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Study on the Anti-Inflammatory Effects of Acemannan and Aloesin Isolated from Jeju Aloe Ferox *In Vitro* Macrophages

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Abstract
This study was conducted using Aloe ferox leaves supplied by KJMBio Research Institute (Seoul, Korea) and Kim Jeong Moon Aloe Jeju Agricultural Factory (Jeju-do, Korea). Acemannan and aloesin, which have proven anti-inflammatory efficacy, were isolated and mixed in various ratios to determine their anti-inflammatory effects. Anti-inflammatory activity was assessed by measuring the production of NO, PGE2, TNF-α, and IL-6, as well as the expression of the inflammatory proteins iNOS and COX-2. When comparing the NO production inhibition activity of acemannan and aloesin individually with that of the mixed samples (AA-1, AA-2, AA-3, AA-4, and AA-5), the mixed sample demonstrated superior NO production inhibition activity. To determine the optimal mixing ratio, PGE2, TNF-α, and IL-6 production were measured. As a result, all five samples showed an inhibitory effect compared to the LPS only treatment group at the final concentration of 100 μg/mL, and among them, AA-2 was confirmed to have the highest inhibitory effect, so the expression level of inflammation-related proteins through western blot was conducted using AA-2. As a result of measuring the expression levels of inflammation-related proteins iNOS and COX-2, AA-2 was confirmed to decrease in a concentration-dependent manner, and an inhibitory effect of more than 25% was confirmed compared to the LPS only treatment group at the final concentration of 100 μg/mL. Based on these results, it was confirmed that acemannan and aloesin had anti-inflammatory activity when mixed at various ratios, and among them, the highest anti-inflammatory activity was shown when acemannan and aloesin were mixed at a ratio of 1:2. In conclusion, it was confirmed that the sample mixed with acemannan and aloesin of Aloe ferox can be utilized as a variety of functional materials, and can also be used as basic data for future anti-inflammatory activity experiments.

KEYWORDS: *Aloe ferox, acemannan, aloesin, anti-inflammatory efficacy, NO, PGE2, TNF-α, IL-6, iNOS, COX-2*

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I. Introduction

The inflammatory response, a defense mechanism within the body, is triggered by harmful external substances such as microbial infections and chemical stimuli, as well as pathological irritants. [1] If the cause of inflammation is not removed and the body is excessively exposed to inflammatory mediators, it can eventually lead to chronic conditions such as cell damage, tissue necrosis, and even cancer over time. [2] Macrophages, immune defense cells, play a pivotal role in the inflammatory process. A well-known example of an inflammatory stimulus is lipopolysaccharide (LPS), which activates various immune cells, including macrophages. [3] Stimulated immune cells activate inflammatory signaling pathways, such as nuclear transcription factor E2-related factor 2/heme oxygenase-1, mitogen-activated protein kinases, and nuclear factor-kappa B, leading to the production of inflammatory enzymes like inducible nitric oxide synthase (iNOS) and cyclooxygenase-2 (COX-2), as well as inflammatory mediators such as interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α), nitric oxide (NO), and prostaglandin E2 (PGE_2). [4,5]

The inflammatory enzyme iNOS is induced and expressed not under normal healthy conditions but rather in response to stimuli like LPS. iNOS is known to exacerbate inflammation by producing large amounts of NO, which damages healthy cells and tissues within the body. [6,7] Similarly, COX-2, associated with PGE production, is acutely expressed due to the triggering of signaling cascades and increased catalytic activity, leading to the generation of large amounts of PGE_2. [8] Excess PGE contributes to tumor growth by promoting angiogenesis and

inhibiting apoptosis while sensitizing pain receptors, thereby maintaining the body in an inflammatory state. [9,10] Efforts to develop anti-inflammatory drugs have focused on targeting and regulating the production of these inflammatory mediators and their associated gene expression. [11]

