

Carpinus turczaninowii extract modulates arterial inflammatory response: a potential therapeutic use for atherosclerosis

Youn Kyoung Son¹, So Ra Yoon^{2,4}, Woo Young Bang¹, Chang-Hwan Bae¹, Joo-Hong Yeo¹, Rimkyo Yeo², Juhyun An², Juhyun Song⁵ and Oh Yoen Kim^{2,3,5}

¹Biological and Genetic Resources Assessment Division, National Institute of Biological Resources, Incheon 22689, Republic of Korea

²Department of Food Science and Nutrition, Dong-A University, Busan 49315, Republic of Korea

³Center for Silver-targeted Biomaterials, Brain Busan 21 Plus Program, Dong-A University, 37 Nakdongdae-ro, 550beon-gil Saha-gu, Busan, 49315, Republic of Korea

⁴Institute of Health Insurance and Clinical Research, National Health Insurance Service Ilsan Hospital, Gyeonggi, 10444, Republic of Korea

⁵Departments of Anatomy, Chonnam National University Medical School, Gwangju, 61469, Republic of Korea

BACKGROUND/OBJECTIVES: Vascular inflammation is an important feature in the atherosclerotic process. Recent studies report that leaves and branches of *Carpinus turczaninowii* (*C. turczaninowii*) have antioxidant capacity and exert anti-inflammatory effects. However, no study has reported the regulatory effect of *C. turczaninowii* extract on the arterial inflammatory response. This study therefore investigated modulation of the arterial inflammatory response after exposure to *C. turczaninowii* extract, using human aortic vascular smooth muscle cells (HAoSMCs).

MATERIALS/METHODS: Scavenging activity of free radicals, total phenolic content (TPC), cell viability, mRNA expressions, and secreted levels of cytokines were measured in LPS-stimulated (10 ng/mL) HAoSMCs treated with the *C. turczaninowii* extract.

RESULTS: *C. turczaninowii* extract contains high amounts of TPC (225.6 ± 21.0 mg of gallic acid equivalents/g of the extract), as well as exerts time- and dose-dependent increases in strongly scavenged free radicals (average 14.8 ± 1.97 $\mu\text{g/mL}$ IC₅₀ at 40 min). Cell viabilities after exposure to the extracts (1 and 10 $\mu\text{g/mL}$) were similar to the viability of non-treated cells. Cytokine mRNA expressions were significantly suppressed by the extracts (1 and 10 $\mu\text{g/mL}$) at 6 hours (h) after exposure. Interleukin-6 secretion was dose-dependently suppressed 2 h after incubation with the extract, at 1-10 $\mu\text{g/mL}$ in non-stimulated cells, and at 5 and 10 $\mu\text{g/mL}$ in LPS-stimulated cells. Similar patterns were also observed at 24 h after incubation with the extract (at 1-10 $\mu\text{g/mL}$ in non-stimulated cells, and at 10 $\mu\text{g/mL}$ in the LPS-stimulated cells). Soluble intracellular vascular adhesion molecules (sICAM-1) secreted from non-stimulated cells and LPS-stimulated cells were similarly suppressed in a dose-dependent manner after 24 h exposure to the extracts, but not after 2 h. In addition, sICAM-1 concentration after 24 h treatment was positively related to IL-6 levels after 2 h and 24 h exposure ($r = 0.418$, $P = 0.003$, and $r = 0.524$, $P < 0.001$, respectively).

CONCLUSIONS: This study demonstrates that *C. turczaninowii* modulates the arterial inflammatory response, and indicates the potential to be applied as a therapeutic use for atherosclerosis.

Nutrition Research and Practice 2019;13(4):302-309; <https://doi.org/10.4162/nrp.2019.13.4.302>; pISSN 1976-1457 eISSN 2005-6168

Keywords: Antioxidants, inflammation, cytokines, atherosclerosis, arteries

INTRODUCTION

Oxidative stress, inflammation and vascular dysfunction are major pathological features of the atherosclerotic process, distinctly observed in cerebro- and cardiovascular diseases [1-3]. Excess free radicals (i.e., reactive oxygen species, ROS) produced in the body cause lipid peroxidation, protein alterations and DNA damages leading to oxidative stress, thereby stimulating various cytokine productions from inflammatory and immune cells, and finally dysregulating the vascular function [4-7].

Many studies have endeavored to reveal natural candidate molecules which are capable of regulating the oxidative stress

and inflammatory processes [8-14]. Among the natural candidates, the abundant phytochemicals present in plant sources have attracted great attention because of their pleiotropic functions including antioxidant capacity, enzyme modulation and gene transcription [11-14]. Recently, it is reported that leaves and branches of *Carpinus turczaninowii* (*C. turczaninowii*) have antioxidant capacity and anti-inflammatory effects [15-17]. As reported by Ko *et al.* [15,16] the extract from *C. turczaninowii* branches inhibits the production of interleukin (IL)-6 and nitric oxide (NO) in RAW264.7 cells. Kang *et al.* [17] also reported antioxidant, skin-whitening (reduced cellular melanogenesis in murine melanoma cells), anti-inflammation (reduced NO

This study was supported by a grant from the National Institute of Biological Resources (NIBR), funded by the Ministry of Environment of the Republic of Korea (NIBR201527101).

⁵ Corresponding Author: Oh Yoen Kim, Tel. 82-51-200-7326, Fax. 82-51-200-7353, Email. oykim@dau.ac.kr

Received: December 16, 2018, Revised: March 9, 2019, Accepted: May 14, 2019

This is an Open Access article distributed under the terms of the Creative Commons Attribution Non-Commercial License (<http://creativecommons.org/licenses/by-nc/3.0/>) which permits unrestricted non-commercial use, distribution, and reproduction in any medium, provided the original work is properly cited.

production in RAW264.7 cells) and antibacterial properties of the *C. turczaninowii* leaf extract. The roots and leaves of *C. turczaninowii* have been used for improvement of fatigue from excessive physical labor as well as the treatment of bruises or abscesses [from Illustration book of Korean medicinal herb]. The species *C. turczaninowii* is a deciduous tree belonging to the Betulaceae family, found endemic in Korea (Korean hornbeam) [18,19]. Although few studies have identified chemical compounds from *C. turczaninowii* branches, these are limited to naringenin and quercetin glycosides which belong to flavonoids [18], and carpinontriols A and B which have a diarylheptanoid skeleton bearing on carbonyl and five hydroxyl functional groups. The compounds isolated from the extract are potential pharmaceutical agents [15,16].

