Original Article

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Anti-inflammatory and Anti-oxidative Effects of *Alpiniae Oxyphyllae Fructus* Hot Aqueous Extract in Lipopolysaccharide (LPS)-stimulated Macrophages

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[Abstract]

Objectives : *Alpiniae oxyphyllae Fructus* (AOF) is an herbal medicine, which has been used for the treatment of fatigue, chills, and poor physical conditions. The objective of this study was to investigate the anti-inflammatory and anti-oxidative effects of AOF hot aqueous extract.

Methods : The cytotoxicity of AOF extract was evaluated using the MTT assay. Nitric oxide (NO) production was measured by the Griess reaction. Prostaglandin E_2 (PGE_2) production was measured by a commercial competitive enzyme immunoassay. Cytokine production (IL- 1tion co6, and TNF- F- was measured by ELISA.

The anti-oxidative effect of AOF extracts was measured by the DPPH method. Polyphenol and flavonoid contents were measured by Folin-Ciocalteu's phenol reagent and aluminum chloride, respectively.

Results : AOF hot aqueous extract did not show toxicity at doses of 25, 50, 100, and 200 μg/mL. AOF extract significantly inhibited NO production at doses of 100 and 200 μg/mL.PGE_2 production was inhibited by AOF extract treatment at doses of 100 and 200 μg/mL. AOF extracts reduced IL- 6 production in a dose-dependent manner. IL- 1ent maTNF- F- 1ent mannerd IL-6 production in uction at doses of 100 and μg/mL. The DPPH free radical scavenging capability was above 50% at 200 μg/mL.

Conclusion : This study suggests that AOF hot aqueous extract may exert anti-inflammatory and anti-oxidative effects in a dose-dependent manner. Further studies are required for validating the safety and efficacy of AOF.

Key words :
Alpiniae oxyphyllae Fructus;
Anti-inflammation;
Antioxidant;
Hot Aqueous Extract;
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I. Introduction

Inflammation is an important immune response that occurs when the body tissues are damaged. This reaction is a defense process against pathogens. Inflammatory reactions are accompanied by pain, fever, and edema[1], and can lead to pain in sprains, tendinitis, aponeurositis, gout, and osteoarthritis[2].

Alpiniae oxyphyllae Fructus (AOF) is the mature fruit of *Alpinia Oxyphylla* Miq. belonging to the family Zingiberaceae. The main constituents of AOF are Yakuchinone A and Yakuchinone B. In Korean medicine, AOF has been widely used for the treatment of fatigue, chills, and wasting disease[3].

Previous studies have reported muscle-relaxing[4], anti-allergic[5], nephroprotective[6], neuronal cell protective[7-10], bone loss inhibitory[11], anti-cancer[12,13], and anti-aging[14] effects of AOF.

Many studies have suggested that AOF might exert anti-inflammatory and anti-oxidative effects. However, researches on the anti-inflammatory and anti-oxidative effects of AOF are scarce. In this study, we aimed to investigate the effects of AOF hot aqueous extract by evaluating nitric Oxide (NO) and Prostaglandin E_2 (PGE_2) production. To understand the mechanism underlying the anti-inflammatory activity of AOF clearly, the levels of interleukin 1 beta (IL-1terleuki, and tumor necrosis factor a (TNF-NF--tumor necrosis factor atoryOF hot aquethe anti-oxidative effect, we assessed 1,1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging activity in macrophages. The amount of polyphenol and flavonoid present in AOF were also measured. We observed that AOF extract exerted significant anti-inflammatory and anti-oxidative effects.

II. Experimental materials and methods

1. Materials

AOF used in the experiment was purchased from Omniherb (Kyongbuk, Korea). The method of extraction was as follows: AOF (200 g) was mixed with 2 L distilled water and boiled for 4 h at 100℃. The extract was filtered through a filter paper and centrifuged at 3000 x *g*. Then, a secondary filtration was carried out through a 0.03 mm filter paper (Nalgene, New York, USA). The filtrate was concentrated to 100 mL and frozen at −80℃. The filtrate was freeze-dried for 7 days using a freeze-drying system (Labconco, USA). AOF extract (15.5 g) was obtained and the yield was 7.75%.

