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Anti-inflammatory Potential of *Artemisia capillaris* and Its Constituents in LPS-induced RAW264.7 Cells

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Abstract – *Artemisia capillaris* has been widely used as an alternative therapy for treating obesity and atopic dermatitis. It has been used as a hepatoprotactant. It is also used for ameliorating inflammatory reactions. Although there are several investigations on other *Artemisia* species, there is no systematic study describing the role of *A. capillaris* MeOH extract, its solvent soluble fractions, or derived anti-inflammatory principal components in regulating inflammatory conditions. Therefore, the objective of this study was to elucidate anti-inflammatory mechanisms of *A. capillaris*. Results revealed that MeOH extract of *A. capillaris* could decrease LPS-stimulated NO secretion. Of tested fractions, CH_2Cl_2, EtOAc, and *n*-BuOH strongly inhibited NO release from RAW264.7 cells. Bioactive mediators derived from CH_2Cl_2 and *n*-BuOH fractions elicited potent anti-inflammatory actions and strikingly abrogated LPS-triggered NO accumulation in RAW264.7 cells. Of particular interest, capillin and isoscopoletin possessed the most potent NO suppressive effects. Western blot analysis validated the molecular mechanism of NO inhibition and showed that capillin and isoscopoletin significantly down-regulated iNOS and COX-2 protein expression. Taken together, our results provide the first evidence that MeOH extract, CH_2Cl_2, EtOAc, and *n*-BuOH fractions from *A. capillaris* and its derived lead candidates can potently suppress inflammatory responses in macrophages by hampering NO release and down-regulating iNOS and COX-2 signaling.
Keywords – *Artemisia capillaris*, Inflammation, iNOS, COX-2, Coumarin, Flavonoids

Introduction

Experimental studies have gathered growing evidence that inflammation is the cornerstone in the evolution of many multifaceted diseases and disorders, including metabolic syndromes, cancer, autoimmune, neurodegenerative, and cardiovascular diseases.[1] Macrophage-derived nitric oxide (NO) is an essential intercellular messenger molecule that has been recognized as one of the most versatile player in the pathogenesis of inflammation. In the inflammatory state, activated macrophages secrete a variety of effector molecules including prionflammatory cytokines and NO. NO is believed to induce vasodilatation in cardiovascular system. Furthermore, cytokine-activated macrophages can release NO in high concentrations as immune responses. In addition, NO is a potent neurotransmitter at neuron synapses. It contributes to the regulation of apoptosis. NO is involved in the pathogenesis of inflammatory disorders of the joint, gut, and lungs.[2] Exposure to inflammatory stimuli such as lipopolysaccharide (LPS) causes activation of macrophages and the release of NO. Activated macrophages secrete various proinflammatory mediators, including cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) which in turn will result in accumulation of cellular NO that contributes to inflammation.[3,4] With the onset of inflammation, signaling of iNOS and COX-2 is also activated. The emergence of iNOS and COX-2 appears to represent crucial effector of inflammatory responses. Numerous experimental models have been designed to mimick inflammatory reactions and provide opportunities for testing new pharmacological components. Research focus has been shifted toward the use of natural products as possible anti-inflammatory agents or the development of targeted hit leads from medicinal plants. Accordingly, this study focused on pharmaceutical and therapeutic use of *Artemisia capillaris* for treatment of inflammation.

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A. capillaris has been frequently used in the treatment of liver diseases, including hepatitis, jaundice, fatty liver, and bilious disorder. Infusions of buds, stems, and leaves of *A. capillaris* have been used in traditional Chinese medicine (TCM) primarily as a choleric, anti-inflammatory, antipyretic, and diuretic agent for treating epidemic hepatitis.[5] A wide variety of pharmacological and biological activities of *A. capillaris* have been reported, including antioxidant,[6,7] cytoprotective,[6] hepatoprotective,[8,9] antimicrobial,[10] anticancer,[11] anti-obesity,[12] and choleretic effects.[13] Ethyl acetate fraction of *A. capillaris* has shown antioxidative and cytoprotective effects by scavenging ROS with protective effect against oxidative DNA damage.[6] In additions, ethyl acetate fraction of *A. capillaris* can induce cytoprotective effects against oxidative stress-induced apoptosis in V79 cells by enhancing antioxidative activity.[12] Phytochemical analysis of the genus *Artemisia* has revealed that it embraces diverse classes of novel compounds such as terpenoids, flavonoids, coumarins, caffeoylquinic acids, sterols, and acetylenes. Coumarins isolated from *A. capillaris* has demonstrated inhibitory potential against advanced glycation end product formation.[14] Aerial parts of *A. capillaryis* and its constituents can suppress 5-lipoxygenase and skin inflammation in rat basophilic leukemia-1 (RBL-1) and male ICR mice.[15] *A. capillaris* and its derived constituents can strongly inhibit α-glucosidase and protein tyrosine phosphatase 1B (PTP1B) activities.[16] Therefore, multiple investigations have ascertained the therapeutic potential of *A. capillaris*. However, there is no systematic study that explores the anti-inflammatory therapeutic potential of *A. capillaris* extract, its fractions, or its constituents responsible for its anti-inflammatory effects. Therefore, the objective of the current study was to identify lead anti-inflammatory mediators from *A. capillaris* through bioguided extraction and fractionation.