Contrarily, there are no studies determining the regulatory effect of *C. turczaninowii* extract on the arterial inflammatory response, which is a major pathological process observed in atherosclerosis [1-3]. Therefore, this study was undertaken to investigate if *C. turczaninowii* extract regulates the arterial inflammatory response, using human aortic vascular smooth muscle cells (HAoSMCs).

MATERIALS AND METHODS

Preparation of *Carpinus turczaninowii* extract

Complete plants (branches, leaves, bodies and roots, etc.) of *C. turczaninowii* were collected in January 2015 from Suin Mountain in GangjinGun, Korea, and identified by Heung Su Lee, the plant taxonomist and curator at the Natural History Museum of Hannam University, Daejeon, Korea. A voucher specimen was deposited at a publicly available herbarium (specimen deposition number: NIBRVP0000519846). The plant material was air-dried, ground and extracted three times with 70% ethanol for 24 h at room temperature. The extract was filtered, evaporated under reduced pressure, and freeze-dried to obtain a powder. It was stored in a deep freezer (-80°C) until further testing. For experiments, the lyophilized extract powder was dissolved in 70% ethanol (final concentration: 30 mg/mL). The extract stock solution was filtered through 0.2 µm Minisart® syringe filter (Sartorius Stedim Biotech GmbH, Goettingen, Germany) for molecular experiments. The filtered extract stock solution was then aliquoted and stored at -80°C till further analysis.

DPPH free radical scavenging assay

2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich Co., LLC., St. Louis, MO, USA), L-ascorbic acid (Tokyo Chemical Industry Co., Ltd., Tokyo, Japan) and ethanol (Duksan pure chemical Co., Ltd., Ansan, Korea) were used for the DPPH free radical scavenging assay. This assay was performed as per the method described by Brand-Williams *et al.*, with slight modifications [20]. The antiradical activity of ascorbic acid (50 µM) was used as the positive control. Briefly, 1 mL control + 1 mL 70% ethanol was used as the control solution (Ac), whereas 1 mL DPPH solution (290 µM) + 1 mL 70% ethanol was used as the blank solution (A0). For the assay, 1 mL DPPH solution (290 µM) was mixed with 1 mL varying concentrations of the sample (As) (5-100 µg/mL). For the assay, all mixtures were shaken and

stood at room temperature in the dark before measurement (up to 5 min). The absorbance was measured at 517 nm, every 5 min for 40 min, using the UV-Visible spectrophotometer (OPTIZEN POP, Mecasys Co., Ltd., Daejeon, Korea). All experiments were repeated four times. The scavenging activity of DPPH radicals was calculated by the following equation:

$$\begin{aligned} \text{Scavenging activity of DPPH radicals (\%)} \\ = [1-(As-A0)/Ac] \times 100. \end{aligned}$$

Quantification of total phenolic content

Folin-Ciocalteu reagent, gallic acid (Sigma-Aldrich Co., LLC., St. Louis, MO, USA) and sodium carbonate (Acros Organics Co., Ltd., NJ, USA) were applied for quantifying the total phenolic content (TPC) in the *C. turczaninowii* extract solution, using a modified method from that described by Deshpande & Cheryan [21]. Serial dilutions of gallic acid (0.5-1,000 µg/mL) in 70% ethanol were used as standard solutions. The absorbance was measured using a microplate absorbance reader (iMark™, Bio-Rad Laboratories, Hercules, CA, USA). The wavelength correction was set to 655 nm. Total phenolic content of the *C. turczaninowii* extract solution is expressed as gallic acid equivalents (mg of GAEs/g of extract). All experiments were repeated four times.

Cell culture

Primary human vascular aortic smooth muscle cells (HAoSMCs) (American Type Culture Collection, ATCC, Manassas, VA, USA) were maintained in a humidified atmosphere at 37°C with 5% CO₂, and cultured in VSMC basal medium supplemented with 5 ng/mL rhFGF-basic, 5 µg/mL rhinsulin, 5 ng/mL rhEGF, 10 mM L-glutamine, 50 µg/mL ascorbic acid, 5% fetal bovine serum, 10 µg/mL gentamicin, 10 Units/mL penicillin, 10 µg/mL streptomycin, 0.28 µg/mL amphotericin B, and 33 µM phenol red (ATCC, Manassas, VA, USA).

Cell viability assay

Cell viability was measured using the Cyto X™ Cell viability assay kit (LPS solution, Daejeon, Korea), with a slight modification of the manufacture's protocol. The absorbance (A) was measured at 450 nm using the microplate absorbance reader (iMark™, Bio-Rad Laboratories, Hercules, CA, USA). All experiments were repeated four times.

Quantitative real time-PCR

Quantitative real time-PCR (qPCR) was performed to determine if the extract modulates mRNA expression of IL-6 and tumor necrosis factor-α (TNF-α). Briefly, HAoSMCs were treated with the extract (1 and 10 µg/mL) and incubated for 6 h. Total cellular RNA was extracted from the treated HAoSMCs using Trizol reagent (Invitrogen, Carlsbad, CA), following the manufacturer's protocol. Poly (A) was subsequently added using poly (A) polymerase (Ambion, Austin, TX). One Step SYBR® Prime Script™ RT-PCR Kit II (Takara, Japan) was used to conduct qPCR. The PCR was performed using the following primers (5' to 3'): TNF-α (F): CGT CAG CCG ATT TGC TAT CT, (R): CGG ACT CCG CAA AGT CTA AG; IL-6 (F): GTT GCC TTC TTG GGA CTG AT, (R): CTG GCT TTG TCT TTC TTG TTA T, and β-actin (F): TCT

GGC ACC ACA CCT TCT A, (R): AGG CAT ACA GGG ACA GCA
 C. Denaturing was carried out at 95°C for 3 min, 40 cycles of 95°C for 20 sec, annealing at 60°C for 20 sec, and extension at 72°C for 20 sec. At each 72°C extension step, the fluorescence was measured at 585 nm. Expression of each factor was measured using an ABI prism 7500 Real-Time PCR System (Life Technologies Corporation, CA), and analyzed with comparative Ct quantification. β -actin was amplified as an internal control. The values are presented as relative quantity (RQ). All experiments were repeated four times.

