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Original Research Article

DNA Damage Protection and Anti-inflammatory Activity of Different Solvent Fractions from *Aruncus dioicus* var. *kamtschaticus*

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Abstract - This study investigated DNA damage protection and anti-inflammatory activity of different solvent fractions from *Aruncus dioicus* var. *kamtschaticus* (*A. dioicus*) aerial parts water extract. As for DNA damage protection, distilled water (H_2O) fraction displayed the most powerful protection for DNA damage at a concentration of 1 ㎎/㎖. As for anti-inflammatory activity, dichloromethane (CH_2Cl_2) fraction exhibited the highest NO inhibition activity, ranging from 61% to 19% (10-40 ㎍/㎖). Furthermore, the levels of pro-inflammatory cytokines mRNA expressions and intracellular reactive oxygen species (ROS) were employed to verify the anti-inflammatory activity of the CH_2Cl_2 fraction on further researches. It could be concluded that *A. dioicus* had a significantly effect of DNA damage protection and anti-inflammatory activity which also as an essential edible vegetable and medicinal species.

Key words - *Aruncus dioicus* var. *kamtschaticus*, DNA damage, Reactive oxygen species, Anti-inflammation

Introduction

Free radicals or, more generally, ROS and reactive nitrogen species (RNS) are regarded as two products of normal cellular metabolism, which play dual roles in human health development (Valko *et al*., 2006). When ROS and RNS exist in a moderate concentration, they have beneficial physiological effects on cellular responses to noxia, such as resisting infectious agents, regulating cellular signaling systems and inducting mitogenic responses (Jayakumar *et al*., 2009; Valko *et al*., 2007). In contrast, overproduction of ROS and RNS involve deleterious roles in cell structure including proteins, DNA and lipids, resulting in a variety of diseases such as inflammation, cancer, gastric ulcers, cardiovascular disease and diabetes mellitus (Li *et al*., 2010). Lipopolysaccharide (LPS) is a primary constituent of the outer cell walls of Gram-negative bacteria which could activate an exaggerated inflammatory response (Park *et al*., 2014). Macrophages play a key pivotal in inflammatory response linking with excessive production of pro-inflammatory cytokines as for an example of interleukin-1β (IL-1β), and inflammatory mediators as for instance of ROS and NO, resulted from activated inducible nitrite oxide synthase (iNOS) and cyclooxygenase-2 (COX-2) (Heo *et al*., 2010). Thus, anti-inflammatory activity can be examined by measuring the suppression activities of pro-inflammatory cytokines iNOS, COX-2 and IL-1β mRNA expressions and the inhibition levels of inflammatory mediators ROS and NO.

Aruncus dioicus var. *kamtschaticus* is widely grown in mountainous areas in Korea and is known as an important kind of edible vegetable and medicinal species, which belongs to the Rosaceae family. *A. dioicus* has been employed in traditional medicine system to treating cancer, obesity, diabetes mellitus, tonsillitis, brain injury and skin aging (Ahn *et al*., 2014; Han and Lee, 2011; Jeong *et al*., 2011; Kim *et al*., 2012). Although the published works have reported foresaid pharmacological activities, in the scope of my knowledge, there is little information involved in its anti-inflammatory activity. Furthermore, *A. dioicus* is edible, inexpensive and easily available. The focus of study shifted to its inflammation become more valuable. In this regard, the goal of this study was to investigate DNA damage protection and anti-inflammatory activity of different fractions from the *A. dioicus* water extract.

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Materials and Methods

Samples preparation

A. dioicus plant was obtained in spring from JeongSun, Korea. The voucher specimen (ADK 2013) was deposited in the lab of molecular of plant biotechnology. The aerial parts were dried in the shade and macerated with distilled water at room temperature for 72 h. The water extract was got by concentrating the filtrates with a rotary evaporator (CCA-110, Eyela, Tokyo, Japan). The water extract (30 g) was dissolved into distilled water and fractionated with dichloromethane, ethyl acetate, *n*-butanol and distilled water to get dichloromethane (CH_2Cl_2) (0.15 g), ethyl acetate (EtOAc) (1.28 g), *n*-butanol (*n*-BuOH) (5.03 g), and distilled water (H_2O) (21.45 g) fractions, respectively.