Experimental

Chemicals and Reagents – LPS from *Escherichia coli*, Griess reagent, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT), ethylene diamine tetraacetic acid (EDTA), 2-amino-5,6-dihydro-6-methyl-4H-1,3-thiazine hydrochloride (AMT), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), fetal bovine serum (FBS), and antibiotics were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA), and Dulbecco's Modified Eagle's Medium (DMEM) from Hyclone (Logan, Utah, USA). Various primary antibodies (iNOS, COX-2, and β-actin) were obtained from Cell Signaling Technology, Inc. (Beverly, MA, USA). Polyvinylidene fluoride (PVDF) membrane (Immobilon-P) was obtained from Millipore Co. (Billerica, MA, USA). Unless otherwise indicated, all chemicals were purchased from Sigma-Aldrich Co. (St Louis, MO).

Plant Materials – Whole plants of *A. capillaris* were collected from Cheongsong, Kyungpuk Province, Korea, in August 2009 and were authenticated by Prof. Je-Hyun Lee, College of Oriental Medicine, Dongguk University, Gyeongju, Korea. A voucher specimen (20093008) was deposited in the laboratory of one of the authors (J. S. Choi) for future reference.

Extraction, Fractionation and Isolation of Compounds from *A. capillaris* – The extraction, fractionation and isolation of compounds from *A. capillaris* were carried out as previously described.[14,16] Isolated compounds were identified by spectroscopic methods, including ^{1}H- and ^{13}C-NMR, as well as by comparison with published spectral data and TLC analysis. The structures of compounds are shown in Fig. 1.

Cell Culture – RAW 264.7 macrophage cells (ATCC, Rockville, MD, USA) were cultured at 37 ºC in DMEM supplemented with 10% FBS in a humidified atmosphere with 5% CO_2. Medium was changed every 24 h.

Cell Viability in RAW264.7 Cells – The macrophage RAW264.7 cell line was incubated in Dulbecco's modified Eagle's medium (DMEM) at 37 ºC under 5% CO_2 humidified air. The cells were seeded onto a 96-well plate at a density of 1.0×10^4 cells per well and incubated at 37 ºC for 24 h. The cells were then treated with various concentrations of test samples. After an additional 24 h of incubation at 37 ºC, 100 μL of MTT (0.5 mg/mL in PBS) was added to the wells and mixed well. The resulting color was assayed at 570 nm using a microplate reader (VERSA max Molecular Devices).

RAW264.7 Cell Culture and Measurement of NO Concentration – RAW264.7 cell culture and measurement of NO concentration RAW264.7 cells obtained from the American type culture collection (ATCC) were cultured with DMEM supplemented with 10% FBS and 1% antibiotics in a 5% CO_2 atmosphere at 37 ºC. The cells were activated with LPS (1 μg/mL). Briefly, the cells were plated in 96-well plates (2×10^5 cells/well). After pre-incubation for 2 h, test compounds and LPS (1 μg/mL) were added and incubated for 18 h unless otherwise specified. The test compounds dissolved in DMSO were diluted to appropriate concentrations with serum-free DMEM. The final concentration of DMSO was adjusted to 0.1%. To assess NO production, the stable conversion product of NO was measured using Griess reagent and the optical density was determined at 540 nm.

