

Original Research Article

Anti-inflammatory effect of neo-lignan isoamericanin A via suppression of NF- κ B in liposaccharide-stimulated RAW 264.7 cells

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Abstract

Purpose: To investigate the potential anti-inflammatory effects of the seeds of *Opuntia humifusa* and its active constituents.

Methods: The extract of *O. humifusa* seeds was tested for the inhibition of nitric oxide (NO) production in liposaccharide (LPS)-stimulated RAW 264.7 cells using Griess reagent. The active constituents were isolated using bioassay-guided isolation methods. The effects of the active constituent on NO, pro-inflammatory cytokines, nuclear factor-kappa B (NF- κ B) and nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor (I κ B) were evaluated by enzyme-linked immunosorbent assay (ELISA) and western blot analysis.

Results: The seed extract of *O. humifusa* significantly attenuated LPS-induced NO production in RAW 264.7 cells ($p < 0.05$). Bioassay-guided fractionation resulted in the isolation of isoamericanin A as an active constituent. Isoamericanin A reduced LPS-induced production of NO, iNOS, and pro-inflammatory cytokines (TNF- α and IL-6) in a concentration-dependent manner ($p < 0.05$). Furthermore, the effect was accompanied by decreased translocation of NF- κ B from the cytosol to the nucleus and the decreased phosphorylation of I κ B in the cytosol induced by LPS ($p < 0.05$).

Conclusion: The seed extract of *O. humifusa* and its active constituent, isoamericanin A, have anti-inflammatory effects in LPS-stimulated RAW 264.7 cells, suggesting that they have potentials as anti-inflammatory agents.

Keywords: *Opuntia humifusa* seeds, Isoamericanin A, Nitric oxide, RAW 264.7 cells, NF-kappa B

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INTRODUCTION

Opuntia humifusa Raf. (Cactaceae) is a cactus known as the eastern prickly pear. It is widely cultivated in the southern parts of Korea and is locally called Cheonnyuncho. The stems and fruits of *O. humifusa* are used as food worldwide

in the form of juice or freeze-dried powder because they are rich in polyphenols, flavonoids, minerals, and dietary fiber. Traditionally, it has been used to treat diabetes, inflammation, and rheumatoid arthritis [1]. In addition, there are reports on its anti-cancer effects on diverse cancer cells including glioblastoma, cervical

carcinoma, and gastric adenocarcinoma [2,3]. *O. humifusa* has antibacterial [4], and anti-fungal [5] effects, and increases insulin sensitivity [6]. The inhibitory effect of *O. humifusa* against LPS-induced inflammation has also been reported [7,8].

However, studies on the biological activities of *O. humifusa* focused mainly on the stems and fruits; the properties of its seeds have not been studied yet. Thus, in this study, the anti-inflammatory effects of *O. humifusa* seeds were examined and their active constituent was isolated using bioassay-guided fractionation. The anti-inflammatory mechanisms of the active constituents were also elucidated.

EXPERIMENTAL

Chemicals and reagents

Dulbecco's modified Eagle's medium (DMEM), and fetal bovine serum (FBS) were obtained from Welgen (Korea). Griess reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT), LPS, and dimethyl sulfoxide (DMSO) were purchased from Sigma Aldrich (CA, USA).

Preparation of the seed extract and isolation of isoamericanin A

The seeds of *O. humifusa* were collected after removing the flesh of the fruits. The seeds (3 kg) were pulverized and extracted with 80% methanol (MeOH). The MeOH filtrate was concentrated under vacuum, yielding 150 g of MeOH extract. The MeOH extract was suspended in distilled water and sequentially partitioned into *n*-hexane (2.2 g), chloroform (6.7 g), EtOAc (7.2 g), *n*-BuOH (8.0 g) and water (19.3 g). Each fraction was dissolved in DMSO for the bioassay. The chloroform fraction was subjected to open column chromatography and eluted with chloroform-MeOH (100:0 to 1:1) to give 12 sub-fractions (OCC1~OCC12). The sub-fraction OCC5 (0.2 g) was further purified by semi-preparative HPLC and eluted with acetonitrile (ACN)-H₂O (30 to 60% ACN gradient) to yield compound **1** (15.2 mg). Compound **1** was identified as isoamericanin A based on the NMR and MS data compared with previously published data [9].