2. Cell culture

RAW 264.7 macrophages purchased from ATCC (Manassas, USA) were used in this study. They were placed in Dulbecco' s modified Eagle' s medium (DMEM) containing 10% fetal bovine serum (FBS) and cultured in an incubator at 37℃ under 5% CO_2.

3. Cytotoxicity evaluation

Cytotoxicity was evaluated using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. RAW 264.7 macrophages were seeded in a 96-well plate at a concentration of 1×10^5/well and cultured for 18 h at 37℃ under 5% CO_2. The macrophages were divided into five experimental groups as follows: control (non-treated), AOF 25 (25 μg/mL AOF hot aqueous extract-treated); AOF 50 (50 μg/mL AOF hot aqueous extract-treated); AOF 100 (100 μg/mL AOF hot aqueous extract-treated), and AOF 200 (200 μg/mL AOF hot aqueous extract-treated) groups.

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Stable cells were treated with the MTT reagent and absorbance was measured at 570 nm.

4. Measurement of NO production

RAW 264.7 macrophages were seeded in a 96-well plate at a concentration of 1×10^5/well and cultured for 18 h at 37℃ under 5% CO_2. The macrophages were divided into five groups (Control, AOF 25, 50, 100, and 200 μg/mL) and treated with 1 μg/mL of lipopolysaccharide (LPS) reagent. After mixing 100 μL of supernatant with Griess reagent (100 μL), absorbance was measured at 540 nm. Griess reagent was prepared by mixing 0.1% naphthylethylenediamine dihydrochloride (50 μL) and 1% sulfanilamide 50 (μL) in 5% phosphoric acid (H_3PO_4).

5. Measurement of PGE_2 production

PGE_2 levels were measured using a commercial competitive enzyme immunoassay kit (R&D systems, Minneapolis, USA). The cells were divided into five groups (control, AOF 25, 50, 100, and 200 μg/mL) and treated with 1 μg/mL of LPS reagent. LPS-treated RAW 264.7 macrophages were cultured for 18 h at 37℃ under 5% CO_2. The culture fluid (100 μL each) was loaded in a goat anti-mouse coated IgG 96 well plate, mixed with a primary antibody solution (50 μL) and PGE_2 conjugate (50 μL), and stabilized at 4℃ overnight. The treated culture fluid was mixed with the substrate solution (200 μL). After 5-20 min of waiting, stop solution (50 μL) was added. The absorbance was measured at 450 nm.

6. Measurement of cytokine production

The amount of IL-1amount , and TNF-N produced was measured by an Elisa kit (R&D systems, Minneapolis, USA). The macrophages were treated with 1 μg/mL of LPS reagent. LPS-treated RAW 264.7 macrophages were cultured for 18 h at 37℃ under 5% CO2. The culture fluid (50 μL each) was loaded in a goat anti-mouse coated IgG 96 well plate and stabilized at 4℃ overnight. The culture fluid was then washed thrice with washing buffer. Then, antibody reagent (100 μL) was loaded into each well, followed by washing thrice with washing buffer. Subsequently, streptavidin-HRP solution (100 μL) was added to the wells and incubated for 1 h at room temperature, followed by washing three times with washing buffer. Then, TMB substrate solution (100 μL) was added, incubated for 30 min at room temperature, followed by the addition of stop solution (100 μL). The absorbance was measured at 450 nm.

7. Measurement of anti-oxidative effect

To investigate the anti-oxidative ability of AOF, DPPH free radical scavenging ability was measured. RAW 264.7 macrophages were seeded in a 96-well plate at a concentration of 1 es w^5/well and cultured for 16 h at 37℃ under 5% CO_2. The cells were divided into four experimental groups (AOF 25, 50, 100, and 200 μg/mL) and diluted with MeOH (Methanol) and 50 μL diluent mixed with 80 μL of 0.15 mM DPPH (Sigma, USA) in a 96-well plate. Isolate the light at room temperature for 3 min. Absorbance was measured using a microplate reader (Tecan, Italy) at 520 nm. DPPH free radical scavenging ability was evaluated using the following formula:

$$\text{DPPH free radical scavenging activity (\%)} = \frac{\textit{Absorbance of Control} - \text{Experimental}}{\textit{Absorbance of Control}} \times 100$$

8. Amount of polyphenol in AOF

Folin & Ciocalteu's phenol regent method was

used for measuring the polyphenol content in AOF. The AOF hot aqueous extract was dissolved in methanol at a concentration of 1 mg/mL. The extract (100 mL) was mixed with 50 mL of Folin & Ciocalteu's phenol regent and incubated for 5 min. Then, 300 μL of 20% (w/v) sodium carbonate was added and incubated for 15 min, followed by the addition of 1 mL distilled water. Absorbance was measured using a microplate reader at 725 nm. The total amount of polyphenol was calculated as tannic acid equivalents.