Aloe is widely distributed in hot and arid regions such as North Africa, the Middle East, and the southern Mediterranean, with around 500 known species. Notable varieties include Aloe arborescens, Aloe vera, Aloe saponaria, and Aloe ferox. Aloe is reported to have various benefits, including improving and preventing constipation, antioxidant effects, anticancer properties, antifungal activity, antihyperlipidemic effects, and antidiabetic effects. [12, 13] Aloe contains polysaccharides and anthraquinones such as aloesin, aloin, aloenin, and aloe-emodin, along with vitamins, amino acids, tocopherols, fatty acids, and minerals. [14,15] Mannose, comprising over 70% of aloe ferox polysaccharides, has been extensively studied for its pharmacological and biological properties in medical and industrial fields. [16] It is known to exhibit beneficial effects across various health conditions, including cancers and inflammatory diseases by promoting macrophage proinflammatory activation and triggering metaflammation. [17,18]

Aloesin, a natural polyphenol derived from aloe plants, exhibits strong antioxidant effects, promotes wound healing, regulates immunity, and has antimicrobial properties. It also directly impacts melanin production. Furthermore, its anti-inflammatory activity, achieved by inhibiting the mitogen-activated protein kinase (MAPK) pathway and reducing pigmentation, demonstrates its diverse biological effects. [19-21] As a result, acemannan and aloesin each have various effects, and both have anti-inflammatory activity in the body. However, aloesin has the disadvantage of being high cost, [22] so combining acemannan, which is more cost-effective, can enhance economic viability.

In this study, we aim to determine the respective effects of mannose and aloin, the key components of Aloe ferox leaves, and to investigate the anti-inflammatory activity based on their mixing ratios. Through this, we aim to identify the optimal ratio and validate its potential use as a functional material.

II. Experimentation

Experimental Materials and Extraction

The materials used in this experiment, mannose and aloin, were supplied from Aloe ferox leaves from KJM Bio Research Institute (Seoul, Korea) and Kim Jeong Moon Aloe Jeju Agricultural Factory (Jeju-do, Korea). Mannose was prepared by removing the outer peel of the *Aloe ferox* leaves, treating them with cellulase, filtering the supernatant, then filtering it with activated carbon, concentrating it, and finally freeze-drying it. Aloin was obtained by hot-water extraction of the *Aloe ferox* leaves, followed by cooling, immersion, and precipitation, then purified using column chromatography. Pure mannose, pure aloin, and five types of their mixtures in ratios of 1:1 (MA_1), 1:2 (MA_2), 1:3 (MA_3), 2:1 (MA_4), and 3:1 (MA_5) were prepared as samples of the experiments.

Reagents and Equipment

For cell culture, fetal bovine serum (FBS), Dulbecco Modified Eagle Medium (DMEM), and 100 U/mL penicillin/streptomycin were purchased from Thermo Scientific Hyclone (Waltham, MA, USA). To measure cell toxicity, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma Chemical Co. (St. Louis, MO, USA).

LPS, Griess reagent, etc. used in the subsequent cell experiments were purchased from Sigma Chemical Co., and Indomethacin, which exhibits anti-inflammatory effects, was also obtained from Sigma Chemicals Co. and used to compare the efficacy of the sample. [22] An enzyme-linked immunosorbent assay (ELISA) kit from R&D Systems, Inc. (Minneapolis, MN, USA) was used to measure the amount of PGE_2 produced and the proinflammatory cytokines TNF-α and IL-6 produced. Cell Signaling (Danvers, MA, USA) was used to detect the protein expression of inflammatory mediators, iNOS, anti-rabbit IgG, and HRP-linked antibody, while β-actin, COX-2, and anti-mouse (m-IgGκ BP-HRP) were obtained from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA). immobilon western chemiluminescent HRP substrate, a reagent for protein expression, was purchased from Merck Millipore (Burlington, MA, USA) and used for enhanced chemiluminescence (ECL).

The equipment used included a pH meter (STARA2110, Thermo Scientific), CO2 incubator (MCO-18AC, Panasonic Healthcare Co., Kadoma-shi, Osaka, Japan), micro refrigerated centrifuge (Smart R17, Hanil Scientific Inc., Gimpo, Gyeonggi-do, Korea), microplate reader (SPECTROstar Nano, BMG LABTECH, Allmendgrün, Ortenberg, Germany), and ChemiDoc imaging system (BIO-RAD, Hercules, CA, USA).

