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Anti-cancer and anti-inflammatory activities of aronia (*Aronia melanocarpa*) leaves

Nhuan Do Thi, Eun-Sun Hwang✉

Department of Nutrition and Culinary Science, Hankyong National University, 327 Chungang-ro, Anseong-si, Gyeonggi-do, 456-749, Korea

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ABSTRACT

Objective: To determine the anti-cancer effect of aronia leaf extract on SK-Hep1 cells using migration, metallo metrix proteinase-2/-9 (MMP-2/-9) and MT-1 MMP expression and to evaluate the anti-inflammatory activities of the leaf extract. **Methods:** The effect of aronia leaf extract on cancer prevention was investigated. SK-Hep1 human liver cancer cell line was treated with aronia leaf extract at various concentractions. MTT assay was used to measure cancer cell growth inhibition, and wound migration assay was used for metastasis determination. The expression of MMP-2/-9 was measured at the protein level using zymography and the expression of MMP-2/-9 and MT-1 MMP was examined at the gene level by RT-PCR. Raw 264.7 macrophage cells were stimulated with lipopolysaccharides to induce inflammation, and then the inhibition of inflammation was evaluated by treatment of aronia leaf extract. Expressions of interleukin-6, tumor factor- α , and nitric oxide (NO) were also determined. **Results:** SK-Hep1 cell growth was inhibited in proportion to the concentration of aronia leaf extract. In migration assay, aronia leaf extract showed 61.3%-96.3% wound size inhibtion after treating 50-200 μg/mL of aronia leaf extract for 24 h. At the protein level, the expression of MMP-2 and MMP-9 decreased as the concentration of aronia leaf extract treated with SK-Hep1 cells increased. In addition, the same pattern as in the protein was also observed in the mRNA levels. The expressions of MMP-2 and MMP-9 protein were inhibited by 92.2% and 53.8%, respectively after treatment with 200 μg/mL aronia leaf extract. In addition, Raw 264.7 cells treated with aronia leaf extract did not affect cell survival. There was dose dependent inhibition of interleukine-6, tumor necrosis factor- α and nitric oxide after treating aronia leaf extract in lipopolysaccharides-treated Raw 264.7 cell. **Conclusions:** The results show that aronia leaf has anticancer and and antimetastatic properties in SK-Hep1 and Raw 264.7 cells.

1. Introduction

Aronia (*Aronia melanocarpa*) was grown in Poland, North America, and Europe, but now it is cultivated worldwide, including Korea[1]. Aronia contains many health-promoting substances such as anthocyanins, proanthocyanidins, vitamin C, pectins, tannins, flavonoids, and phenolic acids[2,3]. Aronia is also known as 'black chokeberry' and is considered by many to be the healthiest fruit in the world, offering more health benefits than any other superfood[4]. Aronia fruits have been reported to have antioxidant, anticancer, hypoglycemic, hypolipidemic, hypotensive, anti-inflammatory and antibacterial properties[3-7].

✉Corresponding author: Eun-Sun Hwang, Department of Nutrition and Culinary Science, Hankyong National University, 327 Chungang-ro, Anseong-si, Gyeonggi-do, 456-749, Korea.
Tel: +82-31-670-5182
Fax: +82-31-670-5187
E-mail: ehwang@hknu.ac.kr

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Aronia leaves have been reported to contain a variety of physiologically active substances such as chlorogenic acid, its isomers, caffeic acid, quercetin 3-*O*-glucopyranoside and rutin[4]. In particular, aronia young leaves contain a variety of phenolic compounds, such as caffeic acid, neochlorogenic acid and chlorogenic acid, compared to old leaves[8]. In some published studies, there is evidence that leaves of plants have physiological potency over fruit[9]. Denev *et al.*[10] reported aronia leaf extract contains a combination of antioxidant and strongly inhibited peroxyl radical formation *in vitro* with the same order of that for α-tocophenol and significantly reduced CCl_4-induced hepatic lipid peroxidation *in vivo*. In addition, streptozotocin was administered to rats to induce diabetes mellitus, and then it was confirmed that blood glucose was decreased at a significant level when the exract of aronia leaf was consumed[11]. Aronia leaves have been studied and reported to be effective in the prevention and treatment of cancer, leukemia and other chronic diseases[9]. Aronia leaf extract is known to be involved in the death of human leukemia cell line, HL60 cell and human promyelocytic leukemia cells, which is thought to be due to the action of polyphenol compounds contained in the extract of aronia leaf[9].