9. Amount of flavonoid in AOF

Aluminum chloride method was used for measuring the flavonoid content. The AOF hot aqueous extract was dissolved in methanol at a concentration of 1 mg/mL. Then, 20 μL of 10% (w/v) aluminum, 20 μL of 1 M potassium acetate, and 860 μL of methanol were added and incubated for 40 min. Absorbance was measured using a microplate reader at 415 nm. The total amount of flavonoid was calculated as quercetin equivalents.

10. Statistical analysis

The experimental results were presented as means ± standard deviations using the SPSS for Windows program (Ver. 21.0). Statistical significance was confirmed by using Student's *t*-test. $p<0.05$ was considered statistically significant.

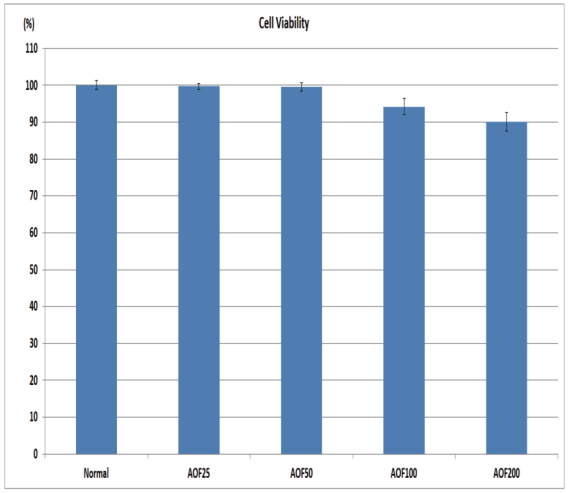

Fig. 1. Cytotoxic effects of *Alpiniae oxyphyllae Fructus* hot aqueous ethanol extract in RAW 264.7 macrophages

No significant toxicity was observed up to 200 μg/mL.

Normal: Non-treated group
AOF 25: 25 μg/mL AOF hot aqueous extract- treated group
AOF 50: 50 μg/mL AOF hot aqueous extract-treated group
AOF 100 100 μg/mL AOF hot aqueous extract- treated group
AOF 200: 200 μg/mL AOF hot aqueous extract- treated group
Values are represented as mean ± SD.
* $p < 0.05$ was considered a statistically significant difference from the control group, as determined by the student's *t*-test.

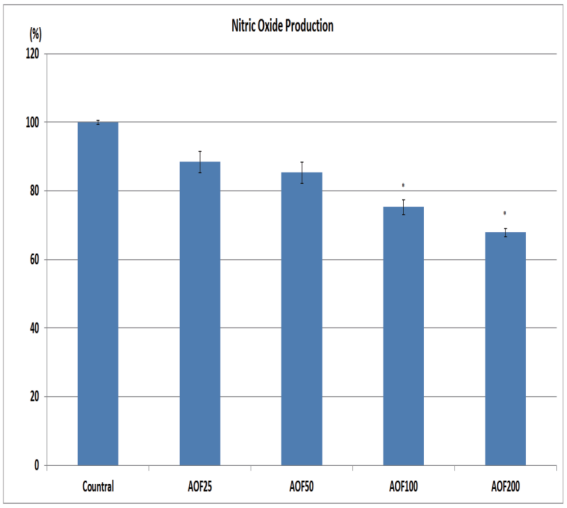

Fig. 2. Effect of *Alpiniae oxyphyllae Fructus* hot aqueous extract on NO production in RAW 264.7 macrophages

NO production rate was significantly decreased in the AOF 100 and 200 groups.