Assessment of inflammatory markers in cell supernatants

HAoSMCs were plated at a density of 1.3×10^4 cells/well in 24-well plates. At approximately 80% cell confluency, the culture media was changed to serum-free medium. Cells were subsequently stimulated with or without LPS (10 ng/mL) for 15 min, after which varying concentrations of the extract (1, 5, and 10 μ g/mL) were added to the wells, and incubated for either 2 h or 24 h. After the appropriate incubation time, cell-free supernatants were collected. Concentrations of IL-6, TNF- α and soluble intracellular adhesion molecule-1 (sICAM-1) were measured using enzyme linked immunosorbent assay kits (PEPROTECH, Inc., Seoul, Korea), as per the manufacturer's protocol. The absorbance was read at 450 nm using the microplate absorbance reader (iMark™, Bio-Rad Laboratories, Hercules, CA, USA). All experiments were repeated four times.

Statistical analysis

Statistical analyses were performed using the Win SPSS ver22.0 (Statistical Package for the Social Science, SPSS Inc., Chicago, IL, USA). Data are expressed as the means \pm standard deviation (SD) of four independent experiments. For descriptive purposes, the mean values are presented as untransformed values. The differences between groups were determined by independent t-test. Pearson correlation analysis was also performed. A two-tailed value of $P < 0.05$ is considered to be statistically significant.

RESULTS

Antioxidant activity and total phenolic content of *C. turczaninowii* extract

The antioxidant potential of *C. turczaninowii* extract was determined by the DPPH assay. As shown in Fig. 1, DPPH free radicals are scavenged by the *C. turczaninowii* extract in time- and dose-dependent manners. The average IC₅₀ value for 40 min is 14.8 ± 1.97 μ g/mL (i.e. from 17.5 IC₅₀ value at 5 min to 12.4 μ g/mL IC₅₀ value at 40 min). Indeed, the radical scavenging activities of the extract at 100 and 50 μ g/mL were found to be stably high at 40 min (average, $90.7 \pm 0.03\%$ and $89.7 \pm 0.24\%$, respectively). The activities of the extract were similarly high and increasing in a time-dependent manner at 30 and 20 μ g/mL (76.4-87.6% and 62.2-74.8%, respectively). Time-dependent increase in activity for 40 min was also observed for 10, 5 and 1 μ g/mL (37.0-45.5%, 18.8-24.2%, and 8.8-9.4%, respectively). In addition, total phenolic content (TPC) was determined to be 225.6 ± 21.0 mg of GAE/g of the extract.

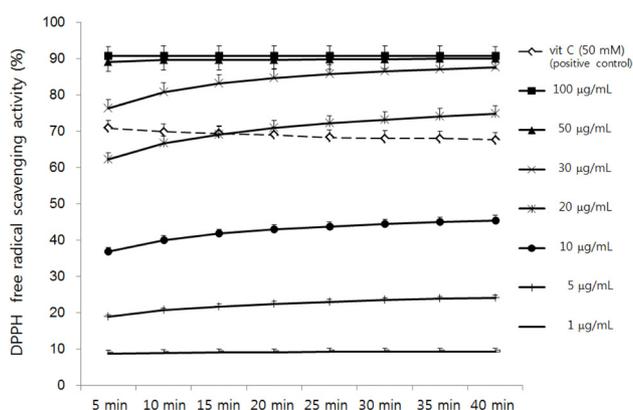


Fig 1. Time- and dose-dependent DPPH free radical scavenging activities (%) of *C. turczaninowii* extract. Data are expressed as means \pm SD. The experiment includes 4 repeats per condition. *C. turczaninowii*: *Carpinus turczaninowii*

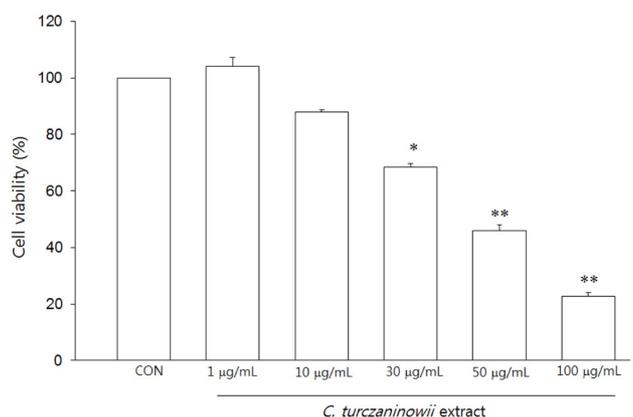


Fig 2. Cell viability (%) of human aortic vascular smooth muscle cells (HAoSMCs) treated with *C. turczaninowii* extract. Data are expressed as means \pm SD. * $P < 0.05$, ** $P < 0.001$ compared to CON (non-treated control cells). Cells were treated with various concentrations of *C. turczaninowii* extract. After 24 h, cell viabilities were measured using the Cytotoxicity assay. The experiment includes 4 repeats per condition. *C. turczaninowii*: *Carpinus turczaninowii*

Cell viability

Cells viabilities were measured after 24 h incubation with varying concentrations of the *C. turczaninowii* extract (1-100 μ g/mL). The cell viabilities of HAoSMCs incubated with 1 and 10 μ g/mL of the extract were not significantly different from those of non-treated cells (control, CON) (about 104% and 88%, respectively) (Fig. 2), but cells incubated with 30, 50 and 100 μ g/mL were dose-dependently reduced compared to CON (about 80%, 46%, and 23% viable, respectively). Based on the above result, further molecular experiments were performed using extract concentrations between 1 and 10 μ g/mL.