Cell culture

RAW 264.7 cells obtained from Korea Cell Line Bank (Seoul, Korea) were grown in DMEM supplemented with 10% FBS under a humidified 5% CO₂ atmosphere at 37 °C. The cells were

treated with LPS (1 µg/mL) in the presence or absence of test samples for 24 h.

Evaluation of cell viability

RAW 264.7 cells plated on 96-well plates (5×10⁴ cells/well) were pre-treated with test samples for 1 h and incubated with LPS for an additional 24 h. Then, the cells were incubated with MTT (5 mg/mL) for 3 h followed by DMSO for 30 min after removing the media. The absorbance was measured at 570 nm (Molecular Devices, CA, USA). All measurements were done in triplicate and repeated at least three times.

Determination of NO production

The production of NO was determined using Griess reagent. The cells pre-treated with test samples were incubated with LPS for 24 h. Then, the supernatants were centrifuged at 12,000 rpm for 15 min and incubated with Griess reagent at 37 °C for 20 min in the dark. The absorbance was measured at 540 nm using a microplate reader.

Western blot analysis

Proteins (30 µg) in cell lysates were separated using denaturing polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred onto a PVDF membrane (Millipore, Billerica, MA, USA). The membranes blocked with 5 % nonfat dry milk were incubated with the following primary antibodies: anti-IκB (1:1000, Cell Signaling, Denver, MA, USA), anti-p-IκB (1:1000, Cell Signaling), anti-lamin A/C (1:1000, Cell Signaling), anti-iNOS (1:1000, Cell Signaling), anti-NF-κB p65 (1:1000, Santa Cruz, Santa Cruz, CA, USA), anti-NF-κB p50 (1:1000, Santa Cruz), and anti-α-tubulin (1:1000, Sigma Aldrich). Horseradish peroxidase-conjugated secondary antibodies (1:2000, Santa Cruz), and enhanced chemiluminescence (ECL) reagents (Advantag, CA, USA) were used for detection with the Bio-Rad Imager (Bio-Rad, CA, USA). Densitometric values were normalized with those of lamin A/C and α-tubulin as internal controls. The cytoplasmic and nuclear extracts were prepared with a Nuclear and Cytoplasmic Kit (Thermo Scientific, CA, USA) based on the manufacturer's instructions.

Enzyme-linked immunosorbent assay (ELISA)

The supernatants were collected from RAW cells after treatment with LPS for 24 h in the presence or absence of isoamericanin A (1, 2, or 4 µg/mL), and the production of cytokines was evaluated using ELISA [10] employing the following capture

antibodies: TNF- α and IL-6 (BD Biosciences, San Jose, CA, USA). The absorbance was measured at 405 nm using a microplate reader. Cytokines were quantified based on a standard curve.

Statistical analysis

All data are presented as mean \pm SD ($n = 3$). One-way analysis of variance (ANOVA) followed by Tukey's post-hoc test was employed for comparisons of two or more groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Inhibitory effect of *O. humifusa* seed extract on NO production

The possible cytotoxicity of the methanol (MeOH) extract of *O. humifusa* seeds was tested by MTT assay. As shown in the results, the MeOH extract of *O. humifusa* seeds did not decrease the viability of RAW cells at up to 20 $\mu\text{g/mL}$. However, 20 $\mu\text{g/mL}$ MeOH extract reduced LPS-induced NO production by 50% compared to that in the LPS-treated control groups (Figure 1). Thus, the MeOH extract was further partitioned by solvent polarity into four sub-fractions (*n*-hexane, chloroform, ethyl acetate and water). The sub-fractions were subjected to the MTT assay to evaluate possible cytotoxicity. Three sub-fractions (chloroform, ethyl acetate and water) did not alter the viability of RAW cells, whereas the *n*-hexane fraction significantly reduced the viability (Figure 1 A). Thus, the *n*-hexane fraction was excluded from further studies. Then, three sub-fractions (20 $\mu\text{g/mL}$) were evaluated for their effects on LPS-stimulated NO production in RAW cells. The chloroform fraction significantly reduced NO production compared to that of the control group (Figure 1 B), whereas the ethyl acetate fraction exhibited only a weak inhibitory effect. Thus, the chloroform fraction was studied further to identify the active components.

