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Anti-inflammatory and anticancer effects of methanol, ethanol and water extracts of *Asiasarum heterotropoide.*

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Abstract

The radix of *Asiasarum heterotropoides var. mandshuricum* F. Maekawa (*A. radix*) is called *seshin* in Korean, *saishin* in Japanese, *xì xīn* in Chinese and Chinese wild ginger in English and is widely used to treat various diseases. The aim of this study was to determine the dose-dependent anti-inflammatory and anticancer effects using methanol, ethanol and water extracts of *A. radix*.

Methanol, ethanol and water extracts of *Asiasarum heterotropoides* were obtained. A mouse leukaemic monocyte macrophage cell line (RAW 264.7) was exposed to *A. radix* at final concentrations that ranged from 25 to 200 µg/ml. The production of nitric oxide (NO) in lipopolysaccharide-induced RAW 264.7 cells was quantified. The inducible nitric oxide synthase (iNOS), interleukin-1β (IL-1β), interleukin-6 (IL-6) and cycloxygenase-2 (COX-2) mRNA expressions were evaluated using lipopolysaccharide (LPS)-stimulated RAW 264.7 cells with semi-quantitative reverse transcription-polymerase chain reaction. Human lung carcinoma cell lines (A549 cells) were treated with *A. radix* and superoxide dismutase-2 (SOD-2), and caspase-3 expression was tested.

Higher cytotoxicity was noted after treatment with methanol, ethanol and water extracts of *A. radix*. Stimulation with LPS for 24 h led to a robust increase in NO production, but *A. radix* significantly suppressed NO by the LPS-stimulated RAW 264.7 cells in all groups. Pretreatment with absolute ethanol extract of *A. radix* suppressed the LPS-stimulated iNOS, IL-1β, IL-6, and COX-2 expressions. The results showed that *A. radix* extract showed significantly higher cytotoxicity when compared with the untreated control. The expression of SOD-2 and caspase-3 increased with the increase of exposure time to *A. radix* extracts.

Within the limits of this study, *A. radix* showed an anti-inflammatory effect using RAW 264.7 cell lines and anticancer effects on A549 cells. These effects were influenced by extraction methods. Absolute ethanol extract showed the highest anti-inflammatory and anticancer effects in these experimental settings.

Keywords: Anti-inflammatory agents, Antineoplastic agents, Herbal medicine, Plant roots.

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Introduction

Medicinal herbs used in traditional oriental medicine are attractive sources for developing novel therapeutics or preventives since they have been used for thousands of years in clinics [1]. The radix of *Asiasarum heterotropoides var. mandshuricum* F. Maekawa (*A. radix*) is called *seshin* in Korean, *saishin* in Japanese, *xì xīn* in Chinese and Chinese wild ginger in English and is widely used to treat various diseases [2]. *A. radix* has been used for its anti-inflammatory, antiallergy, antibacterial, and anticancer effects [1, 3-5].

Inflammatory responses are recognized as natural defense mechanisms critical for the recruitment of a variety of immune cells and molecules to sites with infectious microbes or injured tissues [6]. Inflammation is a complex process characterized by the contributions of mediators,

including nitric oxide (NO) and free radicals [7]. Blocking the expression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) can restrain the production of high-output NO, and inhibiting COX-2 and iNOS expressions have been used as functional criteria for developing anti-inflammatory agents [6, 7].

In cancer, mutations in progenitor cells most likely undergo uncontrolled proliferation [8]. Apoptosis is important for controlling cell numbers and proliferation, but cancer cells do not undergo apoptosis [9]. Thus, drugs and agents that can restore the normal apoptotic pathway may have potential for treating cancers.

The aim of this study was to determine the dose-dependent anti-inflammatory and anticancer effects using methanol, ethanol and water extracts of *A. radix*. To our knowledge, this investigation is the first to elucidate the comparative effects of methanol, ethanol and water extracts of *A. radix* on mouse leukaemic monocyte macrophage cell lines (RAW 264.7) and human lung carcinoma cell lines (A549 cells).

Materials and Methods

Chemicals and Reagents

All chemicals and reagents used in this study were purchased from Sigma (St. Louis, MO, USA) unless otherwise specified.