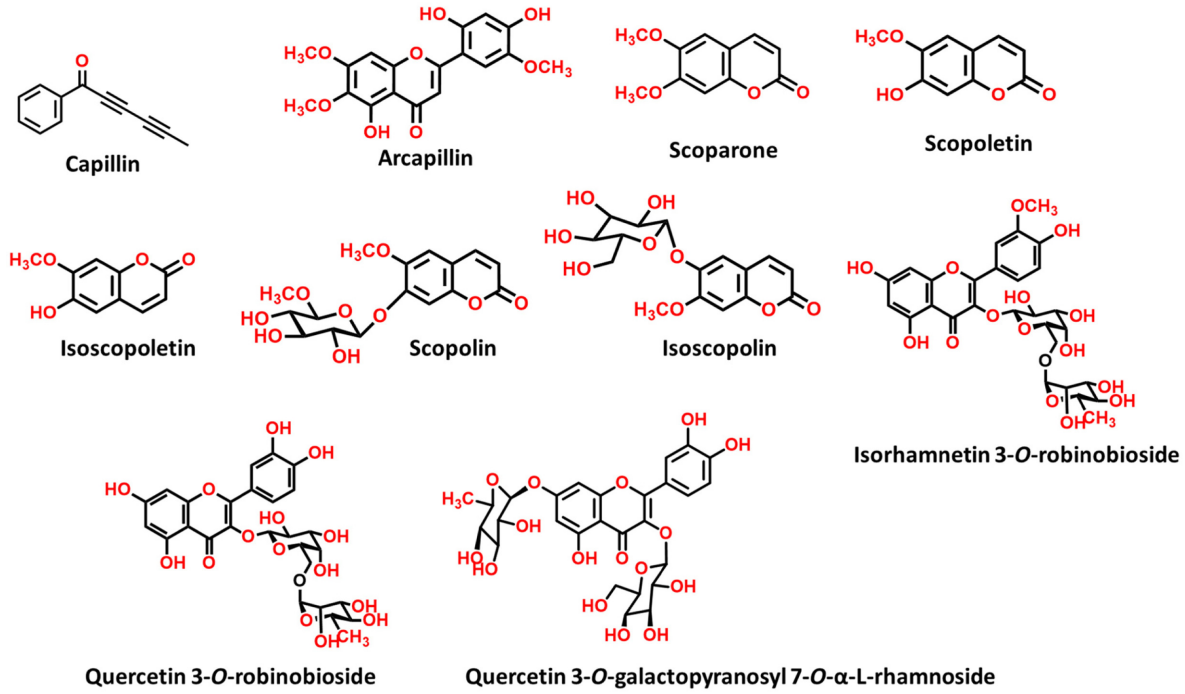

Fig. 1. Structures of compounds isolated from *Artemisia capillaris.*

Analysis for Inhibition of iNOS and COX-2 Protein Expression – Western blotting was used to measure the protein levels of iNOS and COX-2. RAW264.7 cells were cultured in 100 mm culture dishes in the presence or absence of LPS (1.0 μg/mL) and with/without test compounds for 18 h. Afterwards, the cells were washed twice with ice-cold PBS and lysed with buffer on ice for 30 min. Cell extracts were obtained by centrifugation at 14,000 × g and 4 °C for 20 min. Cytosolic proteins were electrophoretically separated by SDS-PAGE and transferred onto PVDF membranes. The membranes were immediately blocked with 5% (w/v) non-fat dry milk in Tris-buffered saline containing 0.1% Tween-20 (pH 7.4) (TBST) buffer at room temperature for 1 h. The membranes were washed three times (10 min) in TBST buffer and incubated with primary antibody, diluted 1:1000 in 5% (w/v) non-fat dry milk in TBST buffer, at 4 °C overnight. After three washings in TBST buffer (10 min), the membranes were incubated with horseradish peroxidase-conjugated secondary antibody, diluted 1:2000 in 5% (w/v) non-fat dry milk in TBST buffer, at room temperature for 1 h. After three washings in TBST buffer (10 min), the antibody was visualized using Supersignal West Pico Chemiluminescent Substrate (Pierce, Rockford, IL, USA), according to the manufacturer's instructions and exposed to an X-ray film (GE Healthcare Ltd., Amersham, UK). Pre-stained blue protein markers were used for molecular weight determination.

Statistical Analysis – The results were analyzed by one-way analysis of variance (ANOVA) followed by Dunnet's post tests for multiple comparisons. Differences were considered significant at $p < 0.05$. Results of anti-inflammatory effects were expressed as respective mean ± standard deviation (STDEV) in triplicate experiments.

Result

Effect of MeOH Extract of *A. capillaris* on LPS-induced NO Production – To determine whether *A. capillaris* MeOH extract could suppress LPS-induced NO production, RAW264.7 cells were pretreated with indicated concentrations (2.5, 5, 10, 20, and 40 μg/mL) of MeOH extract at 2 h prior to induction by LPS (1 μg/mL). As shown in Fig. 2, LPS induction abruptly generated nitrite in culture media up to a level of 100% compared to non-treated control cells. However, exposure of RAW264.7 cells to different concentrations of MeOH extract significantly overturned such increase of NO production mediated by LPS in a dose-dependent manner. Relative NO suppression rates achieved by treatment with MeOH extract at indicated concentrations of 2.5, 5, 10, 20, and 40 μg/mL were 8.85%, 18.14%, 37.17%, 40.54%, and 53.47%, respectively. There were no significant difference in NO suppression rate between 10 and 20 μg/mL of

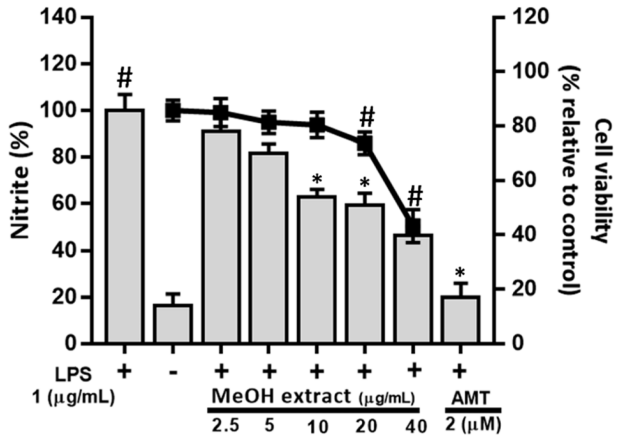

Fig. 2. Effects of MeOH extract on cell viability and LPS-induced nitrite formation in RAW264.7 cells. Cell viability was measured by MTT assay.

MeOH extract, although concentration at 20 μg/mL exhibited slight toxicity. Exposure of RAW264.7 cells to 40 μg/mL resulted in 53.47% of NO inhibition. It might be due to cytotoxic effect of MeOH extract at this concentration. Therefore, NO inhibition at 40 μg/mL might not be due to its anti-inflammatory action, but due to its cytotoxic effect. Cell survival rate at this concentration was observed to be close to 50%. As a positive control inhibited NO production by about 80% at 2 μM.

Effect of MeOH Extract of *A. capillaris* on Cell Viability – To determine whether the anti-inflammatory effect of MeOH extract of *A. capillaris* was due to its ability to scavenge nitrite production, cytotoxicity of methanolic extract was measured using MTT assay. Murine macrophage RAW264.7 cells were treated with *A. capillaris* MeOH extract at different concentrations (2.5, 5, 10, 20, and 40 μg/mL) and incubated for 24 h. As illustrated in Fig. 2, MeOH extract at concentration up to 10 μg/mL did not show any significant cytotoxic effects, with cell survival rate of more than 90%, comparable to that of normal control. However, MeOH extract at 20 μg/mL exerted a slight cytotoxicity. MeOH extract at 40 μg/mL showed obvious cytotoxic effects, with cell death rate of 50%.