Cell culture

The macrophage cell line used in the experiment was obtained from the Korean Cell Line Bank (KCLB40071). Cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin under conditions of 5% CO2 and 37°C. Subculturing was performed after at least 18 hours of incubation.

MTT Assay

To assess the cytotoxicity of the macrophage cell, RAW 264.7 cells, the experiment followed the method by Carmichael et al. [23] After counting the cells, 1×10^4cells/well were seeded in a 96-well plate for culture. Mannose, aloin, and their mixture samples were added at concentrations of 5, 10, 50, 100, 500, and 1,000 μg/mL. After culturing for 24 hours with the sample-added culture medium, 40 μL of MTT reagent at a concentration of 2.5 mg/mL was added to each well and incubated for 3 hours and 30 minutes. Then, all supernatants were removed, 100 μL of DMSO was added at a time, shaking was performed for 10 minutes, and the absorbance was measured at 540 nm using a microplate reader.

NO Production Inhibition Assay

The NO production inhibitory activity was tested using RAW 264.7 cells using the method of Green et al. [24] RAW 264.7 cells were seeded in a 6-well plate at a concentration of 3×10^5 cells/well, and incubated for 24 hours. LPS was added to the culture medium at a concentration of 10 μg/mL, and the diluted samples were treated at 100 μg/mL 2 hours later. After 18 hours of incubation, 100 μL of culture medium was mixed with 100 μL of Griess reagent in a 96-well plate and reacted for 10 minutes. Afterwards, absorbance was measured at 540 nm using a microplate reader.

PGE_2 Production Inhibition Assay

RAW 264.7 cells were distributed in a 6-well plate at 3×10^5 cells/well and cultured for 24 hours to stabilizer the cells. Then, LPS was added at 10 μg/mL and reacted for 2 hours. After inducing inflammation, each sample was added and cultured for 18 hours, and 800 μL of culture solution was centrifuged at 16,305 × g for 5 minutes to remove sediment. After centrifugation, 500 μL of supernatant was collected to measure PGE_2 content using an ELISA kit according to the provided manufacturer's instructions. PGE_2 content was measured at absorbance of 450 nm using a microplate reader.

Measurement of Proinflammatory Cytokines (TNF-α, IL-6)

Production of inflammation mediated cytokines, TNF-α and IL-6, was assessed using RAW 264.7 cells. Cells were seeded at 3×10^5 cells/well in a 6-well plate and cultured for 24 hours. LPS, a stimulant, was added at a concentration of 10 μg/mL, and reacted for 2 hours, followed by the addition of the samples at varying concentrations. After 18 hours, the culture medium was collected and centrifuged at 16,305×g for 5 minutes. The supernatants were analyzed using ELISA kits according to the method provided by the manufacturer. TNF-α and IL-6 contents were measured at absorbance of 450 nm using a microplate reader.

Protein Expression Analysis via Western Blot

To confirm the expression of iNOS and COX-2 proteins, which are inflammatory mediators, inflammation was induced in RAW 264.7 cells with LPS and western blot was performed. RAW 264.7 cells (1×10^6 cells/well) were seeded in a 100 mm cell culture dish and stabilized for 24 hours. After confirming the stabilized cells, the stimulant LPS was treated at a concentration of 10 μg/mL in each well and allowed to react for 2 hours to induce inflammation. After 2 hours, each sample was treated with non-toxic concentrations of 10, 50, and 100 μg/mL and incubated for 16 hours. After removing the culture medium of the cells that had completed culture, they were washed twice with PBS and lysed using a lysis buffer (a solution containing RIPA buffer and protease & phosphatase single-use inhibitor cocktail 100X). The lysed cells were centrifuged for 20 minutes (4°C, 16,305 ×g) and the supernatant was collected. The collected supernatant was quantified using a BCA protein assay kit, and then the proteins were separated by electrophoresis on a 10% acrylamide gel. The separated proteins were transferred to a membrane (polyvinylidene fluoride), and the skim milk was dissolved in tris-buffered saline & tween 20 (TBST) to prepare 5% skim milk, which was then blocked for 1 hour. After that, the primary antibody was diluted in 3% skim milk and reacted overnight at 4°C, and the membrane was washed three times for 10 minutes with TBST. The secondary antibody was added to the washed membrane, reacted for 1 hour and 30 minutes, then removed, and washed three times for 10 minutes with TBST. Then, the proteins on the membrane were reacted with ECL solution, and the results were confirmed with a Chemidoc imaging system.