Control: 1 μg/mL LPS-treated group
AOF 25: 1 μg/mL LPS and 25 μg/mL AOF hot aqueous extract-treated group
AOF 50: 1 μg/mL LPS and 50 μg/mL AOF hot aqueous extract-treated group
AOF 100: 1 μg/mL LPS and 100 μg/mL AOF hot aqueous extract-treated group
AOF 200: 1 μg/mL LPS and 200 μg/mL AOF hot aqueous extract-treated group
Values are represented as mean ± SD.
* $p < 0.05$ was considered a statistically significant difference from the control group, as determined by the student's *t*-test.

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III. Results

1. The toxicity of AOF hot aqueous extract in the control, AOF 25, 50, 100, and 200 μg/mL groups was 100 ± 1.23, 99.68 ± 0.76, 99.52 ± 1.12, 94.21 ± 2.26, and 90.04 ± 2.49%, respectively. This shows that there was no significant toxicity up to 200 μg/mL of AOF (Fig. 1).

2. The production rate of NO in the control, AOF 25, 50, 100, and 200 μg/mL groups was 100.00 ± 0.61, 88.42 ± 3.12, 85.28 ± 3.06, 75.62 ± 2.14, and 67.97 ± 1.16%, respectively. NO production decreased significantly in the AOF 100 and 200 μg/mL groups (Fig. 2).

3. The production rate of PGE_2 in the control, AOF 25, 50, 100, and 200 μg/mL groups was 100.00 ± 5.14, 99.14 ± 4.72, 94.07 ± 3.24, 82.33 ± 4.96, and 76.31 ± 5.51%, respectively. PGE_2 production decreased significantly in the AOF 100 and 200 μg/mL groups (Fig. 3).

4. IL-1).us extract in the control, AOF 25, 50, 100, and 200 μg/mL groups was 100.00 ± 0.19, 91.21 ± 8.41, 80.39 ± 3.57, 75.50 ± 7.45, and 60.33 ± 4.39%, respectively. IL-1β production decreased significantly in the AOF 100 and 200 μg/mL groups (Fig. 4).

5. The rate of IL-6 production in the control, AOF 25, 50, 100, and 200 μg/mL groups was

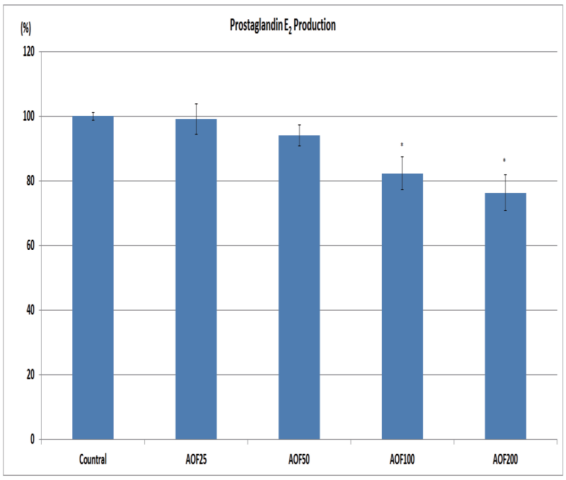

Fig. 3. Effect of *Alpiniae oxyphyllae Fructus* hot aqueous extract on PGE2 production in RAW 264.7 macrophages

PGE_2 production rate decreased significantly in AOF 100 and 200 groups.

Control: 1 μg/mL LPS-treated group
AOF 25: 1 μg/mL LPS and 25 μg/mL AOF hot aqueous extract-treated group
AOF 50: 1 μg/mL LPS and 50 μg/mL AOF hot aqueous extract-treated group
AOF 100: 1 μg/mL LPS and 100 μg/mL AOF hot aqueous extract-treated group
AOF 200: 1 μg/mL LPS and 200 μg/mL AOF hot aqueous extract-treated group
Values are represented as mean ± SD.
* $p < 0.05$ was considered a statistically significant difference from the control group, as determined by the student's *t*-test.