Modulatory effect of *C. turczaninowii* extract on mRNA expression of pro-inflammatory cytokines in HAoSMCs

To examine if pro-inflammatory cytokine expressions are modulated by the *C. turczaninowii* extract, IL-6 and TNF- α mRNA levels expressed in HAoSMCs were measured at 6 h after the extract treatment (1 and 10 μ g/mL). As shown in Fig. 3, exposure to the extract significantly suppresses the mRNA expressions of IL-6 and TNF- α in the cells.

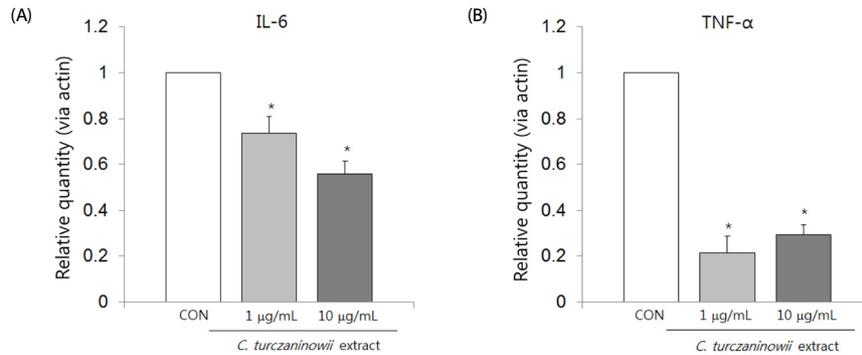


Fig 3. *C. turczaninowii* extract modulates mRNA expression of pro-inflammatory cytokines in HAoSMCs. The mRNA levels of IL-6 (A) and TNF-α (B) in HAoSMCs were measured using quantitative real time PCR (qPCR). HAoSMCs were treated with the extract (1 and 10 µg/mL) and incubated for 6 h. β-actin was amplified as an internal control. The values are presented by relative quantity (RQ). Data are expressed as means ± SD, and each experiment includes 4 repeats per conditions. * $P < 0,05$ compared with CON (non-treated control cells); *C. turczaninowii*, *Carpinus turczaninowii*; IL-6, interleukin-6; sICAM-1, soluble intracellular adhesion molecule-1; TNF-α, tumor necrosis factor-α

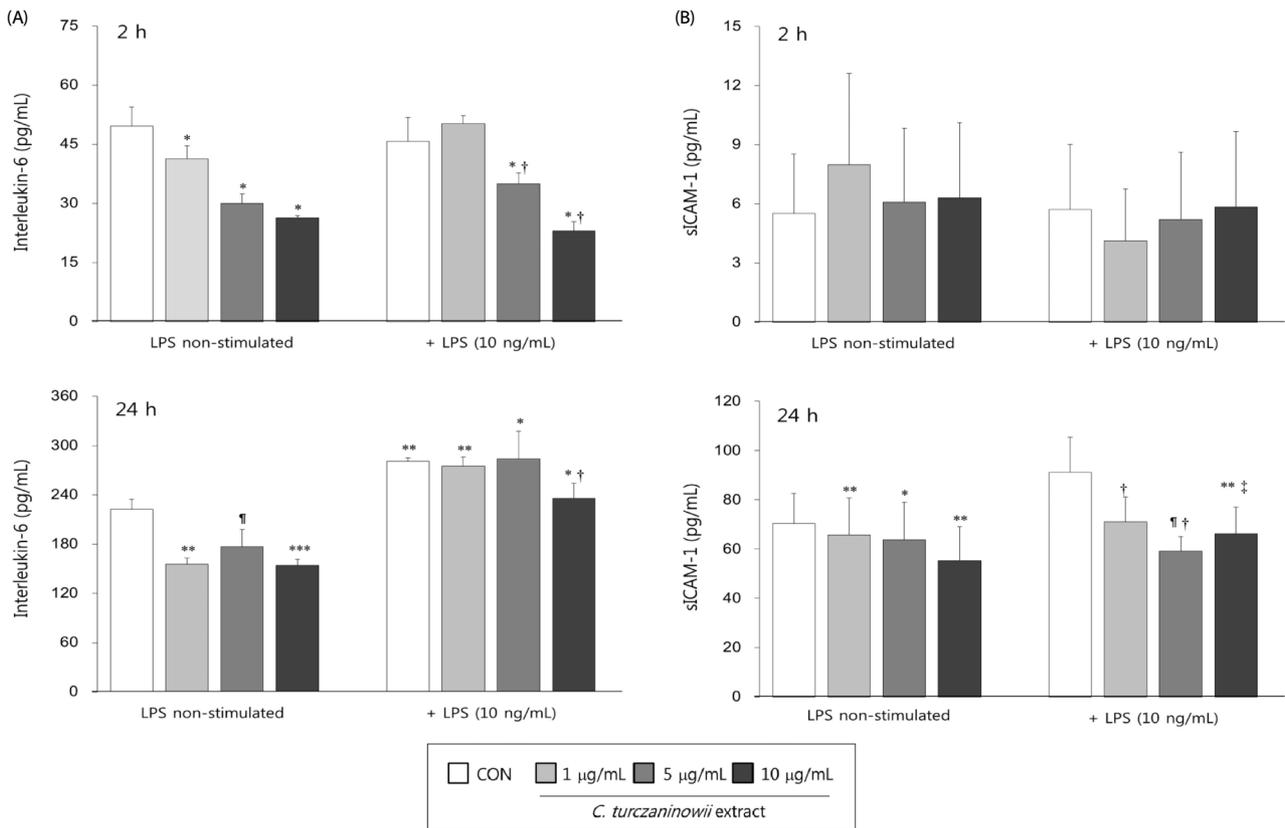


Fig. 4. Suppressive effect of *C. turczaninowii* extract on the productions of IL-6 and sICAM-1 secreted from LPS-stimulated HAoSMCs. HAoSMCs were treated with the extract 15 min after LPS stimulation or not, and then incubated for further 2 h and 24 h. The experiment includes 4 repeats per conditions. Concentrations of IL-6 (A) and sICAM-1 (B) in cell culture supernatants were measured by the ELISA assay. Data are expressed as means ± SD. † $P < 0,1$, * $P < 0,05$, ** $P < 0,01$, *** $P < 0,001$ compared to non-stimulated CON; † $P < 0,05$, †† $P < 0,001$ compared to LPS-stimulated CON; CON, non-treated control; *C. turczaninowii*, *Carpinus turczaninowii*; IL-6, interleukin-6; sICAM-1, soluble intracellular adhesion molecule-1

