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Immunomodulation potential of *Artemisia capillaries* extract in rat splenocytes

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Abstract

Theextract of *Artemisia capillaris*has been used as a traditional medicine for hepatitis or bilious disorder, and some recent studies have revealed its antimicrobial, antioxidant, antiobesity, anticarcinogenic, antioxidant, and anti-inflammatory potential. The current study was designed to evaluate the potential immunomodulatory effects of *A. capillaris*methanol extract on quiescent- and concanavalin A (Con A)-stimulated rat splenocytes. Proliferation of splenocytes was enhanced in response to the Con A mitogen but decreased significantly following *A. capillaris* extract treatment compared to that in the control and the Con A only treatment. *A. capillaris* extract at 10 µg/mL significantly reduced Con A-induced proliferation, and the expression of cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (iNOS), and proinflammatory cytokines as tumor necrosis factor-alpha (*TNF-*) and interleukin-6 (*IL-6*). Another proinflammatory cytokine, interferon-gamma (*IFN-γ*), was not significantly altered by either Con A or *A. capillaris* extract treatments when compared with the control. iNOS expression was largely blocked, whereas COX-2 expression was significantly down-regulated by*A. capillaris* extract treatment. This study was carried out to investigate the potential immunomodulatory activities of*A. capillaris* extract in a Con A induced immune condition to explore its potential pharmacological activity.

Keywords: *Artemisia capillaris*, immunomodulation, cyclooxygenase-2, inducible nitric oxide synthase, cytokine

Introduction

Many plant extracts are traditionally used to treat various diseases. Considerable attention has been focused on the beneficial effects of various plant extracts because of their limited side effects and the wide array of prospective biomedical usages. According to the World Health Organization, > 65% of the global population relies on medicinal plants for primary health care[1]. *Artemisia capillaries* (*A. capillaris*) is a bushy perennial shrub found in East Asia, including China, Korea, Japan, Taiwan, and the Philippines[2]. It has been used as a traditional medicine for jaundice, hepatitis, and bilious disorder. Its profound hepatoprotective function in chemical-induced liver injury has been revealed in some recent studies [3]. Some other studies have shown that *A. capillaris* possesses anticarcinogenic [4], antiobesity [5], antioxidant, antimicrobial [6], and anti-inflammatory properties [3]. *A. capillaris* has essential oils and aromatic components, including capelin, 6,7-dimethylaescletin, benzaldehyde, pinene, myrcene, cineole, 2-pyrrolidinone, camphor, thujone, 1-acethlpiperidine, caryophyllene, coumarine, and farnesol [7]; as well as saccharides such as glucose, xylose, manose and ramnose [8]; and flavonoids such as artemisidin, atemicapins, and coumarins [9].

The immune system plays a pivotal role in protecting the host organism from environmental insult through a network of cellular responses, cytokines, and humoral factors. Many plant extracts are involved in the immunomodulatory activities of the immune system, and, thus, play a key role in immune suppression. However, there is a lack of evidence on modulation of the immune response by*A. capillaris* extract. We used concanavalin A (Con A)-stimulated rat splenocytes as a model in this study. Our first objective was to determine the effect of*A. capillaris* extract on the proliferation of rat splenocytes in response to Con A. Our second objective was to determine whether*A. capillaris* extract exerts any effect on the expression of immune-related cytokines and enzymes in response to Con A. Taken together, our data suggest a direct inhibitory effect of *A. capillaris* extract on Con A induced immune function.

Materials and methods

Preparation of the *A.capillaris* extract

A. capilllaris was purchased from Kwangmyungdang Medicinal Herbs Co. (Ulsan, Korea). One hundred grams of *A.capillaris* was added to a 2 L vessel containing 1 L methanol. The mixture was heated at 64.9 C for 8 h, and the extract was filtered by 100 µm filter paper. Subsequently, the crude filtrate was evaporated and concentrated under vacuum (VacuubrandGmbhand Co. KG, Wertheim, Germany). As preparation for further analysis, the extract was reconstituted in 0.1% dimethylsulfoxide in phosphate

buffered saline (PBS), and filtered through a 0.45 µM syringe filter (Millex® Syringe Filter Units, Millipore, Milford, MA, USA) for use.