Inflammation, which occurs as a tissue damage or as an early symptom of infection, initially occurs locally, but spreads to the surroundings over time[12]. The immune response is promoted by bacterial products and substances such as endogenous toxins or lipopolysaccharide. Previously published studies have shown that MMPs act as regulators in both increasing or decreasing inflammation[13]. In general, MMPs are known to increase in diseases where tissue damage or inflammation occurs, and to break down proteins and regulate cytokine/chemokine activity in the body. There is increasing evidence that it works in inflammation to regulate the inflow of white blood cells[13-15]. Epidemiologic studies show that many cancers such as lung, stomach, colon, bladder cancers are associated with microbial infection and these cancers and inflammation are related by epidemiology, histopathology, inflammatory profiles[16-18]. To study the inflammatory response, Raw 264.7 cells are generally treated with lipopolysaccharides (LPS) known to cause inflammation, which is widely used and evaluated as the most reliable method to date. LPS, called endotoxin, is a macromolecule composed of lipids and polysaccharides. Raw 264.7 cells are able to predict the extent of inflammation when stimulated with nitric oxide (NO) synthase secretion by measuring the amount of NO produced at that time[19,20].

In this study, the effect of aronia leaf extract on cancer cell metastasis was investigated by measuring the degree of inhibition of cancer cell migration and MMP-2/-9 and MT-1 MMP expression after treatment of aronia leaf extract in SK-Hep1 cancer cells. Raw 264.7 cells were treated with LPS to induce inflammation, and then treated with different concentrations of aronia leaf extract. The amount of inflammation was measured to evaluate the anti-inflammatory effect of aronia leaf.

2. Materials and methods

2.1. Chemicals

Cell culture supplies and chemicals were purchased from Sigma Chemical (St. Louis, MO, USA) and Gibco Life Technologies, Inc. (Paisley, UK). Organic solvents were purchased from Burdick & Jackson (Batavia, IL, USA).

2.2. Cell culture

SK-Hep1 human hepatocellular carcinoma cells and Raw 264.7 murine cells were obtained from the Korean Cell Line Bank (Seoul, South Korea). Cells were cultured in a 37 ℃ incubator containing 5% CO_2. The cell culture medium, Dulbeco's Modified Eagle's Media, was supplemeted with 10% fetal bovine serum and penicillin/streptomycin.

2.3. Aronia leaf extraction

Aronia leaves were used in the experiment by receiving leaves of 'Nero' varieties harvested in June at aroina farm in Korea. The leaves were washed in flowing water, frozen at -80 ℃ for 24 h, then placed in a freeze dryer and dried for 2 d. The dried sample was put into a food grinder and made into a fine powder and stored at -80 ℃. The samples were extracted as follows; The powder sample (5 g) was mixed with 125 mL of 80% ethanol and shaken for 2 h in a 85 ℃ water bath to extract the bioactive substances. This procedure was repeated three times. The supernatant was collected separately from the mixture and filtered through Whatman #2 filter paper under the vaccum. The extracted and filtered samples were evaporated under the vacuum at 40 ℃ using a rotary evaporator. After evaporation, 50 mL of water was added to the sample to completely dissolve the sample. The sample was then lyophilized to prepare powder, which was then stored at -20 ℃ for subsequent experiments.

