Flavonoids from Scutellaria baicalensis inhibit senescence-associated secretory phenotype production by interrupting IκBζ/C/EBPβ pathway: Inhibition of age-related inflammation

Hyun Lim , Yong Soo Kwon , Donghoon Kim , Jongkook Lee , Hyun Pyo Kim

PII: S0944-7113(20)30086-6
DOI: https://doi.org/10.1016/j.phymed.2020.153255
Reference: PHYMED 153255

To appear in: *Phytomedicine*

Received date: 23 December 2019
Revised date: 6 May 2020
Accepted date: 23 May 2020

Please cite this article as: Hyun Lim , Yong Soo Kwon , Donghoon Kim , Jongkook Lee , Hyun Pyo Kim , Flavonoids from Scutellaria baicalensis inhibit senescence-associated secretory phenotype production by interrupting IκBζ/C/EBPβ pathway: Inhibition of age-related inflammation, *Phytomedicine* (2020), doi: https://doi.org/10.1016/j.phymed.2020.153255

Flavonoids from *Scutellaria baicalensis* inhibit senescence-associated secretory phenotype production by interrupting IκBζ/C/EBPβ pathway: Inhibition of age-related inflammation

Hyun Lim, Yong Soo Kwon, Donghoon Kim, Jongkook Lee, and Hyun Pyo Kim*

College of Pharmacy, Kangwon National University, Chuncheon 24341, Republic of Korea

*Corresponding author

Hyun Pyo Kim, College of Pharmacy, Kangwon National University, 1, Gangwondaehak-gil, Chuncheon 24341, Republic of Korea,

Tel.: +82 33 250 6915, Fax.: +82 33 259 5631

E-mail address: hpkim@kangwon.ac.kr (H.P. Kim).

Word count: 7,072

ABSTRACT

Background: Prolonged exposure to the senescence-associated secretory phenotype (SASP) with age leads to chronic low-grade inflammation in neighboring cells and tissues, causing many chronic degenerative diseases.

Purpose: The effects on SASP production of the ethanol extract from Scutellaria radix and 17 isolated flavonoid constituents were examined *in vitro* and *in vivo*.

Methods: Cellular senescence was induced by bleomycin. Expression of the SASP and cell signaling molecules was detected using ELISA, RT-qPCR, Western blotting, and immunofluorescence staining. To investigate the *in vivo* effects, 21-month-old aged rats were used.

Results: The ethanol extract and 5 compounds including **1** (Oroxylin A; 5,7-dihydroxy-6-methoxyflavone), **5** (2‘,6’,5,7-tetrahydroxy-8-methoxyflavone), **8** (2‘,5,7-trihydroxyflavone), **10** (2’,5,7-trihydroxy-8-methoxyflavone) and **11** (2‘,5,7-trihydroxy-6-methoxyflavone) potently reduced IL-6 and IL-8 production and gene expression of the SASP, including IL-1α, IL-1β, IL-6, IL-8, GM-CSF, CXCL1, MCP-2, and MMP-3. This finding indicates the important role of the B-ring 2'-hydroxyl group in flavonoid molecules. Furthermore, compounds **8** and **11**, the strongest SASP inhibitors, decreased the expression of IκBζ and C/EBPβ protein without affecting either BrdU uptake or the expression of senescence markers, such as pRb and p21. Finally, the oral administration of compound **8** to aged rats at 2 and 4 mg/kg/day for 10 days significantly inhibited the gene expression of SASP and IκBζ in kidneys. This is the first report of the strong SASP inhibitory action of flavonoids from Scutellaria radix on *in vitro* and *in vivo* senescence models. The inhibitory action was shown

to be mediated mainly by interfering with the IκBζ/C/EBPβ signaling pathway.

Conclusion: Targeting production of the SASP using flavonoids from Scutellaria radix or its extract might help reduce low-grade sterile inflammation and control age-related diseases.

Keywords: Scutellaria baicalensis; Senescence; SASP; IκBζ; C/EBPβ; Chronic low-grade inflammation

Abbreviations:

BrdU, 5-bromo-2'-deoxy-uridine; C/EBPβ, CCAAT/enhancer-binding protein beta; CXCL1, chemokine (C-X-C motif) ligand 1; ELISA, enzyme-linked immunosorbent assay; GM-CSF, granulocyte-macrophage colony-stimulating factor; IL, interleukin; IRAK1, interleukin-1 receptor-associated kinase; MCP-2, monocyte chemoattractant protein-2; MMP-3, matrix metalloproteinase-3; RT-qPCR, reverse transcription-quantitative polymerase chain reaction

