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Suppression of Primary Splenocyte Proliferation by *Artemisia capillaris* and Its Components

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The host immune system is the first line of host defense, consisting mainly of innate and adaptive immunity. Immunity must be maintained, orchestrated, and harmonized, since overactivation of immune responses can lead to inflammation and autoimmune diseases, while immune deficiency can lead to infectious diseases. We investigated the regulation of innate and adaptive immune cell activation by *Artemisia capillaris* and its components (ursolic acid, hyperoside, scopoletin, and scopolin). Macrophage phagocytic activity was determined using fluorescently labeled *Escherichia coli*, as an indicator of innate immune activation. Concanavalin A (ConA)- and lipopolysaccharide (LPS)-induced splenocyte proliferation was analyzed as surrogate markers for cellular and humoral adaptive immunity, respectively. Neither *A. capillaris* water extract (WAC) nor ethanol extract (EAC) greatly inhibited macrophage phagocytic activity. In contrast, WAC suppressed ConA- and LPS-induced proliferation of primary mouse splenocytes in a dose-dependent manner. Similarly, EAC inhibited ConA- and LPS-induced splenocyte proliferation. Oral administration of WAC in mice decreased ConA- and LPS-induced splenocyte proliferation, while that of EAC suppressed LPS-induced splenocyte proliferation. Repeated administration of WAC in mice inhibited ConA- and LPS-induced splenocyte proliferation. Ursolic acid, scopoletin, and scopolin reduced ConA- and LPS-induced primary mouse splenocyte proliferation, while hyperoside did not show such activity. These results indicate that *A. capillaris* and its components, ursolic acid, scopoletin, and scopolin, suppress ConA- and LPS-induced adaptive immune cell activation. The results suggest that *A. capillaris* is useful as a regulator of adaptive immunity for diseases involving excessive immune response activation.

Key words: Immunosuppression, Innate immunity, Adaptive immunity, Natural product, Immune therapy

INTRODUCTION

Innate immune responses are the first line of defense against antigen invasion and include phagocytic activity and antigen presentation, which guide and determine the adaptive immune response to antigen challenge. Host-pathogen interactions are typically initiated through host recognition of conserved molecular structures, known as pathogen-associated molecular patterns (PAMPs), which are essential for the pathogen life cycle. However, these PAMPs are deficient or compartmentalized in the host cell and are detected by the host's germline-encoded pattern recognition receptors (PRRs), which are expressed on the surface of innate immune cells, such as dendritic cells, macrophages, and neutrophils (1). Effective PAMP detection facilitates pathogen eradication by rapidly inducing host immune responses through the activation of complex signaling pathways to induce cytokine and chemokine-mediated inflammatory responses (2,3). A characteristic of adaptive immunity is the induction of a target effector response in two steps using antigen-specific receptors in T and B cells. First, antigens are presented to and recognized by antigen-specific T or B

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Abbreviations: ConA, Concanavalin A; LPS, lipopolysaccharide; WAC, *Artemisia capillaris* water extract; EAC, *Artemisia capillaris* water ethanol extract; PAMPs, pathogen-associated molecular patterns; PRRs, pattern recognition receptors; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MTT, methylthiazol tetrazolium.

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cells, leading to cell priming, activation, and differentiation, which usually occur in specific lymphoid tissue environments. Secondly, the activated T cells leave the lymphatic tissue at the infected site and return to the disease site, or the effector reaction occurs due to antibody release from activated B cells (plasma cells) into the blood and tissue fluid (4). Overactivation of the immune system can lead to inflammation and autoimmune disease. On the contrary, when the immune system is suppressed, it becomes vulnerable to infection and tumor development. It is therefore important to maintain an immune balance.

Artemisia capillaris is an herbal plant well-known for its various beneficial properties. It has been widely used as a hepatoprotective, analgesic, and antipyretic agent (5). Many studies have shown that *A. capillaris* has various biological activities, such as hypoglycemic (6), hypolipidemic (7), anti-inflammatory (8), and anti-carcinogenic (9) effects. However, its immune-modulatory properties have not been fully examined. Therefore, we investigated whether a water or ethanol extract of *A. capillaris* and its components had immunosuppressive effects by modulating innate and adaptive immune cell activation using *in vitro* and *ex vivo* models. Our results may provide important information regarding immune balance regulation when consuming natural plant sources.

