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Chemical Constituents from the Aerial Parts of *Artemisia capillaris* Thunb. and Their Anti-allergic and Anti-inflammatory Effects

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Abstract – *Artemisia capillaris* Thunb. (Compositae) is a traditional medicinal plant with various pharmacological activities. To elucidate new anti-allergic and anti-inflammatory constituents, the aerial parts of *A. capillaries* were investigated to afford a new compound, (6*E*,8*E*)-6-methylundeca-6,8-diene-2,5,10-trione (**17**) together with 19 known compounds (**1 - 16**, **18 - 20**). The structures of these compounds were determined by extensive spectroscopic analyses including 1D, 2D NMR, HREIMS, and optical rotation $[\alpha]_D$. The absolute configuration of compound **2** was determined to be *S* form for the first time. All isolates (**1 - 20**) were tested their inhibitory effects on interleukin 2 (IL-2) expression in T cells and NO production in lipopolysaccharide (LPS)-stimulated RAW246.7. Among them, compounds **10**, **11**, **19**, and **20** reduced IL-2 expression in a dose-dependent manner. In addition, compound **10** also inhibited NO production with an IC_{50} value of 37.3 ± 0.4 μM.
Keywords – *Artemisia capillaris* Thunb., (6*E*,8*E*)-6-methylundeca-6,8-diene-2,5,10-trione, anti-allergic, anti-inflammatory, IL-2, NO inhibition

Introduction

Allergic and inflammatory diseases are among the most common diseases all over the world. The prevalence and severity of these diseases have increased and caused the burden of health-care costs. Their treatment is just symptomatic treatment using steroid agents, anti-histamine agents, immunomodulators, and antibiotics. It is well-known but that prolonged use of steroids causes a variety of side effects.[1] Thus, medicinal plants come into the spotlight as alternative therapeutics in immune disorders due to their proven safety with potent immunomodulatory effects.

Inflammation is the body's responses to an injurious stimulus, such as physical damage, ultraviolet irradiation, bacteria, virus, and immune reactions.[2] In this process, the inflammatory mediators, including nitric oxide (NO), prostaglandin E2, as well as cytokines such as interleukin (IL)-1β, IL-6, and tumor necrosis factor-α are abundantly produced by activated inflammatory cells like neutrophils, eosinophils, mononuclear phagocytes, and macrophages.[3] Thus, the inflammatory mediators are important targets for the discovery of new anti-inflammatory drugs. NO is known as a critical cellular signaling molecule involved in many physiological and pathological processes of both acute and chronic inflammatory disorders.[4] Overproduction of NO results in the development of inflammatory diseases such as rheumatoid arthritis and autoimmune disorders.[5] Therefore, screening of anti-inflammatory agents from natural products by evaluation of their inhibition of NO production has been investigated.

Allergy or hypersensitivity is an exaggerated immune response to contact with a harmless substance, generally called as an allergen.[6] Immunological responses involving IgG antibodies[7] or specific T cells[8] can also cause adverse hypersensitivity reactions. Regulation T-cell is the key to the maintenance of immune self-tolerance and also regular immune responses.[9] Increase T-cell number and function have been demonstrated in allergic patients.[10] While IL-2 is critical for T cell differentiation, development, activity, and survival, which has been implicated in the control of allergic disease.[11] Thus, the discovery of

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natural compounds for the reduction of IL-2 expression in T-cells may be useful in the prevention and treatment of allergic diseases.

Artemisia capillaris Thunb. belongs to the Compositae family and is widely distributed in Eastern Asia, especially in Korean and China. Previous studies have reported that coumarins, flavonoids, organic acids, polyacetylenes, and chromones were main constituents of *A. capillaris*[12,13] with diverse pharmacological functions, such as antioxidant,[14] cytoprotective,[14] anti-inflammatory,[15] hepatoprotective,[16] anticarcinogenic,[17] and antiobesity[18] effects.