2.4. MTT assay

MTT assay was used to determine proliferation of SK-Hep1 and Raw 264.7 cells. SK-Hep1 cells were grown in 96-well plate at a concentration of 1×10^5 and Raw 264.7 cells at a density of 5×10^3 cells per 200 μL of medium. Cells were cultured for 2 h, then the medium in the 96-well plate was removed and replaced with 100 μL of fresh medium containing aronia leaf extract diluted to different concentrations (0-400 μg/mL). The cell viability was determined after 24 or 48 hours of incubation by the method of Hwang and Lee[21].

2.5. Wound migration assay

SK-Hep1 cells were treated with 50-200 μg/mL of aronia leaf

extract and cultured for 12-48 h to observe the migration rate of cancer cells. The cells were incubated in a 6-well plate, and then a line was drawn in the middle of the well with a thin plastic pipette tip to induce the wound, and the migration of the attached cells was observed under a microscope. The wound migration distance by the effect of aronia leaf extract was measured by the method of Thi and Hwang[7].

2.6. Determination of MMP-2/-9 expression with zymography

SK-Hep1 cells were treated with aronia leaf extract at various concentrations of 50-400 μg/mL and cultured for 48 h in serum-free DMEM. After 48 hours of culture, the medium was collected and the protein content contained in the medium was measured. The MMP-2 and MMP-9 proteins were separated using sodium dodecyl sulfate (SDS) gel electrophoresis in a 10% polyacrylamide gel. The MMP-2 and MMP-9 levels were determined by the method of Hwang and Lee[21].

2.7. Determination of MMP-2/-9 and MT-1 MMP with RT-PCR

SK-Hep1 cells were treated with aronia leaf extract at various concentrations of 50-200 μg/mL and cultured for 24 h in DMEM. Total RNA was isolated using TRIzol reagent, RNA purity and concentration were measured, and cDNA was synthesized after mixing with oligo-dT primer. After denaturation at 95 ℃ for 2 min, PCR was carried out for 30 cycles of 3 steps at 92 ℃ for 1 min, 55 ℃ for 60 s, and 73 ℃ for 1 min, and the final extension was carried out at 73 ℃ for 10 min. The amplified PCR product was electrophoresed on 1.5% agarose gel and the amount of expressed *MT1-MMP* and *MMP-2/-9* messenger RNA (mRNA) was quantitated by the method of Hwang and Lee[21].

2.8. NO determination

In a 96-well plate, Raw 264.7 cells (at a concentration of 5×10^5 cell/well) were plated and stabilized. After the medium was removed and LPS was dissolved in fresh medium at a concentration of 2 mg/mL, 100 μL was added to each well. After 1 hour of incubation, different concentrations of aronia leaf extract (50-200 μg/mL) were added. Then, the media were collected and used for NO determination after 12 hours of incubation. Since NO production is directly correlated with the amount of nitrite concentration in the medium, the amount of nitrite was calculated by Griess reaction in this experiment. After adding 50 μL of the Griess reagent to 50 μL of the collected medium and incubating at room temperature for 15 min, the absorbance was measured at 540 nm. Sodium nitrite ($NaNO_2$) standard solution was diluted by different concentrations to make a standard curve, and the NO content in each sample was calculated.

2.9. Interleukin 6 (IL-6) and tumor necrosis factor (TNF- α) measurement

IL-6 and TNF- α were measured after LPS treated Raw 264.7 cells were cultured and incubated for a certain period of time with different concentrations of aronia leaf exteact. Cells treated with different concentrations of aronia leaf extract were mixed with 100 μL of capture antibody solution, reacted at 4 ℃ for 18 h, and then 100 μL of mixed samples were added to a 96-well plate. The samples were incubated for 2 h at room temperature with 100 rpm shaing device, 50 μL of detection antibody solution was added and incubated another 1 h at the same speed of shaker. To the diluted antibody solution, 50 μL of diluted Avidin-HRP solution was added and reacted at room temperature for 30 min. Then, 50 μL of TMB substrate solution was added and incubated for 20-30 min while shaking at 100 rpm in a dark place. The stop soluation of 50 μL was added to terminate the reaction. IL-6 and TNF- α conents were determined using ELISA reader at 570 nm and 450 nm, and the the amount of IL-6 and TNF- α contained in the samples was calculated based on the standard curve.