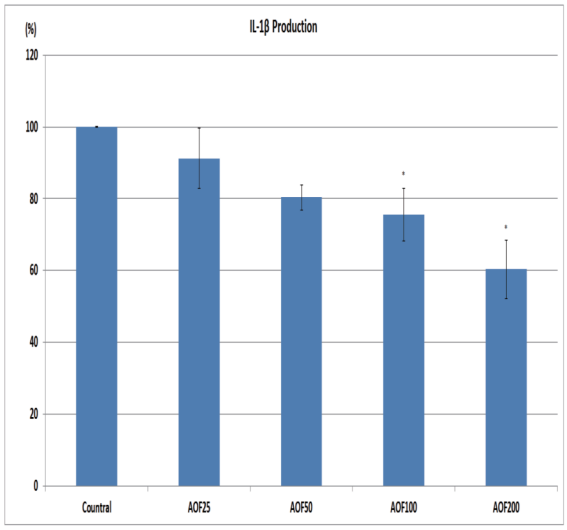

Fig. 4. Effect of *Alpiniae oxyphyllae Fructus* hot aqueous extract on IL- 1graph descri in RAW 264.7 macrophages

IL-1AW 264.7 macrophagdecreased significantly in AOF 100 and 200 groups.

Control: 1 μg/mL LPS-treated group
AOF 25: 1 μg/mL LPS and 25 μg/mL AOF hot aqueous extract-treated group
AOF 50: 1 μg/mL LPS and 50 μg/mL AOF hot aqueous extract-treated group
AOF 100: 1 μg/mL LPS and 100 μg/mL AOF hot aqueous extract-treated group
AOF 200: 1 μg/mL LPS and 200 μg/mL AOF hot aqueous extract-treated group
Values are represented as mean ± SD.
* $p < 0.05$ was considered a statistically significant difference from the control group, as determined by the student's *t*-test.

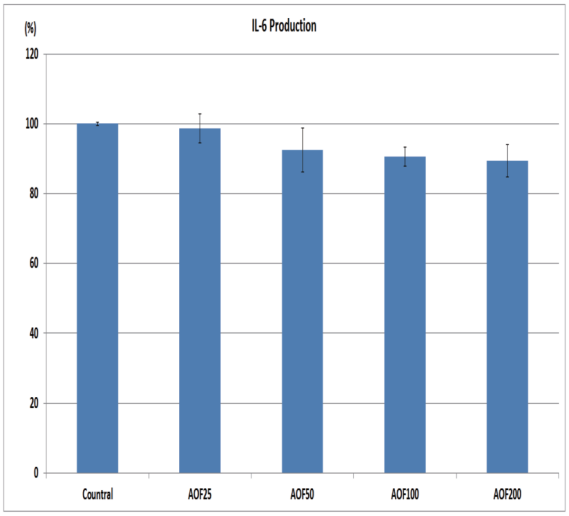

Fig. 5. Effect of *Alpiniae oxyphyllae Fructus* hot aqueous extract on IL-6 production in RAW 264.7 macrophages

IL-6 production rate was decreased in a concentration dependent manner, but there was no statistical significance.

Control: 1 μg/mL LPS-treated group
AOF 25: 1 μg/mL LPS and 25 μg/mL AOF hot aqueous extract-treated group
AOF 50: 1 μg/mL LPS and 50 μg/mL AOF hot aqueous extract-treated group
AOF 100: 1 μg/mL LPS and 100 μg/mL AOF hot aqueous extract-treated group
AOF 200: 1 μg/mL LPS and 200 μg/mL AOF hot aqueous extract-treated group
Values are represented as mean ± SD.
* $p < 0.05$ was considered a statistically significant difference from the control group, as determined by the student's *t*-test.

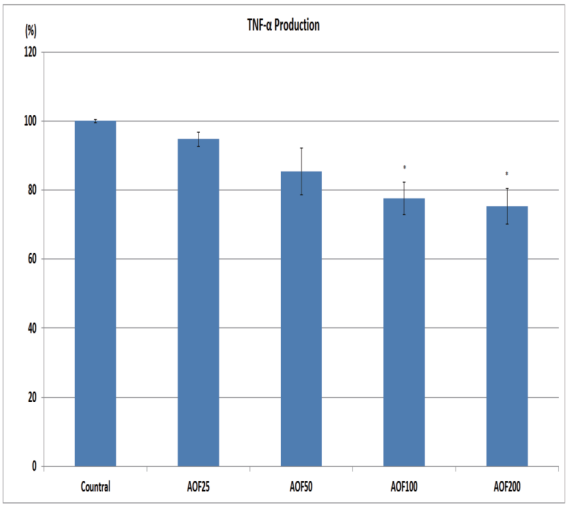

Fig. 6. Effect of *Alpiniae oxyphyllae Fructus* hot aqueous extract on TNF-α production in RAW 264.7 macrophages

TNF-NF-AW 264.7 mdecreased significantly in AOF 100 and 200 groups.