Plant Material and Preparation of the Extracts

The dry roots of *Asiasarum heterotropoides* were obtained from Chungju Hospital of Korean Medicine (Jecheon, Korea). The roots of *A. radix* were chopped to a small size of 0.5 cm long, dried in the shade and powdered in a mechanical grinder. The pulverized roots were extracted for absolute ethanol, 70% ethanol, absolute methanol, 70% methanol, water and boiling water for 3 hours, and finally the extractions were dried under a vacuum rotary evaporator (CCA-1110, Eyela, Tokyo, Japan). Twenty grams of dry roots in each group were used, and 1.544, 3.704, 1.575, 3.852, 4.105, and 5.181 g were obtained for the absolute ethanol extract, 70% ethanol extract, absolute methanol extract, 70% methanol extract, water extract, and boiling water extract, respectively. The yields were 7.72, 18.52, 7.88, 19.26, 20.53, and 25.91% (w/w), respectively.

Anti-inflammatory Assay

Cell line and cell culture

A RAW 264.7 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). RAW 264.7 cells were maintained in RPMI 1640 medium supplemented with 10% FBS, 100 U/ml of penicillin, and 100 μg/ml of streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cytotoxicity assay

The cytotoxicity of samples of RAW 264.7 cells was tested. Cells were seeded into 96-well plates at a density of 1×10^5 cells/well. After incubation for 18 h, cells were exposed to medium along with samples at different concentrations for 24 h. The supernatant was removed from each well, and 10 μl of MTT solution (5 mg/ml in phosphate-buffered saline) and 90 μl of FBS-free medium were added to each well and incubated for 4 h at 37°C. Then, the supernatant was sucked out and 200 μl of DMSO were added to each well. The plate was vibrated slightly for 10 min, and the amount of MTT formazan was quantified by measuring the absorbance at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (ELx800TM, Bio-Tek, Winooski, VT, USA). The optical density of formazan formed in the control cells was considered 100% viability. Cell viability was expressed as a percentage of the control culture value.

Quantification of NO production in lipopolysaccharide (LPS)-induced RAW 264.7 cells

The inhibitory effect of NO production in LPS-induced RAW 264.7 cells was determined according to the method of Jiang *et al*. [10]. RAW 264.7 cells were plated in 96-well cell plates and incubated for 18 h. Then, cells were stimulated with LPS (2 μg/ml) in the presence or absence of samples with various concentrations for 24 h. Aliquots of 100 μl of cell culture medium were mixed with 100 μl of Griess reagent [0.1% aqueous solution of naphthyl-ethylenediamine dihydrochloride, 50 μl; 1% sulfanilamide (in 5% phosphoric acid), 50 μl]. The absorbance was determined at 550 nm using an ELISA plate reader (ELx800TM).

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RAW 264.7 cells (1×10^6) were grown in 6-well plates for 18 h. Then, cells were treated with various concentrations of samples for 30 min, and LPS (2 μg/ml) was added. After incubation for 24 h, the total RNA of the cells was isolated with a Trizol RNA isolation kit (Invitrogen, Carlsbad, CA, USA). The total RNA was reverse-transcribed to cDNA and used as the template for PCR amplification. The iNOS, IL-1β, and COX-2 primers (Table 1) were used

Genes		Primer
GAPDH	F	5'-CACTCACGGCAAATTCAACGGCA-3'
	R	5'-GACTCCACGACATACTCAGCAC-3'
iNOS	F	5'-CCCTTCCGAAGTTTCTGGCAGCAG-3'
	R	5'-GGCTGTCAGAGCCTCGTGGCTTTGG-3'
IL-1β	F	5'-TGGACGGACCCCAAAAGATG-3'
	R	5'-AGAAGGTGCTCATGTCCTCA-3'
IL-6	F	5'-GTTCTCTGGGAAATCGTGGA-3'
	R	5'-TGTACTCCAGGTAGCTATGG-3'
COX-2	F	5'-CACTACATCCTGACCCACTT-3'
	R	5'-ATGCTCCTGCTTGAGTATGT-3'

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

in the PCR. The amplified PCR products were separated on 1% agarose gel, and the gel was stained with ethidium bromide. The gel was photographed with a Mini BIS Image Analysis System (DNR Bio-Imaging Systems Ltd., Jerusalem, Israel).