Animals

Male Sprague–Dawley rats (weight, 190–220 g) were purchased from Nara Biotech Ltd. (Pyeongtaek-si, Korea). They were housed in solid bottom cages, maintained on a 12 h light-dark cycle, and fed standard rodent chow(Purina Rodent Chow, Purina Co., Seoul, Korea) and water *ad libitum* throughout the experiment. This study followed the guidelines of the U.S. National Institutes of Health, and was approved by the Soonchunhyang University Animal Care Committee.

Preparation of primary splenocytes

Rats were sacrificed by cervical dislocation, and their spleens were aseptically collected, gently dissected, and immersed in RPMI 1640 culture media supplemented with 10% (v/v) heat-inactivated fetal bovine serum and 1% (v/v) antibiotic/antimycotic cocktail (100 U/mL penicillin, 100 µg/mL streptomycin, and 0.25 µg/mL amphotericin B; Invitrogen, Carlsbad, CA, USA). A single cell suspension was prepared by passing the cells through a cell strainer (BD Biosciences, Durham, NC, USA). The resulting cell suspension was briefly exposed to lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA, and pH 7.3) to exclude erythrocytes. The cells were washed once, and cultured at 10^6 cells/mL by counting with a hemocytometer, and cell viability was checked using the trypanblue (Sigma-Aldrich, St. Louis, MO) dye exclusion technique.

Cell proliferation assay

Splenocyte proliferation wasassessedby the MTS assay, which is based on the conversion of tetrazolium salt into a colored, aqueous soluble formazan crystal by mitochondrial dehydrogenase enzyme activity in viable cells. The assay was performed according to a protocol described previously [10]. Briefly, isolated splenocytes were treated with 5 µg/mL Con A (Sigma), and/or 10 µg/mL *A. capillaris* extract for 24 h at 37 C in a 5% CO_2 incubator. The detection reagents were composed of a 20:1 ratio of MTS and phenazinemethosulfate (Sigma). The reagent mixture was prepared immediately before being added to the cell culture at a 1:5 ratio (reagent mixture: cell culture), and absorbance was measured at 492 nm using a Victor X3 multilabel reader (Perkin Elmer, Waltham, MA, USA).

Analysis of gene expression by quantitative reverse transcription-polymerase chain reaction (qRT-PCR)

Total RNA was isolated with an RNeasy Mini kit (Qiagen, Valencia, CA), and concentration and purity were evaluated with an Agilent 2100 Bioanalyzer(Agilent Technologies Korea Ltd, Seoul, Korea). RNA (1 µg) was reverse transcribed into complementary DNA using a Random Primer Premix kit (Maxime RT Premix Kit, Intron Biotechnology, Daejeon, Korea) in a Veriti® 96-Well Thermal Cycler (Applied Biosystems, Carlsbad, CA, USA). qRT-PCR was performed using specific primers (Table 1), and an iQ™ SYBR Green Supermix kit (Bio-Rad, Hercules, CA, USA) in the CFX96 Real-Time PCR Detection System (Bio-Rad). The reactions were carried out in the following order in triplicate: 95 C for 5 min followed by 40 cycles of 95 C for 10 s, 42 C for 10 s, and 72 C for 20 s. The assays were normalized to GAPDH as an endogenous control.

Table 1. The primer pair sequences used for *IL-6*, *TNF-* , *IFN-γ*, and *GAPDH* gene expression.

