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Inhibitory effects of IL-6-induced STAT3 activation of bio-active compounds derived from *Salvia plebeia* R.Br

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ABSTRACT

Salvia plebeia R.Br. (SPRB) is a natural resource that exhibits various biological activities. However, its effects on inflammatory diseases are not yet well characterized. This study aimed to evaluate the inhibitory effects of a compound derived from SPRB on interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) activation. The chemical structure of active compounds isolated from the EtOAc-soluble fraction of SPRB were elucidated by comparison with spectroscopic nuclear magnetic resonance (NMR) and electrospray ionization mass spectrometry (ESI–MS) data in the literature, which resulted in the identification of 22 known compounds. All of the isolated compounds were evaluated for potentially higher inhibitory effects on IL-6-induced STAT3 activation in Hep3B cells using a luciferase reporter assay. Furthermore, the protein levels of p-STAT3, p-ERK, and p-JAK2 in IL-6 induced U266 cells were regulated in the presence of cirsimaritin from SPRB by western blot assay. Based on these findings, we suggest that SPRB has the potential to inhibit IL-6-induced STAT3 activation and should be considered in functional food and pharmaceutical applications.

1. Introduction

Salvia plebeia R.Br. (SPRB) belongs to the *salvia* family and is an annual or biennial plant that is found in Korea, China, Japan, and many East Asian countries [1,2]. Because of the pharmacological properties of SPRB, it has been used as a traditional medicine for the treatment of arthritis, asthma, atopic dermatitis, inflammation, and gastric ulcers [3–6]. Previous phytochemical investigations of SPRB indicated the presence of various chemical components, such as sesquiterpenes [7], diterpenes [8,9], flavonoids [10], phenolic compounds [11], and aliphatic compounds [12].

Excessive inflammatory cytokine production underlies many diseases [13]. Notably, interleukin-6 (IL-6) is a multifunctional cytokine that plays an important role in the pathogenesis of various inflammatory diseases [14]. Elevated IL-6 levels can lead to abnormal Janus kinase (JAK) and signal transducer and activator of transcription 3 (STAT3) activation and are involved in rheumatoid arthritis, asthma, autoimmune encephalitis, and Crohn's disease [15]. Therefore, the regulation of IL-6-stimulated signaling cascades may represent an effective therapeutic approach for the treatment of these diseases.

In our search for an IL-6/STAT3 inhibitor derived from this Korean medicinal plant, SPRB ethanol extract exhibited a significant inhibitory effect on IL-6-induced STAT3 expression in Hep3B cells. Bioactivity-guided fractionation and repeated column chromatography purification of the SPRB EtOAc-soluble fraction resulted in the isolation of 22 known compounds, including eight flavone (**1**–**8**), three flavanone (**9**–**11**), one monoterpene (**17**), two triterpene (**18**–**19**), three aliphatic (**20**–**22**), and five phenolic compounds (**12**–**16**).

This study aimed to elucidate the basis of the spectroscopic data obtained by ^{1}H and ^{13}C nuclear magnetic resonance (NMR) and electrospray-ionization mass spectrometry (ESI–MS) as well as inhibitory activities on IL-6/STAT3 activation, which were measured by a luciferase bioassay, for all of the isolated compounds.

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2. Materials and methods

2.1. General experimental procedures

Optical rotation was measured using a Jasco P-2000 polarimeter (Jasco Corp., Tokyo, Japan). UV spectra were recorded on a Spectramax M_2^e spectrometer (Molecular Devices, Sunnyvale, CA, USA). NMR spectra were recorded on JEOL JNM-EX400 and JNM-ECA600 (JEOL, Tokyo, Japan) instruments using TMS as a reference and DMSO-d_6, chloroform-d, pyridine-d_5, and methanol-d_4 as solvents (Cambridge Isotope Laboratories, Inc., Andover, MA, USA). ESI–MS was obtained on an Agilent 1100 series MSD mass spectrometer (Agilent Technologies, Wilmington, DE, USA). Open column chromatography was performed with silica gel (Kieselgel 60, 230–400 mesh, Merck, Darmstadt, Germany), Diaion HP-20 (Mitsubishi Chemical Corp., Tokyo, Japan), and Sephadex LH-20 (GE, New York, NY, USA). Medium pressure liquid chromatography (MPLC) was performed on Combiflash RF (Teledyne Isco, Lincoln, NE, USA), and preparative HPLC was performed using a Shimadzu LC-6AD (Shimadzu Corp., Tokyo, Japan) instrument equipped with a SPD-20A detector with YMC ODS-H80 (YMC Co., Tokyo, Japan, 250 × 20 mm, 4 μm), Luna C_{18} (Phenomenex, Torrance, CA, USA, 250 × 21.2 mm, 5 μm), and Kinetex C_{18} (Phenomenex, Torrance, CA, USA, 150 × 21.2 mm, 5 μm) columns using a mixed solvent system of H_2O—CH_3CN (JT Baker Chemicals, Center Valley, PA, USA) at a flow rate of 6.0 mL/min. The EtOAc sub-fraction was subjected to thin layer chromatography (TLC) profiling using silica gel 60 F_{254} and RP-18 F_{254}s (Merck, Darmstadt, Germany).

2.2. Plant material

SPRB was collected in Jangheung, Korea, in April 2014. One of the authors (M.-C. Rho) performed the botanical identification, and a voucher specimen (KRIB-KR2013-600) has been deposited at the laboratory of the Natural Product Research Center, Jeonbuk Branch of the Korea Research Institute of Bioscience and Biotechnology.

2.3. Extraction and isolation

The aerial portions of SPRB were air-dried, pulverized (30 kg), and extracted with 95% EtOH (300 L) at 70 °C for 5 h using an equipped extractor. After filtering the extract *in vacuo*, the filtrates were concentrated by evaporation to obtain 2.6 kg of extract. The residue (2.0 kg) was suspended in distilled water (2 L), and it was progressively partitioned using n-hexane (30 L) and EtOAc (30 L) to give the n-hexane- (779 g) and EtOAc-soluble fractions (541 g), respectively. The EtOAc-soluble fraction was subjected to silica gel column chromatography and eluted with a step-wise gradient of increasing CH_3OH in $CHCl_3$ (100:0, 50:1, 25:1, 10:1, 5:1, 2:1, 1:1, and 0:1) to obtain 14 sub-fractions (SPE1–SPE14) that were combined for TLC analysis.