Inhibition of NO production by isoamericanin A

To obtain active constituents in the chloroform fraction of the *O. humifusa* seed extract, bioassay-guided isolation was performed using silica gel column chromatography and semi-preparative HPLC. Based on these results, isoamericanin A was isolated as an active constituent from the chloroform fraction (Figure 2 A). The possible cytotoxicity of isoamericanin A was determined by the MTT assay. At up to 4

$\mu\text{g/mL}$, isoamericanin A did not reduce the viability of RAW cells (Figure 2 B). Treatment of cells with isoamericanin A significantly attenuated LPS-induced NO production in RAW cells (Figure 2 B) in a concentration-dependent manner (1, 2, and 4 $\mu\text{g/mL}$). Notably, 4 $\mu\text{g/mL}$ isoamericanin A reduced NO production by 60% compared to that in the LPS-treated group.

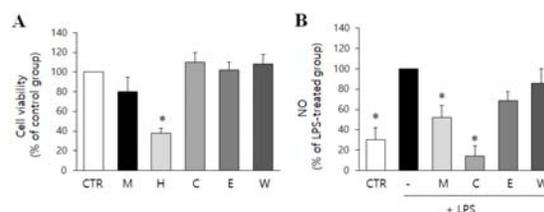


Figure 1: Effect of *O. humifusa* seed extract on the viability of RAW 264.7 cells and NO production. (A) The viability of RAW cells treated with *O. humifusa* seed extract and its four solvent-partitioned fractions (20 $\mu\text{g/mL}$) for 24 h was determined by MTT assay. Values are a percentage of the values for the DMSO-treated control groups. (B) NO production with *O. humifusa* seed methanol extract and its sub-fractions (20 $\mu\text{g/mL}$) of *O. humifusa* seeds was measured with Griess reagent (M: methanol, H: *n*-hexane, C: chloroform, E: ethyl acetate, W: water). Values are a percentage of the values for the LPS-treated groups. Data are presented as mean \pm SD of three different experiments. * $P < 0.05$, significantly different from LPS-treated groups, # $p < 0.05$, significantly different from DMSO-treated control groups

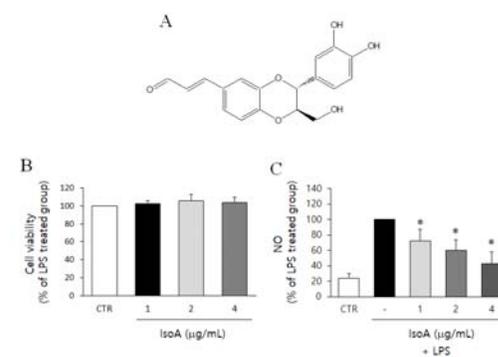


Figure 2: Effect of isoamericanin A (IsoA) on LPS-induced NO production in RAW cells. (A) The structure of isoamericanin A isolated from *O. humifusa* seeds based on bioassay-guided isolation. (B) The viability of RAW cells in the presence of isoamericanin A (1, 2, and 4 $\mu\text{g/mL}$) was determined by MTT assay. Values are expressed as a percentage of values for the DMSO-treated cells. (C) NO production with or without isoamericanin A (1, 2, and 4 $\mu\text{g/mL}$) in LPS-stimulated RAW cells was determined with Griess reagent. Values are a percentage of the values for the LPS-treated groups. Data are presented as mean \pm SD of three different experiments. * $P < 0.05$, significantly different from the LPS-treated groups

Inhibition of iNOS and pro-inflammatory cytokine expression by isoamericanin A

iNOS is an enzyme responsible for NO production in RAW cells. The possible change in iNOS expression was determined by western blot analysis. The cells treated with LPS significantly increased the expression of iNOS whereas pre-treatment of cells with isoamericanin A prior to LPS treatment reduced the expression of iNOS in a concentration-dependent manner (Figure 3 A and B) compared to that in the LPS-treated group. In particular, 4 $\mu\text{g}/\text{mL}$ of isoamericanin A decreased iNOS levels to close to the control-group level. This result is consistent with the inhibitory effects of isoamericanin A on NO production as described above.