Anticancer Assay

Cell line and cell culture

A lung cancer A549 cell line was purchased from the Korean Cell Line Bank (Seoul, Korea). Cells were maintained in DMEM medium supplemented with 10% FBS, 100 U/ml of penicillin, and 100 µg/ml of streptomycin. The cells were incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2.

Cytotoxicity assay

The cytotoxicity of samples on A549 cells was detected by MTT assay. Cells were seeded into 96-well plates and incubated with samples for 24, 48 or 72 h. Then, the supernatant was removed and 100 µl of MTT solution were added to each well and incubated for 4 h at 37°C. The supernatant was sucked out, and 200 µl of DMSO were added to each well. The amount of MTT formazan was quantified by measuring absorbance at 550 nm.

RT-PCR analysis

A549 cells (1×10^6) were grown in 6-well plates for 24 h. Then, cells were treated with samples for different amounts of time (0, 3, 6, 12, 24 and 48 h). The total RNA of the cells was isolated with a Trizol RNA isolation kit (Invitrogen, Carlsbad, CA, USA). The total RNA was reverse-transcribed to cDNA and used as the template for PCR amplification. The forward and reverse primers were as follows: 5'-TGTTACCAACTGGGACGACA-3' and 5'-CTCTCAGCTGTGGTGGTGAA-3' for β-actin; 5'-TGGTGGAGAACCCAAAGG-3' and 5'-GTCAAAGGAACCAAAGTCACG-3'for superoxide dismutase-2 (SOD-2); and 5'AGTGGAGGCCGACTTCTTGT-3' and 5'-CTGTTGCCACCTTTCGGTTA-3' for caspase-3. The amplified PCR products obtained by PCR were separated on 1% agarose gel electrophoresis, and the gel was stained with ethidium bromide. The gel was photographed with a Mini BIS Image Analysis System (DNR Bio-Imaging Systems Ltd.).

Statistical Analysis

The data are represented as means ± standard deviations of the experiments. A one-way analysis of variance (ANOVA) with a post hoc test was performed to determine the differences between the groups using a commercially available program (SPSS 12 for Windows, SPSS Inc., Chicago,mIL, USA). The level of significance was 0.05.

Results

Anti-inflammatory Assay

Cytotoxicity assay: The effects of *A. radix* on RAW 264.7 cells are presented in Figure 1. The results showed that the 70% ethanol extract and the boiling water extract did not exhibit any statistically significant toxicity on RAW 264.7 cells ($P>0.05$). The absolute ethanol extract at concentrations of 25, 50, 100, and 200 µg/ml reduced cell viability to 90.3 ± 1.3%, 80.3 ± 1.4%, 73.8 ± 0.9%, and 71.8 ± 0.2 %, respectively, when the control was considered 100% (100.0 ± 0.8 %) ($P<0.05$).

Quantification of NO production in LPS-induced RAW 264.7 cells: Stimulation with LPS for 24 h led to a robust increase in NO production. However, *A. radix* significantly suppressed NO in the LPS-stimulated RAW 264.7 cells in all groups (70% ethanol, ethanol, 70% methanol, methanol,

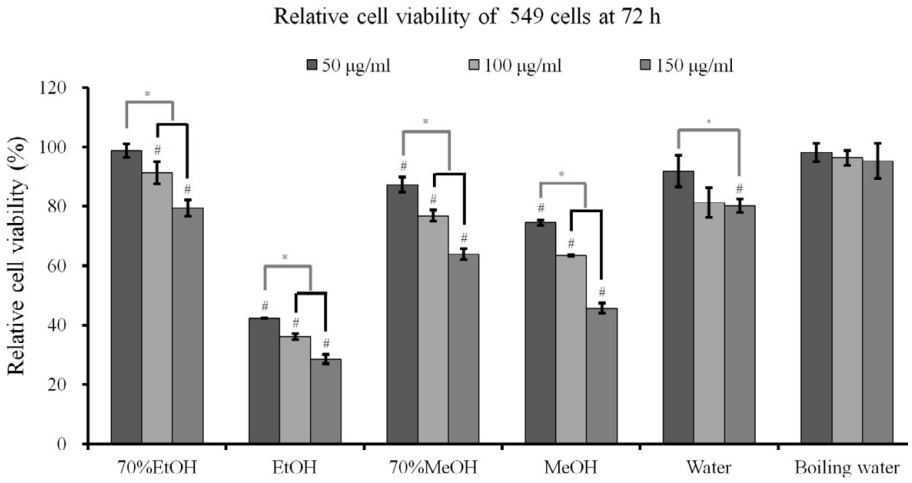

Figure 1. *Cell viability of RAW 264.7 cells after incubation in the presence of different extracts from A. radix for 24 h. Each value is expressed as the mean ± SD (n=3)*