SPE4 (2.4 g) was separated by MPLC (silica gel, 120 g, $CHCl_3$/CH_3OH, 1:0/0:1) to generate 13 sub-fractions (SPE4A–SPE4M) along with a purified compound **18** (SPE4I, 1.5 g). SPE4J (21 mg) was further purified by preparative HPLC (YMC J'sphere ODS-H80, 250 × 20 mm, 25% CH_3CN) to yield compound **17** (11.4 mg). SPE5 (27.8 g) was chromatographed using MPLC a gradient elution of H_2O and CH_3OH (C18, 130 g, 9:1/0:1) to yield 12 sub-fractions (SPE5A–SPE5 K), and SPE5J (102.4 mg) was separated by preparative HPLC (YMC J'sphere ODS-H80, 250 × 20 mm, 35% CH_3CN) to yield compound **8** (3.8 mg). SPE 6 (9.5 g) was chromatographed on a silica MPLC column (silica gel, 120 g, $CHCl_3$/CH_3OH, 1:0/0:1), and then, SPE6D (1.39 g) was separated using MPLC instrument-based elution with a gradient of increasing CH_3OH in H_2O (C18, 130 g, 9:1/0:1) to generate 19 fractions (SPE6D1–SPE6D19). Compounds **9** (2.3 mg) and **14** (7.3 mg) were isolated from SPE6D6 (0.12 g) using preparative HPLC (YMC J'sphere ODS-H80, 250 × 20 mm, 35% CH_3CN). SPE8 (7 g) was subjected to silica gel column chromatography and eluted with a step-wise gradient of EtOAc in *n*-hexane (100:0, 50:1, 25:1, 10:1, 5:1, 2:1, 1:1, and 0:1) to yield 9 fractions (SPE8A–SPE8I), and SPE8F (1.5 g) was further separated by MPLC (silica gel, 120 g, *n*-hexane/acetone, 1:0/0:1) to yield 12 sub-fractions (SPE8F1–SPE8F12); compound **19** (SPE8F5B, 32.3 mg) was then further purified by preparative HPLC (Phenomenex Luna C18, 150 × 21.2 mm, 65% CH_3CN). SPE9 (79.4 g) was chromatographed on a silica gel column and was eluted with *n*-hexane and acetone as step-wise gradient solvents (100:0, 50:1, 25:1, 10:1, 5:1, 2:1, 1:1, and 0:1) to generate 14 fractions (SPE9A–SPE9N). SPE9J (11.4 g) was separated using a MPLC instrument and eluted using a gradient elution system (silica gel, 120 g, *n*-hexane/EtOAc, 19:1/0:1) to yield 20 sub-fractions (SPE9J1–SPE9J20). Compounds **1** (7.7 mg), **5** (12.0 mg), **10** (13.4 mg), **11** (29.3 mg), and **12** (12.2 mg) were isolated from SPE9J4 (231.2 mg), and compounds **4** (3.1 mg) and **13** (133.9 mg) were obtained from SPE9J5 (418.1 mg) using preparative HPLC (YMC J'sphere ODS-H80, 250 × 20 mm, 25% CH_3CN). SPE9L (4.5 g) was chromatographed on Diaion HP-20 resins and eluted with a mixture of H_2O and CH_3OH (9:1, 8:2, 7:3, 5:5, 3:7, 2:8, 1:9, and 0:1) in a step-wise gradient manner to yield 14 sub-fractions (SPE9L1–SPE9L14). SPE9L7 (340 mg) was isolated by preparative HPLC (YMC J'sphere ODS-H80, 250 × 20 mm, 25% CH_3CN) to yield compounds **7** (11.2 mg) and **16** (214.2 mg). Compound **6** (208 mg) was isolated from SPE11 by recrystallization in methanol, and isolated crystals were further purified by preparative HPLC (Phenomenex Luna C_{18}, 150 × 21.2 mm, 35% CH_3OH). SPRB ethanol concentrate (100 g) was chromatographed on Diaion HP-20 resins with a step-wise gradient of H_2O and CH_3OH (9:1/0:1) to generate 11 sub-fractions (SP1–SP11) together with compound **15** (SP4, 501 mg). SP5 (2.5 g) was subjected to Sephadex LH-20 column chromatography with CH_3OH, yielding 9 sub-fractions (SP5A–SP5I) together with compound **3** (SP5C, 10.2 mg). SP7 (3.8 g) was separated by MPLC (silica gel, 120 g, *n*-hexane/acetone, 19:1/0:1) to yield 11 sub-fractions (SP7A-SP7 K), and SP7F was identified as compound **2** (12.9 mg). Compounds **20** (3.4 mg), **21** (5.1 mg), and **22** (14.7 mg) were isolated from SP10 (1.3 g) by preparative HPLC (Phenomenex Kinetex C18, 150 × 21.2 mm, 75% CH_3CN). Compound **18** (0.2 g) was reacted with acetic anhydride (0.1 mL, Sigma, St. Louis, MO, USA) in pyridine (1.5 mL) and CH_2Cl_2 (2.0 mL) at room temperature (RT) for 1 day. The mixture was separated by MPLC (silica gel, 40 g, CH_2Cl_2/CH_3OH, 100:1), and sub-fraction 1 was identified as compound **18a** (57.4 mg).

2.3.1. Apigenin (**1**)

Yellow amorphous powder; $[\alpha]^{25}_D = -210.6$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 271 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ_H: 12.96 (1H, s, OH-5), 7.92 (2H, d, *J* = 8.8 Hz, H-2′,6′), 6.92 (2H, d, *J* = 8.8 Hz, H-3′,5′), 6.77 (1H, s, H-3), 6.47 (1H, s, H-8), 6.19 (1H, s, H-6); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 181.7 (C-4), 164.2 (C-2), 163.7 (C-7), 161.4 (C-5), 161.1 (C-4′), 157.3 (C-9), 128.4 (C-2′,6′), 121.1 (C-1′), 115.9 (C-3′,5′), 103.6 (C-10), 102.8 (C-3), 98.8 (C-6), 93.9 (C-8).

2.3.2. Luteolin (**2**)

Yellow amorphous powder; $[\alpha]^{25}_D = -11.6$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 287 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ_H: 12.97 (1H, s, OH-5), 7.42 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′), 7.39 (1H, d, *J* = 2.0 Hz, H-2′), 6.89 (1H, d, *J* = 8.0 Hz, H-5′), 6.67 (1H, s, H-3), 6.44 (1H, d, *J* = 2.0 Hz, H-8) 6.19 (1H, d, *J* = 2.0 Hz, H-6); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 181.6 (C-4), 164.1 (C-7), 163.9 (C-2), 161.5 (C-5), 157.3 (C-9), 149.7 (C-4′), 145.7 (C-3′), 121.5 (C-1′), 119.0 (C-6′), 116.0 (C-5′), 133.4 (C-2′), 103.7 (C-10), 102.9 (C-3), 98.8 (C-6), 93.8 (C-8).

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*2.3.3. Luteolin-7-O- glucoside (**3**)*

Yellow amorphous powder; $[\alpha]^{25}_D = -19.8$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 449 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ_H: 12.99 (1H, s, OH-5), 7.45 (1H, dd, *J* = 8.0, 2.0 Hz, H-6′), 7.42 (1H, d, *J* = 2.0 Hz, H-2′), 6.90 (1H, d, *J* = 8.0 Hz, H-5′), 6.79 (1H, d, *J* = 2.0 Hz, H-8), 6.76 (1H, s, H-3), 6.44 (1H, dd, *J* = 2.0 Hz, H-6), 5.08 (1H, d, *J* = 7.2 Hz, H-1″), 3.71 (1H, d, *J* = 11.2 Hz, H-6a″), 3.49 (1H, d, m, H-6b″), 3.44 (1H, m, H-5″), 3.34 (1H, m, H-3″), 3.27 (1H, m, H-2″), 3.19 (1H, m, H-4″); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 181.9 (C-4), 164.4 (C-2), 162.9 (C-7), 161.1 (C-5), 156.9 (C-9), 149.9 (C-4′), 145.8 (C-3′), 121.4 (C-1′), 119.2 (C-6′), 116.0 (C-5′), 113.6 (C-2′), 105.3 (C-10), 103.2 (C-3), 99.9 (C-1″), 99.5 (C-6), 94.7 (C-8), 77.2 (C-5″), 76.4 (C-3″), 73.1 (C-2″), 69.5 (C-4″), 60.6 (C-6″).