Thus, to find new anti-allergic and anti-inflammatory agents from natural products, the aerial parts of *A. capillaries* were investigated to isolate a new compound (**17**) and 19 known ones (**1 - 16**, **18 - 20**). All isolates (**1 - 20**) were evaluated the abilities to inhibit IL-2 expression in T-cells as well as NO production in LPS-induced RAW246.7 macrophages.

Experimental

General experimental procedures – The optical rotations were measured using a JASCO DIP-1000 spectropolarimeter. The UV spectra were recorded using an Agilent 8453 UV-visible spectrophotometer. The IR spectra were recorded using a Bruker IFS-66/S Fourier transform (FT)-IR spectrometer. The NMR spectra were recorded using a Bruker Advance Digital 500 MHz NMR spectrometer using TMS as the internal standard. High-resolution electron impact mass spectra (HREIMS) were recorded on a JEOL JMS-700 mass spectrometer in the Korea Basic Science Institute, Daegu. Silica gel 60 (Merck, 60 - 200 μm) and reversed-phase (RP)-C_{18} silica gel (Merck, 40 - 63 μm) were used for column chromatography (CC). TLC was performed using Merck precoated silica gel F_{254} plates and RP-18 F_{254s} plates. HPLC was performed with an HPLC Waters system (Waters, Middleton, USA): 1525 Binary pump; Water 2998 photodiode array detector; YMC Pak ODS column (20 × 250 mm, 5 μm); t_R in min. HPLC solvents were purchased from Burdick & Jackson (USA).

Plant materials – The aerial parts of *A. capillaris* were purchased from Wansan traditional market in Jeonju (July 2016) and identified by Professor Byung Sun Min at Daegu Catholic University. It has been deposited at Pharmacognosy laboratory (voucher no. 20B-AC) in College of Pharmacy, Kyungpook National University, Korea.

Extraction and isolation – The dried aerial parts of *A. capillaris* (9.7 kg) were extracted three times with MeOH (20 L × 3) at 50 °C. After evaporation of the solvents under reduced pressure, the residue (500.0 g) was suspended in H_2O and partitioned with organic solvents, then afforded extracts of *n*-hexane (210.6 g), methylene chloride (MC, 72.5 g), ethyl acetate (EtOAc, 30.2 g), *n*-butanol (49.0 g), and aqueous extract (127.4 g), successfully. The MC extract was chromatographed on a silica gel vacuum liquid chromatography (VLC, 63 - 200 μm particle size, Merck) and eluting with a gradient mixture of MeOH-MC (0 - 100%) to obtain six fractions (1A - 1F). Fraction 1C (47.18 g) was fractionated on silica gel CC with gradient mixtures of EtOAc-*n*-hexane (10 - 40%) and MeOH-MC (5 - 100%) to afford eleven fractions (2A - 2L). Fraction 2G (6.57 g) was continuously separated on silica gel CC with gradient elution of EtOAC-*n*-henxane (10 - 40%) and MeOH-MC (5 - 100%) to give eleven sub-fractions (3A - 3L). Compound **8** (3.5 mg, R_f = 0.67) was purified by RP-TLC with a mixed solvent of MeOH-H_2O (80%) from fraction 3B (24.4 mg). Compound **4** (3.4 mg, t_R = 27.0 min) was isolated from 3D (71.2 mg) by using preparative HPLC with an isocratic elution of MeOH-H_2O (35%, 6 mL/min, 60 min). Fraction 3E (89.9 mg) was applied to preparative HPLC with an isocratic mixture of MeOH-H_2O (45%, 6 mL/min, 60 min) to afford compounds **12** (4.0 mg, t_R = 12.5 min), **17** (2.0 mg, t_R = 23.5 min), **5** (11.0 mg, t_R = 27.4 min), and a mixture compound 3E2 (39.5 mg). This mixture was further separated by NP-TLC with a mixed solvent of EtOAC-*n*-hexane (30%) to obtain compounds **1** (4.9 mg, R_f = 0.70) and **7** (16.2 mg, R_f = 0.20). Fraction 3G (265.6 mg) was purified by using preparative HPLC with an isocratic elution of MeOH-H_2O (45%, 6 mL/min, 60 min) to yield compounds **16** (54.8 mg, t_R = 23.6 min) and **13** (39.0 mg, t_R = 44.2 min). Compound **18** (47.6 mg, t_R = 20.0 min) was isolated from fraction 3H (148.7 mg) by HPLC and eluting with a mixed solvent of MeOH-H_2O (45%). Fraction 3L (2.05 g) was subjected to reverse phase gel CC and eluted with a gradient of MeOH-H_2O (35 - 100%) to obtain eight fractions (4A-4H). Compound **10** (1.4 mg, t_R = 10.5 min) was purified from fraction 4E (282.2 mg) by preparative HPLC with a gradient of MeOH-H_2O (73% - 100%, 6 mL/min, 60 min). Compounds **2** (14.4 mg, R_f = 0.65) and **14** (3.9 mg, R_f = 0.60) were isolated by RP-TLC with an elution of MeOH: H_2O (80%) from 1D (35.1 mg) and 1C (149.5 mg), respectively. Fraction 2E (3.63 g) was separated by RP-C18 CC with an isocratic elution of MeOH-H_2O to obtain five fractions (5A-5E) and compound **19** (49.7 mg). Compound **6** (2.1 mg, t_R = 27.1 min) and two mixture compounds 5C3 (7.7 mg, t_R = 25.0 mg) and 5C5 (8.4 mg, t_R = 33.5 min) were isolated from 5C (57.3 mg) by HPLC