Gene List	Primers
IL-6	5′-AGAGACTTCCAGCCAGTTGC-3′ 5′-AGCCTCCGACTTGTGAACTG-3′
TNF-	5′-ACTCCCAGAAAAGCAAGCAA-3′ 5′-CGAGCAGGAATGAGAAGAGG-3′
IFN-γ	5′-TTTTGCAGCTCTGCCTCATG-3′ 5′-CTGTGGGTTGTTCACCTCGA-3′
GAPDH	5′-GGTGGACCTCATGGCCTACA-3′ 5′-GCAACTGAGGGCCTCTCTCT-3′

Western blot analysis

Following desired treatments, cells were washed with PBS, and lysed with RIPA lysis buffer containing a protease inhibitor cocktail (Santa Cruz Biotechnology, Santa Cruz, CA, USA). Total proteins were extracted following centrifugation and quantified with a BCA Protein Assay kit (Pierce, Rockford, IL, USA). An equal amount (30–40 µg) of protein was separated on 4–20% sodium dodecyl sulfate polyacrylamide gradient gels (Mini-PROTEAN TGX Precast Gel, Bio-rad) at 100 V for 1.30 h and transferred onto a PVDF membrane (Trans-Blot SD Semi-Dry Cell, Bio-Rad). Following transfer, the membranes were blocked with 5% skim milk in Tris-buffered saline containing 0.1% Tween-20 for 1 h at room temperature. The membranes were then incubated overnight at 4 C with primary antibodies for cyclooxygenase-2 (COX-2) (Santa Cruz Biotechnology), inducible nitric oxide synthase (iNOS) (Santa Cruz Biotechnology), and β-actin (Abcam, Cambridge, MA, USA). A second incubation was performed for 1.30 h with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology). Protein bands were visualized using Enhanced Chemiluminescence (ECL) Western Blotting Detection reagents (Bio-Rad) in a ChemiDoc Imaging system (ChemiDoc XRS+ System with Image Lab Software, Bio-Rad).

Statistical analysis

Experiments were repeated two or three times, and the differences between groups were analyzed by Student's t-test with a p-value < 0.05 considered significant.

Results

A. capillaris extract reducedCon A-induced splenocyte proliferation

Splenocyte proliferation following treatment with *A. capillaris* extract and Con A was evaluated with the MTS assay. *A. capillaris* itself did not enhance proliferation of splenocytes under basal conditions when compared to that in the control. The *A. capillaris* extract (10μg/mL) reduced splenocyte proliferation by about 20% in comparison with 5 μg/mL Con A following a 24 h exposure. As expected, 5 μg/mL Con A increased splenocyte proliferation (Figure. 1). Increasing *A. capillaris* extract concentration (50 and 100 μg/mL) with/without Con A inducedsplenocyteproliferation (data not shown).

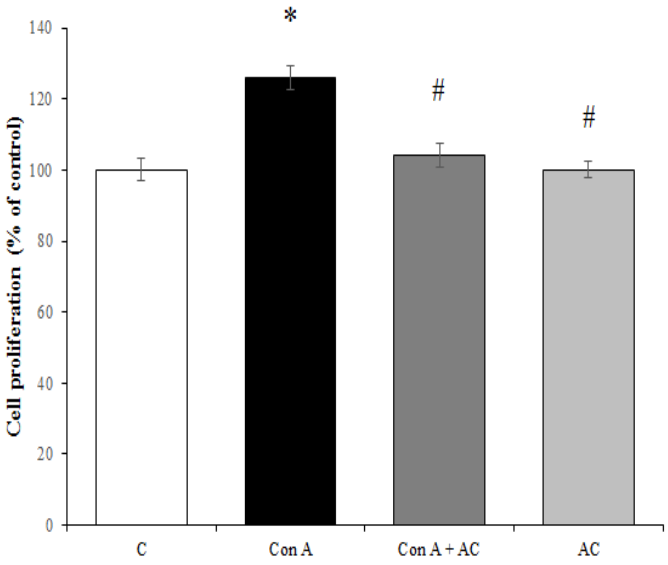

Figure. 1 Cell proliferation assay of rat splenocytes exposed to 5 μg/mL of Con A and/or 10μg/mL of the *A.capillaris* extract for 24 h by MTS assay. Con A, concanavalin A; AC, *A. capillaris* extract. Asterisk (*) denotes a significant difference relative to control (p<0.05) and the pound sign (#) denotes a significant difference relative to Con A treatment (p<0.05).