*2.3.4. Isorhamnetin (**4**)*

Yellow amorphous powder; $[\alpha]^{25}_D = -17.6$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 317 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ_H: 12.70 (1H, s, OH-5), 7.54 (1H, d, *J* = 2.0 Hz, H-2′), 7.44 (1H, dd, *J* = 8.4, 2.0 Hz, H-6′), 6.89 (1H, d, *J* = 8.4 Hz, H-5′), 6.39 (1H, s, H-8), 6.17 (1H, s, H-6), 3.77 (3H, s, 3′-OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 177.7 (C-4), 164.6 (C-7), 161.2 (C-5), 156.3 (C-9), 155.5 (C-4′), 148.8 (C-3′), 145.3 (C-2), 137.5 (C-3), 120.6 (C-1′), 120.5 (C-6′), 115.7 (C-5′), 115.3 (C-2′), 103.9 (C-10), 98.6 (C-6), 93.6 (C-8), 59.6 (3′-OCH_3).

*2.3.5. Hispidulin (**5**)*

Yellow amorphous powder; $[\alpha]^{25}_D = -18.6$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 301 $[M+H]^+$; ^1H NMR (600 MHz, acetone-d_6) δ_H: 7.92 (2H, d, *J* = 8.4 Hz, H-2′,6′), 7.00 (2H, d, *J* = 8.4 Hz, H-3′,5′), 6.62 (1H, s, H-3), 6.60 (1H, s, H-8), 3.87 (3H, s, 6-OCH_3); ^{13}C NMR (125 MHz, acetone-d_6) δ_C: 183.6 (C-4), 165.2 (C-2), 162.1 (C-4′), 158.0 (C-7), 154.0 (C-5), 132.3 (C-6), 129.3 (C-2′,6′), 123.3 (C-1′), 117.0 (C-3′,5′), 105.7 (C-10), 103.5 (C-3), 94.9 (C-8), 60.7 (6-OCH_3).

*2.3.6. Homoplantaginin (**6**)*

Yellow amorphous powder; $[\alpha]^{25}_D = -95.8$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 463 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ_H: 7.88 (2H, d, *J* = 8.8 Hz, H-2′,6′), 6.96 (1H, s, H-8), 6.92 (1H, d, *J* = 8.8 Hz, H-3′,5′), 6.65 (1H, s, H-3), 5.13 (1H, d, *J* = 7.2 Hz, H-1″), 3.96 (1H, dd, *J* = 12.0, 1.6 Hz, H-6a″), 3.89 (3H, s, 6-OCH_3), 3.72 (1H, dd, *J* = 12.0, 6.0 Hz, H-6b″), 3.58 (1H, m, H-5″), 3.57 (1H, m, H-2″), 3.52 (1H, t, *J* = 9.2 Hz, H-3″), 3.42 (1H, t, *J* = 9.2, H-4″); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 184.5 (C-4), 167.1 (C-2), 163.4 (C-4′), 158.0 (C-7), 154.4 (C-9), 154.2 (C-5), 134.4 (C-6), 129.8 (C-2′,6′), 123.0 (C-1), 117.3 (C-3′,5′), 107.7 (C-10), 103.8 (C-3′), 102.2 (C-1″), 96.0 (C-8), 78.7 (C-3″),78.2 (C-5″), 74.9 (C-2″), 71.5 (C-4″), 62.7 (C-6″), 61.6 (6-OCH_3).

*2.3.7. Nepetin (**7**)*

Yellow amorphous powder; $[\alpha]^{25}_D = -8.8$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 317 $[M+H]^+$;^1H NMR (400 MHz, DMSO-d_6) δ_H: 13.08 (1H, s, OH-5), 7.42 (1H, dd, *J* = 8.0, 2.0, H-6′), 7.39 (1H, br s, H-2′), 6.88 (1H, d, *J* = 8.0 Hz, H-5′), 6.66 (1H, s, H-3), 6.54 (1H, s, H-8), 3.74 (6-OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 182.2 (C-4), 164.1 (C-2), 154.5 (C-7), 149.8 (C-5), 149.7 (C-9), 146.4 (C-4′), 145.9 (C-3′), 130.0 (C-6), 121.8 (C-1′), 119.0 (C-6′), 116.1 (C-5′), 113.5 (C-2′), 105.1 (C-10), 102.6 (C-3), 91.1 (C-8), 56.3 (6-OCH_3).

*2.3.8. Cirsimaritin (**8**)*

Yellow amorphous powder; $[\alpha]^{25}_D = -8.0$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 315 $[M+H]^+$; ^1H NMR (400 MHz, DMSO-d_6) δ_H: 13.01 (1H, s, 5-OH), 7.92 (1H, d, *J* = 8.8 Hz, H-2′,6′), 6.92 (1H, s, H-8), 6.85 (1H, d, *J* = 8.8 Hz, H-3′,5′), 6.79 (1H, s, H-3), 3.92 (3H, s, 7-OCH_3), 3.73 (3H, s, 6-OCH_3); ^{13}C NMR (100 MHz, DMSO-d_6) δ_C: 182.0 (C-4), 164.4 (C-2), 163.6 (C-4′), 158.4 (C-7), 152.5 (C-5), 152.1 (C-9), 131.8 (C-6), 128.5 (C-2′,6′), 119.2 (C-1′), 116.5 (C-3′,5′), 105.0 (C-10), 101.8 (C-3), 91.5 (C-8), 60.0 (6-OCH_3), 56.4 (7-OCH_3).

*2.3.9. 6-Methoxy-naringenin (**9**)*

Yellow amorphous powder; $[\alpha]^{25}_D = -11.4$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 303 $[M+H]^+$; ^1H NMR (400 MHz, CD_3OD) δ_H: 7.31 (1H, d, *J* = 8.4 Hz, H-2′,6′), 6.81 (1H, d, *J* = 8.4 Hz, H-3′,5′), 5.97 (1H, s, H-8), 5.33 (1H, dd, *J* = 12.8, 1.6 Hz, H-2β), 3.79 (3H, s, 6-OCH_3), 3.12 (1H, dd, *J* = 17.2, 13.2 Hz, H_2-3α) 2.70 (1H, dd, *J* = 17.2, 1.6 Hz, H_2-3β); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 198.7 (C-4), 161.0 (C-7), 160.4 (C-9), 159.2 (C-4′), 156.7 (C-5), 131.2 (C-6), 130.6 (C-1′), 129.2 (C-2′,6′), 116.5 (C-3′,5′), 103.6 (C-10), 96.4 (C-8), 80.8 (C-2), 61.1 (6-OCH_3), 44.2 (C-3).

*2.3.10. Eriodictyol (**10**)*

Light yellow amorphous powder; $[\alpha]^{25}_D = -18.2$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 289 $[M+H]^+$; ^1H NMR (400 MHz, acetone-d_6) δ_H: 12.18 (1H, s, OH-5), 7.04 (1H, s, H-2′), 6.87 (2H, s, H-5′,6′), 5.96 (1H, s, H-8), 5.94 (1H, s, H-6), 5.39 (1H, dd, *J* = 12.8, 3.2 Hz, H-2β), 3.13 (1H, dd, *J* = 17.2, 12.8, H-3α), 2.72 (1H, dd, *J* = 17.2, 3.2 Hz, H-3β); ^{13}C NMR (100 MHz, acetone-d_6) δ_C: 197.3 (C-4), 167.5 (C-7), 165.4 (C-5), 164.4 (C-9), 146.5 (C-4′), 146.1 (C-3′), 131.7 (C-1′), 119.3 (C-6′), 116.1 (C-5′), 114.8 (C-2′), 103.3 (C-10′), 96.9 (C-8′), 96.0 (C-6), 80.1 (C-2), 43.7 (C-3).