[#] A statistically significant difference was seen when compared with the control (non-treated group) at 24 h ($P<0.05$)

[*] There was a statistically significant difference when compared with the 25 µg/ml group for each extraction method

EtOH: ethanol

MeOH: methanol

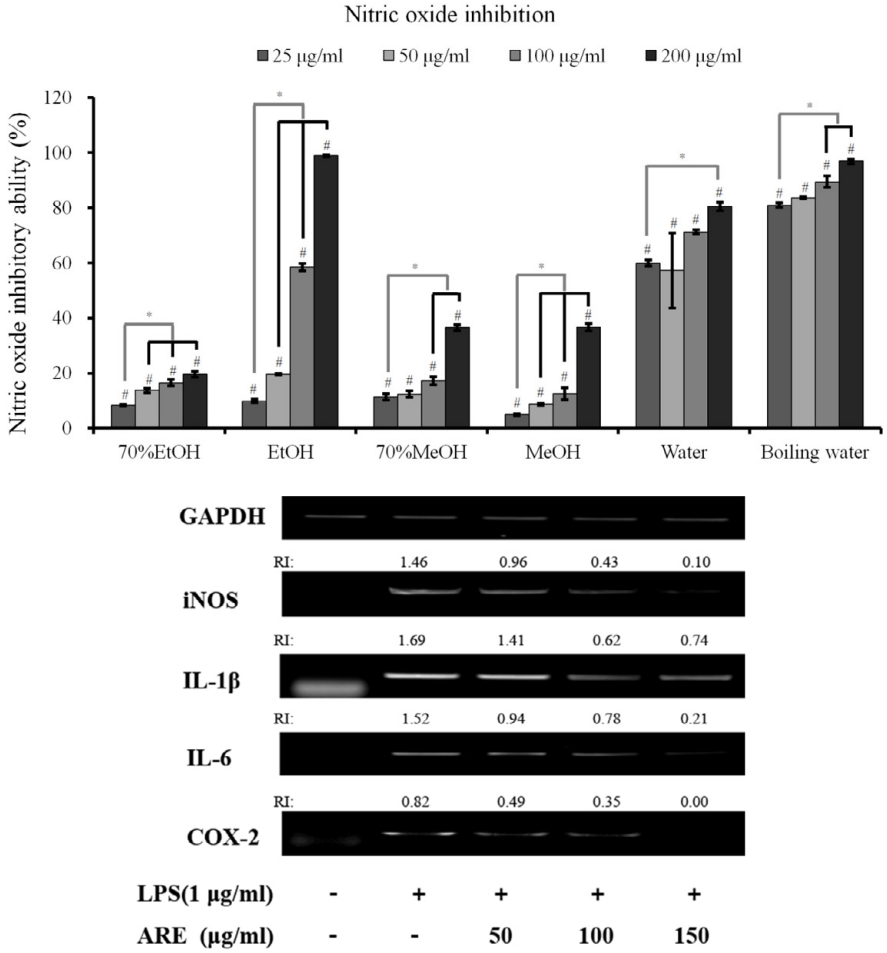

Figure 2. *(A) Nitric oxide (NO) inhibition of different extracts from A. radix on LPS-stimulated RAW 264.7 cells. Each value is expressed as the mean ± SD (n=3)*
#A statistically significant difference was seen when compared with the control at 24 h (P<0.05)
** A significant difference was seen when compared to the control (nonloaded group) (P<0.05)*
EtOH: ethanol
MeOH: methanol
(B) Effects of absolute ethanol extract of A. radix (AHE) on nitric oxide synthase (iNOS), interleukin 1β (IL-1β), interleukin 6 (IL-6) and cyclooxygenase-2 (COX-2) mRNA expression in lipopolysaccharide (LPS)-stimulated RAW 264.7 cells

water and boiling water extracts) (Figure 2A). The boiling water extract showed an 80.9 ± 0.8 % reduction of NO production at 25 μg/ml. The highest reduction of NO production was achieved in the ethanol extract group at 200 μg/ml with 98.9 ± 0.4 %.