MATERIALS AND METHODS

Preparation of A. capillaris Thunb extracts. Whole *A. capillaris* plants were purchased from a local retailer and authenticated by Prof. J. H. Lee (Dongguk University, Kyeongju, Korea). The voucher specimen (No. 20090920) was deposited at the laboratory of Prof. J. S. Choi, a co-author of this study. Whole plants were dried and ground into powder. The dried powder (each 100.0 g) was then separately refluxed with 70% (v/v) aqueous ethanol and water for 3 hr (2 × 0.5 L) at 95°C. The total filtrate was then concentrated to dryness *in vacuo* at 40°C to yield a 70% ethanol extract (EAC, 18.0 g, yield: 18.0%) and a water extract (WAC, 22.0 g, yield: 22.0%), respectively, which were then used in the pharmacological study.

Animals and cell culture. Male Balb/c mice, 7 weeks old, were obtained from Orientbio Inc (Seongnam, Gyeonggi, Korea). Animals were housed in groups of 5 per cage under standard animal housing conditions (23°C, 12 hr/12 hr light/dark cycle, light on at 08:00) in the animal facility at the Catholic University of Korea. Mice were allowed to acclimatize for at least 1 week before experiments. Cell culture procedures were performed as previously described (10). Briefly, primary splenocytes isolated from mice and a murine monocytic cell line (RAW264.7; ATCC TIB-71, American Type Culture Collection, Manassas, VA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. Cells were maintained in a humidified atmosphere under 5% CO_2 at 37°C.

Phagocytosis assays. This was performed as described previously (11). RAW264.7 cells were plated in 96-well plates at 1×10^5 cells/well and incubated overnight. To quantify phagocytosis, cells were incubated with fluorescently-labeled *Escherichia coli* (K-12) BioParticles (Life Technologies Inc., Grand Island, NY, USA). Engulfed particles were enumerated after 1 hr by measuring fluorescence emission per well at 480 nm.

Splenocyte proliferation assay. This was performed as described previously (11). Primary splenocytes were isolated from Balb/c mouse spleens. Cells were seeded in 96-well plates at 1×10^4 cells/well in complete DMEM. After stimulation with concanavalin A (ConA, 5 μg/mL) or lipopolysaccharide (LPS, 10 μg/mL), methylthiazol tetrazolium (MTT) assays (Sigma-Aldrich, St. Louis, MO, USA) were performed. MTT formazan product was determined using a microplate reader at an absorbance of 560 nm (Molecular Devices, San Francisco, CA, USA).

Histological assessment. Spleens were isolated from mice and fixed in 10% buffered formalin solution, dehydrated in ethanol, embedded in paraffin wax, sectioned, and stained with hematoxylin and eosin.

Statistical analyses. Data are presented as mean ± SEM for the indicated numbers. Data comparisons between groups were examined using a student's *t*-test (significant when $p < 0.05$).

RESULTS

A. capillaris does not suppress macrophage phagocytic activity. To investigate the effects of *A. capillaris* on innate immunity, WAC and EAC were prepared and their effects on macrophage phagocytic activity were determined as a representative indicator of innate immune response. Phagocytic activity was determined by the uptake of fluorescently-labeled *E. coli* in a mouse monocytic cell line (RAW 264.7). WAC treatment resulted in a slight decrease in phagocytic activity, by 20% maximum (Fig. 1A). EAC treatment reduced phagocytic activity to a similar degree (Fig. 1B). These results indicate that *A. capillaris* extracts do not significantly affect macrophage activity.