1. Introduction

The identification of anti-aging agents that extend the lifespan of organisms is not a simple task. The compounds such as resveratrol, curcumin, and rapamycin have been shown to prolong lifespan of fruit flies *Drosophila melanogaster* and *Caenorhabditis elegans* (Bass et al., 2007; Bjedov et al., 2010; Lee et al., 2010). Recently, potential targets such as senolytic activity of fisetin, microbiome modulation of rapamycin, and proteostatic maintenance of rapamycin with or without metformin have been demonstrated to be associated with lifespan extension in mice (Bitto et al., 2016; Reid et al., 2020; Yousefzadeh et al., 2018). However, evidence demonstrating the extension of the human lifespan is lacking. Agents that inhibit cellular senescence may have some deleterious effects, possibly resulting in cancer cell formation, because cellular senescence is one way to eliminate potential cancer-transforming cells. Instead, eliminating the harmful effects of cellular senescence is an option for healthy aging. As organisms age, cells can become damaged or telomerase shorten that triggers activation of a signaling cascade that results in cell senescence, a permanent cell cycle arrest. The senescent cells express molecules known as senescence-associated secretory phenotype (SASP) during this process (Coppé et al., 2008; 2010). Although the SASP, consisting of the proinflammatory cytokines, proteases, and growth factors, has some beneficial effects, such as normal development and tissue remodeling (Coppé et al., 2010; Storer et al., 2013), prolonged exposure to SASP factors can cause chronic low-grade sterile inflammation during aging, also known as inflammaging, which facilitates aging and transformation of the surrounding cells, leading to age-related chronic inflammatory diseases, such as type 2 diabetes, cardiovascular diseases, neurodegenerative diseases, and cancer (Campisi et al.,

2011; Kumar et al., 2014; Onat and Can 2014; Singh and Newman 2011). In this regard, finding SASP-inhibiting agents without affecting cellular senescence is a current issue in the field of healthy aging.

Recently, several natural and synthetic flavonoids were shown for the first time to inhibit SASP production without affecting the senescence process itself. The optimum chemical structures are found to be basic flavone structures having hydroxyl and/or methoxyl substitutions on C-5,7,2',3' and/or 4' (Lim et al., 2015). The inhibitory activity was shown to be associated with the activation of transcription factors, such as nuclear factor-kappa B (NF-κB) and IkappaB zeta (IκBζ) (Lim et al., 2015). Moreover, one of the strong inhibitors, apigenin, showed a similar response in aged-rats by the oral administration of doses as low as 2 – 4 mg/kg/day (Lim et al., 2015). The similar suppressive effect of apigenin on expression of the SASP was also confirmed in fibroblasts and breast cancer cells (Perrott et al., 2017). During our preliminary experiment to screen various herbal products, the alcohol extract of the radix of *Scutellaria baicalensis* Georgi strongly inhibited SASP production. It is well-known that the chemical structures of the main flavonoids in the radix of *S. baicalensis* are flavones with hydroxy/methoxyl substitution(s) at C-5,6,7 or 5,7,8 in the A-ring and C-2' and/or 6' in the B-ring (Liu et al., 2009). However, the effects of these flavonoids on SASP production are not known. Based on the potential importance of this SASP inhibitor, we isolated 17 flavonoids from *S. baicalensis* and their inhibitory action against SASP production, as well as the mechanisms of action, were evaluated in the present investigation.

2. Materials and methods

102 *2.1. Chemicals and animals*

103

104 Bleomycin and apigenin were purchased from Enzo Life Sciences (Farmingdale, NY,
105 USA). Fetal bovine serum (FBS) and Dulbecco's Modified Eagle Medium (DMEM) were
106 bought form MP biomedicals (Santa Ana, CA, USA) and Hyclone Laboratories (South Logan,
107 UT, USA). Phenylmethylsulfonyl fluoride (PMSF), sodium orthovanadate, sodium fluoride,
108 protease inhibitor cocktail and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
109 (MTT) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Human IL-6 and IL-8
110 ELISA kits were bought from BD Biosciences (San Jose, CA, USA). The origins and grades
111 of other chemicals, primers and antibodies were described in detail in supplementary file.
112 Male Sprague Dawley (SD) rats (5-month-old young and 21-month-old) were purchased
113 from Samtako (Osan, Korea). The animals were maintained in the animal facility at 20 - 22^{o}C
114 under 40 - 60% relative humidity and a 12 h light/dark cycle for at least 7 days prior to the
115 experiment. The experimental design using the animals was approved by the local committee
116 for animal experimentation, KNU (KW-190927-3). In addition, the ethical guidelines
117 described in the Korean Food and Drug Administration guide for the care and use of
118 laboratory animals was followed throughout the experiments.