Suppression of IL-6 and sICAM-1 production from LPS-stimulated HAoSMCs by *C. turczaninowii* extract

Fig. 4A and 4B present the effects of *C. turczaninowii* extract on the levels of IL-6 and sICAM-1 secreted from non-stimulated (CON) and LPS-stimulated HAoSMCs. Briefly, cells were treated with varying concentrations of the extract (1, 5, and 10 µg/mL) 15 min after stimulation with (or without) LPS (10 ng/mL), and further incubated for 2 h and 24 h. The IL-6 secretion was

dose-dependently suppressed after 2 h incubation with the extract, at 1, 5 and 10 µg/mL in CON cells, and at 5 and 10 µg/mL in LPS-stimulated cells (upper panel, Fig. 4A). Similar patterns were observed after 24 h incubation with the extract (at 1, 5 and 10 µg/mL in CON cells, and at 10 µg/mL in LPS-stimulated cells) (lower panel, Fig. 4A). In addition, sICAM-1 production in both CON and LPS-stimulated cells was dose-dependently suppressed by the extracts after 24 h exposure,

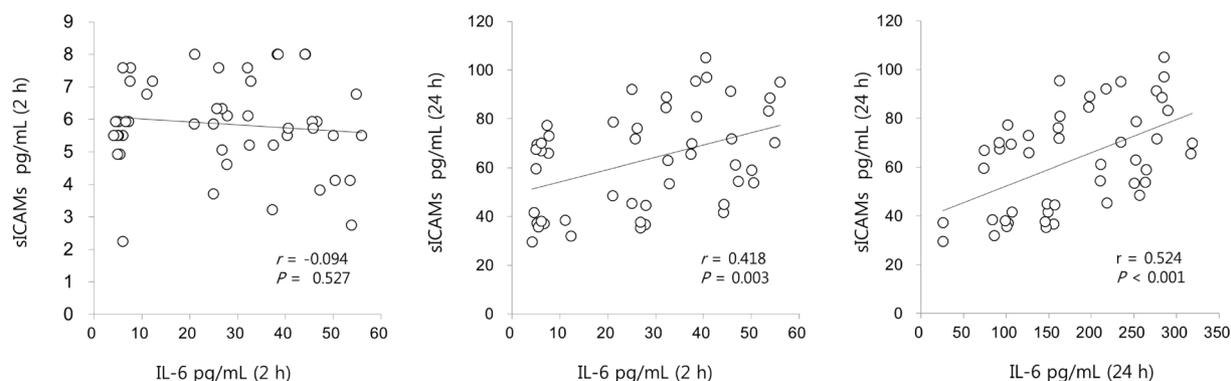


Fig. 5. Relationship between IL-6 and sICAM-1 concentrations in the cell culture supernatants from HAoSMCs. HAoSMCs were treated with *C. turczaninowii* extract (0, 1, 5 and 10 $\mu\text{g}/\text{mL}$) 15 min after LPS stimulation or not, and then incubated for further 2 h and 24 h. IL-6 and sICAM-1 concentrations in the cell culture supernatants were measured by the ELISA assay. Tested by *Pearson* correlation analysis; r = correlation coefficient. The experiment includes 4 repeats per conditions, *C. turczaninowii*, *Carpinus turczaninowii*, IL-6, interleukin-6; sICAM-1, soluble intracellular adhesion molecule-1

but not in 2 h incubation with the extract (Fig. 4B). Furthermore, TNF- α levels in CON or the LPS-stimulated cells were not detected at both 2 h and 24 h after exposure to the extract.

Relationship between IL-6 and sICAM-1 levels secreted from HAoSMCs treated with *C. turczaninowii* extract

Fig. 5 shows the relationship between sICAM-1 and IL-6 levels secreted from HAoSMCs after 2 h or 24 h incubation with the extract. After 2 h incubation, sICAM-1 levels did not significantly correlate with IL-6 levels ($r = -0.094$, $P = 0.527$). However, sICAM-1 levels after 24 h exposure to the extract were positively correlated to IL-6 levels at 2 h exposure ($r = 0.418$, $P = 0.003$) and 24 h ($r = 0.524$, $P < 0.001$).

DISCUSSION

Results from this study indicate that *C. turczaninowii* extract modulates the arterial inflammatory response in LPS-stimulated HAoSMCs, and can be a potential therapeutic agent for atherosclerosis. This is further supported by the fact that *C. turczaninowii* extract has a high amount of TPCs, and has a strong ability to scavenge the DPPH free radicals. Interestingly, the radical scavenging capacities show a time-dependent increase. Consequently, this high antioxidant capacity might be the basis of suppressing the inflammatory response in arterial cells, and thereby ameliorating the vascular function.

C. turczaninowii belongs to the Betulaceae family; it is a deciduous woody plant usually grown in the Jeju islands of Korea [19]. Very few recent studies have reported the antioxidant capacity and anti-inflammatory effects of leaves and branches of *C. turczaninowii*, thereby suggesting the potential use as a therapeutic agent for inflammatory diseases [15-17,22]. As reported in other studies [15-17,22], the current study also evaluates the antioxidant capacity of the *C. turczaninowii* extract. Oxidative stress caused by free radicals due to various intrinsic and extrinsic factors (cigarette smoking, metals, saturated fats, lipid peroxidation, glycosylated proteins, hyperglycemia, etc.) [3-7] stimulate cytokine production from immune cells in circulation and body tissues [1,2,6], consequently leading to vascular dysfunction or directly harming the vascular endothelium,

which thereby increases the risk of atherogenesis not only in the blood vessels, but also in the brain [1,4-7].