Statistical Analysis

All experimental results were analyzed using data from at least three repetitions. Data were presented as mean ± standard deviation (SD) of each item. Significance was assessed using one-way ANOVA and Duncan's multiple range test ($p<0.05$) or t-test ($p<0.001$) as post hoc tests via IBM SPSS Statistics (23, IBM Corp., Armonk, NY, USA).

III. Result and Discussion

Cell Viability by MTT Assay

The buff substrate MTT undergoes a reduction reaction in the mitochondrial respiratory chain enzymes within cells, forming formazan. The carmine formazan is produced only in living cells and not in dead cells, exhibiting maximum absorbance near 550 nm. [25]

The cell viability of mannose, aloin, and the mixed samples (MA-1, MA-2, MA-3, MA-4, MA-5) in RAW 264.7 macrophages was evaluated using the MTT assay (Fig. 1). Mannose, aloin, and MA-1 through MA-5 were tested at concentrations ranging from 5 to 1,000 μg/mL. The results showed that at a concentration of 100 μg/mL, all samples exhibited over 90% cell viability. Therefore, subsequent cell experiments related to anti-inflammatory activity were conducted at a concentration of 100 μg/mL, where cell viability exceeded 90%.

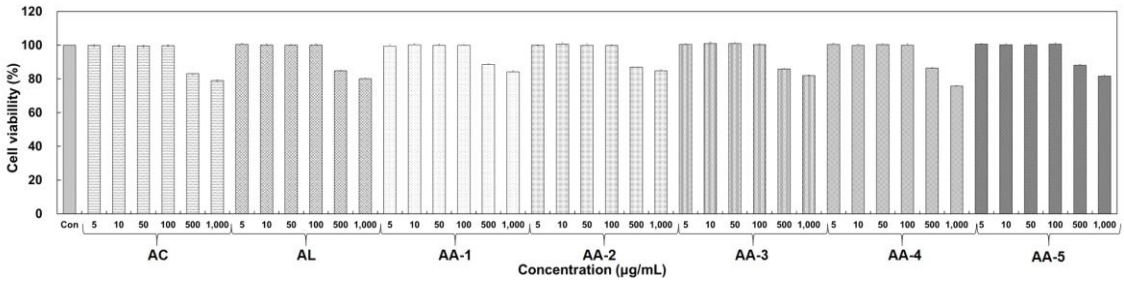


Fig. 1. Cell viability results of samples according to mixing ratio for RAW 264.7 cells. RAW 264.7 cells were seeded in 96-well plates at 1×104 cells/well for 24 h. Five types of samples mixed with acemannan and aloesin were treated and cultured for 24 h, and cell viability was measured using the MTT assay. AC, acemannan; AL, aloesin; AA-1, the ratio of acemannan to aloesin was 1:1; AA-2, the ratio of acemannan to aloesin was 1:2; AA-3, the ratio of acemannan to aloesin was 1:3; AA-4, the ratio of acemannan to aloesin was 2:1; AA-5, the ratio of acemannan to aloesin was 3:1. All values are mean±SD (n=3).