with a gradient of MeOH-H_2O (50% - 100%, 6 mL/min, 60 min). Mixtures 5C3 and 5C5 were further subjected to NP-TLC with elution of 30% EtOAc in *n*-hexane to obtain compounds **3** (2.5 mg, R_f = 0.60) and **15** (4.5 mg, R_f = 0.50). Compounds **9** (5.6 mg, t_R = 12.0 min), **11** (4.4 mg, t_R = 21.5 min), and **20** (1.8 mg, t_R = 34.6 min) were isolated from 5E (358.9 mg) by HPLC with elution mixture of MeOH-H_2O (77%, 6 mL/min, 60 min).

(*S*)-4-(1-Methoxyethyl)phenol (2) – White solid; $[\alpha]_D^{20.6}$ +20.45 (*c* 0.04, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.18 (2H, d, *J* = 8.5 Hz, H-2′, H-6′), 6.81 (2H, d, *J* = 8.5 Hz, H-3′, H-5′), 4.26 (1H, q, *J* = 6.5 Hz, H-1), 3.21 (3H, s, 1-OCH_3), 1.43 (3H, d, *J* = 6.5 Hz, H-2), ^{13}C NMR (125 MHz, $CDCl_3$) δ 155.5 (C-4′), 135.3 (C-1′), 127.8 (C-2′, C-6′), 115.4 (C-3′, C-5′), 79.4 (C-1), 56.3 (1-OCH_3), 23.7 (C-2).

(6*E*,8*E*)-6-Methylundeca-6,8-diene-2,5,10-trione (17) – Pale amorphous solid; UV (MeOH) λ_{max} (log ε) 202.5 (4.14), 286 (4.15) nm; IR (ν_{max}) 1671 (C=O), 1637 (C=C) cm^{-1}; ^{1}H (500 MHz) and ^{13}C (125 MHz) NMR data (CD_3OD) see Table 1; HREIMS (molecular ion peak) *m/z* 208.1099 $[M]^+$ (Calcd for $C_{12}H_{16}O_3$, 208.1099).

