produces a large amount of NO concentration for a long period of time, thereby promoting an inflammatory reaction. The expression of COX-2 and iNOS in the LPS-treated group was inhibited in all the combined extracts of Aj and Ac compared to that in the control group. COX-2 and iNOS were inhibited in a concentration-dependent manner in the ratios 1 : 3, 2 : 1, and 3 : 1 (Aj : Ac).

As a result of treatment with 50 μg/mL of 1 : 1, 1 : 3 and 1 : 5 ratios of Aj and Ac, iNOS expression was 421.4, 526.3, and 645.7% lower than the control group, respectively (Figures 4A, 4B, and 4C). The expression of COX-2 was 55.6, 55.6, and 10% when treated with 50 μg/mL of 2 : 1, 3 : 1, and 5 : 1 ratios of Aj and Ac. (Figures 4D, 4E, and 4F), respectively. In particular, at the 2 : 1 ratio, the iNOS expression was 37.7% lower than that in the control group. From these results, it can be considered that the expression of COX-2 and iNOS are effectively inhibited at a ratio of 2 : 1.

4. Conclusion

In order to develop anti-inflammatory functional foods and medicines, continuous studies have been conducted to identify active substances that inhibit inflammatory cytokines from natural plant resources and identify their physiochemical characteristics. Kang et al.[28] reported that in RAW264.7 cells induced with LPS, *Aralia cordata* var extracts suppressed the expression of inflammatory cytokines. Lee et al.[29] reported that the ethanol extracts of *Aralia cordata* var. showed strong anti-inflammatory activities. Kang et al.[30] reported that applying the water extracts of five medicinal herbs suppressed NO production and reduced the production of IL-6 and IL-2 with statistical significance. However, no previous research has been conducted to identify anti-inflammatory effects using the combined extracts of Aj and Ac. Therefore, additional studies are required to identify active substances and understand the mechanisms by which the combined extracts of Aj and Ac suppress inflammation. The results of this study suggest that the combined extracts of Aj and Ac control the formation of NO and inflammatory cytokines. Therefore, these extracts can effectively suppress macrophage-induced inflammatory responses and have outstanding effects on the treatment of inflammatory mediator-induced diseases. In addition, these extracts may be used as preventive substances.

Acknowledgement

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