RT-PCR analysis

The iNOS, IL-1β, IL-6 and COX-2 mRNA expressions in the unstimulated RAW 264.7 cells were minimal, but their mRNAs were profoundly induced after the treatment with LPS (Figure 2B). Pretreatment with the absolute ethanol extract of *A. radix* suppressed the LPS-stimulated iNOS, IL-1β, IL-6 and COX-2 expression. The suppression of inflammatory-related genes increased with the increasing concentration of *A. radix* extract.

Anticancer assay

Cytotoxicity assay and RT-PCR analysis: The results of cell viability of A549 cells after incubation in the presence of different extracts from *A. radix* for 24 h, 48 h and 72 h are shown in Figures 3A-3C. The results at 24 h showed that *A. radix* extracts significantly reduced cellular viability when compared with the untreated control (P<0.05) (Figure 3A). The results at 48 h and 72 h showed similar trends with 24 h data (Figures 3B and 3C). The absolute ethanol extract showed the most powerful effects of 42.3 ± 0.1%, 36.1 ± 1.0%, and 28.5 ± 1.5% with concentrations of 50, 100, and 150 μg/ml, respectively, at 72 h when the control was considered 100 (100.0 ± 2.7)% (P<0.05). The expression of SOD-2 and caspase-3 increased with the increase of exposure time to the absolute ethanol extract (Figure 3D).

Discussion

In this study, we examined the effects of different extracts of *A. radix* on RAW 264.7 and A549 cells under predetermined concentrations. The absolute ethanol extract showed the highest anti-inflammatory and anticancer