119

120 *2.2. Preparation of 80% ethanol extract from Scutellaria radix and isolation of flavonoid*
121 *constituents*

122

123 The dried radix of *Scutellaria baicalensis* was purchased in local herbal market and
124 taxonomically identified by one of the authors (Dr. Y. S. Kwon). A voucher specimen

(KNUH-R-15-1) was deposited at the Herbarium of the College of Pharmacy (KNU, Korea). The dried radix of *S. baicalensis* (1 kg) was refluxed in 80% ethanol three times and filtered. The evaporation gave the dried ethanol extract (2.8 kg) and the extract was used for the assay. Part of the ethanol extract (2.7 kg) was suspended in water and fractioned with *n*-hexane, ethylacetate and *n*-butanol, successfully. The evaporation of each fraction under reduced pressure provided *n*-hexane (38.7 g), ethylacetate (84.4 g) and *n*-butanol (106.0 g) fractions.

The flavonoid constituents were isolated with repetitive column chromatography. Briefly, ethylacetate fraction (80.0 g) was separated in silica gel chromatography using chloroform:methanol mixture as mobile phase, yielding fractions (S-1 to S-6). Fraction S-1 (12.9 g) was separated with Buchi MPLC, Rdisep silica gel, ODS and Sephadex LH20 column chromatography, yielding compound **1** (70 mg), **2** (110 mg) and **3** (160 mg). Using similar column chromatography, fraction S-2 gave compounds **4** (65 mg), **5** (5 mg), **6** (20 mg), **7** (7 mg), **8** (6 mg), **9** (4.2 mg), **10** (4 mg) and **11** (2.4 mg). From S-3 (19 g), consecutive silica gel and ODS column chromatography produced compounds **12** (350 mg) and **13** (1.3 g). Fraction S-4 (11 g) was separated successfully with silica gel flash column and ODS column chromatography, yielding compounds **14** (365 mg) and **15** (2.3 g). From *n*-butanol fraction (30 g), repetitive silica gel column chromatography yielded compound **16** (40 mg) and compound **17** (1.98 g). All the chemical structures of the isolated compounds are shown in Fig. 1A and confirmed by the comparison of the previously reported data. The detailed separation procedures and instrumental data are represented in supplementary file. The isolated compounds showed one spot in two-dimensional thin-layer chromatography and the purities (> 95%) were determined by Thermo Dynamic HPLC system using a intersil column (ODS-2, 150 x 4.5 mm) with acetonitrile:water (4:6) as a mobile phase.

148 For *in vivo* study, compound **8** was chemically synthesized. 2',5,7-Trihydroxyflavone (**8**)
149 was synthesized from commercially available 1[2,4-bis(benzyloxy)-6-
150 hydroxyphenyl]ethanone via a aldol condensation-oxidative cyclization-debenzylation
151 sequence as shown in Fig. 1B.
152
153 *2.3. Bleomycin-induced cellular senescence and SASP production*
154
155 BJ cells (human foreskin fibroblast) purchased from the American Type Culture
156 Collection (ATCC, Manassas, VA) were maintained in DMEM containing 10% FBS,
157 glutamine, and penicillin/streptomycin. Damage-induced cellular senescence was produced
158 by treating BJ cells with bleomycin according to a protocol in previous reports (Lim et al.,
159 2015; Robles and Adami, 1998). In brief, the cells were treated with bleomycin (50 μg/ml)
160 for 24 h and washed twice with phosphate-buffered saline (PBS), followed by further
161 incubation for 6 days. Completion of cellular senescence was determined by the ratio of
162 incorporated BrdU into cellular DNA using the BrdU labeling and detection kit using anti-
163 BrdU antibody. Percentages of BrdU-positive cells of 10% or less indicate that the cellular
164 senescence is complete (Laberge et al., 2012).
165 To identify the effect of the test compounds after the completion of senescence, the cells
166 were treated with test compounds for the last 24 h after the completion of senescence by
167 bleomycin. To examine simultaneous effects, the cells were treated with the test compounds
168 with bleomycin (50 μg/ml) for 24 h at the initial time of senescence induction and incubated
169 for 6 days with the test compounds. On the 6^{th} day, the IL-6 and/or IL-8 (representative
170 markers of SASP molecules) production in the media was quantified with an ELISA kit. The