Quantitative PCR and Real-time PCR – Jurkat T cells (1×10^6) were incubated with indicated concentrations of compounds for 30 min at 37 ºC. Incubated cells were stimulated with PMA (200 nM) and A23187 (1 μM) for 6 h for PCR. In the case of quantitative PCR experiments, cells were harvested and total RNAs were isolated with TRIZOL reagent (JBI, Korea). Reverse transcription of the RNA was performed using RT PreMix (enzynomics, Korea). The primers and PCR conditions for each gene were used as following: human IL-2, 5′-CAC GTC TTG CAC TTG TCA C-3′ and 5′-CCT TCT TGG GCA TGT AAA ACT-3′. Human GAPDH, 5′-CGG AGT CAA CGG ATT TGG TCG TAT-3′, and 5′-AGC CTT CTC CAT GGT GGT GAA GAC-3′. The amplification profile was composed of denaturation at 94 ºC for 30 s, annealing at 60 ºC for 20 s, and extension at 72 ºC for 40 s. The 30 cycles were preceded by denaturation at 72 ºC for 7 min. For Real-time PCR, amplification was performed in DNA Engine Opticon 1 for a continuous fluorescence detection system (MJ Research, Waltham, MA) in a total volume of 10 μL containing 1 μL of cDNA/control and gene-specific primers using SYBR Premix Ex Taq (Takara, Japan). Each PCR reaction was performed using the following conditions: 94 ºC 30 s, 60 ºC 30 s, 72 ºC 30 s, plate read (detection of fluorescent product) for 40 cycles followed by 7 min extension at 72 ºC 17. Melting curve analysis was done to characterize the dsDNA product by slowly raising the temperature (0.2 ºC/s) from 65 ºC to 95 ºC with fluorescence data collected at 0.2 ºC intervals. The levels of IL-2 mRNA normalized for GAPDH were expressed as fold changes relative to that of the untreated controls. The fold change in gene expression was calculated using the following equation: Fold change = $2^{-\Delta\Delta CT}$, where ΔΔCT = (CT, Target − CT, GAPDH) Time 24 h − (CT, Target − CT, GAPDH) Time 0 h and Time 0 h represents the 1X expression of the target gene of untreated cells, which was normalized to GAPDH. All experiments were performed at least three times unless otherwise indicated.

Cell viability assay – Jurkat T cells (3×10^5) were seeded in a 24 well-plate and incubated with isolates (**1** - **20**) for 24 h. After incubation, cells (180 μL) were added with MTT solution (20 μL, 5 mg/mL). After 2 h of incubation on 37 ºC incubator, cells were centrifuged and

Table 1. NMR data of compound **17** (CD_3OD)

Positions	δ_H[a] (*J* in Hz)	δ_C[b], type	COSY	HMBC
1	2.21 (3H, s)	12.4, CH_3		C-2, C-3
2		210.3, C		
3	2.81 (2H, dd, 5.2, 6.7)	38.0, CH_2	H-4	C-2, C-4, C-5
4	3.05 (2H, m)	32.8, CH_2	H-3	C-2, C-3, C-5
5		201.8, C		
6		144.4, C		
7	7.28 (1H, d, 11.2)	136.1, CH	H-8	C5, C-9
8	7.63 (1H, dd, 11.2, 15.5)	139.1, CH	H-7, H-9	C-7, C-10
9	6.55 (1H, d, 15.5)	136.6, CH		C-10
10		200.9, C		
11	2.37 (3H, s)	27.7, CH_3		C-9, C-10
12	2.03 (3H, d, 1.3)	29.8, CH_3		C-5, C-6, C-7

[a] ^{1}H-NMR measured at 500 MHz. [b] ^{13}C-NMR measured at 125 MHz.

supernatants were taken out. 150 μL of DMSO was added and incubated for 15 min in RT. After incubation, absorbance was detected at 590 nm wavelength.