A. capillaris suppresses ConA- and LPS-induced proliferation of primary mouse splenocytes. Next, we investigated the effect of *A. capillaris* on the activation of adaptive immune cells, such as T and B lymphocytes. ConA is known as a T lymphocyte division promoter whereas LPS is a B

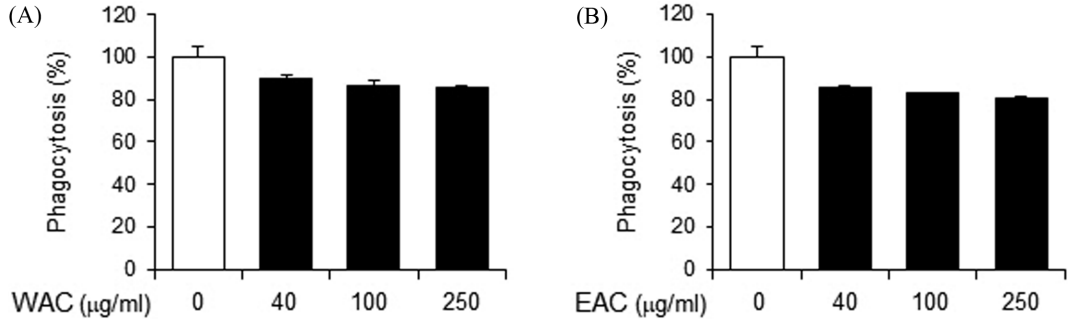

Fig. 1. *Artemisia capillaris* does not suppress phagocytic activity of macrophages. RAW264.7 cells were treated with (A) a water extract (WAC) or (B) an ethanol extract (EAC) of *A. capillaris*. Phagocytic activity was measured as the uptake of fluorescently-labeled *Escherichia coli* (K-12). Data are mean ± SEM (n = 3).