171 gene expression of the SASP (IL-6, IL-8, IL-1α, IL-1β, GM-CSF, CXCL1, MCP-2, and
172 MMP-3) was evaluated using RT-qPCR with specific primers on an C1000 Touch Thermal
173 Cycler (Biorad Lab., Hercules, CA, USA) according to protocols described in a previous
174 report (Lim et al., 2015). The primer sequences for the human genes are shown in Table 1 of
175 the supplementary file. Senescence-associated-β-galactosidase (SA-β-gal) activity was
176 determined by counting the number of blue-stained cells versus the number of total cells
177 using senescence cells histochemical staining kit (Sigma Aldrich, St. Louis, MO, USA). Cells
178 are considered to be senescent if >80% of cells are stained in blue (Itahana et al., 2013).
179 Cellular viability was examined using the MTT assay (Mosmann, 1983) and all tested
180 compounds were used at non-cytotoxic concentrations.
181
182 *2.4. Cellular mechanisms associated with SASP production*
183
184 To investigate the cellular mechanisms related to the inhibitory effects on SASP
185 production, compound **8** and **11** were selected due to their strong SASP inhibitory activity
186 among the 17 compounds and the mechanism study was carried out by simultaneous
187 treatment with bleomycin. On the 6^{th} day, the total proteins in the cell lysates were extracted
188 with Proprep solution (iNtRON Biotechnology, Korea). All Western blot procedures,
189 including the preparation of nuclear protein, were essentially the same as previously
190 described (Lim et al., 2015). Each protein band on the blots was visualized using an enhanced
191 chemiluminescence (ECL) system (GE Healthcare, UK) with ImageQuant LAS4000 mini
192 and analyzed using Image J (NIH, USA).). Images of the cells immunostained with anti-IκBζ
193 or CCAAT/enhancer-binding protein beta (C/EBPβ) antibody on cells were obtained using a

super-sensitive high resolution confocal laser scanning microscope (SR-CLSM) (Carl Zeiss, Oberkochen, Germany). The detailed procedures are found in the supplementary file.

2.5. siRNA transfection against C/EBPβ

siRNA against C/EBPβ and non-targeting siRNA were prepared in 1 × siRNA buffer (60 mM KCl, 6 mM HEPES and 0.2 mM $MgCl_2$) as suggested in the manufacturer's protocol (Dharmacon Inc, Lafayette, CO, USA). siRNA against C/EBPβ or non-targeting siRNA and DharmaFECT reagent were mixed and incubated for 20 min. To increase the transfection efficiency, each siRNA (25 nM) was pre-transfected for 24 h, washed out with PBS twice, and re-transfected with bleomycin (50 μg/ml) for 24 h. The gene expressions of C/EBPβ, IκBζ, p21, and the SASP were detected by RT-qPCR.

2.6. SASP production in aged rats

Compound **8** was dissolved in 0.3% carboxymethyl cellulose (CMC) and orally administered to 21-month-old aged rats at 2 and 4 mg/kg/day once daily for 10 consecutive days. For the control group, only 0.3% CMC was administered to 5- and 21-month-old rats for 10 days. Five-month-old rats were used as young controls for comparison with 21-month-old aged rats. After 10 days, the rats were euthanized under CO_2. Organs, including the kidneys, liver, and lung were excised and blood was collected. To measure the expression of the SASP genes in the kidneys, kidney tissue (50 mg) was homogenized in 1 ml of Trizol reagent (Invitrogen, Carlsbad, CA, USA) at 50 Hz for 2 min using TissueLyser LT (Qiagen,

Hilden, Germany). Total RNA was extracted with chloroform and purified with a NucleoSpin Clean-up kit (MACHEREY-NAGEL GmbH & Co. KG, Düren, Germany). RT-qPCR was performed by the same method used in the BJ cells above. The primer sequences and manufacturer's information are shown in Table 1 of the supplementary file.

2.7. Statistics

Experimental values were represented as arithmetic mean ± SD. One way analysis of variance (ANOVA) followed by Dunnett's *post hoc* test was used to determine the statistical significance (IBM SPSS Statistics, version 24).

3. Results

3.1. Effects of the ethanol extract of Scutellaria radix on cellular senescence and SASP production

Bleomycin treatment provoked cellular senescence with SASP production. Treatment with the extract after senescence inhibited SASP production (IL-6 and IL-8, 59.5% and 41.5%, respectively, at 100 μg/ml), whereas the level of BrdU uptake was not changed (Fig. 2A). When the expression levels of the SASP genes (IL-6 and IL-8), IκBζ and p21 (senescence marker) were examined using RT-PCR, the extract significantly inhibited the expression of IL-6 and IL-8 (55.5% and 24.3% at 100 μg/ml), and IκBζ expression was slightly decreased by 18.3% at 100 μg/ml, although not significant. However, the mRNA

level of p21 was not changed (Fig. 2B).