Effect of Solvent Soluble Fractions of *A. capillaris* MeOH Extract on LPS-induced NO Production and Cell Viability – Since MeOH extract of *A. capillaris* demonstrated promising NO inhibitory activity, it was sequentially partitioned with different organic solvents to yield CH_2Cl_2, EtOAc, *n*-BuOH, and H_2O fractions. These fractions were successively screened for their NO suppressing abilities in LPS-activated RAW264.7 cells. After treatment with LPS for 18 h, NO level was significantly increased to 15.27 ± 0.70 μM compared to normal control group (0.57 ± 0.06 μM). Among obtained fractions, CH_2Cl_2 fraction inhibited LPS-stimulated NO production at 10, 50, and 100 μg/mL, with inhibition rates of 43.73%, 61.75%, and 55.93%, respectively. There was no significant difference in NO inhibition rate between concentrations of 50 and 100 μg/mL (Fig. 3). There was a slight increase in cell viability at 100 μg/mL compared to that at 50 μg/mL. However, the difference between the two was not statistically significant. In case of EtOAc fraction, tested concentrations at 10, 50, and 100 μg/mL profoundly inhibited NO production, with average inhibition rates of 21.66%, 53.78%, and 56.15%, respectively (Fig. 3). Unexpectedly, concentration at 100 μg/mL did not show significant

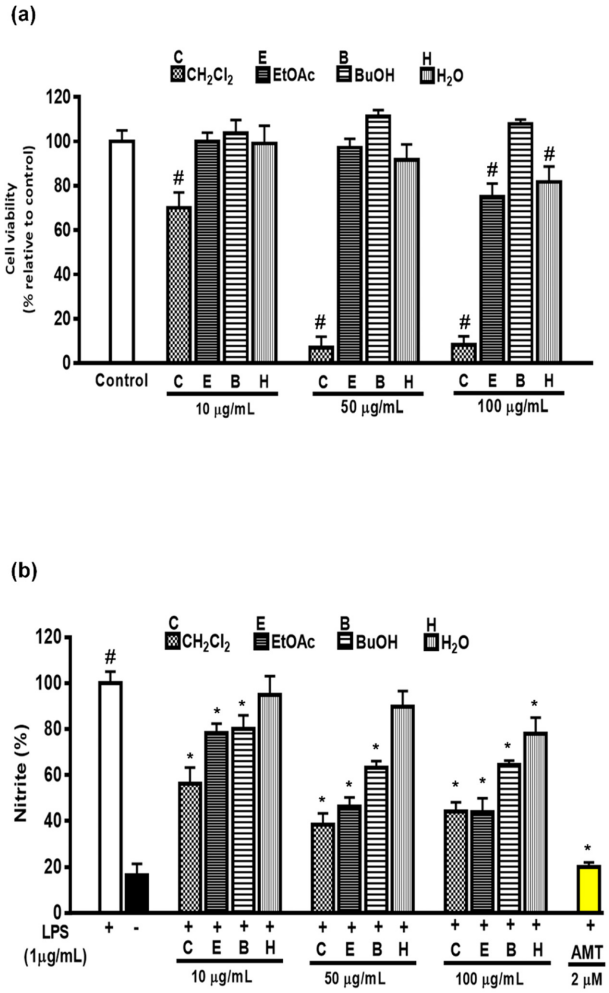

Fig. 3. Effects of solvent soluble fractions of MeOH extract from *Artemisia capillaris* on cell viability (a) and LPS-induced nitrite formation (b) in RAW264.7 cells. Cells were pre-treated with different concentrations (10, 50 and 100 μg/mL) of CH_2Cl_2 (C), EtOAc (E), BuOH (B), or H_2O (H) fraction of MeOH extract from *Artemisia capillaris* followed by treatment with LPS (1 μg/mL) and incubation for 18 h.

difference in NO suppression compared to concentration at 50 μg/mL. Such result might be due to cell toxicity at 100 μg/mL. Regarding *n*-BuOH fraction, at concentrations of 10, 50, and 100 μg/mL, it strongly decreased LPS-induced NO production at 20%, 37%, and 36%, respectively (Fig. 3). However, aqueous fraction of MeOH extract failed to potently attenuate LPS-triggered NO production at concentrations of 10, 50, or 100 μg/mL, with NO suppression rates of 5%, 10%, and 23%, respectively (Fig. 3). After treatment with aqueous fraction at concentrations of 10, 50, or 100 μg/mL, cell viabilities were 100%, 90%, and 20%, respectively (Fig. 3). As a positive control, AMT (2 μM) inhibited LPS-induced NO production by ~80%.