2.10. Statistical analysis

All measurements were expressed as mean ± SD and statistical analysis was performed using the SPSS software package (Ver. 17). All results were compared using one-way ANOVA analysis and significance was shown at the $P < 0.05$ level.

3. Results

3.1. Effect of aronia leaf extract on SK-Hep1 cell proliferative activity

To investigate the effect of aronia leaf on SK-Hep1 cell proliferation, the exract was diluted to various concentrations (0-400 μg/mL) and cultured for 24 or 48 h. Aronia leaf extract significantly inhibited SK-Hep1 human hepatoma cancer cell growth in both dose- and time-dependent manner (Figure 1). SK-Hep1 cancer cell growth was significantly reduced by 4.4%-27.6% after treating 12.5-400 μg/mL of aronia leaf extract for 24 h compared with the control. Similar to the results of 24 h, SK-Hep1 cell proliferation decreased with increasing concentration of aronia leaf extract even in the samples cultured for 48 h. As the concentration of aroina leaf extract increased from 12.5 to 400 μg/mL, the proliferation of cancer cells decreased by 8.9%-50.4%. The growth of SK-Hep1 cells was reduced by 13.4% and 18.7%, respectively, when 100 and 200 μg/mL of aronia leaf extract were added for 24 h. In addition, when SK-Hep1 cells were cultured for 48 h under the same conditions, the numbers of cells were decreased by 29.7% and 39.8%, respectively, compared to the control.

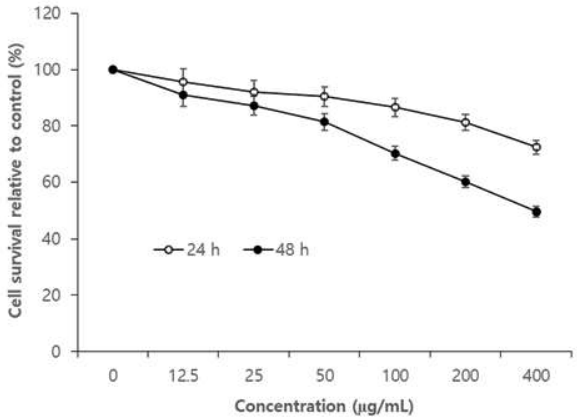

Figure 1. Effect of aronia leaf extract on cell viability in SK-Hep1 human hepatoma cells.

3.2. Effect of aronia leaf extract on migration

As shown in Figure 2, the migration distance of cancer cells was shorter as the concentration of aronia leaf extract was higher and the incubation time was longer. There was dose dependent inhibition of migration distance with 70.6%-98.4% wound size inhibtion after treating 50-200 μg/mL of aronia leaf extract for 12 h. In the case of treatement with 100 μg/mL aronia leaf extract, the mobility of SK-Hep1 cells was controlled in proportion to the treated time, and the wound size in cells cultured for 12, 24 and 48 h were 91.5%, 89.6% and 76.3%. As the treated time of aronia leaf extreact increased, the cells recovered mobility.

3.3. Effect of aronia leaf extract on MMP

As shown in Figure 3, cell culture media were collected and separated the MMP-2 and MMP-9 protein with gelatin zymography. The quantification of MMP-2 and MMP-9 by gelatin gel staining and de-staining revealed that SK-Hep1 cells secreted the largest amount of MMP-2 and MMP-9 in the control group without aronia leaf extract. After 48 h, aronia leaf extract treatment at 50 and 400 μg/mL decreased MMP-9 expression by 15.5% and 75.8%, respectively. Aronia leaf extract at 50 and 400 μg/mL decreased MMP-2 expression by 37.7% and 100.0%, respectively and aronia leaf extract at 400 μg/mL clearly suppressed MMP-2 activity. These results suggested that the expression of MMP-2 and MMP-9 protein in SK-Hep1 cells was inhibited by higher concentration of aronia leaf extract.