Ko *et al.* [15] reported that IL-6 production from LPS-stimulated RAW264.7 cells (1 $\mu\text{g}/\text{mL}$ LPS) was significantly reduced when treated with the extract of *C. turczaninowii* branches (50 to 500 $\mu\text{g}/\text{mL}$). They further suggested that from among the 8 compounds isolated from *C. turczaninowii* branches, carpinontriols A and B may be major contributors to higher antioxidant capacities [16]. Considering that NO is one of the inflammatory mediators, NO generation from the cells were slightly, but dose-dependently decreased, after 24 h *C. turczaninowii* extract treatment (25 to 200 $\mu\text{g}/\text{mL}$) [15]. Kang *et al.* [17] also showed dose-dependent reduction of NO production from LPS-stimulated RAW264.7 cells (1 $\mu\text{g}/\text{mL}$ of LPS) after exposure to the extract (12.5 to 100 $\mu\text{g}/\text{mL}$). In addition, in their study of NO inhibition by 260 types of Jeju plant extracts, Yang *et al.* reported about 69% inhibition of NO and 80% viability of RAW264.7 cells after exposure to 100 $\mu\text{g}/\text{mL}$ of *C. turczaninowii* extract [22].

The current study measured the mRNA levels and secreted concentrations of IL-6 and TNF- α from HAoSMCs. IL-6 and TNF- α are well-known pro-inflammatory cytokines that promptly respond to inflammatory stimuli [23]. In this study, increased productions of IL-6 from the non-stimulated cells were significantly and dose-dependently attenuated in 2 h after treatment of *C. turczaninowii* extracts, at concentrations of 1, 5 and 10 $\mu\text{g}/\text{mL}$. Interestingly, IL-6 production in the LPS-stimulated cells were also dose-dependently suppressed at 2 h after *C. turczaninowii* treatment (5 and 10 $\mu\text{g}/\text{mL}$). Furthermore, secretion of IL-6 from non-stimulated or LPS-stimulated cells were significantly suppressed at 24 h after treatment. Conversely, TNF- α levels from non-stimulated and LPS-stimulated HAoSMCs were not detected at both 2 h and 24 h after treatment, even though significant reduction of mRNA expressions of IL-6 and TNF- α were detected 6 h after exposure (1 and 10 $\mu\text{g}/\text{mL}$). These phenomena may be partly explained by the report of Schildberger *et al.* [24], which states that cytokine secretion patterns following LPS-stimulation differ according to the type of cytokine (i.e., TNF- α , IL-6, IL-8 and IL-1 β) and cells (i.e., monocytes, peripheral blood mononuclear cells (PBMCs), and

THP-1). For example, IL-6 secretion after LPS stimulation is not detected in THP-1 cells, but gradually increases in PBMCs (up to 500 pg/mL), and is gradually but promptly increased in monocytes (up to 600 pg/mL, peaks at 8 h and subsequently plateaus) for 24 h [24]. Secreted TNF- α levels were also greater in monocytes than in PBMCs and THP-1 cells. However, unlike IL-6, secreted TNF- α levels peaked at 4 h after stimulation, and subsequently decreased time- dependently, returning almost to baseline or non-detectable levels after 24 h. Furthermore, secreted TNF- α levels were not detected within 2 h after LPS stimulation [24]. Therefore, the report by Schildberger *et al.* [24] partly supports our results, where the secreted TNF- α levels were not detected at 2 h and 24 h after treatment, but mRNA levels of TNF- α were detected at 6 h post-exposure. This result indicates that the *C. turczaninowii* extract significantly suppresses the TNF- α production, together with IL-6 levels in human arterial cells. Secretion of pro-inflammatory cytokines may be mediated by nuclear factor kappaB (NF- κ B) signaling in vascular cells [25]. Although we did not directly study the activation of NF- κ B signaling in this study, our results may provide the possibility that *C. turczaninowii* extract modulates NF- κ B activation, thereby attenuating pro-inflammatory cytokine expression in human arterial cells. Further studies are required for determining the modulation effect of *C. turczaninowii* extract on NF- κ B signaling-mediated cytokine secretion in human arterial cells.

The circulating form of ICAM-1 (sICAM-1) is constitutively expressed or induced on the surface of various cells [26-29]. It is found in body fluids such as the blood stream, cerebrospinal fluids, bronchoalveolar lavage fluids and urine [30-32]. In clinical settings, elevated levels of sICAM are associated with cardiovascular risk factors (hypertension, cigarette smoking and alcohol consumption) [33-35]. Increased sICAM-1 levels are also observed in hyperlipidemic subjects (hypertriglyceridemia and hypercholesterolemia) [36,37], and are positively correlated with triglycerides, fibrinogen and tissue-type plasminogen activator antigen, which lead to the development of atherosclerosis [38,39]. Secretion of sICAM-1 is regulated by various factors including cytokines (increased by IL-6, TNF- α , IL-1, interferon- γ , angiotensin II, saturated fatty acids and alcohol; decreased by IL-10, insulin, n3-fatty acids and antioxidants) [40-45]. Although two possible mechanisms for sICAM-1 production have been considered (reflection of ICAM-1 expressed on the cell surfaces, and direct-coding by mRNA transcripts), it has yet to be clearly elucidated. In our study, sICAM-1 production from non-stimulated cells and LPS-stimulated cells were dose-dependently suppressed by the *C. turczaninowii* extract at 24 h after treatment, but no significant alterations were observed at 2 h after exposure. Based on the previous reports and our results, we believe the sICAM-1 levels may be mediated by the inflammatory cytokines (i.e., increased by IL-6, TNF- α etc.) and modulated by *C. turczaninowii* extract.

This study has a few limitations. The secreted cytokines produced from the cells were measured only at 2 h and 24 h after treatments. In addition, the mRNA expressions of cytokines were measured only in non-stimulated cells at 6 h after treatment. Therefore, further studies are required to confirm if production of cytokines and vascular function markers in the non-stimulated and LPS-stimulated cells are modulated by *C.*

turczaninowii extract between 2 h and 24 h after treatment. Furthermore, all previous studies have been performed only in RAW264.7 macrophages and not in vascular cells. Considering this, our study using human arterial cells demonstrates that *C. turczaninowii* extract has the potential to attenuate pro-inflammatory responses in human arterial conditions. However, to elucidate the anti-atherogenic effect of *C. turczaninowii* extract in the human artery, we need to undertake further studies using other parts of arterial tissue. Also, metabolite profiling of chemical compounds in the *C. turczaninowii* extract (antioxidants and anti-inflammation) is required, as well as comparison with other well-known bioactive compounds exerting anti-inflammatory effects.