When the extract (5, 10, and 20 μg/ml) was simultaneously treated with bleomycin from the initial point of senescence induction, the expression of IL-6 and IL-8 in the media was inhibited by 80.1% and 80.3%, respectively, at 20 μg/ml (Fig. 2C). The gene expression of IL-6, IL-8 and IκBζ was strongly inhibited by 86.3%, 85.1%, and 58.8%, respectively, at 20 μg/ml (Fig. 2D). The ratio of BrdU uptake and the p21 gene level were not changed by treatment with the extract (Fig. 2C and 2D). These results showed that the ethanol extract of Scutellaria radix had strong SASP inhibitory activity without affecting cellular senescence.

3.2. Effects of flavonoid constituents isolated from the extract of Scutellaria radix on SASP production

To identify the specific constituents responsible for SASP inhibitory activity in the extract of Scutellaria radix, 17 flavonoid derivatives were isolated from the extract (Fig. 1A). The effects of all flavonoids isolated on IL-6 production were examined at 10 μM, a pharmacologically-relevant concentration (Rodriguez-Mateos et al., 2014). When treated after senescence completion, only compound **5** (5,7,2',6'-tetrahydroxy-8-methoxyflavone) significantly inhibited IL-6 production (Fig. 3A). However, when treated concomitantly with bleomycin from the initial point of senescence induction, eight compounds (**1**, **2**, **5**, **8**, **9**, **10**, **11** and **16**) significantly inhibited IL-6 production (Fig. 3B). Compound **1** (oroxylin A), **2** (wogonin), **5** (5,7,2',6'-tetrahydroxy-8-methoxyflavone), **8** (2',5,7-trihydroxyflavone), **9** (norwogonin), **10** (2',5,7-trihydroxy-8-methoxyflavone), **11** (2',5,7-trihydroxy-6-methoxyflavone), and **16** (baicalin) inhibited IL-6 production by 107.5%, 69.9%, 73.0%,

153.9%, 42.6%, 96.9%, 136.3%, and 41.6%, respectively, at 10 μM. Among them, five compounds (compounds **1**, **5**, **8**, **10** and **11**) showing strong inhibitory activity were selected to further examine the effects on IL-8 production and the ratio of incorporated BrdU into cells. When treated once for the last 24 h after reaching senescence, the compounds slightly reduced IL-8 production, although not significantly (Fig. 3C). On the other hand, in the case of simultaneous treatment, compounds **1**, **5**, **8**, **10**, and **11** inhibited IL-8 production by 55.3%, 69.1%, 76.7%, 44.8% and 71.8%, respectively, at 10 μM (Fig. 3D). The ratio of BrdU uptake into cells was not changed in either experiments (Fig. 3C and 3D). Thus, these results indicate that certain flavonoid constituents were responsible for the strong SASP inhibitory activity exerted by Scutellaria radix without affecting cellular senescence itself. Like the extract, these flavonoids also showed more potent activity when simultaneously treated with bleomycin.

3.3. Effects on gene expression of SASP molecules

Each compound (**1**, **5**, **8**, **10**, and **11**) was simultaneously treated with 50 μg/ml of bleomycin. At 10 μM, all five compounds strongly suppressed the gene expression of SASP molecules including IL-6, IL-8, IL-1α, IL-1β, GM-CSF, CXCL1, MCP-2, and MMP-3 (Fig. 4A). Among them, compounds **8** and **11** most strongly suppressed the gene expression of IL-6, GM-CSF, IL-1α, IL-1β, and MCP-2 by more than 90% at 10 μM. Apigenin, a potent inhibitor (Lim et al., 2015), also strongly inhibited the gene expression of all tested SASP molecules at 10 μM. Expression of the IκBζ gene showed a close relationship with the cellular signaling pathway for SASP production and was also significantly decreased by these five compounds

and apigenin (Fig. 4B). In contrast, none of the compounds changed the gene expression level of p21, a senescence marker. Furthermore, compound **8** and **11** having potent activity on SASP inhibition, did not affect SA-β-gal activity compared to bleomycin-treated positive control (Fig. 4C). These results clearly showed that certain flavonoids from Scutellaria radix inhibited the expression of SASP molecules and these inhibitory effects coincided with the reduced expression of the IκBζ gene without changing the p21 gene expression and SA-β-gal activity.

*3.4. Cellular mechanisms related to SASP inhibition by compound **8** and **11***

Compounds **8** and **11** were selected for further investigation of the cellular signaling mechanism of SASP inhibition. Bleomycin treatment reduced the expression of IRAK1 and IκBα protein and phosphorylation of signal transducer and activator of transcription 3 (STAT3) (Fig. 5A). Among them, the degradation of IRAK1 protein was blocked by 56.3% and 32.0% by treatment with 10 μM **8** and **11** compounds, respectively. Also, the phosphorylation level of STAT3 was slightly increased by 24.5 % following treatment with compound **11** at 10 μM. The decreased expression of IκBα protein provoked by bleomycin was not recovered by these compounds. Compounds **8** and **11** did not affect either the degradation of pRb protein or the expression of p21 protein, senescence markers. Among the transcription factors involved in SASP production, the elevated expression of IκBζ in the nuclear fraction by bleomycin was decreased by 65.1% and 79.2% by treatment with compounds **8** and **11,** respectively, at 10 μM (Fig. 5B). Whereas the expression of C/EBPβ protein was increased 3.9-fold in the nuclear fraction by bleomycin, compounds **8** and **11** at