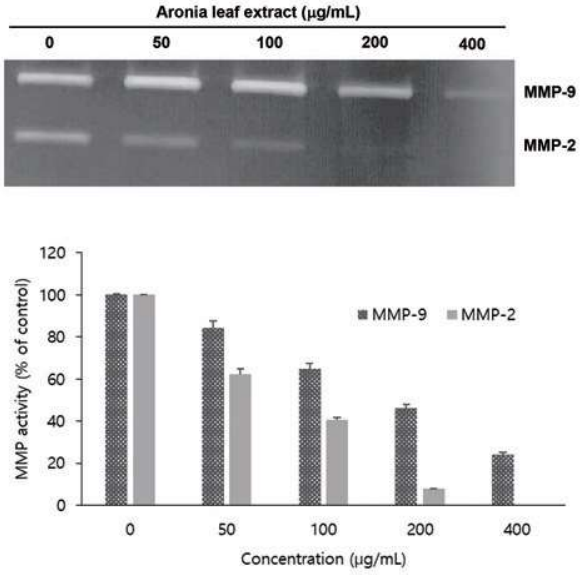

Figure 3. Effect of aronia leaf extract on MMP-2/-9 expression in SK-Hep1 human hepatoma cells.

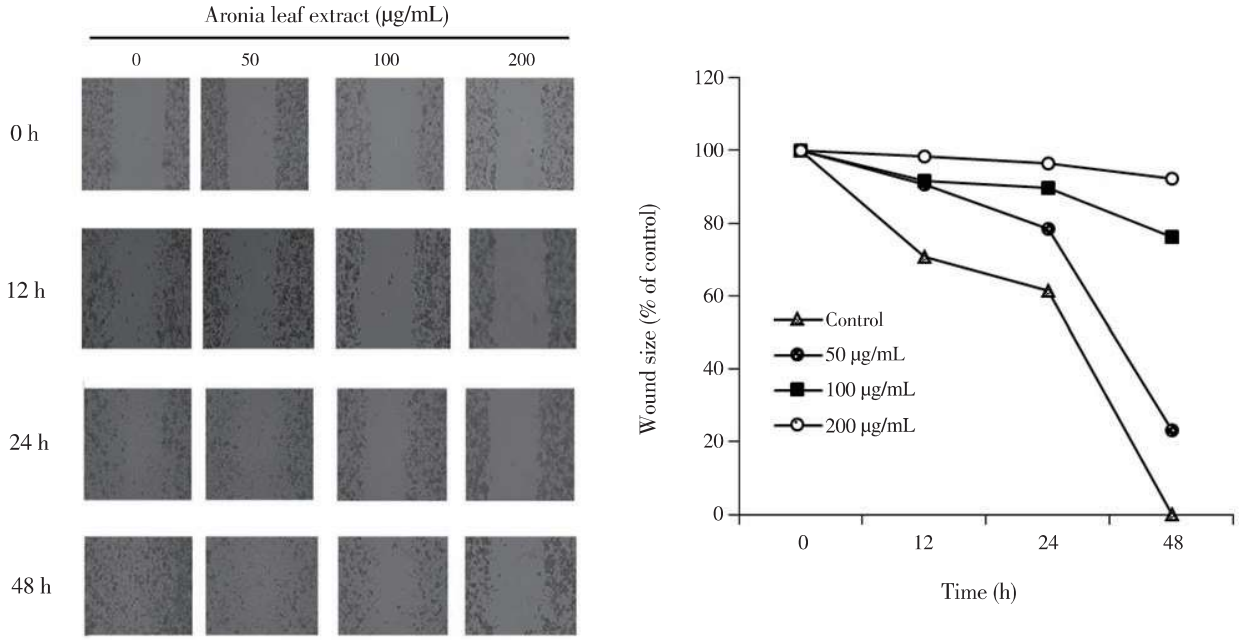

Figure 2. Effect of aronia leaf extract on wound healing migration of SK-Hep1 human hepatoma cells.