Protective Effects of Compounds Isolated from *A. capillaris* against LPS-induced NO Production and Structure Activity Relationship – Bioactivity guided fractionation revealed that CH_2Cl_2, EtOAc, and *n*-BuOH fractions could strongly suppress LPS-induced NO accumulation. Therefore, systematic chromatographic techniques were used to isolate scopoletin, isoscopoletin, capillin, scoparone, and arcapillin from CH_2Cl_2 fraction and scopolin, isoscopolin, quercetin 3-*O*-robinobioside (Q3R), isorhmanetin 3-*O*-robinobioside (IR3R), and quercetin 3-*O*-galactopyranosyl 7-*O*-α-L-rhamnoside (QGR) from *n*-BuOH fraction. Anti-inflammatory activities of isolated compounds from both fractions were further assessed based on their suppressive effects on inflammatory mediators released from macrophages upon exposure to LPS (1 μg/mL). As shown in Fig. 4, stimulation of cells with LPS induced a dramatic increase in NO production from the basal level. However, pre-treatment with most of these compounds decreased inflammatory reactions by hampering NO release. Compound capillin possessed the most potent NO inhibitory effects even at low concentrations of 0.062, 0.125, 0.25, 0.5 μM, with NO suppression rates of 20%, 23%, 65%, and 83.36%, respectively. Scopoletin also demonstrated NO inhibitory effects, with NO suppression rates of 5% and 35% at 50 and 100 μM, respectively. Although scopoletin at higher concentration (100 μM) showed promising NO inhibition effect, it failed to inhibit NO accumulation at lower concentrations (12.5 or 25 μM). Scopoletin at 100 μM displayed minute level of cell toxicity, although it was non-toxic at other concentrations (12.5 - 50 μM). On the other hand, isoscopoletin profoundly hindered NO release in a dose-dependent manner. NO inhibition rates by isoscopoletin at concentrations of 12.5, 25, 50, and 100 μM were 12%, 24%, 46%, 70%, respectively. Isoscopoletin at 100 μM showed minor cell toxicity, although it was non-toxic at concentrations of 12.5, 25, or 50 μM. Although scopolin, isoscopolin, or scoparone was not toxic to RAW264.7 cells, they could not profoundly scavenge NO release at concentrations of 12.5 to 100 μM. AMT, a positive control, inhibited NO accumulation by ~80%. Arcapillin isolated from CH_2Cl_2 fraction significantly prevented NO release at 12.5 μM, with inhibition rate of 38%. At concentrations of 1.56, 3.12, 6.25 μM, NO inhibition rates were 18%, 23%, and 28%, respectively. It showed no cytotoxic effects at tested concentrations (1.56,

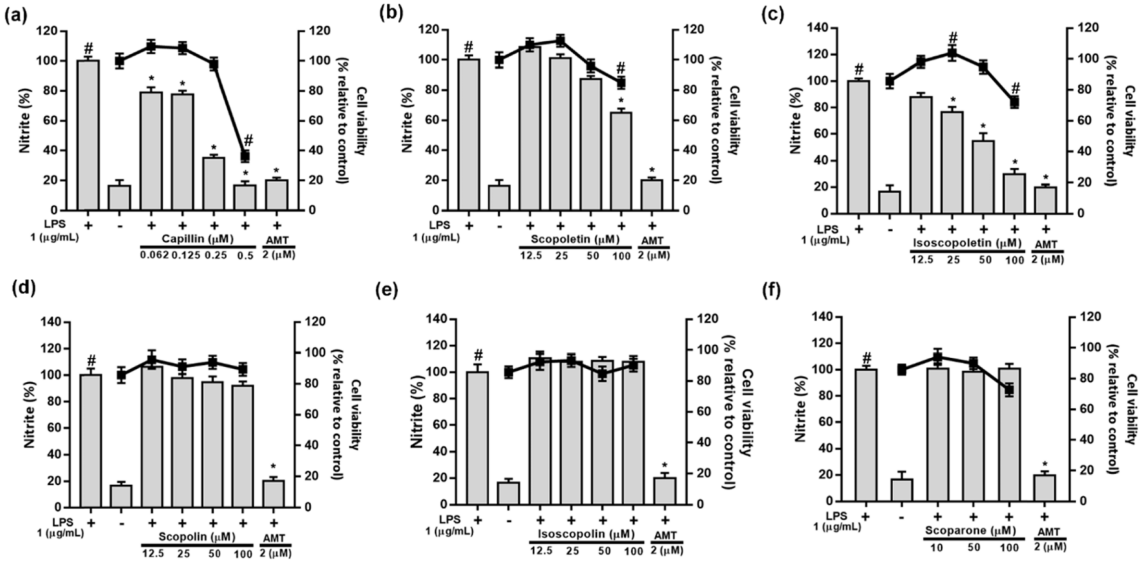

Fig. 4. Effects of capillin (a), scopoletin (b), isoscopoletin (c), scopolin (d), isoscopolin (e), or scoparone (f) isolated from *Artemisia capillaris* on cell viability and LPS-induced NO production in RAW264.7 cells.