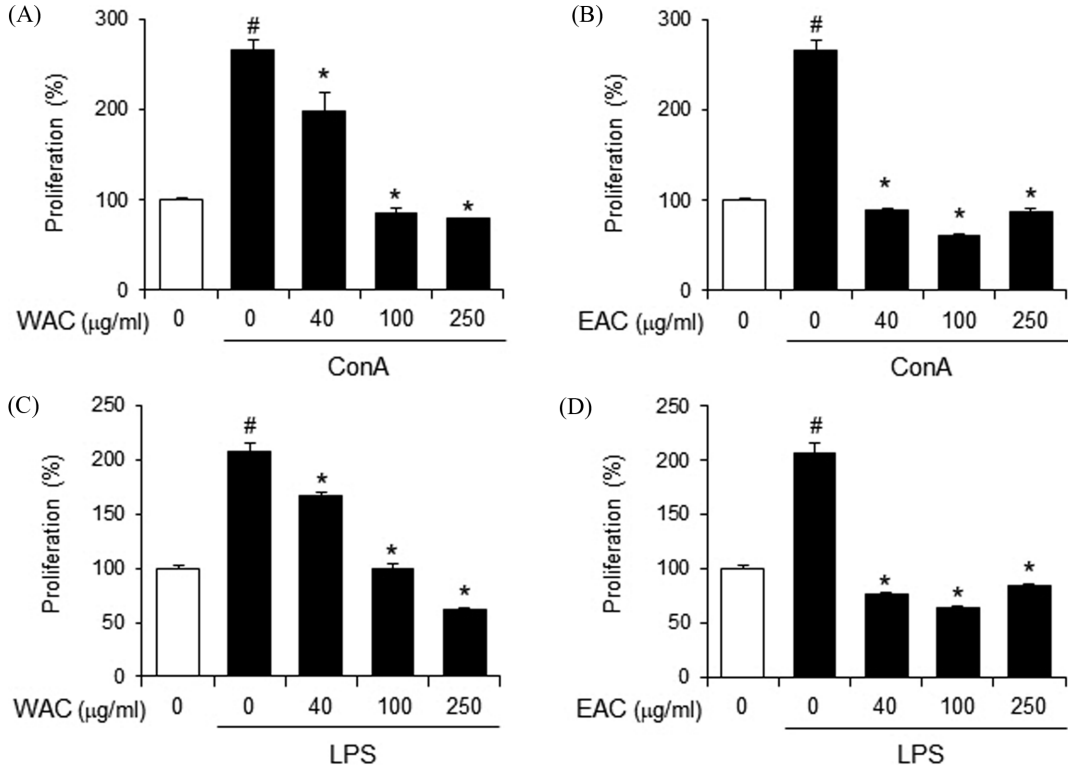

Fig. 2. *Artemisia capillaris* suppresses concanavalin A- and LPS-induced proliferation of primary mouse splenocytes. Primary splenocytes from Balb/c mice were stimulated with (A), (B). concanavalin A (ConA; 5 μg/mL) or (C), (D). lipopolysaccharide (LPS; 10 μg/mL) in the presence or absence of a (A), (C). water extract (WAC) or (B), (D). an ethanol extract (EAC) of *A. capillaris* for 24 hr. Cellular proliferation was measured by methylthiazol tetrazolium (MTT) assay. Data are mean ± SEM (n = 3). #Significantly different from vehicle alone, $p < 0.05$. *Significantly different from ConA or LPS alone, $p < 0.05$. Veh, vehicle.

lymphocyte-activating factor. Therefore, ConA- and LPS-induced splenocyte proliferation was determined as parameters for cellular and humoral immunity, respectively. WAC decreased both ConA- and LPS-induced proliferation of primary mouse splenocytes in a dose-dependent manner (Fig. 2A and 2B). Similarly, EAC suppressed ConA- and LPS-induced splenocyte proliferation more potently than WAC (Fig. 2C and 2D). These results indicate that *A. capillaris* suppresses T and B lymphocyte activation, suggesting that *A. capillaris* treatment leads to a reduction in adaptive immune response, including cellular and humoral immune response.

Oral administration of A. capillaris extract suppresses ConA- and LPS-induced splenocyte proliferation. To confirm the inhibitory activity of *A. capillaris* on adaptive immunity, a single dose of *A. capillaris* extracts was orally

administered to mice. Mouse splenocytes were isolated to examine whether *A. capillaris* intake affected ConA- or LPA-induced proliferation. WAC at 200 and 500 mg/kg reduced both ConA- and LPS-induced primary splenocyte proliferation, while WAC at 100 mg/kg did not inhibit splenocyte proliferation (Fig. 3A, 3B). Histological assessment of the mouse spleens did not show pathological changes in the WAC-treated groups (Fig. 3C). The spleen weight to body weight ratio slightly increased in the WAC-treated groups (Fig. 3D). However, no dose-response relationship