3.4. Effect of aronia leaf extract on mRNA MMP and MT1-MMP

The mRNA expression of *MT1-MMP*, *MMP-2*, and *MMP-9* decreased as the concentration of aronia leaf extract increased (Figure 4). The *MMP-9* mRNA expression was reduced by 34.7% and 63.3%, respectively, in samples treated with 50 and 100 μg/mL of aronia leaf extracts compared to the control. The *MMP-2* mRNA expression was inhibited by 32.4% and 50.0%, respectively, in the same concentration of aronia leaf treatment. When 200 μg/mL of aronia leaf extract was treated, the *MMP-2* mRNA expression was completely inhibited. The expression of *MMP-9* mRNA in leaf extracts was much higher than that of *MMP-2* mRNA. The *MT-1 MMP* expression was reduced by 5.3%, 15.8% and 31.6% compared to the control group after SK-Hep1 cells were treated with 50, 100, and 200 μg/mL of aronia leaf extracts for 24 h.

3.5. Effect of aronia leaf extract on Raw 264.7 cell proliferation

The degree of inhibition of Raw 264.7 cell proliferation was measured (Figure 5). The concentration of aronia leaf extract up to 400 μg/mL used in this experiment did not increase or inhibit the survial of Raw 264.7 cells.

3.6. Effects of aronia leaf extract on NO production

Raw 264.7 cells with LPS (1 μg/mL) stimulation were treated with aronia leaf extract (50-200 μg/mL) and cultured for 12 h to determine NO content. NO production that was in control and LPS treated without aronia leaf extract was 30.3 and 96.6 μM, respectively. It appeared that aronia leaf extract had potential to inhibit LPS-induced production of NO compared to the LPS-treated control in Raw 264.7 cells. Aronia leaf extract at 50, 100, and 200 μg/mL suppressed the production of NO to 85.2, 70.5, and 58.2 μM, respectively in a dose dependent manner.

3.7. TNF-α and IL-6 measurement

The Raw 264.7 cells were inoculated with aronia leaf extract of 0-200 μg/mL and cultured for 12 h. As the concentration of aronia leaf extract increased, TNF-α and IL-6 expression decreased, especially IL-6. These expressions showed aronia leaf extract inhibited the proinflammatory mediators in a dose dependent manner (Figure 6).

TNF-α production that was in control and LPS treated without aronia leaf extract was 260.4 and 1 300.0 μg/mL, respectively. The results also demonstrated that aronia leaf extract had potential to inhibit LPS-induced IL-6 production compared to the LPS-treated control in Raw 264.7 cells. Aronia leaf extract at 50, 100 and 200 μg/mL suppressed dose dependently the production of TNF-α by 10.0%, 20.6%, and 28.4%, respectively compared to LPS-treated control without aronia leaf extract.

IL-6 production that was in control and LPS treated without aronia leaf extract was 296.3 and 9 810.3 μg/mL, respectively. As shown in Figure 6, aronia leaf extract had potential to inhibit LPS-induced IL-6 production compared to the LPS-treated control in Raw 264.7 cells. Aronia leaf extract at 50, 100 and 200 μg/mL suppressed dose dependently the production of IL-6 by 20.1%, 32.9%, and 87.3%, respectively compared to LPS-treated control without aronia leaf extract.