Control: 1 μg/mL LPS-treated group
AOF 25: 1 μg/mL LPS and 25 μg/mL AOF hot aqueous extract-treated group
AOF 50: 1 μg/mL LPS and 50 μg/mL AOF hot aqueous extract-treated group
AOF 100: 1 μg/mL LPS and 100 μg/mL AOF hot aqueous extract-treated group
AOF 200: 1 μg/mL LPS and 200 μg/mL AOF hot aqueous extract-treated group
Values are represented as mean ± SD.
* $p < 0.05$ was considered a statistically significant difference from the control group, as determined by the student's *t*-test.

100.00 ± 0.42, 98.69 ± 4.17, 92.39 ± 6.32, 90.05 ± 2.78, and 98.37 ± 4.75%, respectively. IL-6 production decreased in a concentration-dependent manner, but it was not significant (Fig. 5).

6. The rate of TNF-NF- 5).nl nnin the control, AOF 25, 50, 100, and 200 μg/mL groups was 100.00 ± 0.48, 64.69 ± 2.11, 85.34 ± 6.76, 77.50 ± 4.78, 75.33 ± 5.21%, respectively. TNF-NF-3 eous extdecreased significantly in the AOF 100 and 200 μg/mL groups (Fig. 6).

7. DPPH free radical scavenging ability in the AOF 25, 50, 100, and 200 μg/mL groups was 7.35 ± 0.73, 12.98 ± 1.32, 41.63 ± 1.03, and 55.31 ± 1.16%, respectively. The scavenging ability of AOF 200 μg/mL group was more than 50% (Fig. 7).

8. The amount of polyphenol in AOF is 29.34 ± 3.10 mg/g (Table 1).

9. The amount of flavonoid in AOF is 8.19 ± 1.27 mg/g (Table 2).

IV. Discussion

In BenCao GangMu, it has been reported that

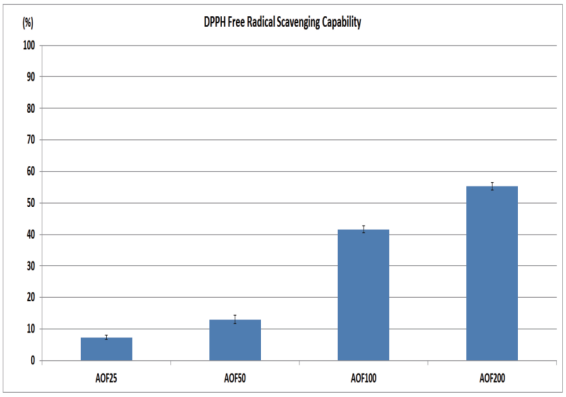

Fig. 7. Effect of *Alpiniae oxyphyllae Fructus* hot aqueous extract on DPPH free radical scavenging capability in RAW 264.7 macrophages

The scavenging ability of the AOF 200 group was more than 50%.

AOF 25: 1 μg/mL LPS and 25 μg/mL AOF hot aqueous extract-treated group
AOF 50: 1 μg/mL LPS and 50 μg/mL AOF hot aqueous extract-treated group
AOF 100: 1 μg/mL LPS and 100 μg/mL AOF hot aqueous extract-treated group
AOF 200: 1 μg/mL LPS and 200 μg/mL AOF hot aqueous extract-treated group

AOF can strengthen weak constitution and eliminate chills[15]. Therefore, in Korean medicine, AOF has been used for the treatment of elderly patients. Previous studies have reported the effect of AOF on cell regeneration and damage inhibition[6-14]. Yakuchinone A and Yakuchinone B[16, 17], the main components of AOF, have been reported to possess NO reduction effect. In addition, a previous study reported the free radical scavenging effect of Yakuchinone B[18].

In Korean medicine, inflammatory response is considered a competing vital force and bad factor, where vital force refers to resistance power against a disease and bad factor refers to the cause of the disease[19]. In pathology, inflammatory response is the process of restoring a damaged area. This process can cause edema, fever, pain, redness, swelling, and functional disability[2].