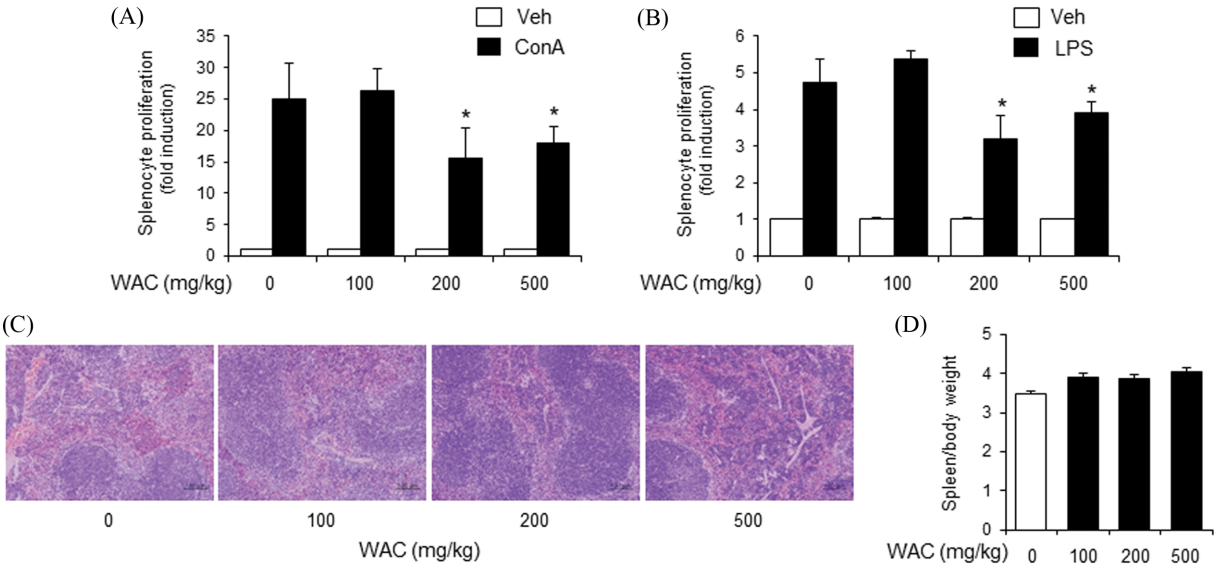

Fig. 3. Oral administration of *Artemisia capillaris* water extract reduces concanavalin A- and LPS-induced proliferation of primary mouse splenocytes. Balb/c mice were orally administered a water extract (WAC) of *A. capillaris*. (A), (B). After 24 hr, splenocytes were isolated and stimulated with concanavalin A (ConA; 5 μg/mL) or lipopolysaccharide (LPS; 10 μg/mL). Cellular proliferation was measured by methylthiazol tetrazolium (MTT) assay. (C) Spleens were stained with hematoxylin and eosin. (D) Spleen weight per body weight. Data are mean ± SEM (n = 3). *Significantly different from ConA or LPS alone, $p < 0.05$. Veh, vehicle.

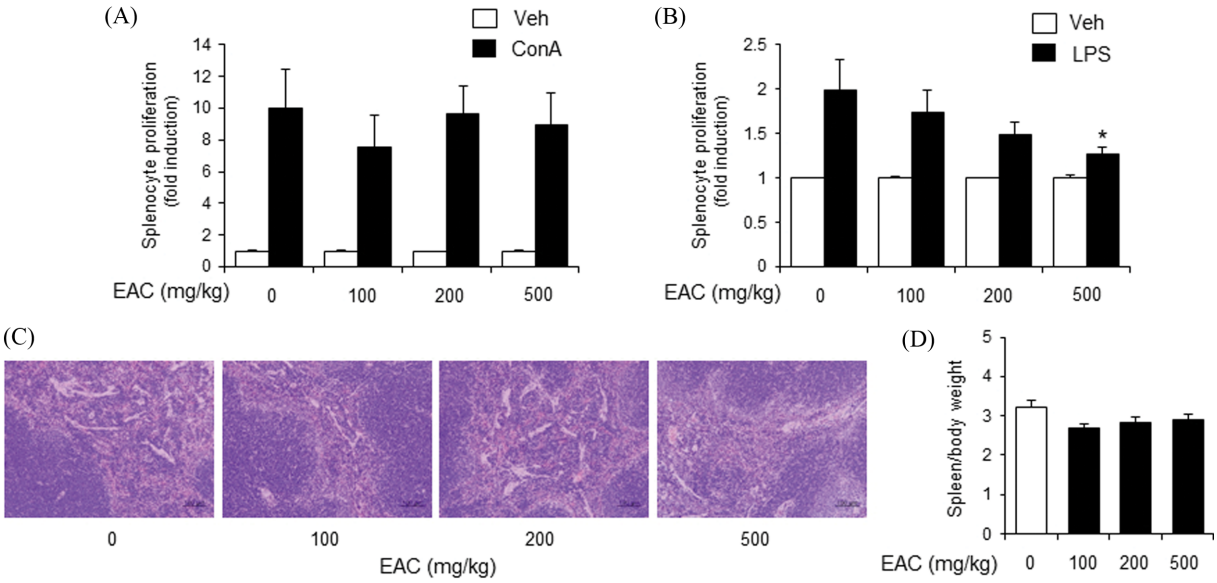

Fig. 4. Oral administration of *Artemisia capillaris* ethanol extract decreases LPS-induced primary mouse splenocyte proliferation. Balb/c mice were orally administered an ethanol extract (EAC) of *A. capillaris*. (A), (B). After 24 hr, splenocytes were isolated and stimulated with concanavalin A (ConA; 5 μg/mL) or lipopolysaccharide (LPS; 10 μg/mL). Cellular proliferation was measured by methylthiazol tetrazolium (MTT) assay. (C) Spleens were stained with hematoxylin and eosin. (D) Spleen weight per body weight. Data are mean ± SEM (n = 3). *Significantly different from ConA or LPS alone, $p < 0.05$. Veh, vehicle.