*2.3.11. Filifolin (**11**)*

Light yellow amorphous powder; $[\alpha]^{25}_D = -10.2$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 319 $[M+H]^+$; ^1H NMR (400 MHz, acetone-d_6) δ_H: 12.31 (1H, s, OH-5), 7.03 (1H, s, H-2′), 6.86 (2H, s, H-5′,6′), 6.00 (1H, s, H-8), 5.37 (1H, dd, *J* = 12.8, 2.8 Hz, H-2β), 3.77 (6-OCH_3), 3.13 (1H, dd, *J* = 17.2, 12.8, H-3α), 2.73 (1H, dd, *J* = 17.2, 2.8 Hz, H-3β); ^{13}C NMR (100 MHz, acetone-d_6) δ_C: 198.1 (C-4), 160.0 (C-7), 159.7 (C-9), 156.4 (C-5), 146.4 (C-4′), 146.1 (C-3′), 131.7 (C-1′), 130.0 (C-6), 119.3 (C-5′), 116.1 (C-6′), 114.8 (C-2′), 103.4 (C-10), 95.8 (C-8), 80.1 (C-2), 60.8 (6-OCH_3), 43.8 (C-3).

*2.3.12. Protocatechuic acid (**12**)*

Brown amorphous powder; $[\alpha]^{25}_D = -31.6$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 155 $[M+H]^+$; ^1H NMR (400 MHz, CD_3OD) δ_H: 7.44 (1H, s, H-2), 7.43 (1H, d, *J* = 8.0 Hz, H-6), 6.80 (1H, d, *J* = 8.0, Hz, H-5); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 170.4 (C-7), 151.6 (C-4), 146.2 (C-3), 124.0 (C-6), 123.4 (C-1), 117.9 (C-2), 115.9 (C-5).

*2.3.13. Caffeic acid (**13**)*

Yellow amorphous powder; $[\alpha]^{25}_D = -3.6$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 179 $[M-H]^-$; ^1H NMR (400 MHz, CD_3OD) δ_H: 7.41 (1H, d, *J* = 16.0 Hz, H-7), 7.02 (1H, d, *J* = 2.0 Hz, H-2), 6.96 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.75 (1H, d, *J* = 8.0 Hz, H-5), 6.17 (1H, d, *J* = 16.0 Hz, H-8); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 171.2 (C-9), 149.6 (C-4), 147.1 (C-7), 146.9 (C-3), 128.0 (C-1), 123.0 (C-5), 116.6 (C-2), 115.8 (C-6), 115.2 (C-8).

*2.3.14. Ethyl caffeate (**14**)*

Yellow amorphous powder; $[\alpha]^{25}_D = -4.4$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 209 $[M+H]^+$; ^1H NMR (400 MHz, CD_3OD) δ_H: 7.53 (1H, d, *J* = 16.0 Hz, H-7), 7.03 (1H, d, *J* = 1.2 Hz, H-2), 6.94 (1H, d, *J* = 8.0 Hz, H-6), 6.77 (1H, d, *J* = 8.0 Hz, H-5), 6.24 (1H, d, *J* = 16.0 Hz, H-8), 4.22 (2H, q, *J* = 7.2 Hz, H_2-1′) 1.30 (3H, t, *J* = 7.2 Hz, H_3-2′); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 169.5 (C-9), 149.7 (C-4), 147.0 (C-7), 146.9 (C-3), 127.9 (C-1), 123.0 (C-5), 116.6 (C-2), 115.4 (C-6), 115.3 (C-8), 61.5 (C-1′), 14.8 (C-2′).

*2.3.15. Rosmarinic acid (**15**)*

Yellow amorphous powder; $[\alpha]^{25}_D = +80.0$ (*c* 0.1, CH_3OH); ESI–MS: *m/z* 361 $[M+H]^+$; ^1H NMR (400 MHz, CD_3OD) δ_H: 7.55 (1H, d, *J* = 16.0 Hz, H-7′), 7.04 (1H, d, *J* = 2.0 Hz, H-2′), 6.95 (1H, dd, *J* = 8.0 2.0 Hz, H-6′), 6.78 (1H, d, *J* = 8.0 Hz, H-5′), 6.75 (1H, d, *J* = 2.0 Hz, H-2), 6.70 (1H, d, *J* = 8.0 Hz, H-5), 6.61 (1H, dd, *J* = 8.0, 2.0 Hz, H-6), 6.27 (1H, d, *J* = 16.0 Hz, H-8′), 5.18 (1H, d, *J* = 8.4, 4.4 Hz, H-8), 3.10 (1H, dd,

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J = 14.0, 4.4 Hz, H_2-7a), 3.01 (1H, dd, J = 14.0 8.4 Hz, H_2-7b); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 173.6 (C-9), 168.6 (C-9′), 149.9 (C-4′), 147.9 (C-3′), 146.9 (C-7′), 146.3 (C-3), 145.4 (C-4), 129.4 (C-1), 127.8 (C-1′), 123.3 (C-6′), 121.9 (C-6), 117.7 (C-2), 116.6 (C-5′), 116.4 (C-5), 115.3 (C-8′), 114.5 (C-2′), 74.7 (C-8), 38.1 (C-7).

2.3.16. Methyl rosmarinate (**16**)

Brown amorphous powder; $[\alpha]^{25}_D$ = +34.0 (c 0.1, CH_3OH); ESI–MS: m/z 373 $[M-H]^-$; ^{1}H NMR (400 MHz, CD_3OD) δ_H: 7.56 (1H, d, J = 16.0 Hz, H-7′), 7.05 (1H, d, J = 2.0 Hz, H-2′), 6.96 (1H, dd, J = 8.0 2.0 Hz, H-6′), 6.78 (1H, d, J = 8.0 Hz, H-5′), 6.71 (1H, d, J = 2.0 Hz, H-2), 6.70 (1H, d, J = 8.0 Hz, H-5), 6.57 (1H, dd, J = 8.0, 2.0 Hz, H-6), 6.26 (1H, d, J = 16.0 Hz, H-8′), 5.19 (1H, d, J = 7.2, 5.2 Hz, H-8), 3.70 (3H, s, $-CO_2$-OCH_3), 3.04 (2H, m, H_2-7); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 172.5 (C-9), 168.6 (C-9′), 150.1 (C-4′), 148.2 (C-7′), 147.1 (C-3′), 146.5 (C-3), 145.7 (C-4), 129.0 (C-1), 127.8 (C-1′), 123.4 (C-6′), 122.0 (C-6), 117.8 (C-2), 116.7 (C-5′), 116.6 (C-5), 115.5 (C-2′), 114.4 (C-8′), 74.9 (C-8), 52.8 (-CO_2-OCH_3), 38.0 (C-7).

2.3.17. 7-Epiloliolide (**17**)

Colorless oil; $[\alpha]^{25}_D$ = −113.6 (c 0.1, CH_3OH); ESI–MS: m/z 197 $[M+H]^+$; ^{1}H NMR (400 MHz, CD_3OD) δ_H: 5.75 (1H, s, H-3), 4.21 (1H, q, J = 3.6 Hz, H-6), 2.42 (1H, dt, J = 13.6, 2.4 Hz, H-8$_a$), 1.99 (1H, dt, J = 14.4, 2.4 Hz, H-8$_b$), 1.76 (3H, s, H_3-12), 1.74 (1H, m, H-6$_a$), 1.53 (1H, dd, J = 14.4, 3.6 Hz, H-6$_b$), 1.46 (3H, s, H_3-11), 1.27 (3H, s, H_3-10); ^{13}C NMR (100 MHz, CD_3OD) δ_C: 185.8 (C-4), 174.5 (C-2), 113.5 (C-3), 89.1 (C-9), 67.4 (C-7), 48.1 (C-6), 46.6 (C-8), 37.3 (C-5), 31.2 (C-11), 27.6(C-10), 27.1 (C-12).