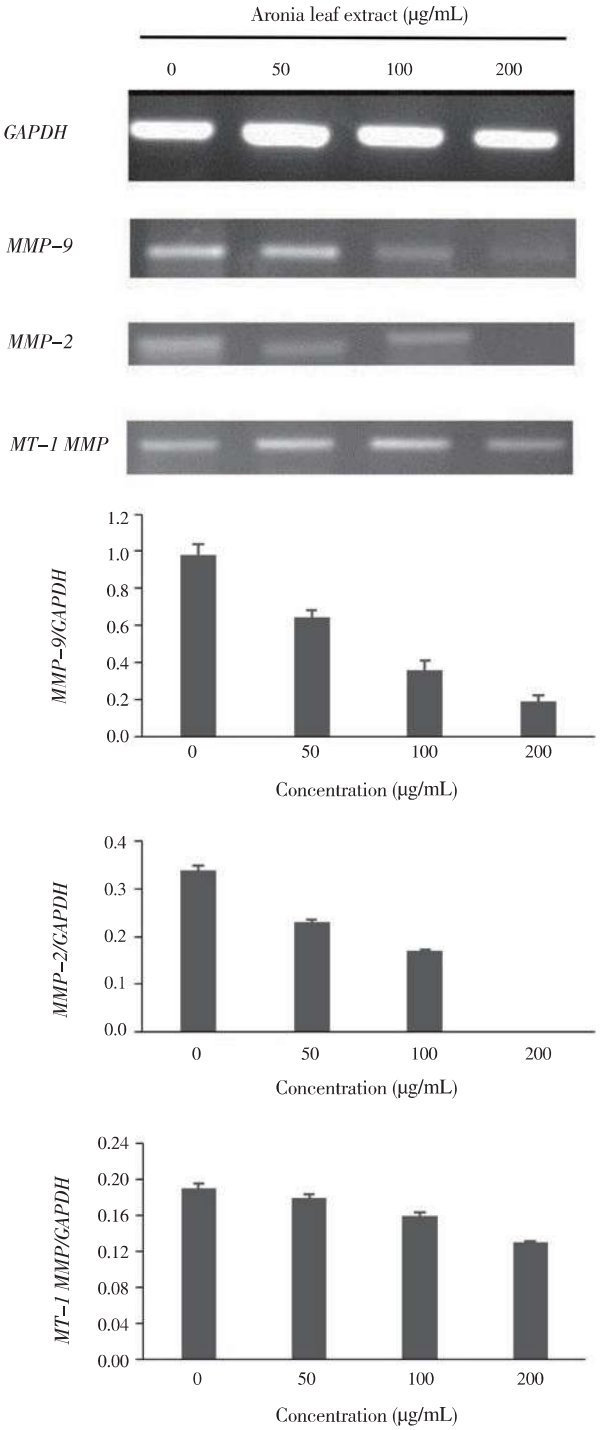

Figure 4. Effect of aronia leaf extract on *MMP-2/-9* and *MT-1 MMP* gene expression in SK-Hep1 human hepatoma cells.

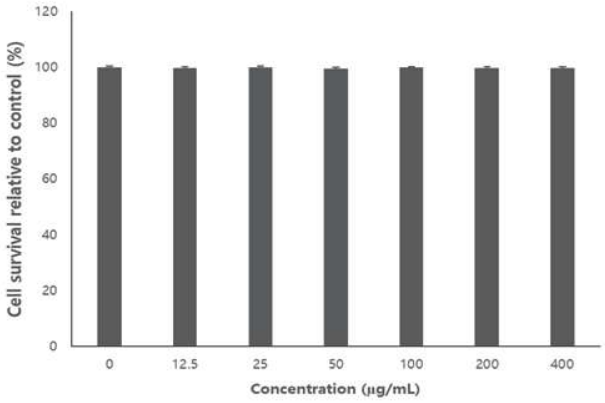

Figure 5. Effect of aronia leaf extract on the cytotoxicity of Raw 264.7 macrophage cells.