Despite the study limitations, this present study indicates that *C. turczaninowii* extract has the ability to modulate the arterial inflammatory response, and is a potential therapeutic agent for atherosclerosis.

CONFLICTS OF INTERESTS

The authors declare no potential conflicts of interests.

ORCID

Youn Kyoung Son: <https://orcid.org/0000-0002-7394-2520>

So Ra Yoon: <https://orcid.org/0000-0003-2277-0594>

Woo Young Bang: <https://orcid.org/0000-0002-2162-4010>

Chang-Hwan Bae: <https://orcid.org/0000-0002-7310-3022>

Joo-Hong Yeo: <https://orcid.org/0000-0001-8132-8208>

Rimkyo Yeo: <https://orcid.org/0000-0002-5193-6672>

Juhyun An: <https://orcid.org/0000-0001-6532-2447>

Juhyun Song: <https://orcid.org/0000-0002-9165-8507>

Oh Yoen Kim: <https://orcid.org/0000-0001-9262-3309>

REFERENCES

- Hansson GK, Hermansson A. The immune system in atherosclerosis. *Nat Immunol* 2011;12:204-12.
- Wright E Jr, Scism-Bacon JL, Glass LC. Oxidative stress in type 2 diabetes: the role of fasting and postprandial glycaemia. *Int J Clin Pract* 2006;60:308-14.
- Ohnuki Y, Nagano R, Takizawa S, Takagi S, Miyata T. Advanced glycation end products in patients with cerebral infarction. *Intern Med* 2009;48:587-91.
- Lönn ME, Dennis JM, Stocker R. Actions of "antioxidants" in the protection against atherosclerosis. *Free Radic Biol Med* 2012;53: 863-84.
- Quagliaro L, Piconi L, Assaloni R, Martinelli L, Motz E, Ceriello A. Intermittent high glucose enhances apoptosis related to oxidative stress in human umbilical vein endothelial cells: the role of protein kinase C and NAD(P)H-oxidase activation. *Diabetes* 2003;52: 2795-804.
- Chae JS, Kim OY, Paik JK, Kang R, Seo WJ, Jeong TS, Sweeney G, Lee SH, Lee JH. Association of Lp-PLA(2) activity and LDL size with interleukin-6, an inflammatory cytokine and oxidized LDL, a marker of oxidative stress, in women with metabolic syndrome. *Atherosclerosis* 2011;218:499-506.
- Förstermann U, Sessa WC. Nitric oxide synthases: regulation and