In addition, the secretion of pro-inflammatory cytokines TNF- α and IL-6 by RAW cells was determined by ELISA. As shown in Figure 3 C and D, LPS treatment of RAW cells significantly increased the concentration of TNF- α and IL-6 in the supernatant whereas pre-treatment with isoamericanin A prior to LPS treatment significantly reduced the levels of cytokines in a concentration-dependent manner.

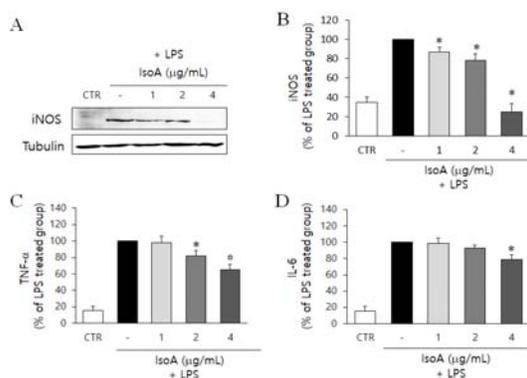


Figure 3: Effect of isoamericanin A (IsoA) on the expression of iNOS and pro-inflammatory cytokines in RAW cells. (A, B) The changes in iNOS expression in the presence of isoamericanin A (1, 2, and 4 $\mu\text{g}/\text{mL}$) were determined by western blot analysis. Graph showing the protein levels of iNOS. (C, D) The changes in TNF- α and IL-6 pro-inflammatory cytokines in the presence of isoamericanin A were determined by ELISA. Values are a percentage of the values for the LPS-treated groups, presented as mean \pm SD of three different experiments. * $p < 0.05$, significantly different from the LPS-treated groups

Inhibition of NF- κ B activation by isoamericanin A

Western blot experiments were performed using specific antibodies to elucidate the effect of isoamericanin A on LPS-induced NF- κ B

activation. As shown in Figure 4A, treatment of cells with LPS significantly increased the amount of NF- κ B (p65 and p50) in the nucleus, indicating that LPS increased the translocation of NF- κ B (p65 and p50) from the cytosol into the nucleus, whereas phosphorylated I κ B (p-I κ B) in the cytosol was significantly increased. However, the pre-treatment of cells with isoamericanin A prior to LPS treatment reversed the effects of LPS on NF- κ B translocation and I κ B phosphorylation. Isoamericanin A decreased the translocation of NF- κ B p65 and p50 and reduced the amount of NF- κ B p65 and p50 in the nucleus compared to LPS-treated control group. The treatment of cells with 4 $\mu\text{g}/\text{mL}$ isoamericanin A decreased the levels of NF- κ B in the nucleus to close to that of the DMSO-treated control groups. In addition, isoamericanin A inhibited the phosphorylation of I κ B located in the cytosol and decreased the amount of p-I κ B in a concentration-dependent manner.