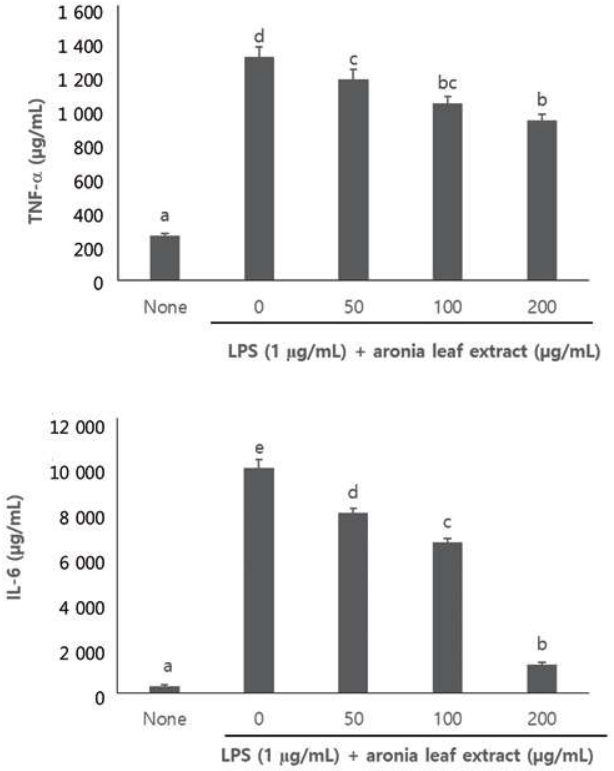

Figure 6. Effect of aronia leaf extract on the level of TNF- α and IL-6 in LPS-stimulated Raw 264.7 macrophage cells.

[a-c]Values with different superscript letters within the same column are significantly different at P <0.05.

4. Discussion

The anticancer activity of aronia leaf extract was investigated by the multiple cellular mechanism assays. The extract of aronia leaf inhibited SK-Hep1 human hepatoma cell growth and metastasis of cancer cells in a dose dependent manner. It has been reported that the growth and migration of cancer cells are very important in the metastasis of cancer, and thus, the effect of aronia leaf extract on SK-Hep1 cancer cell migration was examined. The mechanism of antitumor effect was investigated by measuring the effect of aronia leaf extract on the expression of MMP-2 and MMP-9, which are one of the matrix malloproteinases and are reported to be directly involved in invasion and metastasis of cancer cells[22]. Among the proteolytic enzymes, MMP-2/-9 are the most important enzymes involved in the degradation of basic membranes, and thus they are the enzymes directly related to the invasion and metastasis of cancer cells[20,23]. When SK-Hep1 cells were treated with 200 μg/mL of aronia leaf extract for 24 h, it was confirmed that up to 96.3% of the cells inhibited migration to the wound site. The results of this study demonstrated that aronia leaf extract inhibited extremely cell migration.

The potential anti-inflammatory properties of aronia leaf extract were also investigated. Aronia leaf extract inhibited NO production in a dose-dependent manner in Raw 264.7 cells induced by LPS. In addition, in the Raw 264.7 cell line stimulated with LPS, the aronia leaf extract decreased the expression of TNF- α and IL-6, which are secreted before inflammation, in proportion to the treatment concentration of aronia leaf extract. TNF- α and IL-6 are among the most important cytokines released by activated macrophages. During inflammatory response, TNF- α induces the expression of IL-6 together. Similar to the physiological function of TNF- α , IL-6 is one of the major cytokines and is induced by a variety of stimuli including LPS. Also, IL-6 is one of the major initiators of acute response and plays an important role in the immune response to inhibit chronic inflammation. Studies have shown that IL-6 level is increased in a variety of inflammatory diseases such as arthritis, Crohn's disease, and systemic lupus erythematosus[24,25]. LPS activiates a wide variety of transcription factors and induces a number of genes that are expressed during inflammation[26]. IL-6 induces inflammation and is actually found in serum and tumors of cancer cells and cancer patients[18]. IL-6 has the ability to promote tumors and, in fact, it is known that the increase in IL-6 expression is related to the growth of aggressive tumor cells in most cancers[27,28]. Therefore, inhibition of cancer cell growth and progression is necessary to discover and develop a substance that inhibits IL-6 or IL-6 receptor[18]. Our results indicate that aronia leaf extract inhibits growth and inflammation of cancer cells. These suggest that aronia leaf can be used as a candidate for the development of cancer and inflammation inhibitory food supplement or drug.

Conflict of interest statement

All authors declare that there is no conflict of interest.

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