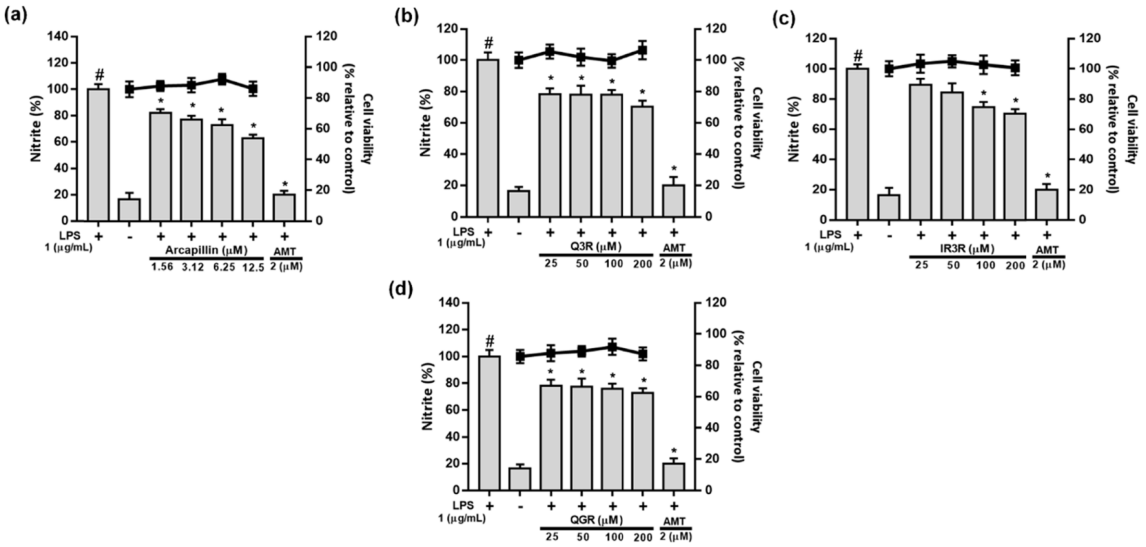

Fig. 5. Effects of arcapillin (a), quercetin 3-*O*-robinobioside (Q3R) (b), isorhmanetin 3-*O*-robinobioside (IR3R) (c), or quercetin 3-*O*-galactopyranosyl 7-*O*-α-L-rhamnoside (QGR) (d) isolated from *Artemisia capillaris* on cell viability and LPS-induced NO production in RAW264.7 cells.

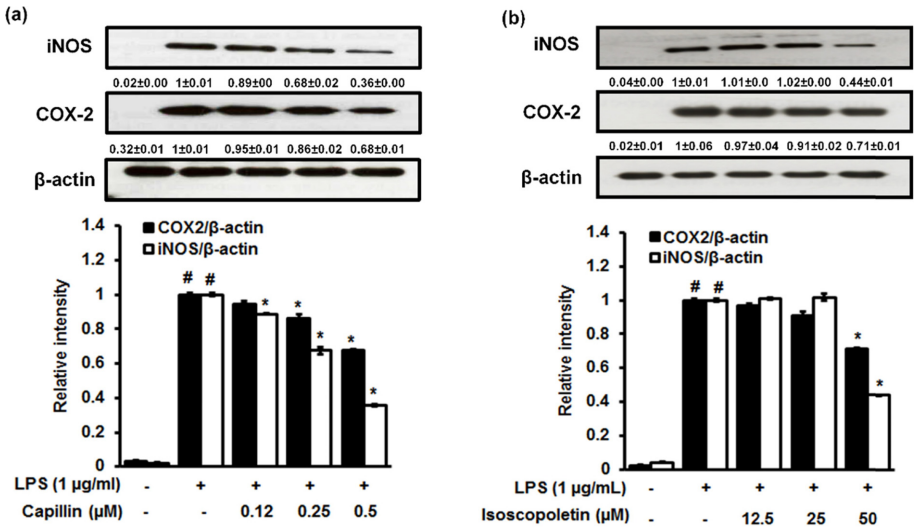

Fig. 6. Effects of capillin (a) or isoscopoletin (b) on the production of iNOS and COX-2 protein in RAW264.7 cells.

3.12, 6.25, 12.5 μM) (Fig. 5). Glucosides (Q3R, IR3R, QGR) demonstrated moderate inhibitory effects on LPS-activated NO accumulation at higher concentrations (25, 50, 100, 200 μM). However, these glucosides were cytotoxic at high concentrations (Fig. 5).

Effects of Compounds Isolated from *A. capillaris* on LPS-induced iNOS and COX-2 Expression in RAW264.7 Cells – Inflammatory markers such as iNOS and COX-2 play mighty roles in the progression of inflammation. To determine whether the inhibition of NO production in RAW264.7 cells was associated with down-regulation of iNOS protein expression, Western blot analysis was performed. As illustrated in Fig. 6, iNOS and COX-2 expression was nearly undetectable in unstimulated cells. In contrast, iNOS and COX-2 band intensities were very high in LPS-treated cells with NO generation. Pretreatment with isoscopoletin or capillin effectively down-regulated iNOS and COX-2 protein expression in a dose response manner. Interestingly, such effect of capillin was more pronounced than that of isoscopoletin because capillin at lower concentrations (0.12 - 0.5 μM) showed apparent iNOS and COX-2 down-regulating effects.

Discussion

Most inflammatory reactions are mediated by cytokines, chemokinesnitric, and interleukins.[12] These mediators from different sources can influence various reactions within the inflammatory process. Among these mediators, production of excess NO is widely accepted as the principal source of inflammatory reactions. Productions of iNOS and NO are positively associated with the pathogenesis of inflammatory and autoimmune diseases.[17] Transcriptional expression of iNOS can be induced by cytokines, microbial products, and radiation. The production and activity of iNOS can be induced in adipocytes by endotoxins and cytokines, leading to significant elevation of NO.[18] Over-expression of NO in obesity appears to contribute to impairment of muscle cell insulin sensitivity, β-cell dysfunction, and suppression of adipocyte differentiation.[19,20] In addition to iNOS, COX-2 is frequently produced during inflammatory reactions.[21] COX-2 expression is induced as pro-inflammatory mediator during an innate immune response.[22] Studies in animal models have shown that pharmacological inhibition or genetic deletions of COX-2 can lead to suppression of tumor growth.[23] Furthermore, inhibition of COX-2 could affect the production of inflammatory mediators.[24]

Narcotic, steroidal, and non-steroidal anti-inflammatory drugs (NSAIDs) are currently treatment options against inflammation. However, these available drugs might have adverse effects, including hypertension due to analogy of steroidal drugs to the steroid hormones. Non-steroidal drugs have also posed adverse effects that include gastrointestinal bleeding and improper clotting of blood.[25] Therefore, the ultimate goal would be to obtain anti-inflammatory agents that are effective with minimal to no adverse effects even when they are administered over prolonged period. Plant derived natural products have proved to be the most reliable source of new therapeutic agents. These phytochemicals could serve as prototypes to develop more effective and less toxic medicines. The low toxicity of plant derived anti-inflammatories is attributed to biological compounds that are present in nature. Novel anti-inflammatory agents from plants have potential to have multiple targets as a phytoconstituent or a mixture. As mixtures, several phytochemicals might be present and act on more than one target at a time which could result in faster and more efficient anti-inflammatory activity. Hence, the role of medicinal plants in the treatment and management as well as possible prevention of inflammation has attracted great attention.