A.capillaris extract modulated interlukin-6 (*IL-6*), tumor necrosis factor- (*TNF-*), and interferon-γ (*IFN-γ*) expression

The pro-inflammatory cytokines *IL-6*, *TNF-*, and *IFN-γ* play a pivotal role in inflammation, immune modulation, and lymphocyte activation. Various flavonoids have potential to modulate the expression and activation of cytokines. Therefore, we observed *IL-6*, *TNF-*, and *IFN-γ* expression at the gene level by qRT-PCR analysis. At 12 and 24 h time points, *IL-6* and *TNF-* expression increased significantly with 5 μg/mL Con A treatment. Interestingly, co-treatment with 5 μg/mL Con A and 10 μg/mL *A. capillaris*extract significantly decreased the elevated expression of *IL-6*and *TNF-* by 5 μg/mL Con A alone treatment at both 12 and 24 h time points. *IL-6* and *TNF-* expression remained at basal level with 10 μg/mL *A. capillaris* extract treatment at both time points (Figure. 2A, 2B). *IFN-γ* expression was induced slightly with 5 μg/mL Con A and/or 10 μg/mL of*A. capillaris* extract treatment at both time points but the expression was not very significant comparative to that of control (Figure. 2C).

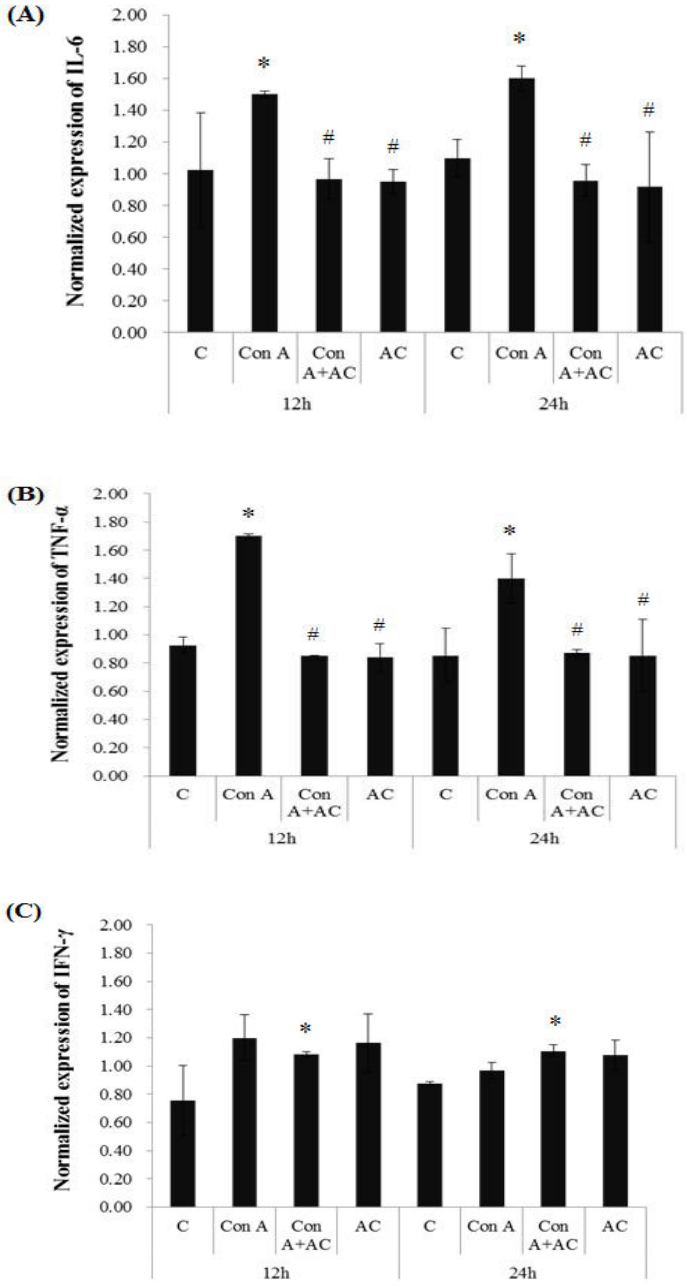