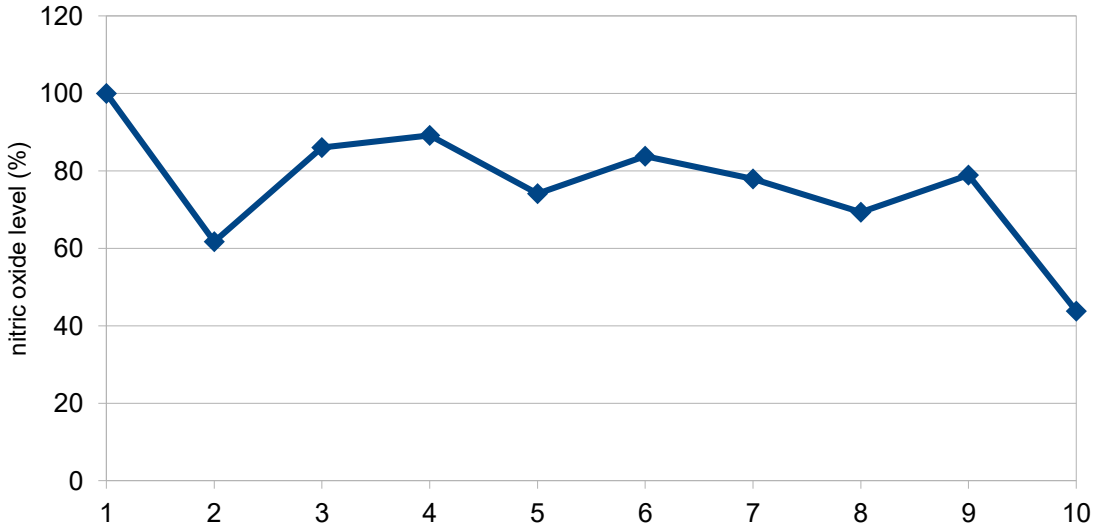
Inhibition of NO production

The inflammatory response is known to be the earliest immune reaction within the innate immune system, demonstrating the close relationship between immunity and inflammation. As one of the most representative substances in the innate immune response, NO plays various roles, including blood clotting, blood pressure regulation, and immune responses related to cancer cells. However, excessive NO production can lead to cell damage and contribute to various diseases. [26]

To evaluate the inhibitory effect of mannose, aloin, and the mixed samples (MA-1, MA-2, MA-3, MA-4, MA-5) on NO production, RAW 264.7 cells induced with LPS were used. Initially, the difference between the untreated group and the LPS-treated group was confirmed. At a concentration of 100 μg/mL, the NO inhibition rates were 13.68% for mannose, 10.95% for aloin, 25.89% for MA-1, 16.61% for MA-2, 21.85% for MA-3, 30.51% for MA-4, and 21.33% for MA-5 (Fig. 2). These results showed that the NO inhibitory effect was enhanced when mannose and aloin were combined, rather than used individually, indicating a synergistic effect. To determine the optimal mixing ratio, additional experiments were conducted on the production of PGE_2, TNF-α, and IL-6.

Table 1, Type of sample treated in RAW 264.7 cells

	1	2	3	4	5	6	7	8	9	10
LPS(10 μg/mL)	+	-	+	+	+	+	+	+	+	+
Sample (100 μg/mL)	-	-	MN	AL	MA_1	MA_2	MA_3	MA_4	MA_5	Indo



1) For the number of samples refer to Table 1.

Fig. 2. Inhibitory effect of samples according to the mixing ratio on NO production in LPS-induced RAW 264.7 cells. RAW 264.7 cells were distributed at 3×105 cells/well in a 6 well plates and stabilized for 24 h. Afterwards, LPS(10 μg/mL) was treated and reacted for 2 h, and then 5 types of samples with different mixing ratios of acemannan and aloesin were treated and reacted for 16 h. AC, acemannan; AL, aloesin; AA-1, the ratio of acemannan to aloesin was 1:1; AA-2, the ratio of acemannan to aloesin was 1:2; AA-3, the ratio of acemannan to aloesin was 1:3; AA-4, the ratio of acemannan to aloesin was 2:1; AA-5, the ratio of acemannan to aloesin was 3:1; Indo, indomethacin. All values are mean±SD(n=3). Different superscripts (a-i) on the bars indicate significant differences among treatments at $p<0.05$ by Duncan's multiple range test.