Artemisia, a widespread and diverse genus in the family of Asteraceae, comprises more than 500 species. It has been used traditionally used as anti-pyretic, anti-diabetic, anti-hypertensive, anti-hepatotoxic, anti-inflammatory, eczema, jaundice, and anti-hemorrhoid. Owing to high number of species with ecological and economic importance, the genus *Artemisia* has been investigated extensively, focusing on its diverse biological activities.[26] However, anti-inflammatory therapeutic potential of *Artemisia* species, particularly *A. capillaris* extract, its fractions, or its anti-inflammatory responsible constituents, has not been explored yet. Therefore, the current study aimed to identify lead anti-inflammatory candidates from *A. capillaris* through bioguided extraction and fractionation. Bioactivity guided extraction revealed that MeOH extract significantly overturned the increased NO production mediated by LPS. In addition, CH_2Cl_2 fractions strongly decreased LPS-stimulated NO production, with inhibition rates of 43.73% - 55.93%. However, such inhibition was closely related to the cytotoxic effect of CH_2Cl_2 fraction. EtOAc fraction was found to be very pragmatic against NO generation. It profoundly inhibited NO release. In addition, *n*-BuOH fraction decreased LPS-activated NO secretion ina dose-dependent manner. Interestingly, these obvious NO suppressive effects by *n*-BuOH fraction were not attributed to its cytotoxic aspects, but due to its protective potential based on cell viability data. Previous reports have also suggested anti-inflammatory potential of *A. capillaris*. Ethanol extract of *A. capillaris* has exerted anti-inflammatory effect by affecting mRNA expression levels of typical inflammatory cytokines (IL-6, IL1β) and COX-2 in LPS-stimulated RAW264.7 cells. Additionally, this extract can inhibit NO production via downregulating iNOS transcription and expression levels of p38 and ERK proteins in LPS-stimulated RAW264.7 cells.[27] It has been reported that *A. capillaris* extract obtained by response surface methodology exerts marked anti-inammatory activity at cellular level.[28] Coumarins from *A. capillaris* and *Angelica decursiva* also exhibit anti-Alzheimer's disease potential.[29] Moreover, tablets of *A. capillaris* have potential antiviral activity.[30] *A. iyawomogi* and *A. capillaris*, referred to as 'Hanjin' and 'Injin', respectively, have been used in traditional medicinal system in China, Korea, and Japan for treatment of dieresis and inflammation related diseases. *A. capillaris* extract can protect beta cells against cytokine-induced beta-cell damage by suppressing NF-kappaB activation.[31] Infusions of buds, stems, and leaves of *A. capillaris* have been used in TCM primarily as a choleric, anti-inflammatory, antipyretic, and diuretic agent for treating epidemic hepatitis.[5] *A. iyawomogi* and *A. capillaris* have been used

in traditional medicinal system in China, Korea, and Japan for the treatment of dieresis and inflammation related diseases. It has been reported that 70% ethanol extract of aerial parts of *A. capillaris* shows potent inhibitory activity against 5-lipoxigenase-catalyzed leukotriene production by ionophore-induced rat basophilic leukemia-1 cells.[15] Hong and Lee[12] have reported that ethyl acetate fraction of *A. capillaris* exhibits excellent protective effect by strengthening the antioxidant defense system, reducing the generation of reactive oxygen species, and damaging oxidative substances in the liver of high-fat diet induced obese mice. Chloroform extract of *A. capillaris* has anticarcinogenic activity against 7,12-dimethylbenzene anthracene (DMBA)-induced mouse epidermal carcinogenesis.[32] MeOH extract of *A. maritime* (L.) displays hepatoprotective activity against acetaminophen and CCl_4-induced hepatic damage.[33] Benli et al.[34] have shown that MeOH, chloroform, and acetone extracts of *A. dracunculus* display antimicrobial activity against nine bacterial and four yeast isolates. Another study has revealed that MeOH extract and chloroform fraction of A. dracunculus can suppress platelet aggregation to laminin coated wells by 50% and 60%, respectively.[35] Extract of *A. campestris* (L.) can scavenge radicals generation and protected liver against CCl4-induced toxicity.[36]