2.3.18. Ursolic acid (**18**)

White amorphous powder; $[\alpha]^{25}_D$ = +33.0 (c 0.1, CH_3OH); FAB-MS: m/z 479 $[M+Na]^+$; ^{1}H NMR (600 MHz, DMSO-d_6) δ_H: 5.11 (1H, t, J = 3.0 Hz, H-12), 3.00 (1H, dd, J = 10.8, 5.4 Hz, H-3α), 2.11 (1H, d, J = 10.8 Hz, H-18), 1.04 (3H, s, H_3-27), 0.91 (3H, m, H_3-30), 0.90 (3H, s, H-23), 0.87 (3H, s, H-25), 0.81 (3H, d, J = 6.6 Hz, H_3-29), 0.75 (3H, s, H_3-26), 0.68 (3H, s, H_3-24); ^{13}C NMR (150 MHz, DMSO-d_6) δ_C: 178.4 (C-28), 138.3 (C-13), 124.4 (C-12), 76.8 (C-3), 54.8 (C-5), 52.4 (C-18), 47.0 (C-9), 46.8 (C-17), 41.7 (C-14), 40.1 (C-8, overlap), 38.5 (C-19), 38.5 (C-1), 38.4(C-4), 38.2 (C-20), 36.5 (C-10), 36.4 (C-22), 32.7 (C-7), 30.3 (C-21), 28.3 (C-23), 27.6 (C-15), 27.0 (C-2), 23.9 (C-16), 23.3 (C-27), 22.8 (C-11), 21.1 (C-30), 18.0 (C-6), 17.1 (C-29), 17.0 (C-26), 16.1 (C-24), 15.2 (C-25).

2.3.19. Ursolic acid-3-acetate (**18a**)

White amorphous powder; $[\alpha]^{25}_D$ = +37.4 (c 0.1, CH_3OH); ESI–MS: m/z 499 $[M+H]^+$; ^{1}H NMR (400 MHz, pyridine-d_5) δ_H: 5.48 (1H, t, J = 3.2 Hz, H-12), 4.70 (1H, dd, J = 11.2, 4.8 Hz, H-3), 2.64 (1H, d, J = 11.2 Hz, H-18), 2.01 (3H, s, OAc-CH_3), 1.24 (3H, s, H_3-27), 1.03 (3H, s, H_3-25), 1.02 (3H, m, H-30), 0.98 (3H, d, J = 6.0 Hz, H-29), 0.92 (3H, s, H_3-23), 0.89 (3H, s, H_3-24), 0.82 (3H, s, H_3-26); ^{13}C NMR (100 MHz, pyridine-d_5) δ_C: 180.3 (C-28), 171.0 (OAc), 139.7 (C-13), 125.9 (C-12), 81.2 (C-3), 56.0 (C-5), 54.0 (C-18), 48.5 (C-9), 48.3 (C-17), 42.9 (C-14), 40.3 (C-8), 39.9 (C-19), 39.8 (C-20), 38.7 (C-1), 38.3 (C-4), 37.9 (C-10), 37.5 (C-22), 33.8 (C-7), 31.5 (C-11), 29.1 (C-21), 28.6 (C-15), 25.3 (C-23), 24.3 (C-2, 16), 24.0 (C-27), 21.8 (OAc-CH_3), 21.5 (C-30), 18.9 (C-6), 17.9 (C-24), 17.8 (C-29), 17.4 (C-26), 16.0 (C-25).

2.3.20. Corosolic acid (**19**)

White amorphous powder; $[\alpha]^{25}_D$ = +36.8 (c 0.1, CH_3OH); FAB-MS: m/z 495 $[M+Na]^+$; ^{1}H NMR (600 MHz pyridine-d_5) δ_H: 5.48 (1H, t, J = 3.6 Hz, H-12), 4.11 (1H, m, H-2), 3.42 (1H, d, J = 9.0 Hz, H-3), 2.64 (1H, d, J = 11.4 Hz, H-18), 1.29 (3H, s, H-23), 1.22 (3H, s, H_3-27), 1.09 (3H, s, H_3-24), 1.06 (3H, s, H-26), 1.00 (3H, d, J = 6.0 Hz, H_3-29), 0.99 (3H, s, H_3-25), 0.96 (3H, s, H_3-30); ^{13}C NMR (150 MHz, pyridine-d_5) δ_C: 180.4 (C-28), 139.7 (C-13), 125.9 (C-12), 84.2 (C-3), 69.0 (C-2), 56.3 (C-5), 53.4 (C-18), 48.5 (C-9), 48.5 (C-17), 48.4 (C-1), 43.0 (C-14), 40.4 (C-8), 40.3 (C-4), 39.9 (C-20), 39.8 (C-19), 38.9 (C-10), 37.9 (C-22), 33.9 (C-7), 31.5 (C-21), 29.8 (C-23), 29.1 (C-15), 25.3 (C-16), 24.4 (C-27), 24.2 (C-11), 21.9 (C-30), 19.3 (C-6), 18.2 (C-24), 18.0 (C-26), 17.9 (C-29), 17.4 (C-25).

2.3.21. α-Linolenic acid (**20**)

Yellow oil; $[\alpha]^{25}_D$ = −1.6 (c 0.1, CH_3OH); ESI–MS: m/z 279 $[M+H]^+$; ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.33 (6H, m, H-9,10,12,13,15,16), 2.79 (4H, m, H_2-11,14), 2.33 (2H, t, J = 7.2 Hz, H_2-2), 2.05 (4H, m, H-8,17), 1.62 (2H, q, J = 7.2 Hz, H_2-3), 1.30 (8H, m, H_2-4,5,6,7), 0.96 (3H, t, J = 7.2 Hz, H-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ_C: 179.9 (C-1), 132.2 (C-9), 130.5 (C-16), 128.5 (C-12), 128.5 (C-13), 128.0 (C-15), 127.3 (C-10), 34.2 (C-2), 29.9 (C-6), 29.8 (C-7), 29.3 (C-5), 29.3 (C-4), 27.4 (C-8), 25.9 (C-14), 25.8 (C-11), 24.9 (C-3), 20.8 (C-17), 14.5 (C-18).

2.3.22. 1-Linolenoyl glycerol (**21**)

Yellow oil; $[\alpha]^{25}_D$ = −3.0 (c 0.1, CH_3OH); ESI–MS: m/z 353 $[M+H]^+$; ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.34 (6H, m, H-9,10,12,13,15,16), 4.19 (1H, dd, J = 11.4, 4.8 Hz, H-1′$_a$), 4.13 (1H, dd, J = 11.4, 6.0 Hz, H-1′$_b$), 3.92 (1H, q, J = 6.0 Hz, H-2′), 3.68 (1H, dd, J = 11.4, 3.6 Hz, H-3′$_a$), 3.58 (1H, dd, J = 11.4, 6.0 Hz, H-3′$_b$) 2.79 (4H, m, H_2-11,14), 2.34 (2H, t, J = 7.2 Hz, H_2-2), 2.05 (4H, m, H-8,17), 1.61 (2H, q, J = 7.2 Hz, H_2-3), 1.30 (8H, m, H_2-4,5,6,7), 0.96 (3H, t, J = 7.2 Hz, H-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ_C: 174.5 (C-1), 132.2 (C-9), 130.5 (C-16), 128.5 (C-12), 128.5 (C-13), 128.0 (C-15), 127.3 (C-10), 70.5 (C-2′), 65.4 (C-1′), 63.6 (C-3′), 34.4 (C-2), 29.9 (C-6), 29.8 (C-7), 29.4 (C-5), 29.3 (C-4), 27.4 (C-8), 25.9 (C-14), 25.8 (C-11), 25.1 (C-3), 20.8 (C-17), 14.5 (C-18).