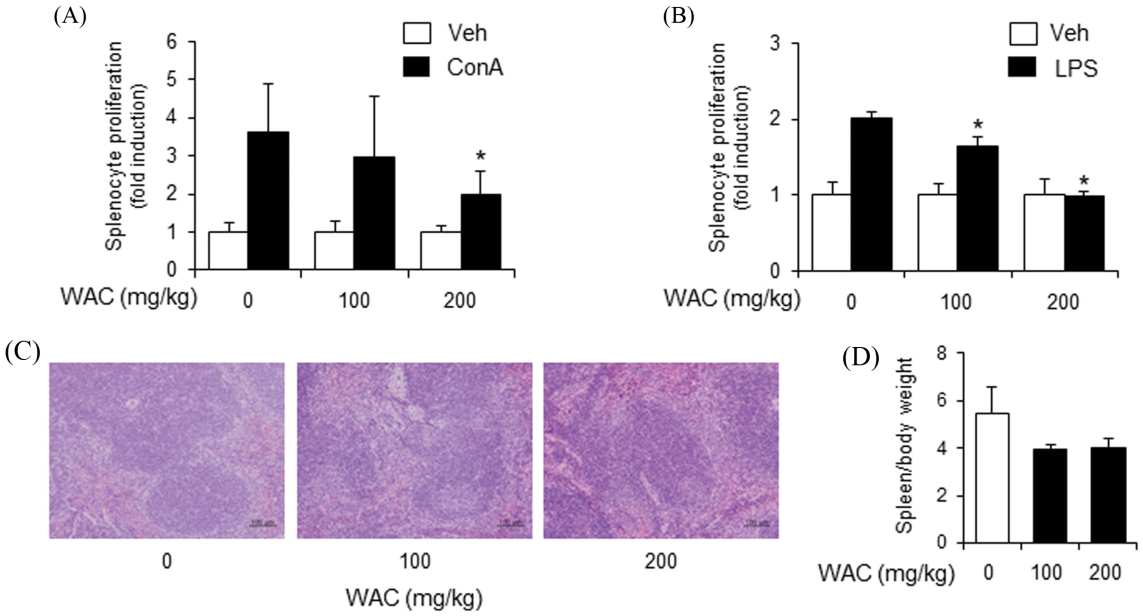

Fig. 5. Repeated administration of *Artemisia capillaris* water extract suppresses concanavalin A- and LPS-induced primary mouse splenocyte proliferation. Balb/c mice were orally administered a water extract (WAC) of *A. capillaris* once per day for 14 days. (A), (B). Splenocytes were isolated and stimulated with concanavalin A (ConA; 5 μg/mL) or lipopolysaccharide (LPS; 10 μg/mL). Cellular proliferation was measured by methylthiazol tetrazolium (MTT) assay. (C) Spleens were stained with hematoxylin and eosin. (D) Spleen weight per body weight. Data are mean ± SEM (n = 3). *Significantly different from ConA or LPS alone, $p < 0.05$. Veh, vehicle.

was observed (Fig. 3D).

EAC treatment did not inhibit ConA-induced primary splenocyte proliferation, but did reduce LPS-induced splenocyte proliferation (Fig. 4A, 4B). There were no significant histopathological changes in mouse spleens from the EAC-treated groups (Fig. 4C). The spleen weight to body weight ratio slightly decreased in the EAC-treated groups (Fig. 4D). However, there was no observable dose-response relationship (Fig. 4D).

We next investigated the effects of multiple *A. capillaris* exposures on adaptive immunity. Since inhibition was more potent with WAC treatment than with EAC treatment, WAC was orally administered to mice once per day for 14 days. Splenocytes were isolated and stimulated with ConA or LPS. ConA and LPS-induced proliferation was significantly attenuated by repeated administration of WAC (Fig. 5A, 5B). Histological evaluation of mouse spleen showed no noticeable changes in the treatment groups (Fig. 5C). The spleen weight to body weight ratio decreased in the EAC-treated groups (Fig. 5D). However, this decrease was not dose-dependent (Fig. 5D).