Chemicals and reagents

1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT), 7-dichlorofluorescein-diacetate (DCFH-DA), dimethyl sulfoxide (DMSO), ethidium bromide (EB), lipopolysaccharide (LPS) (E. *coli* 0111:B4) and phosphate-buffered saline (PBS) were purchased from Sigma (St, Louis, MO, USA). RPMI medium 1640 and fetal bovine serum (FBS) were acquired from Gibco BRL (Grand Island, NY, USA). All culture supplies were obtained from BD-Falcon (BD, Franklin Lakes, NJ). All other chemicals were of analytical grade.

Cell line and cell culture

Murine macrophage cell RAW 264.7 lines were obtained from the Korean Cell Line Bank (Seoul, Korea). The RAW 264.7 cells were cultured in RPMI medium 1640, supplemented with 10% FBS and 100 U/㎖ of penicillin. The cells were incubated at 37℃ in a humidified atmosphere, 5% CO_2.

Genomic DNA isolation

The genomic DNA was isolated from RAW 264.7 cells. The cells were harvested by incubating for three days and washed with PBS. The collected cells were lysed in extraction buffer (1 M Tris-HCl, 0.5 M EDTA, 5 M Sodium chloride and 20% SDS), and then incubated at 64℃ for 10 min. The DNA was centrifuged at 13,000 rpm for 15 min after isolated three times with phenol:chloroform:isoamylalcohol (25:24:1) solution. Finally, the DNA was washed by 70% ethanol and dissolved in distilled water.

Assay of DNA damage protection

Four ㎕ of genomic DNA was added into 3 ㎕ of 50 mM phosphate buffer (pH 7.4). The mixture solution was incubated at 37℃ for 30 min after addition of 3 ㎕ of 1 mM ferrous sulphate, 10 ㎕ of 1 ㎎/㎖ sample and 4 ㎕ of 0.1 mM hydrogen peroxide. The DNA was analyzed using 1% agarose gels containing 1 ㎕ of EB. The band was observed by a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd, Kiryat Anavim, Israel). The protective effect of DNA damage was measured by comparing with the genomic DNA control.

Assay of cell viability

The cytotoxicity of sample on RAW 264.7 cells was evaluated with a conventional MTT assay. The cells were plated at a 96-well plate (1×10^5 cells/well) for 16 h. Sample (0, 10, 20, 40 ㎍/㎖) was added into medium for 24 h. After discarding all medium, 10 ㎕ of MTT solution (5 ㎎/㎖ in PBS) and 90 ㎕ of non-FBS medium were mixed sufficiently and added into each well. The mixture was incubated for 4 h. After removing all the supernatant, 200 ㎕ of DMSO was added into each well for solubilization. The absorbance was measured at 550 ㎚ by using an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek, Winooski, VT, USA).

Determination of NO production

The level of NO production about cell culture supernatant was based on Griess reaction. The RAW 264.7 cells were seeded in 96-well plates (1×10^5 cells/well) for 16 h. The cells were pre-treated with sample (0, 10, 20, 40 ㎍/㎖) and stimulated with LPS (1 ㎍/㎖) for 24 h. Subsequently, 100 ㎕ of each supernatant was removed into a new 96-well plated. 100 ㎕ of Griess reagent (1% sulfanilamide diluted in 5% phosphoric acid, 50 ㎕; 0.1% naphthyl-ethylenediamine dihydrochloride diluted in distilled water, 50 ㎕) was added. Rest for 10 min, the absorbance was measured at 550 ㎚ with an ELISA.

Total RNA extraction and RT-PCR

The total RNA was isolated from the LPS-stimulated RAW 264.7 cells in the pre-treatment of sample (0, 10, 20, 40 ㎍/㎖)

Table 1. PCR primers used in the experiment (F: forward, R: reverse)

Genes		Primer
iNOS[z]	F	5'-CCCTTCCGAAGTTTCTGGCAGCAG-3'
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
COX-2[y]	F	5'-CACTACATCCTGACCCACTT-3'
	R	5'-ATGCTCCTGCTTGAGTATGT-3'
IL-1β[x]	F	5'-TGGACGGACCCCAAAAGATG-3'
	R	5'-AGAAGGTGCTCATGTCCTCA-3'
GAPDH	F	5'-CACTCACGGCAAATTCAACGGCA-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'

[z]iNOS: inducible nitrite oxide synthase.
[y]COX-2: cyclooxygenase-2.
[x]IL-1β: interleukin-1β.