309 concentrations of 5 - 10 μM significantly inhibited the expression by more than 50%. As a
310 reference, apigenin also suppressed the expression of IκBζ and C/EBPβ. Reduction of the
311 expression of IκBζ and C/EBPβ protein by compounds **8** and **11** was also confirmed by
312 immunofluorescence staining using anti-IκBζ and C/EBPβ antibodies (Fig. 5C). These results
313 demonstrate that the inhibitory effect of compounds **8** and **11** on SASP production was
314 closely related to the inhibition of signaling pathways involved in IκBζ and C/EBPβ.

315 To further characterize the essential role of C/EBPβ, an siRNA transfection study was
316 designed, since the correlation between IκBζ expression and SASP production has been
317 shown in previous reports (Alexander et al., 2013; Lim et al., 2015). When siRNA against
318 C/EBPβ (25 nM) was treated with bleomycin (50 μg/ml) for 24 h, the expression of C/EBPβ
319 gene was successfully blocked compared to that of non-targeting siRNA-transfected cells, as
320 expected (Fig. 5D). The increased expression of several SASP genes by bleomycin was
321 significantly suppressed by transfection with C/EBPβ-targeting siRNA. The levels of IL-1α,
322 IL-1β, IL-6, IL-8, GM-CSF, CXCL1, MCP-2, and MMP-3 genes were inhibited by 41.8%,
323 50.5%, 49.1%, 47.5%, 38.9%, 56.8%, 69.5%, and 61.7%, respectively. Moreover, along with
324 the inhibition of SASP expression resulting from blocking the C/EBPβ gene, the IκBζ gene
325 level was inhibited by 32.4%, as well. However, the expression level of the p21 gene
326 (senescence marker) was not changed. All these results indicate that C/EBPβ expression was
327 essential to the signaling pathway for SASP production and IκBζ expression without affecting
328 senescence itself in the bleomycin-induced senescence model.

329

330 *3.5. Effects of compound **8** on SASP production in aged rats*

331

To demonstrate the *in vivo* effect, compound **8** was orally administered to 21-month-old aged rats for 10 days (2 and 4 mg/kg/day). After euthanasia, the expression of SASP genes in the kidney was evaluated using RT-qPCR. The expression of IL-6, IL-1α, IL-1β, GM-CSF, and CXCL1 genes in 21-month-old aged rats was significantly increased by 17.1, 20.7, 5.2, 6.7, and 8.2-fold, respectively, over those in 5-month-old rats used as young controls (Fig. 6A). The elevated expression of these SASP genes was reduced by oral treatment with compound **8** for 10 days. In particular, IL-6 was strongly decreased by 47.1% and 49.6% at the doses of 2 and 4 mg/kg/day, respectively. IL-1β expression was significantly inhibited by 42.9% at 4 mg/kg/day and CXCL1 was inhibited by 40.4% at 2 mg/kg/day. In parallel with the increased SASP expression, the expression of IκBζ found as the transcription factor associated with senescence, was also increased by 6.1-fold in the kidney of a 21-month-old aged rat, whereas this increase was significantly reduced by 48.0% with the administration of compound **8** at 2 mg/kg/day (Fig. 6B). The C/EBPβ gene was also slightly increased by 1.2-fold in 21-month-old aged rats ($P < 0.5$). This increase in the C/EBPβ gene was down-regulated to similar levels as those in 5-month-old aged rats at 4 mg/kg/day, although not statistically significant. All these results showed that compound **8** was a strong SASP inhibitor in the aged animal model. It also demonstrated that the IκBζ and C/EBPβ key factors were downregulated by the oral administration of compound **8** in aged rats.

4. Discussion

The present study demonstrated that the ethanol extract of Scutellaria radix and flavonoid

355 constituents possessed strong inhibitory activities against SASP production without affecting
356 cellular senescence in bleomycin-treated senescence cells. In particular, the study found that
357 flavone derivatives having a B-ring 2'-hydroxyl substitution among the constituents had an
358 enhanced inhibitory activity on SASP production. For the cellular action mechanism, the
359 present study strongly suggests that transcription factors, such as IκBζ and C/EBPβ, are
360 closely involved in SASP production and flavonoids possibly interrupt their expression.