Anti-inflammatory activities of isolated compounds were assessed for their suppressive effects on inflammatory mediators released from macrophages upon exposure to LPS (1 μg/mL). Capillin particularly exhibited strong inhibition effect on NO release from RAW264.7 cells induced by LPS even at lower concentrations. Moreover, capillin was able to effectively defend RAW264.7 cells against LPS-stimulated NO without inducing cytotoxic effects at concentration up to 0.25 μM. Effects of polyacetylene compounds such as capillin, capillene, capillinol, *cis*-dehydromatricaria ester, and transdehydromatricaria ester on *Tribolium castaneum* have been investigated. Results showed that these crude essential oil derived polyacetylenes possessed fair repellent and fumigant activities.[37] Capillin and capillinol from *A. capillaris* have shown promising anti-diabetic effects.[38] Capillin, a component of the essential oil isolated from *A. capillaris*, has exhibited antimicrobial activity against *Trichophyton mentagrophytes*, *Pyricularia oryzae*, *Candida albicans*, *Bacillus subtilis*, *E. coli*, and *Cochliobolus miyabeanus*.[39] Capillin can potently inhibit growth of human leukemia HL-60 cells. Cytotoxic effects of capillin in cells were found to be associated with apoptosis. Capillin induced apoptosis in HL-60 cells via the mitochondrial apoptotic pathway which might be controlled through JNK signaling.[40] In the present study, scopoletin exhibited NO inhibitory effects at higher concentration (100 μM), showing promising NO inhibition. In addition, scopoletin displayed minute cell toxicity at 100 μM. On the other hand, isoscopoletin profoundly blocked NO release in a dose-dependent manner. Interestingly, isoscopoletin also exhibited slight cell toxicity at 100 μM. In parallel, isoscopoletin was found to be more active than scopoletin to counteract NO release from RAW264.7 cells. Although scopolin, isoscopolin, or scoparone was not toxic to RAW264.7 cells, neither of them could profoundly scavenge NO release at tested concentrations (12.5 - 100 μM). Arcapillin significantly prevented NO release at higher does. At other concentrations, it moderately inhibited NO release. Flavonoid glycosides (Q3R, IR3R, QGR) demonstrated moderate inhibitory effects in response to LPS-activated NO accumulation at higher concentrations. However, they showed cell toxicity at such concentrations. Subsequently, the current study validated that flavonoids, coumarins, and polyacetylenes exhibited profound anti-inflammatory properties by blocking NO release from LPS-activated RAW264.7 cells. Arcapillin has shown promising advanced glycation end product formation, showing $IC_{50} = 77.84 \pm 1.07$ μm compared to aminoguanidine, a positive control, with $IC_{50} = 932.66 \pm 4.94$. Isorhamnetin 3-*O*-robinobioside (IR3R) has been evaluated for its ability to induce apoptosis in human lymphoblastoid cells (TK6). The apoptosis was confirmed by DNA fragmentation and poly (ADP-ribose) polymerase (PARP) cleavage, indicating the release of caspase-3 level. Results indicated that the IR3R had great anti-proliferative effect on human lymphoblastoid (TK6) cells which might be due to their involvement in the apoptotic pathway.[41] Furthermore, IR3R can protect against lipid peroxidation induced by H_2O_2. Similarly, other reports have validated that flavonoids and a coumarin (6,7-dimethylesculetin) isolated from buds of *A. capillaris* possess significant antihepatotoxic activity by decreasing carbon tetrachloride-induced liver lesions both *in vivo* and *in vitro*.[42] Structure activity relationship in the present study revealed some interesting facts. Among tested compounds (scopoletin, isoscopoletin, scopolin, isoscopolin, and scoparone), both methoxy and OH groups played important role in hampering NO accumulation. The presence of methoxy group at C-6 and OH group at C-7 seemed to decrease the inhibitory potential of scopoletin. However, alternation of methoxy and OH groups at subsequent C-6 and C-7 positions evidently enhanced the activity of isoscopoletin compared to scopoletin. Although glycosylation significantly hindered the inhibitory potential

as noticed in the case of scopolin and isoscopolin and denied the role of hydorxy and glucose moiety substitution at either C-6 or C-7 positions toward NO inhibition. The presence of additional OCH_3 group in scoparone also diminished its activity. Hence, there was no significant NO inhibition by scoparone even at higher concentration.

Targeting inflammatory mediators has been a means of therapy for inflammatory conditions. Selective inhibition of the expression and activity of iNOS and COX-2 is the current medical intervention strategy for inflammatory conditions. It could result in the reduction of inflammatory response. Therefore, inhibition of iNOS and COX-2 would be ideal to relieve inflammation and possibly prevent disease. Inhibitors could play another role by quenching free radicals to attenuate chronic inflammation. Pretreatment with isoscopoletin or capillin markedly down-regulated iNOS and COX-2 expression in a dose response manner. In particular, capillin was more effective than isoscopoletin because capillin at lower doses (0.12 - 0.5 μM) manifested apparent iNOS and COX-2 down-regulatory effects. Taking together, results of the current investigation provided evidence for the first time that MeOH extract and CH_2Cl_2, EtOAc, *n*-BuOH fractions from *A. capillaris* and its derived mediators could strongly block inflammatory reactions in RAW264.7 macrophages. These effects might be by hampering NO signaling. Of particular interest, capillin from CH_2Cl_2 fraction and isoscopoletin from *n*-BuOH fraction strongly blocked NO accumulation. Furthermore, Western blotting analysis showed that NO suppression was likely associated with regulation of iNOS and COX-2 expression, indicating therapeutic potential of *A. capillaris* for the development of anti-inflammatory lead candidates. These molecules further down-regulated iNOS and COX-2 protein expressions known to play a vital role in the release of NO in response to LPS. Clearly, there is a tremendous need to explore therapeutic and preventive potential of *A. capillaris* with the aim to design new clinical treatment strategies for inflammation. The present investigation provides the first evidence of pharmacological perspectives of *A. capillaris* against inflammation. It might harbor lead anti-inflammatory candidates to prevent inflammation.

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