Figure. 2 Modulated expression of interleukin-6 (*IL-6*) (A), tumor necrosis factor- (*TNF-*) (B), and interferon-γ (*IFN-γ*) (C) as detected by quantitative reverse transcription-polymerase chain reaction following treatment of splenocytes with 5 μg/mL concanavalin A (Con A) and/or 10 μg/mL of *A.capillaris* extract for 12 and 24 h. Asterisk (*) denotes significant difference relative to control (p<0.05) and the pound sign (#) denotes a significant difference relative to Con A treatment (p<0.05).

A. capillaris extract down-regulated Con A-induced COX-2 and iNOS expression

The COX-2 and iNOS enzymes are involved ininflammation. To evaluate the effect of the *A. capillaris* extract on their expression, we treated splenocytes with Con A and/or 10 µg/mL *A. capillaris* extract for 12 and 24 h. Con A treatment induced the expression of both proteins, which were significantly suppressed by treatment with*A. capillaris* extract at both 12 and 24h time points. Interestingly,*A. capillaris* extract treatment alone significantly blocked or reduced iNOS, and COX-2 expression in comparison with the control. These results indicate that*A. capillaris* extract had distinct effects on the expression of the inflammatory proteins,COX-2 and iNOS (Figure. 3).

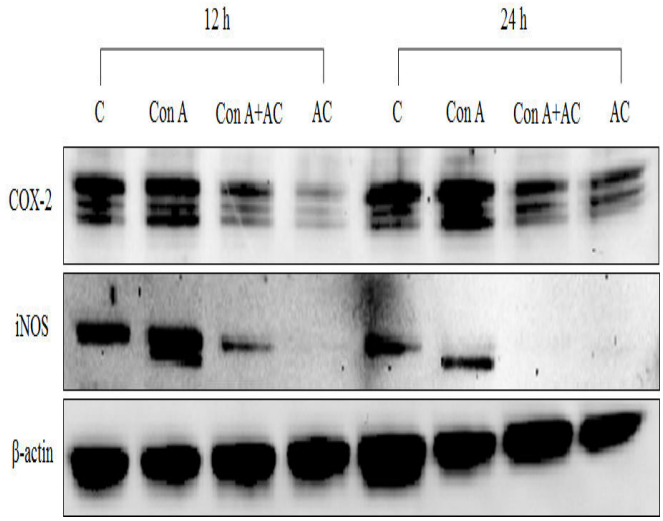

Figure. 3 Cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) expression were examined by Western blot analysis following treatment of rat splenocytes with 5 µg/mL of Con A and/or 10 µg/mL of *A.capillaris* extract for 12 and 24 h. The housekeeping gene β-actin was used as an endogenous control.