- function. *Eur Heart J* 2012;33:829-37.
8. Halford JC. Obesity drugs in clinical development. *Curr Opin Investig Drugs* 2006;7:312-8.
 9. Marazza JA, Nazareno MA, de Giori GS, Garro MS. Enhancement of the antioxidant capacity of soymilk by fermentation with *Lactobacillus rhamnosus*. *J Funct Foods* 2012;4:594-601.
 10. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. *Lancet* 1993;342:1007-11.
 11. Chen Y, Wang J, Ou Y, Chen H, Xiao S, Liu G, Cao Y, Huang Q. Cellular antioxidant activities of polyphenols isolated from Eucalyptus leaves (*Eucalyptus grandis* × *Eucalyptus urophylla* GL9). *J Funct Foods* 2014;7:737-45.
 12. Zheng G, Xu L, Wu P, Xie H, Jiang Y, Chen F, Wei X. Polyphenols from longan seeds and their radical-scavenging activity. *Food Chem* 2009;116:433-6.
 13. Jin T, Kim OY, Shin MJ, Choi EY, Lee SS, Han YS, Chung JH. Fisetin up-regulates the expression of adiponectin in 3T3-L1 adipocytes via the activation of silent mating type information regulation 2 homologue 1 (SIRT1)-deacetylase and peroxisome proliferator-activated receptors (PPARs). *J Agric Food Chem* 2014;62:10468-74.
 14. Moon J, Do HJ, Kim OY, Shin MJ. Antiobesity effects of quercetin-rich onion peel extract on the differentiation of 3T3-L1 preadipocytes and the adipogenesis in high fat-fed rats. *Food Chem Toxicol* 2013;58:347-54.
 15. Ko HN, Oh TH, Baik JS, Hyun CG, Kim SS, Lee NH. Anti-inflammatory activities for the extracts and Carpinontriols from branches of *Carpinus turczaninowii*. *Int J Pharmacol* 2013;9:157-63.
 16. Ko HN, Kim JM, Bu HJ, Lee NH. Chemical constituents from the branches of *Carpinus turczaninowii* with antioxidative activities. *J Korean Chem Soc* 2013;57:520-4.
 17. Kang JM. Identification of anti-oxidative, skin whitening, anti-inflammatory and anti-bacterial constituents from the leaves of *Carpinus turczaninowii* Hance [master's thesis]. Jeju: Jeju National University; 2015.
 18. Jeon JI, Chang CS. Foliar flavonoids of genus *Carpinus* in eastern Asia primarily based on native taxa to Korea. *Korean J Plant Taxon* 2000;30:139-53.
 19. Chang CS, Chang KS. Typification of *Corylopsis coreana* (Hamamelidaceae) and *Carpinus laxiflora* var. *longispica* (Betulaceae). *Shokubutsu Kenkyu Zasshi* 2010;85:270-6.
 20. Brand-Williams W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. *Lebenson Wiss Technol* 1995;28:25-30.
 21. Deshpande SS, Cheryan M. Determination of phenolic compounds of dry beans using vanillin, redox and precipitation assays. *J Food Sci* 1987;52:332-4.
 22. Yang EJ, Yim EY, Song G, Kim GO, Hyun CG. Inhibition of nitric oxide production in lipopolysaccharide-activated RAW 264.7 macrophages by Jeju plant extracts. *Interdiscip Toxicol* 2009;2:245-9.
 23. Libby P, Ridker PM. Novel inflammatory markers of coronary risk: theory versus practice. *Circulation* 1999;100:1148-50.
 24. Schildberger A, Rossmanith E, Eichhorn T, Strassl K, Weber V. Monocytes, peripheral blood mononuclear cells, and THP-1 cells exhibit different cytokine expression patterns following stimulation with lipopolysaccharide. *Mediators Inflamm* 2013;2013:697972.
 25. Brasier AR. The nuclear factor-kappaB-interleukin-6 signalling pathway mediating vascular inflammation. *Cardiovasc Res* 2010;86:211-8.
 26. Rothlein R, Mainolfi EA, Czajkowski M, Marlin SD. A form of circulating ICAM-1 in human serum. *J Immunol* 1991;147:3788-93.
 27. Rieckmann P, Michel U, Albrecht M, Brück W, Wöckel L, Felgenhauer K. Soluble forms of intercellular adhesion molecule-1 (ICAM-1) block lymphocyte attachment to cerebral endothelial cells. *J Neuroimmunol* 1995;60:9-15.
 28. Vejlsgaard GL, Ralfkiaer E, Avnstorp C, Czajkowski M, Marlin SD, Rothlein R. Kinetics and characterization of intercellular adhesion molecule-1 (ICAM-1) expression on keratinocytes in various inflammatory skin lesions and malignant cutaneous lymphomas. *J Am Acad Dermatol* 1989;20:782-90.
 29. Smith CW, Marlin SD, Rothlein R, Toman C, Anderson DC. Cooperative interactions of LFA-1 and Mac-1 with intercellular adhesion molecule-1 in facilitating adherence and transendothelial migration of human neutrophils *in vitro*. *J Clin Invest* 1989;83:2008-17.
 30. Shijubo N, Imai K, Shigehara K, Honda Y, Koba H, Tsujisaki M, Hinoda Y, Yachi A, Ohmichi M, Hiraga Y, Abe S. Soluble intercellular adhesion molecule-1 (ICAM-1) in sera and bronchoalveolar lavage fluid of patients with idiopathic pulmonary fibrosis and pulmonary sarcoidosis. *Clin Exp Immunol* 1994;95:156-61.
 31. Tsukada N, Matsuda M, Miyagi K, Yanagisawa N. Increased levels of intercellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor receptor in the cerebrospinal fluid of patients with multiple sclerosis. *Neurology* 1993;43:2679-82.
 32. Teppo AM, von Willebrand E, Honkanen E, Ahonen J, Grönhagen-Riska C. Soluble intercellular adhesion molecule-1 (sICAM-1) after kidney transplantation: the origin and role of urinary sICAM-1? *Transplantation* 2001;71:1113-9.
 33. Bongard V, Elias A, Bal dit Sollier C, Ruidavets J, Boccalon H, Drouet L, Ferrières J. Soluble intercellular adhesion molecule-1 is associated with carotid and femoral atherosclerosis but not with intima-media thickness in a population-based sample. *Atherosclerosis* 2002;164:297-304.
 34. Blann AD, Steele C, McCollum CN. The influence of smoking on soluble adhesion molecules and endothelial cell markers. *Thromb Res* 1997;85:433-8.
 35. Rohde LE, Hennekens CH, Ridker PM. Cross-sectional study of soluble intercellular adhesion molecule-1 and cardiovascular risk factors in apparently healthy men. *Arterioscler Thromb Vasc Biol* 1999;19:1595-9.
 36. Hackman A, Abe Y, Insull W Jr, Pownall H, Smith L, Dunn K, Gotto AM Jr, Ballantyne CM. Levels of soluble cell adhesion molecules in patients with dyslipidemia. *Circulation* 1996;93:1334-8.
 37. Abe Y, El-Masri B, Kimball KT, Pownall H, Reilly CF, Osmundsen K, Smith CW, Ballantyne CM. Soluble cell adhesion molecules in hypertriglyceridemia and potential significance on monocyte adhesion. *Arterioscler Thromb Vasc Biol* 1998;18:723-31.
 38. Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble intercellular adhesion molecule 1 and risks of future myocardial infarction in apparently healthy men. *Lancet* 1998;351:88-92.
 39. Ross R. Atherosclerosis—an inflammatory disease. *N Engl J Med* 1999;340:115-26.
 40. Marino M, Scuderi F, Mazzarelli P, Mannella F, Provenzano C, Bartocioni E. Constitutive and cytokine-induced expression of MHC and intercellular adhesion molecule-1 (ICAM-1) on human myoblasts. *J Neuroimmunol* 2001;116:94-101.
 41. Ghaisas NK, Shahi CN, Foley B, Goggins M, Crean P, Kelly A, Kelleher D, Walsh M. Elevated levels of circulating soluble adhesion molecules

- in peripheral blood of patients with unstable angina. *Am J Cardiol* 1997;80:617-9.
42. Frohman EM, Frohman TC, Dustin ML, Vayuvegula B, Choi B, Gupta A, van den Noort S, Gupta S. The induction of intercellular adhesion molecule 1 (ICAM-1) expression on human fetal astrocytes by interferon-gamma, tumor necrosis factor alpha, lymphotoxin, and interleukin-1: relevance to intracerebral antigen presentation. *J Neuroimmunol* 1989;23:117-24.
43. Thomson AW, Satoh S, Nüssler AK, Tamura K, Woo J, Gavalier J, van Thiel DH. Circulating intercellular adhesion molecule-1 (ICAM-1) in autoimmune liver disease and evidence for the production of ICAM-1 by cytokine-stimulated human hepatocytes. *Clin Exp Immunol* 1994;95:83-90.
44. Chen NG, Azhar S, Abbasi F, Carantoni M, Reaven GM. The relationship between plasma glucose and insulin responses to oral glucose, LDL oxidation, and soluble intercellular adhesion molecule-1 in healthy volunteers. *Atherosclerosis* 2000;152:203-8.
45. Aljada A, Saadeh R, Assian E, Ghanim H, Dandona P. Insulin inhibits the expression of intercellular adhesion molecule-1 by human aortic endothelial cells through stimulation of nitric oxide. *J Clin Endocrinol Metab* 2000;85:2572-5.