NO is a free radical that plays an important role in the transfer of substances in the cardiovascular, nervous, and immune systems. In addition, it is involved in intracellular homeostasis, transport of

Table 1. Total polyphenolic compounds in *Alpiniae oxyphyllae Fructus* hot aqueous extract

Compounds	AOF
TPC (mg tannic acid equivalents/g)	29.34 ± 3.10

TPC: Total polyphenolic compounds
AOF: *Alpiniae oxyphyllae Fructus* hot aqueous extract

Table 2. Total flavonoid content of *Alpiniae oxyphyllae Fructus* hot aqueous extract

Compounds	AOF
TF (mg quercetin equivalents/g)	8.19 ± 1.27

TF: Total flavonoids
AOF: *Alpiniae oxyphyllae Fructus* hot aqueous extract

neurotransmitters, and anticancer effects[20]. However, excessive NO production causes inflammation and tissue damage. Persistent inflammatory responses can result in myocarditis, chronic arthritis, glomerulonephritis, insulin-dependent diabetes mellitus, and irritable bowel syndrome[21]. Therefore, recent studies have focused on ways to treat inflammation by effectively suppressing the production of NO[22-24].

PGE_2 is an important inflammatory mediator. It is synthesized by cyclooxygenase (COX-2) and activated by macrophages. COX-2 is not expressed in the resting state, but its expression is induced by inflammatory stimulation. Excessive PGE_2 production leads to long-lasting inflammatory reaction resulting in rubefaction, edema, stiffness, and pain[25,26].

In the human body, oxidative stress is caused by an imbalance between oxidants and antioxidants. It results in the decomposition of proteins and inhibition of DNA synthesis, consequently leading to harmful effects in cells and organs[27]. Reactive oxygen species (ROS) and reactive nitrogen species (RNS) are involved in oxidative damage. ROS include free radicals, such as superoxide (O2-), nitric oxide (NO), and hydroxyl radicals (HO-), as well as oxygen-derived species, such as singlet oxygen

(O2) and hydrogen peroxide (H_2O_2)[28].

DPPH is a free radical. It is purple colored and exhibits absorption band at 520 nm. Generally, DPPH is converted to DPPH-H by proton-radical scavengers in various antioxidant mechanisms, which leads to its discoloration. The results can be verified easily by the naked eye. In addition, DPPH has the advantage of being very stable in organic solvents such as alcohol[29].

The viability of RAW 264.7 macrophages did not significantly decrease in the AOF 25, 50, 100, and 200 groups compared to that in the control group. The AOF 100 and 200 groups showed significant inhibition of NO production in LPS-stimulated RAW 264.7 macrophages compared to the control group. PGE_2 production was significantly inhibited in LPS-stimulated RAW 264.7 macrophages treated with 100 and 200 μg/mL of AOF hot aqueous extract compared to that in the control group. IL-1rol group. trwas significantly inhibited in LPS-stimulated RAW 264.7 macrophages treated with 100 and 200 μg/mL of AOF hot aqueous extract compared to that in the control group. AOF decreased the production of IL-6 in a concentration dependent manner; however, it was not statistically significant. TNF-NF- was not swas significantly inhibited in LPS-stimulated RAW 264.7 macrophages treated with 100 and 200 μg/mL of AOF hot aqueous extract compared to that in the control group. The DPPH radical scavenging capability was more than 50% in RAW 264.7 macrophages treated with 200 μg/mL extract. The amount of polyphenol and flavonoid in AOF is 29.34 ± 3.10 and 8.19 ± 1.27 mg/g, respectively.

By analyzing the results of these experiments, we can suggest that AOF hot aqueous extract decreased NO, PGE_2, IL-1eased N, and TNF-NF-TNF--6Oexand DPPH radical scavenging at concentrations of 50, 100, and 200 μg/mL. Therefore, AOF hot aqueous extract is believed to possess anti-inflammatory and anti-oxidative effects. More studies will be needed to assess the safety and efficacy of AOF.

V. Conclusion

In conclusion, AOF hot aqueous extract suppressed NO, PGE_2, IL-1 PGEto , and TNF-NF-TNF-tion and improved DPPH free radical scavenging activity. The polyphenols and flavonoids present in AOF exert antioxidant activity. Thus, we can suggest that AOF hot aqueous extract may exert anti-inflammatory and anti-oxidative activities.

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