2.3.23. 1-Linoleoyl glycerol (**22**)

Yellow oil; $[\alpha]^{25}_D$ = −2.2 (c 0.1, CH_3OH); ESI–MS: m/z 355 $[M+H]^+$; ^{1}H NMR (600 MHz, $CDCl_3$) δ_H: 5.34 (4H, m, H-9,10,12,13), 4.19 (1H, dd, J = 11.4, 4.8 Hz, H-1′$_a$), 4.13 (1H, dd, J = 11.4, 6.0 Hz, H-1′$_b$), 3.92 (1H, q, J = 6.0 Hz, H-2′), 3.68 (1H, dd, J = 11.4, 3.6 Hz, H-3′$_a$), 3.58 (1H, dd, J = 11.4, 6.0 Hz, H-3′$_b$) 2.75 (2H, t, J = 7.2 Hz, H_2-11), 2.34 (2H, t, J = 7.2 Hz, H_2-2), 2.03 (4H, m, H-8,14), 1.62 (2H, q, J = 7.2 Hz, H_2-3), 1.29 (14H, m, H_2-4,5,6,7,15,16,17), 0.87 (3H, t, J = 7.2 Hz, H-18); ^{13}C NMR (150 MHz, $CDCl_3$) δ_C: 174.5 (C-1), 130.5 (C-9), 130.2 (C-13), 128.3 (C-12), 128.1 (C-10), 70.5 (C-2′), 65.4 (C-1′), 63.6 (C-3′), 34.4 (C-2), 31.8 (C-16), 29.9, (C-6), 29.8 (C-7), 29.6 (C-15), 29.4 (C-5), 29.3 (C-4), 27.4 (C-8), 27.4 (C-14), 25.9 (C-11), 25.1 (C-3), 22.8 (C-17), 14.3 (C-18).

2.4. IL-6/STAT3 luciferase reporter assay

Human hepatoma Hep3 B (HB-8064) and myeloma U266 (TIB-196TM) cell lines were obtained from the American Type Culture Collection (ATCC; Rockville, MD, USA). Hep3 B cells stably transfected with pSTAT3-Luc were previously described by Chang et al. [16]. Hep3 B cells were seeded into 96-well culture plates at 2×10^4 cells/well and were treated with 10 ng/mL IL-6 in the presence or absence of compounds. After incubation for 12 h, luciferase activity was evaluated according to the manufacturer's instructions (Promega Corp., Madison, WI, USA). Cell viability was determined based on the MTT (Sigma Chemical Co., St. Louis, MO, USA) assay, as described in previous studies [17]. Recombinant human IL-6 and genistein, used for the control groups, were obtained from R&D System (Minneapolis, MN, USA) and Sigma-Aldrich Ltd (St Louis, MO, USA). All other reagents used were the highest commercially available grade.

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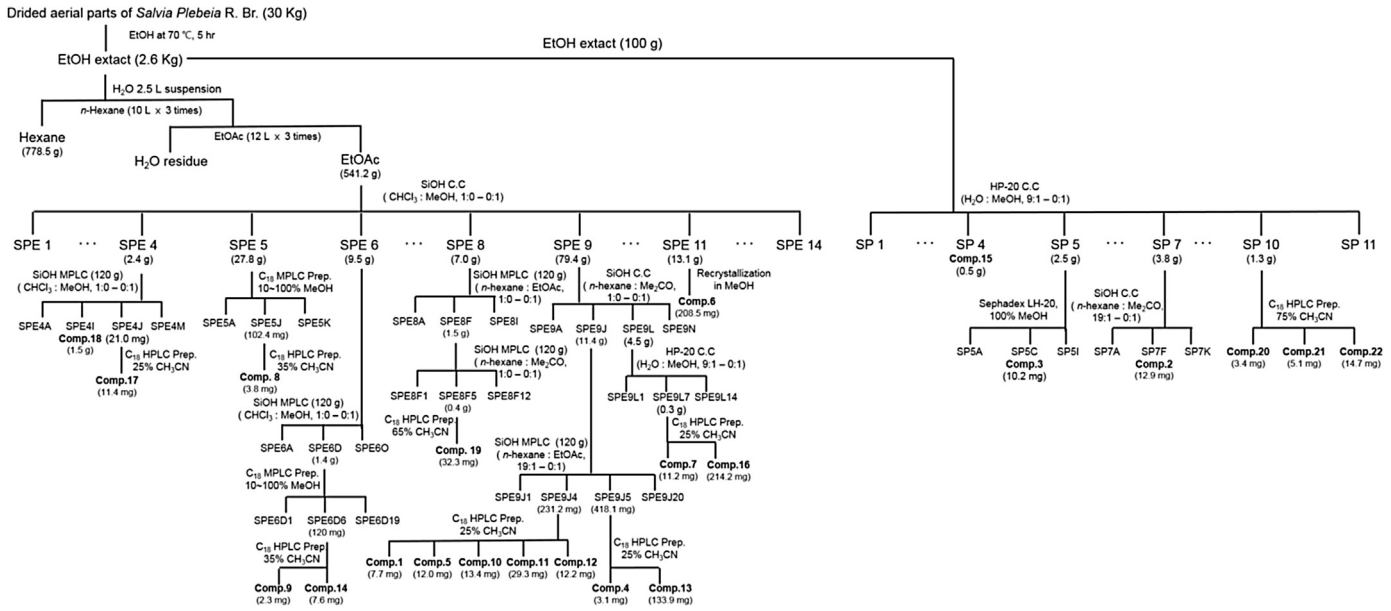

Fig. 1. A schematic diagram of the isolation of bioactive-compounds from *Salvia plebeia* (**1–22**).

Table 1
Inhibitory effects of compounds **1–22** on IL-6/STAT3 activation.

Compound	IC_{50} (μM)	Compound	IC_{50} (μM)
Apigenin (**1**)	10.4 ± 0.48	Caffeic acid (**13**)	>50
Luteolin (**2**)	6.7 ± 1.30	Ethyl caffeate (**14**)	>50
Luteolin-7-*O*-glycoside (**3**)	>50	Rosmarinic acid (**15**)	>50
Isorhamnetin (**4**)	14.7 ± 1.16	Methyl Rosmarinate (**16**)	5.8 ± 0.42
Hispidulin (**5**)	21.0 ± 1.36	7-Epiloliolide (**17**)	>50
Homoplantaginin (**6**)	>50	Ursolic acid (**18**)	3.1 ± 0.23
Nepetin (**7**)	5.5 ± 0.69	Ursolic acid-3-acetate (**18a**)	1.5 ± 0.35
Cirsimaritin (**8**)	1.4 ± 0.40	Corosolic acid (**19**)	0.5 ± 0.14
6-Methoxy-naringenin (**9**)	>50	α-Linolenic acid (**20**)	21.9 ± 3.68
Eriodictyol (**10**)	>50	1-Linolenoyl glycerol (**21**)	28.6 ± 0.20
Filifolin (**11**)	>50	1-Linoleoyl glycerol (**22**)	6.3 ± 1.95
Protocatechuic acid (**12**)	>50	Genistein[a]	15.0 ± 0.53

[a] Genistein was used as a positive control.