Determination of NO production and cell viability – The level of NO production was determined by measuring the amount of nitrite present in cell culture supernatants, as described previously. Briefly, the RAW264.7 cells (1×10^6 cells/well) were stimulated with or without 1 μg/mL LPS (Sigma Chemical Co., St. Louis, MO) for 24 h in the presence or absence of the test compounds (100 μM). The cell culture supernatant (100 μL) was then reacted with 100 μL of Griess reagent (1% sulfanilamide in 5% phosphoric acid and 0.1% naphtylethylenediamine dihydrochloride in distilled H_2O). The absorbance at 540 nm was determined with a microplate reader (Molecular Devices, Emax, Sunnyvale, CA, USA), and the absorption coefficient was calibrated by using a sodium nitrite ($NaNO_2$) solution standard. Cell viability was measured with an MTT-based colorimetric assay. For this experiment, celastrol was used as a positive control.

Statistical analysis – Values are expressed as mean ± S.E.M.

Result and Discussion

The methanol extract of the aerial parts of *A. capillaris* was suspended in water and successively divided into *n*-hexane, MC, EtOAc, *n*-butanol, and water-soluble fractions. A new compound (**17**) along with nineteen known compounds (**1 - 16**, **18 - 20**) were isolated by various

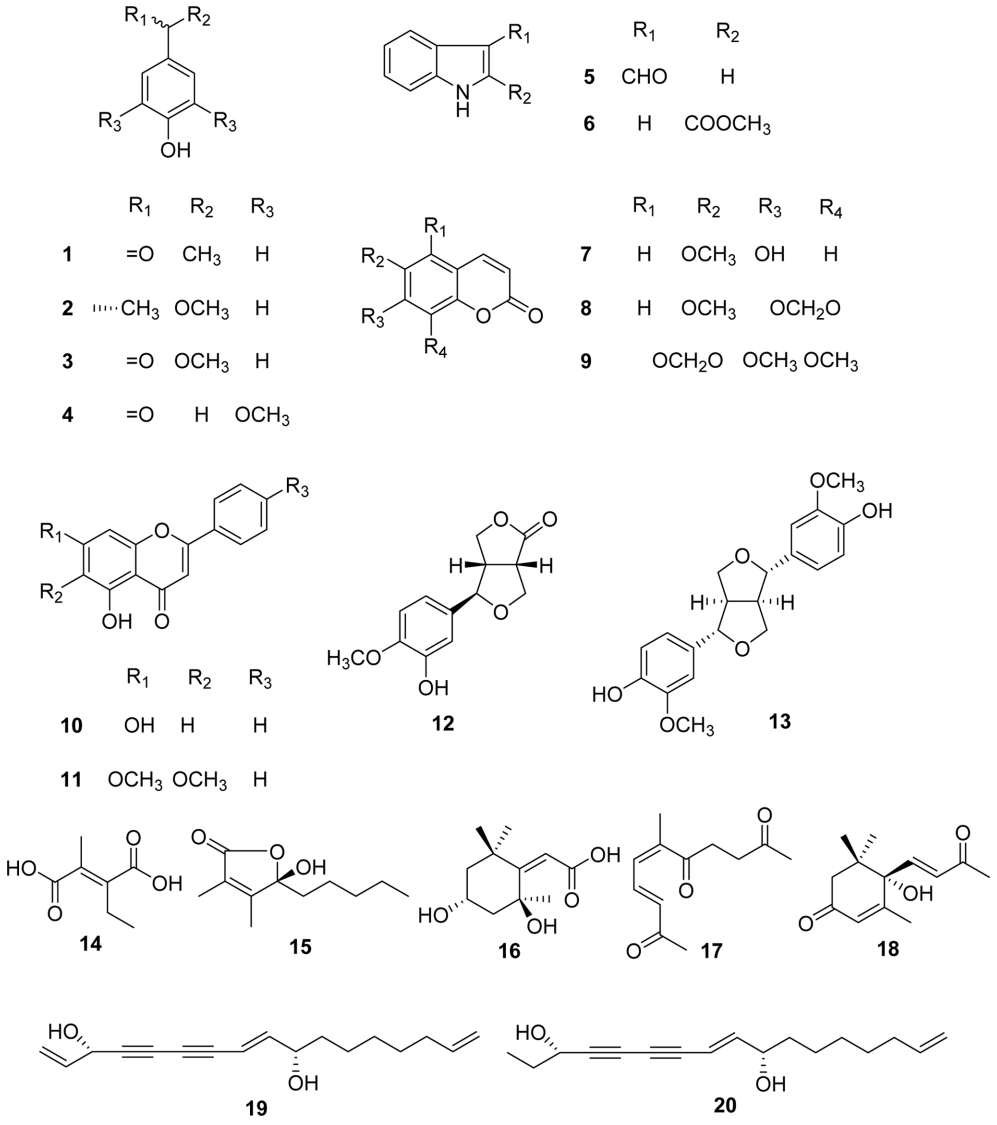

Fig. 1. Structures of compounds isolated from the arial parts of *Artemisia capillaris*.

column chromatographies using silica gel, reverse phase gel, HPLC, and TLC from the MC soluble fraction of *A. capillaris*. The structures of the known compounds were identified by means of spectroscopic analyses (^{1}H and ^{13}C NMR) and by comparing with reported literature data as 4-acetophenol (**1**),[19] (*S*)-4-(1-methoxyethyl)phenol (**2**),[20] 4-hydroxymethyl benzoate (**3**),[21] syringaldehyde (**4**),[22] indole-3-aldehyde (**5**),[23] methyl 2-indolecarboxylate (**6**),[24] scopoletin (7),[25] 6-methoxy-7,8-methylenedioxycoumarin (8),[26] sabandin B (9),[27] chrysin (**10**),[28] cirsimaritin (**11**),[29] salicifoliol (**12**),[30] (+)-pinoresinol (**13**),[31] 2-ethyl-3-methylfumaric acid (**14**),[32] (*S*)-5-hydroxy-3,4-dimethyl-5-pentylfuran-2(5*H*)-one (**15**),[33] (*Z*)-2-(2,4-dihydroxy-2,6,6-trimethylcyclohexylidene) acetic acid (**16**),[34] (+)-dehydrovomifoliol (**18**),[35] dendroarboreol B (**19**),[36] and 1,2-dihydrodendroarboreol B (**20**)[37] (Fig. 1).