Fig. 1. Agarose gel electrophoretic patterns of the DNA damage induced by hydroxyl radicals in the treatment of different fractions from *A. dioicus* water extract. Line 1, DNA incubated without Fenton's reagent; Line 2, DNA incubated with Fenton's reagent; Line 3, 4, 5 and 6, DNA incubated with Fenton's reagent in the presence of 1 ㎎/㎖ CH_2Cl_2 fraction, EtOAc fraction, *n*-BuOH fraction, H_2O fraction.

by using Trizol reagent (Invitrogen, Carlsbad, CA, USA). cDNA was obtained from the reverse-transcribed RNA with oligo (dT) and Moloney murine leukemia virus reverse transcriptase (M-MLV RT, Invitrogen). The primer sequences for iNOS, COX-2, IL-1β and GAPDH were listed in Table 1. The isopyknic of the individual PCR products were analyzed by electrophoresis using 1% agarose gels containing 1 ㎕ of EB. The band was observed by a Mini BIS image analysis system (DNR Bio-Imaging Systems Ltd, Kiryat Anavim, Israel).

Determination of intracellular ROS

Intracellular ROS induced by LPS was evaluated using fluorescent DCFH-DA probe. RAW 264.7 cells were plated at a 6-well plate (1×10^6 cells/well) for 16 h. After removing medium, the cells were cultivated by sample (0, 10, 20, 40 ㎍/㎖) and stimulated with LPS (1 ㎍/㎖) for 24 h. After discarding all of the culture, 1 ㎖ of DCFH-DA solution (20 ㎛) was added into each well, and then incubated at 37℃ for 30 min. The DCFH-DA solution of each well was removed and 0.5 ㎖ of PBS was added. The level of intracellular ROS was evaluated by flow cytometry (Becton-Dichinson, Franklin Lakes, NJ, USA) at excitation and emission wavelengths of 485 ㎚ and 530 ㎚, respectively.

Statistical analyses

Each test was carried out in triplicate (n = 3). The results were expressed as mean ± standard deviation (SD). One-way analysis of variance with Duncan's multiple-range test was used to analyze the significant differences between the groups ($p < 0.05$ was considered significant). All analyses were analyzed using SPSS 21 (SPSS Institute, Cary, NC, USA).

Results and Discussion

DNA damage protection

The normal life cycle of the cell and the stability of the genome are closely related to DNA, which has influenced on repair pathways, cell cycle regulation and cell death through a variety of mechanisms (Packer *et al*., 1999). Oxidative DNA damage by ROS influences replication and transcription, and finally causes cell death, which has initiated various degenerative diseases, for instance, cancer, cardiovascular disease and diabetes mellitus (Rowe *et al*., 2008). It has been generally recognized that hydroxyl radicals are capable of causing strand breaks in isolated DNA (Rhim, 2013). The DNA damage stimulated by hydroxyl radicals in the treatment of the four fractions from *A. dioicus* was shown in Fig. 1. Line 1 and Line 2 was the genomic DNA incubated without or with Fenton's reagent as the control. On the contrary, line 3, line 4 and line 5 were absolutely damaged by hydroxyl radicals. Line 6 exhibited protection for DNA damage. According to the result, except the H_2O fraction, none of the other fractions prevented the DNA from damage, which manifested that the components of protecting DNA from damage may be more likely to dissolve in water.

Cell viability and NO production

The cytotoxicity of the four fractions from *A. dioicus* on RAW 264.7 cells was shown in Fig. 2A. The four fractions at the test concentrations exhibited little toxicity to cell viability.