2.5. Western blot analysis

U266 cells were stimulated with IL-6 (10 ng/mL) for 20 min in the presence or absence of compound. After treatment, cells were collected and lysed in RIPA buffer (Millipore, Billerica, MA). The cell protein concentration was determined by the DC protein assay from Bio-Rad, and 20–40 μg of whole cell lysate was separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred onto PVDF membranes. The membrane was blocked for 1 h with TBS containing 5% fat-free dried milk. Next, the membrane was incubated overnight at 4 °C with the primary antibody. The phosphorylation status of JAK2, STAT3, and ERK was examined using anti-phospho-STAT3 (1:1000), anti-STAT3 (1:1000), anti-phospho-JAK2 (1:1000), anti-JAK2 (1:1000), anti-phospho-ERK (1:1000), and anti-ERK (1:1000) antibodies (Cell Signaling, Beverly, MA, USA) and then were incubated with the appropriate horseradish peroxide-conjugated secondary antibody (1:3000) at RT, triplicate washes were followed with TBS-T, and developed for visualization using an ECL detection kit by Luminescent Image Analyzer, LAS-3000 (Fuji, Tokyo, Japan) [18].

2.6. Statistical analyses

Statistical analyses were performed on data collected in triplicate for all experimental items. Data are expressed as the means ± standard error of mean (SEM). Statistical analyses were assessed by Student's *t*-test for paired data. Statistical analyses were performed using Prism 5 software (GraphPad software, San Diego, CA, USA). Student's *t*-test was used to evaluate data, and differences between the means were considered to be statistically significant at $P < 0.05$ or $P < 0.01$.

3. Results and discussion

3.1. Isolation and identification of compounds from SPRB

As part of our ongoing studies of IL-6/STAT3 inhibitors, an ethanol extract of the aerial parts of SPRB showed a significant inhibitory effect on IL-6-induced STAT3 expression in Hep3 B cells (IC_{50}: 4.3 μg/mL). Bioactivity-guided fractionation and column chromatographic purification of the SPRB EtOAc-soluble fraction resulted in the isolation of 22 known compounds (Fig. 1). We evaluated the structures of SPRB-derived compounds, which were determined to be apigenin (**1**) [19,20], luteolin (**2**) [21], luteolin-7-*O*-glycoside (**3**) [22], isorhamnetin (**4**) [23], hispidulin (**5**) [2], homoplantaginin (**6**) [2], nepetin (**7**) [2], cirsimaritin (**8**) [24], 6-methoxy-naringenin (**9**) [2], eriodictyol (**10**) [25], filifolin (**11**) [26], protocatechuic acid (**12**) [27], caffeic acid (**13**) [2], ethyl caffeate (**14**) [28], rosmarinic acid (**15**) [21], methyl rosmarinate (**16**) [21], 7-epiloliolide [29] (**17**), ursolic acid (**18**) [30], corosolic acid (**19**)

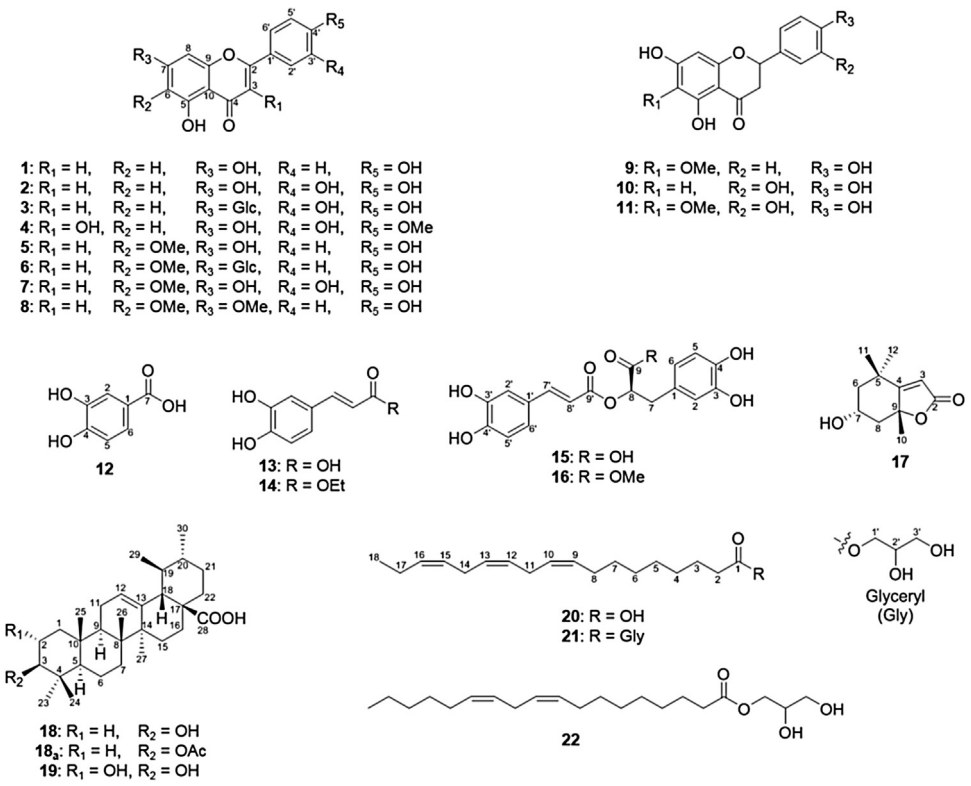

Fig. 2. The chemical structure of the compounds isolated from *Salvia plebeia* (**1–22**).

[31], α-linolenic acid (**20**) [32], 1-linolenoyl glycerol (**21**) [33], and 1-linoleoyl glycerol (**22**) [33] based on comparisons with the spectroscopic data available in the publically available literature.

3.2. Measurement of IL-6/STAT3 inhibitory effects using luciferase

Among the test compounds, compounds **1**, **2**, **4**, **5**, **7**, **8**, **16**, **18**, **18a**, **19**, **20**, **21**, and **22** exhibited inhibitory activity with half-maximal inhibitory concentration (IC_{50}) values ranging from 0.5 to 28.6 μM (Table 1); no cytotoxicity was observed at the IC_{50} concentration value (data not shown). However, additional studies are needed to fully elucidate structure-activity relationships, which is a correlation between the chemical structure and biological activity based on a finite number of possibilities. Luteolin (**2**) and nepetin (**7**), which have a 3′,4′-dihydroxy arrangement in the B ring, exhibited ~1.5- to 4-fold higher inhibitory activities compared with compounds that only contained a 4′-OH group in the B ring, such as apigenin (**1**) and hispidulin (**5**). Additionally, comparisons of cirsimaritin (**8**), which has a 6,7-dimethoxy group in the A ring, with hispidulin (**5**) shows the influence of a methoxylation substitution in flavonoids on the correlation between flavonoid chemical structures and IL-6/STAT3 inhibitory activity. Among the series of flavone compounds (**1**, **2**, **4**, **5**, **7**, and **8**), the order of the observed IL-6/STAT3 inhibitory activities was as follows: cirsimaritin (**8**) > nepetin (**7**) > luteolin (**2**) > apigenin (**1**) > isorhamnetin (**4**) > hispidulin (**5**); by contrast, flavanones (**9**, **10**, and **11**) and glycoside flavonoids (**3** and **6**) did not show an inhibitory effect. Therefore, both the position of the hydroxyl group in the B ring and the number of methoxyl group substitutions in the A ring of these flavone compounds contributed to IL-6/STAT3 inhibition. Rosmarinic acid does not show any inhibitory activity, whereas methyl rosmarinate (**16**) (IC_{50} values = 5.8 μM), which has a 9-methoxy substitution in rosmarinic acid (**15**), exhibited an inhibitory effect on IL-6/STAT3. Thus, the methoxylation substitution of compounds influenced the IL-6/STAT3 inhibitory activity. Ursolic acid-3-acetate (UAA, **18a**), which was synthesized from ursolic acid (UA, **18**), exhibited a slightly higher inhibitory activity than that of ursolic acid obtained from SPRB (IC_{50} values: UAA = 1.5 μM, UA = 3.1 μM). Our bioassay findings also support the hypothesis that corosolic acid (**19**) may be a promising STAT3 inhibitor (IC_{50} values of 0.5 μM). Additionally, the luciferase assay showed that α-linolenic acid (**20**) exhibits inhibitory activity with an IC_{50} value of 21.9 μM, and 1-linoleoyl glycerol (**22**), which possesses a glyceryl group at the C-1 position and is an omega-6 fatty acid, shows more potent IL-6/STAT3 inhibitory activity (IC_{50} values: 6.3 μM) than α-linolenic acid (**20**).