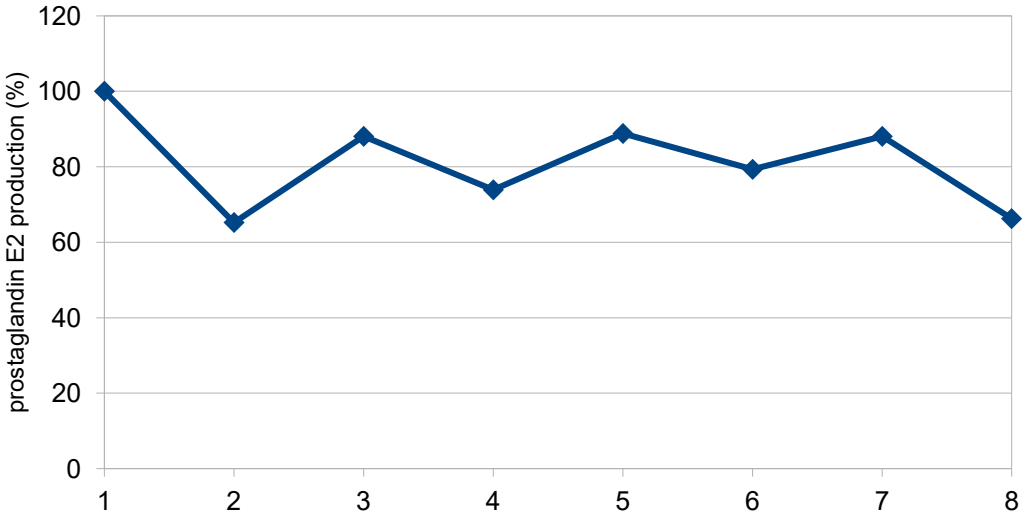
Inhibition of PGE_2 Production

PGE_2, one of the key factors inducing inflammation, is synthesized locally through the action of COX on arachidonic acid. PGE_2 promotes angiogenesis, which can lead to cancer development, and contributes to inflammation-related symptoms such as pain, swelling, and erythema. [26,27]

The inhibitory effect on increased PGE_2 production by LPS treatment in RAW 264.7 cells was evaluated by treating mannose and aloin mixed samples (AA-1, AA-2, AA-3, AA-4, AA-5) (Fig. 3). At a concentration of 100 μg/mL, the inhibition rates for PGE_2 production were 11.97% for MA-1, 26.11% for MA-2, 11.34% for MA-3, 20.53% for MA-4, and 12.21% for MA-5.

Table 2, Type of sample treated in RAW 264.7 cells

	1	2	3	4	5	6	7	8
LPS(10 μg/mL)	+	-	+	+	+	+	+	+
Sample (100 μg/mL)	-	-	MA_1	MA_2	MA_3	MA_4	MA_5	Indo



1) For the number of samples refer to Table 2.

Fig. 3. Inhibition effect on PGE2 production of samples according to the mixing ratio in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were distributed at 3×105 cells/well in a 6 well plates and cultured with DMEM for 24 h. Afterwards, LPS(10 μg/mL) was treated for 2 h, and then 5 types of samples with different mixing ratios of acemannan and aloesin were treated and cultured for 16 h. PGE2 production was measured by collecting the supernatant. AA-1, the ratio of acemannan to aloesin was 1:1; AA-2, the ratio of acemannan to aloesin was 1:2; AA-3, the ratio of acemannan to aloesin was 1:3; AA-4, the ratio of acemannan to aloesin was 2:1; AA-5, the ratio of acemannan to aloesin was 3:1; Indo, indomethacin. All values are mean±SD(n=3). Different superscripts(a-i) on the bars indicate significant differences among treatments at $p<0.05$ by Duncan's multiple range test.

Production of Pro-Inflammatory Cytokines (TNF-α, IL-6)

Pro-inflammatory cytokines such as TNF-α, IL-6, and IL-1β are known to act as inflammatory mediators. These cytokines stimulate the immune system and are involved in chronic inflammation and tumor formation. Overproduction of these cytokines can lead to conditions such as septic shock and inflammatory diseases. [28,29] The inhibitory effects of mannose and aloin mixed samples (MA-1, MA-2, MA-3, MA-4, MA-5) on TNF-α and IL-6 production were measured. As shown in Fig. 4, at 100 μg/mL, the inhibition rates of TNF-α production were 18.13% for MA-1, 26.77% for MA-2, 5.97% for MA-3, 9.79% for MA-4, and 2.58% for MA-5. Similarly, the inhibition rates of IL-6 production were 12.66% for MA-1, 27.33% for MA-2, 13.42% for MA-3, 12.84% for MA-4, and 5.05% for MA-5.