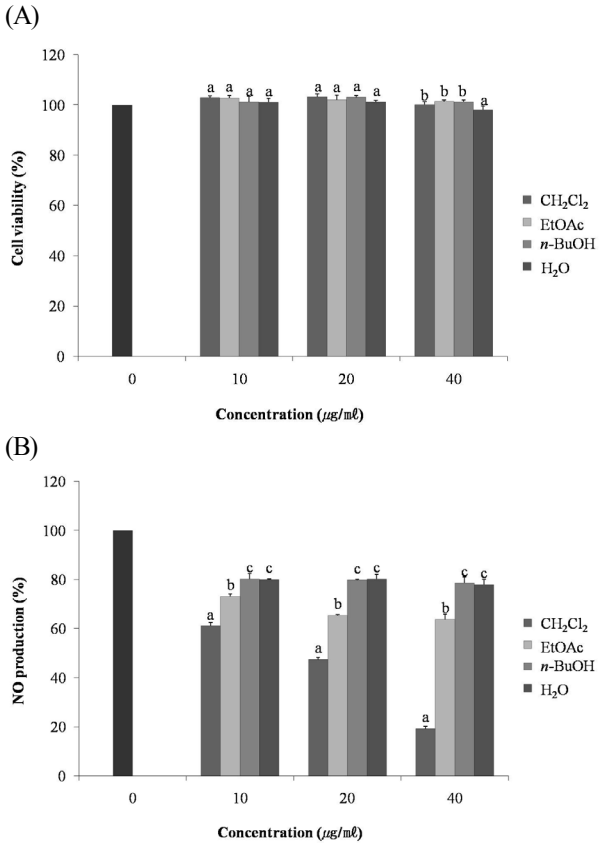

Fig. 2. Cytotoxicity (A) and NO inhibitory ability (B) on LPS-stimulated RAW 264.7 cells of different fractions from *A. dioicus* water extract. Each value is expressed as the mean ± SD (n = 3). Means not sharing the same letter are significantly different at $P < 0.05$ probability level at same concentration by Duncan's multiple-range test.

Inflammation is recognized to implicate for many pathological conditions, such as diabetes, cancer and cardiovascular disease (Jiang *et al*., 2013). In order to investigate the anti-inflammatory effect, NO is regarded as a primary regulator and mediator of inflammatory responses (MacMicking *et al*., 1997). The NO inhibitory ability of the four fractions from *A. dioicus* was evaluated using the LPS-induced RAW 264.7 cells. As shown in Fig. 2B, the cells stimulated by LPS generated excessively high-level of NO. However, the high-level of NO production was suppressed in a dose-dependent trend as the cells were pretreated with different concentrations of the four fractions. Especially, the CH_2Cl_2 fraction exhibited the highest NO inhibition activity, ranging from 61% to 19% (10-40 ㎍/㎖), which reflected unusually outstanding NO inhibition ability.

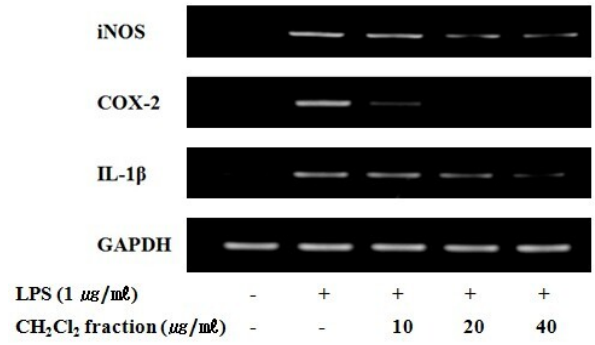

Fig. 3. Effect of CH_2Cl_2 fraction from *A. dioicus* water extract on iNOS, COX-2, and IL-1β expression in the LPS-stimulated RAW 264.7 cells. RAW 264.7 cells were treated with 1 ㎍/㎖ of LPS and different concentrations of CH_2Cl_2 fraction from *A. dioicus* water extract for 24 h. The levels of iNOS, COX-2, IL-1β and GAPDH mRNA expression were determined by RT-PCR.

RT-PCR of inflammatory genes

RT-PCR was employed to determine whether the above NO inhibition ability of CH_2Cl_2 fraction was correlated with the modulation of specific mRNA expression. During the process of anti-inflammation, massive amounts of the pro-inflammatory mediators such as NO are produced by the inducible isoforms of pro-inflammatory cytokines (Kim *et al*., 2007; Nam *et al*., 2013). Housekeeping gene (GAPDH) was essential for basis cell survival and was used to normalize gene expression. Furthermore, we investigated the mRNA expression level of pro-inflammatory cytokines including iNOS, COX-2 and IL-1β. As shown in Fig. 3, in the LPS-unstimulated RAW 264.7 cells, the expression of iNOS, COX-2 and IL-1β mRNA were not detected; however, the expression was markedly up regulated after the pre-treatment with LPS. Meanwhile, the LPS-stimulated expression was considerably suppressed in a concentration-dependent manner of the CH_2Cl_2 fraction. According to the result, it could be concluded that the CH_2Cl_2 fraction had the suppressive activity on iNOS, COX-2 and IL-1β at the transcriptional levels.

Intracellular ROS activity

Inflammatory cells are specifically adept in producing and releasing ROS, which can cause the situation of oxidative stress and is potentially related to a range of diseases such as inflammation, cancer, gastric ulcers (Ramos *et al*., 1992).