Compound **2** was a known compound, which is determined as 4-(1-methoxyethyl)phenol.[20] But its absolute configuration has not demonstrated yet. To determine the absolute configuration of **2**, the optical rotation was checked. As the result, the positive optical rotation value of **2** ($[\alpha]_D^{20.6}$ +20.45 (*c* 0.04, $CHCl_3$)) is similar to that of [(*S*)-2-hydroxy-2-(4-methoxy-phenyl)-ethyl]-carbamic acid benzyl ester $[\alpha]_D^{25}$ +26.39 (*c* 0.52, $CHCl_3$),[37] which confirmed that two compounds possess similar stereochemical character. Thus, the structure of **2** was assigned to be (*S*)-4-(1-methoxyethyl) phenol.

Compound **17** was isolated as yellow amorphous solid. Its UV spectrum exhibited absorption maxima at 202.5 and 286 nm. The IR spectrum of **17** displayed absorption bands for carbonyl (1671 cm^{-1}) and double bond (1637 cm^{-1}) functionalities. The molecular formula of **17** was determined as $C_{12}H_{16}O$ by HREIMS at *m/z* 208.1099 $[M]^+$ (Calcd for $C_{12}H_{16}O_3$, 208.1099). The ^{1}H NMR spectrum of **17** showed signals for three methyl groups at δ_H 2.21 (3H, s, H-1), 2.37 (3H, s, H-11), and 2.03 (3H, d, J = 1.3 Hz, H-1), two methylene groups at δ_H 2.81 (2H, dd, J = 5.2, 6.7 Hz, H-3) and 3.05 (2H, m, H-4), and three olefinic protons at δ_H 7.28 (1H, d, J = 11.2 Hz, H-7), 7.63 (1H, dd, J = 11.2, 15.5 Hz, H-8), and 6.55 (1H, d, J = 15.5 Hz, H-9). The coupling constants of H-7 and H-8 ($J_{7,8}$ 11.2 Hz), H-8 and H-9 ($J_{8,9}$ 15.5 Hz) suggested *Z* and *E* configurations of the double bonds of these protons, respectively. Furthermore, ^{13}C and DEPT spectra of **17** also exhibited the presence of two methyl, three methylene, three methine, and four quarternary carbons (including three ketones at δ_C 210.3, 201.8, and 200.9) (Table 1). The structure of **17** was further supported by COSY correlations between H-3 and H-4 as well as between H-8 and H-7, H-9. The HMBC correlations between H-1 with C-2