Excessive expression of pro-inflammatory cytokines such as TNF-α and IL-6 induces the production of inflammatory mediators like NO and PGE2 by upregulating inflammation-related genes such as iNOS and COX-2. [30] Although MA-2 showed lower NO inhibitory activity compared to MA-4, it exhibited higher inhibitory effects on PGE_2, TNF-α, and IL-6, which are involved in inflammation. Based on these findings, further experiments were conducted on the protein expression levels of iNOS and COX-2 using MA-2. Additional studies will be carried out on other mixing ratios to establish the anti-inflammatory mechanism.

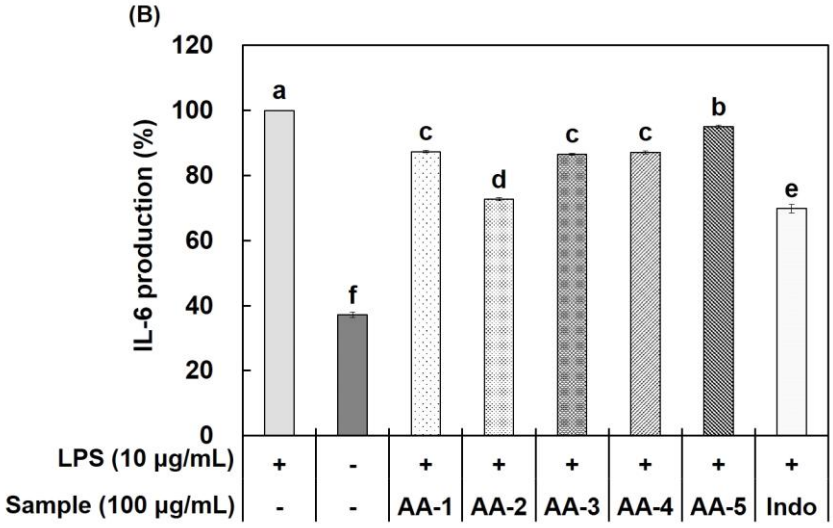


Fig. 4. Inhibitory effects on TNF-α and IL-6 of samples with different mixing ratios in LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were distributed at 3×105 cells/well, stabilized for 24 h, and then stimulated by treatment with LPS (10 μg/mL). Afterwards, 5 types of samples with different mixing ratios of acemannan and aloesin were added and incubated for 16 h. The supernatant was collected and the production amounts of TNF-α and IL-6 were measured. (A) Effect of TNF-α inhibitory activity according to mixing ratio of acemannan and aloesin. (B) Effect of IL-6 inhibitory activity according to mixing ratio of acemannan and aloesin. AA-1, the ratio of acemannan to aloesin was 1:1; AA-2, the ratio of acemannan to aloesin was 1:2; AA-3, the ratio of acemannan to aloesin was 1:3; AA-4, the ratio of acemannan to aloesin was 2:1; AA-5, the ratio of acemannan to aloesin was 3:1; Indo, indomethacin. All values are mean±SD(n=3). Different superscripts(a-f) on the bars indicate significant differences among treatments at $p<0.05$ by Duncan's multiple range test.

Protein Expression via Western Blot

The enzymes iNOS and COX-2 are involved in regulating the production of NO and PGE2 during inflammation. iNOS, a protein induced by inflammatory stimuli, is known to aggravate inflammation by promoting excessive NO production and the biosynthesis of inflammatory mediators, resulting in tissue damage, nerve damage, and genetic mutations. COX-2, expressed in inflammatory regions, inhibits apoptosis, promotes cell proliferation, and plays a critical role in the development of various inflammatory and degenerative diseases, including cancer. Regulating the expression of iNOS and COX-2 is known to be crucial for anti-inflammatory responses. [31-33]

The effect of MA-2, the mixed sample with the most effective inhibitory activity on PGE2, TNF-α, and IL-6 production, on the protein expression of iNOS and COX-2 was examined (Fig. 5). MA-2 was tested at concentrations of 10, 50, and 100 μg/mL, and at the final concentration of 100 μg/mL, the inhibitory effects on iNOS and COX-2 protein expression were 25.01% and 27.27%, respectively. This study confirms that the mixed extract of mannose and aloin has potential as an anti-inflammatory agent through its ability to inhibit the production of inflammatory mediators such as NO, PGE2, and pro-inflammatory cytokines. Based on these results, further validation of the inflammatory mechanism and in vivo studies will be conducted to explore its potential as a functional material.