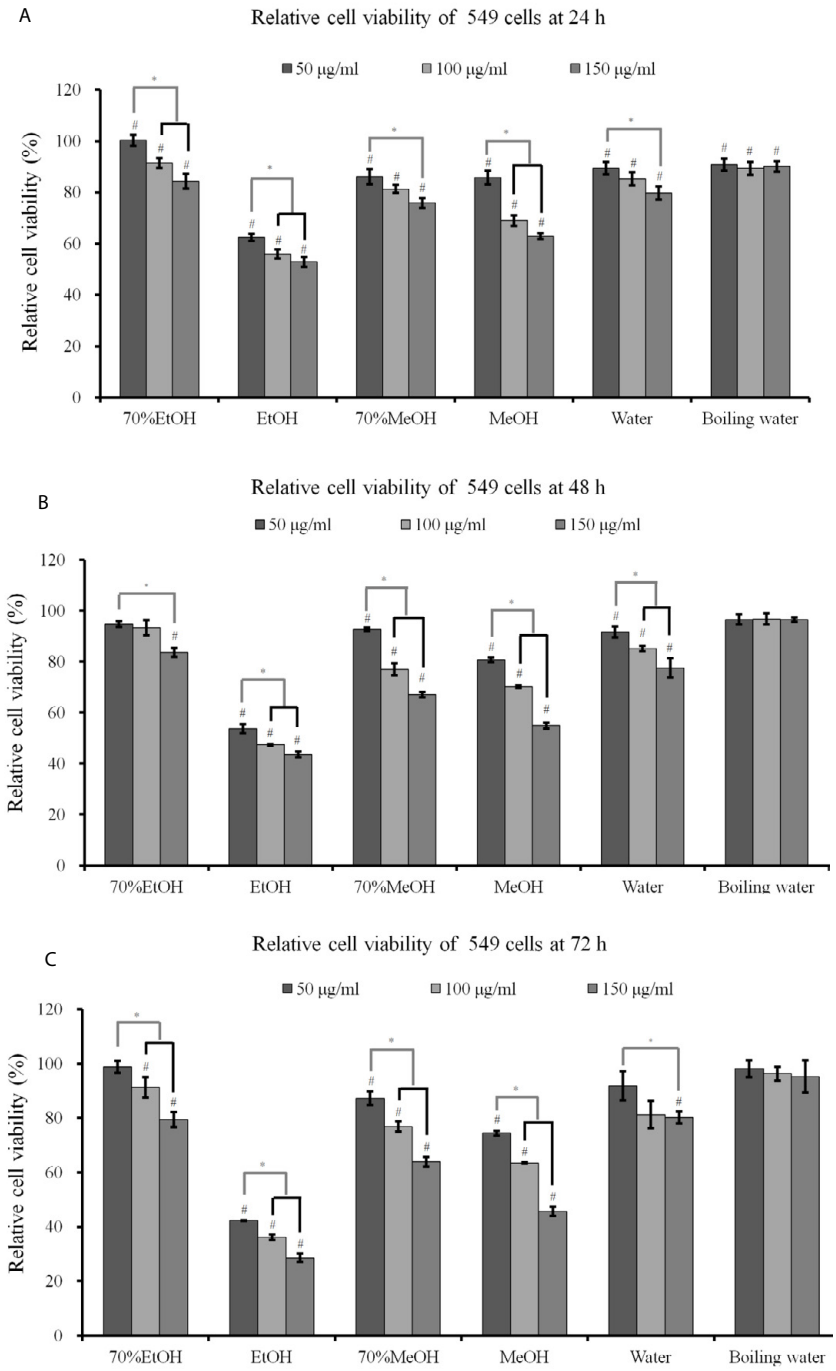

Figure 3. *(A) Cell viability of A549 cells after incubation in the presence of different extracts from A. radix for 24 h. Each value is expressed as the mean ± SD (n=3)*

#A statistically significant difference was seen when compared with the control (nontreated group) at 24 h (P<0.05)

** There was a statistically significant difference when compared with 25 μg/ml group in each extraction method.*

EtOH: ethanol

MeOH: methanol

(B) Cell viability of A549 cells after incubation in the presence of different extracts from A. radix for 48 h. Each value is expressed as the mean ± SD (n=3)

#A statistically significant difference was seen when compared with the control (nontreated group) at 48 h (P<0.05).

**There was a statistically significant difference when compared with 25 μg/ml group in each extraction method.*

(C) Cell viability of A549 cells after incubation in the presence of different extracts from A. radix for 72 h. Each value is expressed as the mean ± SD (n=3)

#A statistically significant difference was seen when compared with the control (nontreated group) at 72 h (P<0.05)

**There was a statistically significant difference when compared with 25 μg/ml group in each extraction method.*

(D) A549 cancer cells were plated in 6-well plates and then exposed to absolute ethanol extract (AHE) for the indicated times. Caspase-3 and superoxide dismutase-2 (SOD-2) mRNA levels in A. radix-stimulated cells were detected by reverse transcription-polymerase chain reaction (RT-PCR)

effects in these experimental settings.

The RAW 264.7 macrophage model is considered a useful model for evaluating anti-inflammatory agents because a number of different inflammatory mediators, including NO, prostaglandin E2 and tumor necrosis factor-α, are generated by the macrophages upon stimulation with LPS (a primary component of the Gram-negative bacteria cell wall) [6]. Increased anti-inflammatory effects of the ethanol extract may be explained by the phenolic and flavonoid compounds in *A. radix* because with typical extraction procedures phenolic compounds are mostly carried out using organic solvents [11,12]. These compounds have reportedly shown various health-promoting biological actions, including anti-inflammatory, anticarcinogenic, and anti-atherosclerotic functions [7]. It was unclear whether the *A. radix*-mediated inhibition of NO production was the consequence of inhibiting iNOS, COX-2, IL-1β, and IL-6 at the transcriptional level or due to some other mechanism. This study clearly suggested that the suppressive activity of *A. radix* extracts on iNOS, COX-2, IL-1β and IL-6 was mediated via transcriptional levels.

Caspases are crucial mediators of programmed cell death (apoptosis), and caspase-3 is a frequently activated protease in mammalian cell apoptosis [13]. Cytotoxic effects on lung carcinoma cell lines are partly explained by the expression of caspase-3. The transcriptional level of caspase-3 was increased with longer exposure to *A. radix* extracts in this study. Additionally, this study showed that the transcriptional level of SOD-2 was greater with an increase in exposure time. The role of SOD in carcinogenesis has been widely studied but remains ambiguous and controversial [16]. A major intracellular form of the SOD enzyme is considered a tumor suppressor [9,14]. A previous report showed that increased manganese SOD expression suppresses the malignant phenotype of human melanoma cells [15].