Discussion

The study was undertaken to demonstrate whether*A. capillaris* extract has immunomodulatoryactivities when treated with Con A mitogen in rat splenocytes. Our results showed that*A. capillaris* extract had distinct effects on splenocyte proliferation, and expression of immune components. We observed that splenocyteproliferation in response toCon A decreased after adding*A. capillaris* extract in comparison with cultures that did not receive the *A. capillaris* extract. *A. capillaris* is comprised of flavonoids such as capillarisin and quercetin, flavonoid glycosides such as hyperoside, phenolic compounds such as chlorogenic acid, and coumarins such as esculetin and scoparone [11, 12, 13]. These active components might be involved in the antioxidative, anti-inflammatory, and hepatoprotective activities of the *A. capillaris* extract. It was reported that ethanol-induced TNF- , and IL-1 secretion decreased in response to *A. capillaris* extract treatment in Hep G2 cells, and subsequently, the cytotoxic effects are controlled [14]. IL-6 is an important cytokine involved in cell growth and inflammation, and exerts its effects during initiation, and maintenance of the acute inflammatory response [15, 16]. Con A increases the serum IL-6 levels associated with liver cell injury in mice [17], which coincided with our data showing that 5 µg/mL Con A up-regulated *IL-6* expression at both 12 and 24 h. According to our data, 10 µg/mL of the *A. capillaris* extract significantly reduced the up-regulated expression of *IL-6* by Con A at both time points. Another study showed that the reduction in Con A-induced liver cell damage by ME3738 is associated with lower IL-6 expression [18]. TNF- has both growth stimulatory and growth inhibitory effects and is involved in systemic inflammation and acute phase reactions [19, 20]. TNF- is triggered at the early time (2 h) following Con A treatment but declines with time [18]. Our data showed that longer exposure of Con A for 24 h also induced *TNF-* expression in splenocytes, although *TNF-* expression was comparatively reduced at 24 h than that of 12 h time point. Treatment of splenocytes with*A. capillaris* extract significantly reduced the up-regulated expression of *TNF-* by Con A. Blocking of liver injury induced by Con A is suppressed by anti-TNF antibody, which leads to lower IL-6 expression [17]. The pleiotropic cytokine IFN-γ is involved in cellular resistance to viral infections and also affects cell growth [21]. According to our data,*IFN-γ* expression was slightly induced following Con A treatment, but*A. capillaris* extract alone or with Con A treatment did not show any significant change in*IFN-γ* expression when compared with that of the Con A treatment alone.

COX-2 is involved in inflammation, as evidenced by relief of inflammation and pain by blocking COX-2 with pharmacological inhibitors [22]. Con A treatment increased COX-2 levels in splenocytes, as expected, but they were dramatically reduced by the *A. capillaris* extract alone or with Con A treatment. iNOS, induced by TNF- and IFN-γ, generates NO, which plays an important role in inflammation [23, 24, 25, 26, 27]. iNOS mRNA was significantly induced by Con A in mice within 3–12 h of treatment [28]. Our data showed induction of iNOS following Con A treatment at 12 h but not at 24 h compared to that in the control. But, the *A. capillaris* extract alone or with Con A greatly reduced iNOS expressionat both time points.

The *A. capillaris* extract had striking inhibitory effects on the expression of inflammation-related cytokines and enzymes that might be associated with cytotoxic/proapoptotic mechanisms, as the cytokine surge is partly dependent on cell proliferation. Our results showed that a lower concentration of*A. capillaris* extract considerably suppressed Con A-induced proliferation of rat splenocytes. A reduction in lymphocyte proliferation following mitogen treatment has been reported by some studies that used several flavonoid derivatives. Here, we used splenocytes, which are a mixed cell population, and, thus, the effect on other cell types such as monocytes cannot be ruled out. Some additional experiments are required to address such issues as the cell separation technique and/or alternative stimuli. Although our results did not completely elucidate the effect of *A. capillaris* extract on immunomodulation, we suggest that this extract is a potential anti-inflammatory candidate. Further detailed studies are required

to explore the potential effects of an *A. capillaris* extract on immunomodulation of diseases.

Conclusion

Our study demonstrated some immunomodulatory properties of the *A. capillaris* extract against mitogen, Con A. However, the mechanism of action remains to be elucidated. In summary, our data showed that *A. capillaris* extract could inhibit Con A-induced splenocyte proliferation and expression of some immune molecules. Our study propose that *A. capillaris* has anti-inflammatory properties that might be considered as a functional remedy to control inflammation although further in-detail study is required to form a definite conclusion.

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