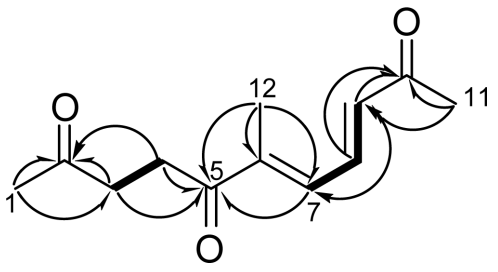

Fig. 2. ^{1}H-^{1}H COSY (—) and key HMBC (→) correlations of compound **17**.

and C-3, H-11 with C-10 and C-9, H-12 with C-5, C-6, and C-7 confirmed the position of three methyl groups at C-2, C-6, and C-10. The other parts were also deducted by HMBC connections between H-3, H-4, H-7 with C-5 and between H-8, H-9 with C-10 (Figure 2). According to the above evidence, the structure of **17** was elucidated as (6*E*,8*E*)-6-methylundeca-6,8-diene-2,5,10-trione.

In this study, compounds (**1 - 20**) were tested to inhibit IL-2 production in Jurkat T cells stimulated with PMA (200 nM) and A23187 (1 μM). Among the isolated compounds, compounds **14** and **17** showed moderate effects whereas two flavonoids (**10** and **11**) and two polyacetylenes (**19** and **20**) moderately inhibited IL-2 production (Figure 3A). The cytotoxic effects of the isolated compounds (**1 - 20**) were evaluated by the MTT assay. As a result, none of the isolated compounds significantly affected the viability of activated Jurkat T-cells, even at a concentration of 100 μM (data not shown). Next, to confirm whether compounds **10**, **11**, **19**, and **20** regulate T cell activity, cells were pre-incubated with **10**, **11**, **19**, and **20** at various concentrations for 30 min before treatment with PMA/A23187. After 6 h, IL-2 mRNA levels were measured by PCR. As shown in Figure 3B, these compounds demonstrated the considerable inhibition of IL-2 in a dose-dependent manner. Our data implied that flavones (**10** and **11**) and polyacetylenes (**19** and **20**) could be the active constituents of *A. capillaris* for inhibiting IL-2 expression. Particularly, compound **11** bearing a hydroxy group at C-4' and two methoxy groups at C-6 and C-7 in its structure showed weaker inhibitory effects than compound **10** which absents these groups. In other hand, by the hydrogenation of double bond at C-1, compound **20** exerted stronger activity than compound **19**, suggesting that the double bond at C-1 of polyacetylenes may influence to their inhibitory effects on IL-2 production. In addition, the inhibitory effects of all isolated compounds (**1 - 20**) on the NO production were tested in LPS-stimulated RAW264.7 cells. RAW264.7 cells were stimulated with 1 μg/mL LPS for 24 h in the presence of