Within the limits of this study, *A. radix* showed anti-inflammatory effects using a RAW 264.7 cell line and anticancer effects on A549 cells. These effects were influenced by extraction methods. The absolute ethanol extract showed the highest anti-inflammatory and anticancer effects in these experimental settings.

Acknowledgement

The authors report no conflicts of interest related to this study. The author does not have any financial interest in the companies whose materials are included in the article.

References

1. Oh SM, Kim J, Lee J, Yi JM, Oh DS, Bang OS. Anticancer potential of an ethanol extract of Asiasari radix against HCT-116 human colon cancer cells in vitro. Oncol Lett 2013; 5: 305-310.
2. Jeong SH, Lee JE, Jin SH, Ko Y, Park JB. Effects of Asiasari radix on the morphology and viability of mesenchymal stem cells derived from the gingiva. Mol Med Rep 2014; 10: 3315-3319.
3. Kamei T, Kondoh T, Nagura S, Toriumi Y, Kumano H, Tomioka H. Improvement of C-reactive protein levels and body temperature of an elderly patient infected with Pseudomonas aeruginosa on treatment with Mao-bushi-saishin-to. J Altern Complement Med 2000; 6: 235-239.
4. Kim HM, Moon YS. Asiasari radix inhibits immunoglobulin E production on experimental models in vitro and in vivo. Immunopharmacol Immunotoxicol 1999; 21: 469-481.
5. Jang JY, Lee JH, Shin HK, Choi YH, Lee JD, Choi BT. Partially purified Asiasari radix inhibits melanogenesis through extracellular signal-regulated kinase signaling in B16F10 cells. Int J Mol Med 2010; 25: 287-292.
6. Jiang Y, Wang MH. Ethanol extract of Synurus deltoides (Aiton) Nakai suppresses in vitro LPS-induced cytokine production in RAW 264.7 macrophages and in vivo acute inflammatory symptoms. Nutr Res Pract 2014; 8: 11-19.
7. Jiang Y, Wang MH. Different Solvent Fractions of Acanthopanax senticosus Harms Exert Antioxidant and Anti-Inflammatory Activities and Inhibit the Human Kv1.3 Channel. J Med Food 2014.
8. Li L, Neaves WB. Normal stem cells and cancer stem cells: the niche matters. Cancer Res 2006; 66: 4553-4557.
9. Li C, Wang MH. Aristolochia debilis Sieb. et Zucc. induces apoptosis and reactive oxygen species in the HT-29 human colon cancer cell line. Cancer Biother Radiopharm 2013; 28: 717-724.
10. Jiang Y, Hu W, Han W, Yeo JH, Wang MH. Antioxidant and nitric oxide production inhibitory activities of scouring rush (Equisetum hyemale L.). Food Science and Biotechnology 2012; 21: 1037-1044.
11. Reis SF, Rai DK, Abu-Ghannam N. Water at room temperature as a solvent for the extraction of apple pomace phenolic compounds. Food Chem 2012; 135: 1991-1998.
12. Mahugo Santana C, Sosa Ferrera Z, Esther Torres Padron M, Juan Santana Rodriguez J. Methodologies for the extraction of phenolic compounds from environmental samples: new approaches. Molecules 2009; 14: 298-320.
13. Porter AG, Janicke RU. Emerging roles of caspase-3 in apoptosis. Cell Death Differ 1999; 6: 99-104.
14. Bravard A, Sabatier L, Hoffschir F, Ricoul M, Luccioni C, Dutrillaux B. SOD2: a new type of tumor-suppressor gene? Int J Cancer 1992; 51: 476-480.
15. Church SL, Grant JW, Ridnour LA, Oberley LW, Swanson PE, Meltzer PS. Increased manganese superoxide dismutase expression suppresses the malignant phenotype of human melanoma cells. Proc Natl Acad Sci USA 1993; 90: 3113-3117.
16. Liu X, Wang A, Lo Muzio L, Kolokythas A, Sheng S, Rubini C. Deregulation of manganese superoxide dismutase (SOD2) expression and lymph node metastasis in tongue squamous cell carcinoma. BMC Cancer 2010; 10: 365.

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