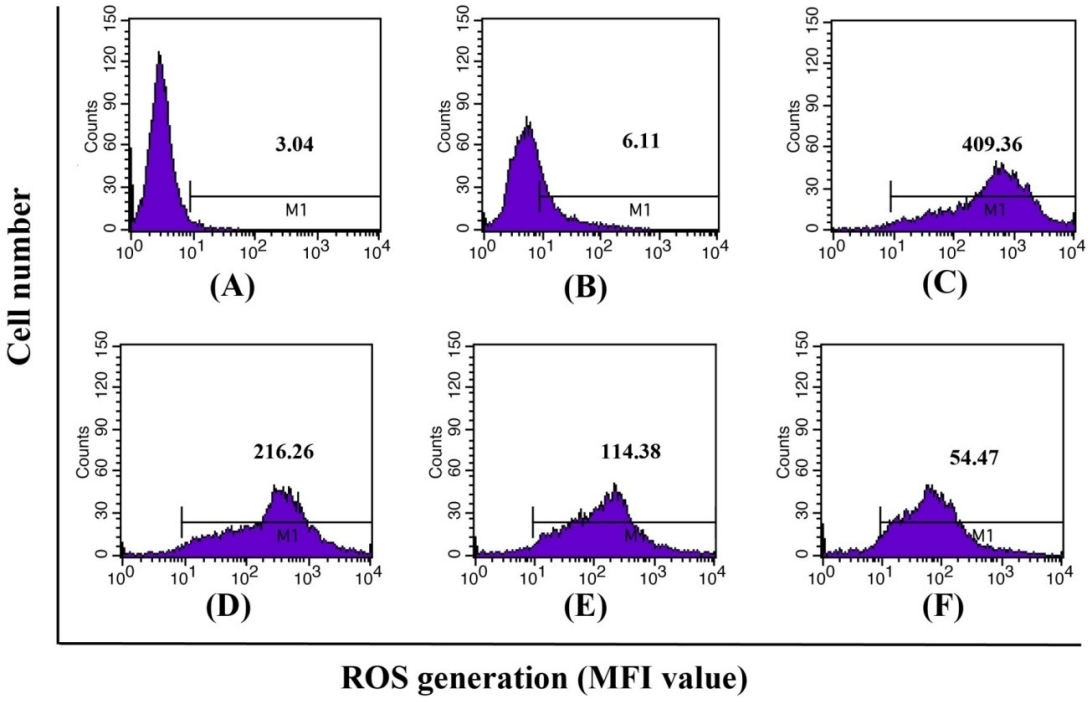

Fig. 4. Flow cytometric analysis of LPS-stimulated ROS production in RAW 264.7 cells. (A) cells treated without LPS and DCFH-DA; (B) cells treated without LPS; (C) cells treated with LPS; (D) (E) (F) cells treated with LPS and different concentrations of CH_2Cl_2 fraction, 10 ㎍/㎖, 20 ㎍/㎖, 40 ㎍/㎖.

Non-fluorescent compound DCFH-DA can freely permeate through the cell membrane and supply DCFH as the intracellular esterases in cells. Since the exposure of intracellular ROS, DCFH is easily oxidized to the fluorescent product DCF (LeBel *et al*., 1992). Therefore, the fluorescence of DHF can be monitored to quantify the overall reactive oxygen in living cells. LPS was used to generate oxidant stress in RAW 264.7 cells. As shown in Fig. 4, the intracellular ROS accumulation in presence of LPS was increased remarkably contrasted with untreated cells. Pretreatment with the CH_2Cl_2 fraction blocked LPS-stimulated intracellular ROS generation in a concentration-dependent trend. This result suggested that CH_2Cl_2 fraction prevented the oxidative stress in cells and therefore protected cells against oxidative damage.

In conclusion, this study demonstrated that DNA damage protection and anti-inflammatory activity of different fractions from *A. dioicus* aerial parts water extract. In the DNA damage protection assay, the H_2O fraction displayed the most powerful protection for DNA damage. In a series of anti-inflammatory assays, the CH_2Cl_2 fraction exhibited significantly anti-inflammatory activity. Moreover, the active ingredients of DNA damage protection and anti-inflammatory activity are needed to do further researches. Additional research can currently underway to identify the active ingredients for DNA damage protection and anti-inflammatory activity in this plant.

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