361 Based on these results, the analysis of the chemical structures of 17 flavonoid derivatives
362 isolated from the extract of Scutellaria radix provided some structure-activity relationships
363 involved in SASP production, despite some limited structural diversities. Among the isolated
364 flavonoids, compounds **8**, **10**, and **11** showed strong inhibitory action. These three
365 compounds have B-ring 2'-hydroxyl substitution in common and showed higher inhibition
366 than flavonoids having the same chemical structures devoid of 2'-hydroxyl moiety
367 (compound **8** vs. chrysin, **10** vs. **2**, **11** vs. **1**). Chrysin did not show significant inhibitory
368 action under the same experimental conditions (Lim et al., 2015). Of note, methoxyl
369 substitution at C-6 in the A-ring maybe favorable (compound **1** vs. **12**, baicalein, 5,6,7-
370 trihydroxyflavone). In our previous report, the results suggested that apigenin, kaempferol,
371 2',3'-dihydroxyflavone, 3',4',7-trihydroxyflavone, and 2',3',5,7-tetrahydroxyflavone were
372 the flavonoid derivatives with optimum structures for SASP inhibition (Lim et al., 2015). In
373 the present study, the importance of a hydroxyl substitution at C-2' in the B-ring and/or a
374 methoxy group substitution at C-6 in the A-ring was revealed.

375 Following the initial discovery of SASP inhibitory action of some flavonoids (Lim et al.,
376 2015), several studies demonstrated the SASP inhibitory action of certain flavonoid and
377 polyphenol derivatives. Quercetin was found to reduce SASP production under various

treatment conditions alone or in combination with dasatinib *in vitro* and *in vivo* (Hickson et al., 2019; Kim et al., 2019; Xu et al., 2018). Epigallocatechin gallate and resveratrol were shown to suppress SASP production in 3T3-L1 preadipocytes and stromal senescent fibroblasts, respectively (Kumar et al., 2019; Menicacci et al., 2017). In the present study, another cluster of flavonoids with B-ring 2'-hydroxyl group was found to be strong SASP inhibitors.

Thus far, the regulatory mechanisms of SASP production have been linked to NF-κB, C/EBPβ, DNA damage response, and the p38 mitogen-activated protein kinase pathway (Freund et al. 2011; Salminen et al. 2012). Mixed lineage linkage, GATA4 and reactive oxygen species-protein kinase Cδ-protein kinase 1 cascade were also proposed to regulate SASP expression (Capell et al., 2016; Kang et al., 2015; Wang et al. 2014). These signaling molecules contribute to SASP production differently depending on the cell type and senescence inducers.

The involvement of the IκBζ transcription factor was demonstrated in the signaling pathway for IL-6 and IL-8 expression in DNA damage- and oncogene-induced senescence (Alexander et al., 2013). IκBζ expression in the kidneys of aged rats was maintained at an elevated level in 17- to 19-month-old aged rats and greatly increased in 21-month-old aged rats (data not shown). Regarding the cellular mechanisms of action of the flavonoids, in our previous report, the inhibitory action of flavonoid derivatives, including apigenin on the SASP production, was shown to be mediated through the IκBζ and NF-κB pathways (Lim et al., 2015). Compound **8** was also found to affect the expression of IκBζ in the present study. Furthermore, in the present study, C/EBPβ expression was suggested to be associated with the cellular mechanism of the SASP production, as well as IκBζ expression. C/EBPβ was

previously reported to regulate IκBζ target genes by interacting with other transcription factors, such as NF-κB and IκBζ (Matsuo et al. 2007). Using microarray analysis of SASP genes by targeting against C/EBPβ, the expression of 127 SASP genes was reported to be C/EBPβ-dependent (Flanagan et al., 2017). In our experimental results, blocking C/EBPβ by siRNA transfection was found to significantly decrease the gene expression of SASP factors (Fig. 5D). Furthermore, the IκBζ mRNA level was inhibited by treatment with C/EBPβ siRNA. These results strongly support that IκBζ and C/EBPβ are involved in the signaling pathway for SASP production in the DNA damage-induced senescence model and present an opportunity for discovering key mediators targeting SASP production.