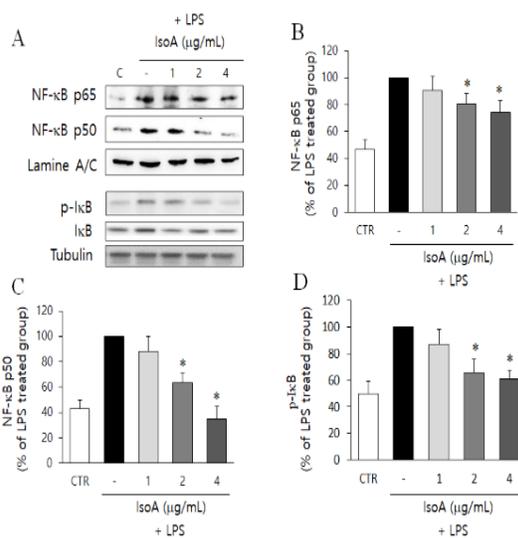


Figure 4: Effect of isoamericanin A (IsoA) on NF- κ B activation in RAW cells. (A) The changes in NF- κ B p65 and p50 in the nucleus, and in p-I κ B and I κ B in the cytosol were determined by western blot analysis. (B, C, D) Graphs showing the protein levels of NF- κ B p65 and p50, p-I κ B, and I κ B. Values are a percentage of the value for the LPS-treated groups. Data are presented as mean \pm SD of three different experiments. * $P < 0.05$, significantly different from the LPS-treated groups

DISCUSSION

The stems and fruits of *O. humifusa* have been widely used as food and traditional medicines due to their beneficial effects on diabetes, arthritis, and inflammation [1]; the related biological activities have been reported. For example, the MeOH extract of *O. humifusa*

stems exhibit anti-nociceptive and anti-inflammatory effects [8]. The stems of *O. humifusa* significantly reduce serum total-cholesterol and blood-sugar levels in streptozotocin-induced diabetic rats [11]. The fruit of *O. humifusa* reduces the morphological changes observed in asthma, and suppresses UVB radiation-induced skin degeneration and non-melanoma skin carcinogenesis due to their anti-inflammatory effects [12,13]. The beneficial effects of *O. humifusa* on inflammation are accompanied by high antioxidant effects due to high levels of vitamin C, polyphenols, and flavonoids present [14]. High levels of antioxidants in *O. humifusa* reduce the production of reactive oxygen species, decrease oxidative damage, and eventually alleviate oxidative stress in inflammation [15].

Conversely, reports regarding the biological activities of *O. humifusa* seeds are very limited. For example, the seed extract of *O. humifusa* alleviates osteoporosis [16] and decreases serum cholesterol and triglyceride contents [17] in ovariectomized rats because of the high polyphenol content in the seeds. Reports on the biological activities of *O. humifusa* are mostly limited to the plant's stems and fruits. Here, we report the anti-inflammatory effects of the seeds of *O. humifusa* and the isolation of the active constituent, isoamericanin A.

Isoamericanin A was first isolated from *Phytolacca americana* L. in 1987 [18]. Additionally, isoamericanin A was isolated from Brazilian *Joannesia princeps* [9] and barley tea [19]. Initially, isoamericanin A was identified as an inducer of prostaglandin I₂ [18] or as an antioxidant constituent [19]. It was also reported to enhance choline acetyltransferase activity [20]. Other than the above, very few biological activities of isoamericanin A have been reported.

To the best of our knowledge, this is the first report regarding the potent anti-inflammatory effects of isoamericanin A against LPS-stimulated RAW cells. Isoamericanin A effectively lowered the production of NO and pro-inflammatory cytokines, that was accompanied by the inhibition of NF- κ B translocation. The NF- κ B pathway plays crucial roles in human inflammatory diseases including rheumatoid arthritis, atherosclerosis, asthma, and inflammatory bowel disease [21-23]. The activation of NF- κ B at inflammation sites increases the transcription of pro-inflammatory cytokines, chemokines, COX-2, and iNOS [24]. Therefore, NF- κ B is a potential therapeutic target for inflammatory diseases.

CONCLUSION

The extract of *O. humifusa* seeds significantly reduces NO production in LPS-stimulated RAW cells, and isoamericanin A was identified as its active constituent. Isoamericanin A effectively lowers the production of NO as well as the expression of iNOS and pro-inflammatory cytokines. These effects are accompanied by decreased translocation of NF- κ B from the cytosol to the nucleus and decreased phosphorylation of I κ B in the cytosol induced by LPS. Thus, the seeds of *O. humifusa* and isoamericanin A have potential as anti-inflammatory agents.

DECLARATIONS

Acknowledgement

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Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors. Ji Yun Yeo performed the experimental work. So-Young Park (SYP) and Kwang Woo Hwang designed the study and discussed the data. SYP analyzed the data and supervised the experimental work. All authors read and approved the final manuscript.

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