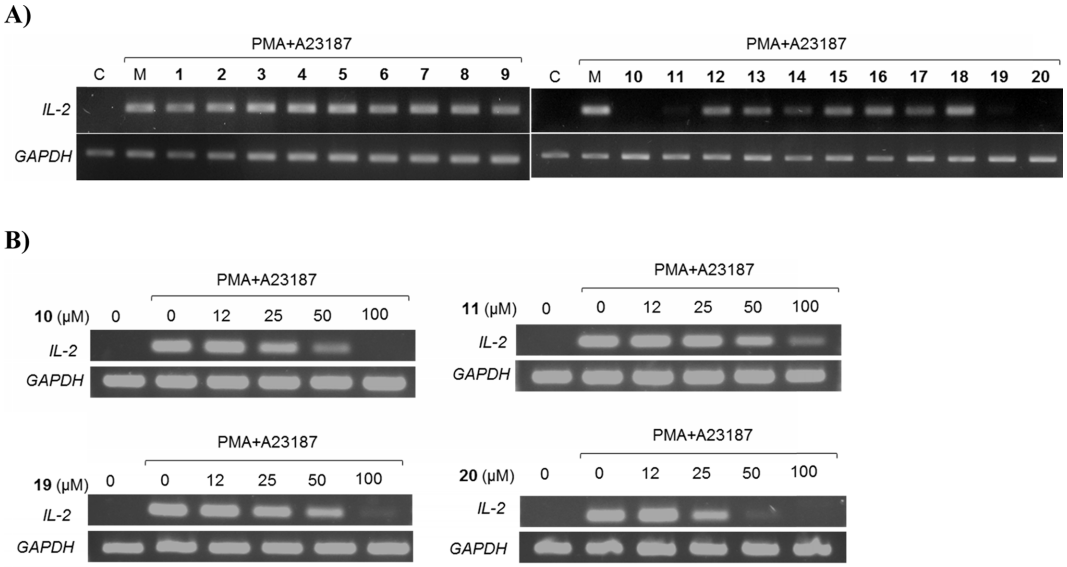

Fig. 3. Inhibition of IL-2 expression by compounds (**1 - 20**) isolated from *A. capillaris*.
Jurkat T cells (1×10^6) were treated with compounds **1 - 20** at 50 μM concentrations (A) and active compounds (**10**, **11**, **19**, and **20**) at different concentrations (from 12 to 100 μM) (B) for 30 min and stimulated with PMA (100 nM) / A23187 (1 μM) for 6 h. After incubation, cells were harvested and total RNA was isolated from harvested cells. Human IL-2 mRNA levels were detected by RT-PCR and real-time quantitative PCR.

Table 2. Inhibition of NO production in macrophage RAW264.7 cells by compounds **1** – **20**

Compounds	NO inhibition, IC_{50} (μM) [a]
1	>50
2	>50
3	>50
4	>50
5	>50
6	>50
7	>50
8	>50
9	>50
10	37.3 ± 0.4
11	>50
12	>50
13	>50
14	>50
15	>50
16	>50
17	>50
18	>50
19	-[c]
20	-[c]
Celastrol[b]	1.0 ± 0.1

[a] The inhibitory effects are represented as the molar concentration (μM) giving 50% inhibition (IC_{50}) relative to the vehicle control. These data represent the average values of three repeated experiments (mean ± S.E.M.). [b] Positive control for NO production. [c] Cytotoxicity

compounds **1 - 20** at various concentrations, and then the levels of NO in the culture supernatants were measured by Griess reaction. Celastrol was used as a positive control with an IC_{50} value of 1.0 μM. The cytotoxic effect of the compounds (**1 - 20**) on RAW264.7 cells was examined using MTT assay to determine whether inhibition of NO production is due to cellular toxicity. As results are shown in Table 2, only compound **10** (Chrysin) exhibited a moderate decrease of NO production with an IC_{50} value of 37.3 μM whereas the other compounds were inactive or showed the cytotoxic activities (compounds **19** and **20**). In the previous studies, chrysin has been demonstrated to be a potent inhibitor of aromatase and of human immunodeficiency virus activation and possessed anti-inflammatory and anti-oxidant effects.[38] This study further confirmed the anti-inflammatory effects of chrysin through inhibition of NO production and suggested a new candidate for the development of anti-inflammatory agents from natural sources.

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