These results indicate that orally administered *A. capillaris* results in the suppression of cellular and humoral adaptive immunity, without affecting spleen integrity.

Ursolic acid, scopoletin, and scopolin suppress ConA- and LPS-induced splenocyte proliferation. We investigated whether *A. capillaris* components would affect splenocyte proliferation. Ursolic acid, scopoletin, and scopolin inhibited ConA-induced primary splenocyte proliferation, whereas hyperoside did not (Fig. 6A). Similarly, ursolic acid, scopoletin, and scopolin suppressed LPS-induced splenocyte proliferation, whereas hyperoside did not show a dose-dependent inhibition (Fig. 6B). These results indicate that ursolic acid, scopoletin, and scopolin inhibit T and B lymphocyte activation, suggesting that these compounds inhibit cellular and humoral adaptive immune responses.

DISCUSSION

Immunosuppressive agents are a mainstay treatment for organ graft patients, and are becoming increasingly important in the treatment of autoimmune diseases (12). The use of immunosuppressive drugs is essential in cases of solid organ transplantation because it prevents an immune response against the graft, or delays the appearance of *de novo* baseline disease. The most frequently used drugs act on pathways that inhibit T cell proliferation and activation, the main mechanisms involved in rejection (13). Calcineurin inhibitors, such as cyclosporin A and tacrolimus, are the most commonly used treatments. Cyclosporin A emerged as an alternative to azathioprine, triggering an important advance in medical transplantation (14). These drugs inhibit the calcineurin pathway, preventing nuclear factor dephosphorylation in active T lymphocytes and subsequent transfer to the nucleus, ultimately blocking upregulation of genes