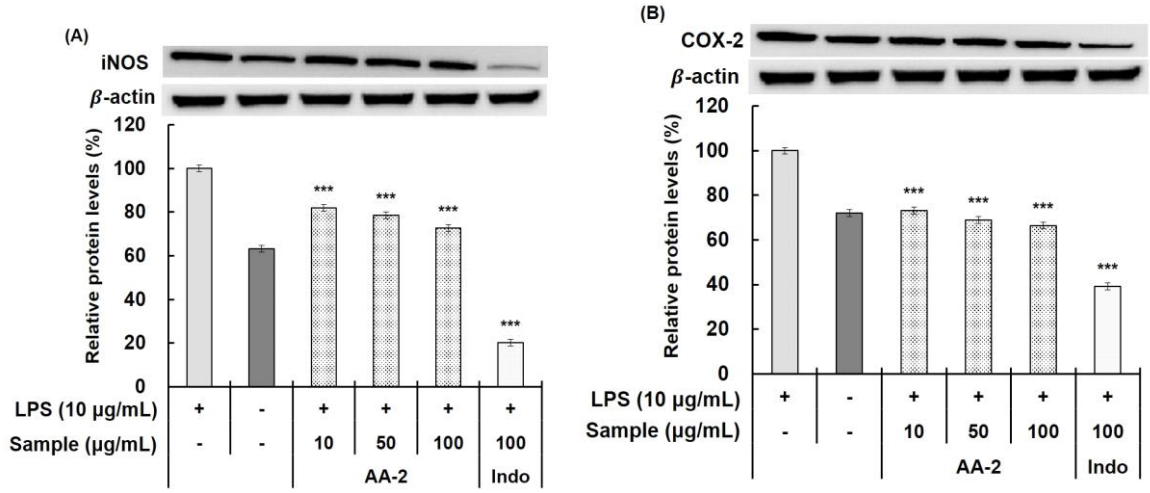


Fig. 5. Inhibitory effects on iNOS and COX-2 protein expression of samples in RAW 264.7 cells stimulated with LPS. RAW 264.7 cells were seeded at 1×106 cells/well and cultured for 24 h. LPS (10 μg/mL) was reacted for 2 h, and 5 samples with different mixing ratios of acemannan and aloesin were treated and incubated for 16 h. Afterwards, proteins were extracted from the cells, and protein expression of iNOS and COX-2 was measured using western blot. (A) When acemannan and aloesin are in a 1:2 ratio, iNOS protein expression level inhibitory activity effect. (B) When acemannan and aloesin are in a 1:2 ratio, COX-2 protein expression level inhibitory activity effect. AA-2, the ratio of acemannan to aloesin was 1:2; Indo, indomethacin. All values are mean±SD(n=3) (Compared to the LPS alone treatment group. ***p<0.001).

IV. Conclusion

This study investigated the anti-inflammatory effects of mannose and aloin, isolated from Aloe ferox leaves, and their mixtures in various ratios. The results demonstrated that the mixed samples exhibited superior anti-inflammatory activity compared to individual components. Among the tested ratios, the 1:2 mixture of mannose and aloin (MA-2) showed the highest efficacy in inhibiting the production of NO, PGE2, TNF-α, and IL-6, as well as suppressing the expression levels of inflammation-related proteins iNOS and COX-2 in a dose-dependent manner. At a final concentration of 100 μg/mL, MA-2 achieved over 25% inhibition of iNOS and COX-2 expression compared to the LPS-only treatment group.

These findings highlight the potential of mannose and aloin mixtures as promising anti-inflammatory agents. The enhanced efficacy observed in the combined formulation suggests a synergistic interaction between these compounds, making them suitable candidates for further exploration as functional materials for anti-inflammatory applications.

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