The biological activities of these compounds on IL-6/STAT3 inhibition were evaluated by a luciferase reporter assay in Hep3 B cells; genistein was used as a positive control, as it has been reported to inhibit STAT3 activation and dimerization [34]. We recently reported that two triterpenoid compounds isolated from *Vigna angularis* could inhibit IL-6/STAT3 signaling [17]. Oleanolic acid-3-acetate (OAA) showed greater effective IL-6-stimulated STAT3 inhibitory activity than oleanolic acid (OA) (IC_{50} values: OAA 0.9 μM, OA 18.6 μM). Therefore, we hypothesized that the biological activity accounting for the anti-inflammatory effect was enhanced by the acetylation of triterpenoids (Fig. 2).

3.3. Inhibitory effects of active compounds at the protein level

Here, we investigated whether cirsimaritin (**8**) could inhibit the phosphorylation of STAT3. Western blot analysis revealed that IL-6-inducible phosphorylation of STAT3 was reduced in U266 cells after pre-treatment with cirsimaritin at 10 or 30 μM (Fig. 3A). Furthermore, we measured the levels of p-JAK2/JAK2 (Fig. 3B) and p-ERK/ERK (Fig. 3C). When cells were treated with the same concentration of cirsimaritin, a lower level of protein expression was observed at 30 μM. Cirsimaritin, a natural flavonoid with a low molecular weight, can induce apoptosis of carcinoma cells [35,36]. Our findings suggested that cirsimaritin induces endoplas-

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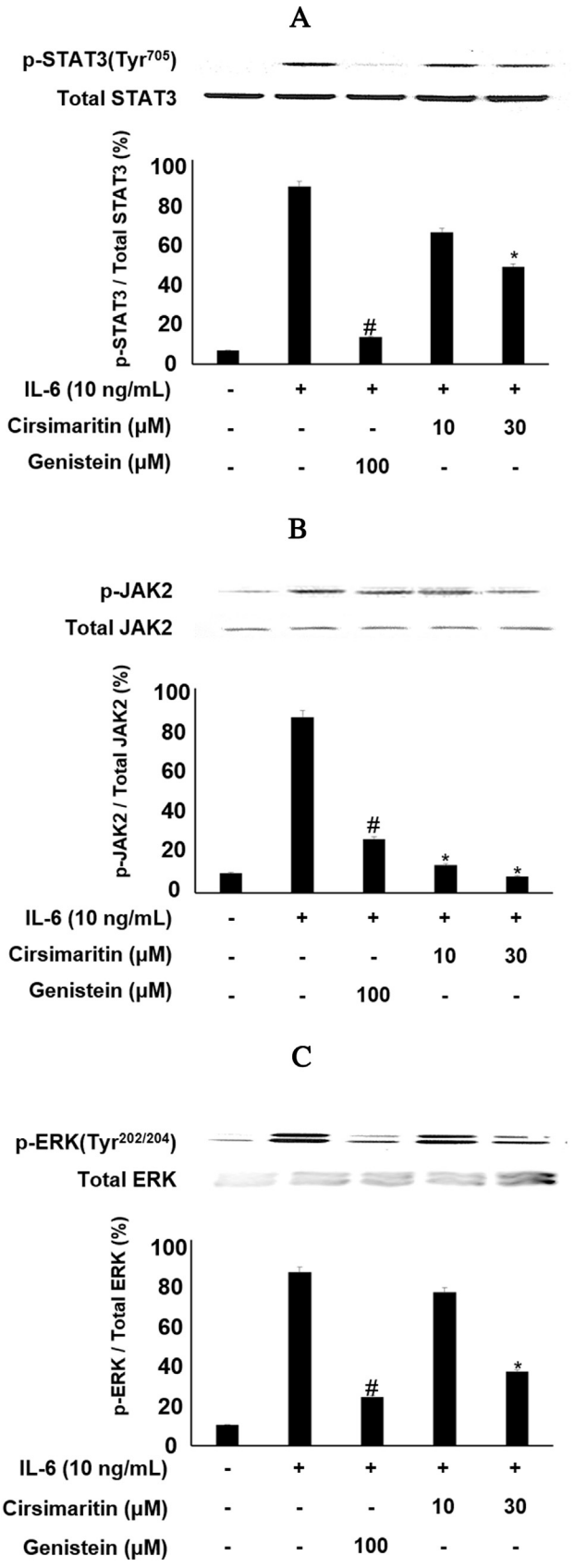

Fig. 3. Inhibitory effects of cirsimaritin (**8**) on STAT3, JAK2 and ERK activation after IL-6 stimulation, shown by western blot analysis of lysates from U266 cells; #Not significantly different from that of the control group. *Denotes a significant difference ($p < 0.05$) as analyzed by paired-samples *t*-test compared with the IL-6-treated group.

mic reticulum stress by inducing the unfolded protein response and activating C/EBP-homologous protein, which is a key transcription factor induced by endoplasmic reticulum stress and contributes to carcinoma cell apoptosis by inhibition of AKT activity. Additionally, cirsimaritin triggers the increased accumulation of reactive oxygen species, and the antioxidant N-acetyl-cysteine suppresses cirsimaritin-induced endoplasmic reticulum stress and mitochondrial dysfunction [36]. It has been previously reported that the *Salvia plebeia* methanolic extract and its active ingredients protect against inflammation. In addition, *Salvia plebeia* methanolic extract significantly reduced inflammatory processes *in vitro* and *in vivo* [37]. According to previously published reports, corosolic acid (**19**) alters tumor cell proliferation by inhibiting STAT3 and NF-κB activation [38]. α-linolenic acid (**20**) is a widely known example of an omega-3 fatty acid that has been reported to exhibit therapeutic activity against inflammatory and cardiovascular diseases [39]. The phosphorylation of STAT3 in response to IL-6 triggers the nuclear translocation of STAT3, which allows it to drive the expression of mRNA transcripts that encode pro-inflammatory cytokines, such as IFN-γ, IL-17, and IL-1β [17,40]. IL-6 is well known for its ability to activate immunity and inflammation by binding to the IL-6 receptor and dimeric gp130, which subsequently drives the phosphorylation of JAK2, STAT3, and ERK [15,41].

Based on the structure-activity relationship inferred from the SPBR constituent chemical compounds, several candidates were identified as effective inhibitors of IL-6-mediated STAT3 activation; our present study also reveals promising drug targets for several diseases, such as cancer and inflammatory pathologies.

4. Conclusions

Based on the present data, we isolated and identified 22 compounds from SPBR, which were identified by HPLC, NMR and ESI–MS. The effectiveness of these compounds was tested for their ability to inhibit IL-6/STAT3 activation using a luciferase reporter assay. Additionally, we measured protein levels by western blot. Other downstream signaling molecules involved in IL-6-triggered responses may be inhibited by the suppression of phosphorylated STAT3, JAK2, and ERK; cirsimaritin, an active compound from SPBR, may be responsible for these effects. However, further studies will be needed to elucidate the precise mechanism *in vivo*.

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