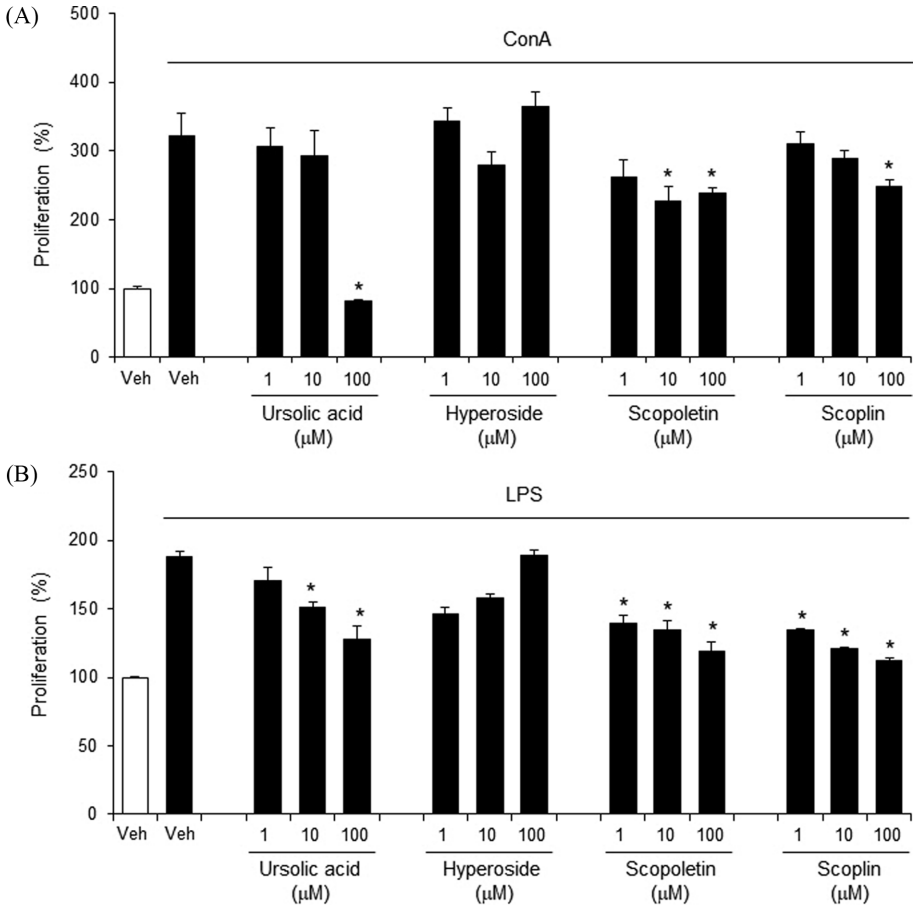

Fig. 6. Ursolic acid and scopoletin suppresses concanavalin A- and LPS-induced proliferation of primary mouse splenocytes. (A), (B). Primary splenocytes isolated from Balb/c mice were stimulated with concanavalin A (ConA; 5 μg/mL) or lipopolysaccharide (LPS; 10 μg/mL) in the presence or absence of ursolic acid, hyperoside, scopoletin, and scopolin for 24 hr. Cellular proliferation was measured by methylthiazol tetrazolium (MTT) assay. Data are mean ± SEM (n = 3). *Significantly different from ConA or LPS alone, $p < 0.05$. Veh, vehicle.

involved in T cell activation, and consequently, immune response (15). Immunosuppressive drug therapy, although necessary after transplantation, has many adverse consequences, including side effects induced by secondary metabolite formation. Calcineurin inhibitors are associated with nephrotoxicity, cardiotoxicity and neurotoxicity; moreover, they increase the risk of many diseases after transplantation (16). Therefore, novel immune suppressants sourced from widely-used, traditional medicinal plants could be beneficial in providing clinically useful and safe treatments, with fewer side effects (17). Our results indicate that *A. capillaris* and its components (ursolic acid, scopoletin, scopolin) inhibit adaptive immune cell activation. These results suggest the potential use of *A. capillaris* and its components as immunosuppressants for diseases involving excessive activation of the adaptive immune system.

The genus *Artemisia* (family *Asteraceae*) includes over 500 species, and is found in Europe, North America, and mainly in Asia (18). Among them, *A. capillaris* is known in Chinese medicine as "Yin Chen Hao" (medicinal term "Injin"), and is the most important herb in East Asia, especially in Korea and China (19). The active components identified in *A. capillaris* extracts are caffeic acid, chlorogenic acid, peroxides, isocuritol, isochlorogenic acid A, and scopolin. Among them, chlorogenic acid, an ester of caffeic acid and quinic acid, has been reported as a main component of *A. capillaris* extract (20). In addition, various compounds have been reported including coumarins (scoparone, scopolin), flavonoids (isorhamnetin, quercetin, isoquercitrin, hyperoside), chromones (capillarisin, 7-methylcapillarisin), phenylpropanoids (caffeic acid, chlorogenic acid, caffeoylquinic acids), lignans ((+)-sesamin, pluviatide, honokiol). and essential oils (β-pinene, β-caryophyllene, capillene) (5,21-24). Among these different constituents, we evaluated four compounds, including ursolic acid, hyperoside, scopoletin and scopolin (25-28), as modulators of adaptive immunity. Jang *et al*. (29) used response surface methodology to optimize the *A. capillaris* extraction parameters (extraction temperature, extraction time, and ethanol concentration) for obtaining an extract with high anti-inflammatory activity at

the cellular level. The ranges used for determining the optimal extraction conditions were extraction temperatures of 57~65°C, ethanol concentrations of 45~57%, and extraction times of 5.5~6.8 hr (29). Yun *et al*. (30) investigated the 13-week subchronic toxicity and genotoxicity of the *A. capillaris extract*. In the 13-week toxicity study, using dosages of 25, 74, 222, 667, and 2,000 mg/kg body weight, oral *A. capillaris* extract administration in male and female rats did not result in any significant adverse effects on food/water consumption, body weight, mortality, hematology, serum biochemistry, organ weight, and histopathology. Accordingly, the no-observed-adverse-effect level in rats of both genders was established for the *A. capillaris* extract at 2,000 mg/kg/day, the highest dose level tested. Yun *et al*. (30) demonstrated that the *A. capillaris* extract is considered safe for human consumption.

Collectively, our results demonstrate that *A. capillaris* and its active components suppress adaptive immune cell activation in *in vitro* and *ex vivo* systems. These results suggest that *A. capillaris* and its active components may be useful as, or in the development of, novel immunosuppressive treatments.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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