To treat age-associated disorders, besides the strategy targeting SASP production, the removal of the senescent cells from the organisms seems to be a possible option. Since high-level activation of the $p16^{INK4a}$ promoter is a feature of cellular senescence and coincided with SASP production (Liu et al., 2019), the clearance of $p16^{INK4a}$-positive cells in a mouse model was shown to slow down age-related symptoms in organs, such as kidneys and heart (Baker et al., 2016). Clearance of senescent cells using the p16-3MR transgenic mouse also attenuated inflammatory markers in degenerative joints of the anterior cruciate ligament transection mouse model (Jeon et al., 2017). However, some studies have reported with conflicting results. Somatic inactivation of $p16^{INK4a}$ did not lower SASP production and did not protect against aging-related osteoarthritis (Diekman et al., 2018). $P16^{INK4a}$ global knockout did not attenuate the lung inflammation induced in a chronic obstructive pulmonary disease mouse model (Sundar et al., 2018). Senescent cells have dual and opposite functions in complex aging processes. Beneficial actions of senescent cells include tissue remodeling by promoting wound healing and anti-cancer mechanisms by recruiting immune cells to

eliminate damaged cells in normal tissues (Campisi, 2005; Storer et al., 2013). Therefore, the approach targeting only SASP factors could be more safe and effective. In this regard, it is important to mention that the properties of flavonoids may fit into a therapeutic role by inhibiting SASP production without interfering with proliferative activity. Thus, SASP inhibitory activity of the extract and certain constituents from the roots of *S. baicalensis* may be good candidates with less toxicity to slow down age-associated chronic inflammation, prolonging the healthy lifespan.

5. Conclusions

In conclusion, our experimental results demonstrate that the extract and certain flavonoids from Scutellaria radix acted as strong SASP inhibitors in bleomycin-induced senescence in BJ fibroblasts. Among the 17 flavonoids isolated from Scutellaria radix, 5 compounds showed strong SASP suppressive action and 2'-hydroxyl substitution in the B-ring of the flavone structure is an important moiety for SASP inhibition. Compounds **8** (2',5,7-trihydroxyflavone) and **11** (2',5,7-trihydroxy-6-methoxyflavone) showed most potent SASP inhibitory activity. Their inhibitory action was possibly mediated by blocking the signaling pathway associated with IκBζ/C/EBPβ expression. Importantly, the oral administration of compound **8** significantly inhibited the gene expression of SASP, IκBζ and C/EBPβ in old-aged rats. Therefore, our data suggest that the extract and certain flavonoids from the roots of *Scutellaria baicalensis* have strong SASP inhibitory activity and that treatment with the compounds or blocking the target molecules can be helpful to prevent or slow down the progression of age-related chronic inflammatory disorders.

447

448 **Conflict of interest statement**

449

450 There are no conflicts of interest.

451

452 **Acknowledgements**

453

454 This research was supported by Basic Science Research Program through the National
455 Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-
456 2017R1A6A3A11028151), NRF grant (NRF-2018R1A2B6001314) funded by the Korean
457 government (MSIT) and BK21-plus by the Ministry of Education. The bioassay facility of
458 New Drug Development Institute (KNU) was used.

459

460 **References**

461

462 Alexander, E., Hildebrand, D.G., Kriebs, A., Obermayer, K., Manz, M., Rothfuss, O.,
463 Schulze-Osthoff, K., Essmann, F., 2013. IκBζ is a regulator of the senescence-associated
464 secretory phenotype in DNA damage- and oncogene-induced senescence. J. Cell Sci. 26,
465 3738-3745.
466 Baker, D.J., Childs, B.G., Durik, M., Wijers, M.E., Sieben, C.J., Zhong, J., Saltness, R.A.,
467 Jeganathan, K.B., Verzosa, G.C., Pezeshki, A., Khazaie, K., Miller, J.D., van Deursen,
468 J.M., 2016. Naturally occurring p16(Ink4a)-positive cells shorten healthy lifespan. Nature
469 530, 184-189.
470 Bass, T.M., Weinkove, D., Houthoofd, K., Gems, D., Partridge, L., 2007. Effects of
471 resveratrol on lifespan in *Drosophila melanogaster* and *Caenorhabditis elegans*. Mech.
472 Ageing Dev. 128, 546-552.
473 Bitto, A., Ito, T.K., Pineda, V.V., LeTexier, N.J., Huang, H.Z., Sutlief, E., Tung, H., Vizzini,
474 N., Chen, B., Smith, K., Meza, D., Yajima, M., Beyer, R.P., Kerr, K.F., Davis, D.J.,
475 Gillespie, C.H., Snyder, J.M., Treuting, P.M., Kaeberlein, M., 2016. Transient rapamycin
476 treatment can increase lifespan and healthspan in middle-aged mice. Elife 23, pii: e16351.
477 Bjedov, I., Toivonen, J.M., Kerr, F., Slack, C., Jacobson, J., Foley, A., Partridge, L., 2010.
478 Mechanisms of life span extension by rapamycin in the fruit fly *Drosophila melanogaster*.
479 Cell Metab. 11, 35-46.
480 Campisi, J., Andersen, J.K., Kapahi, P., Melov, S., 2011. Cellular senescence: a link between
481 cancer and age-related degenerative disease? Semin. Cancer Biol. 21, 354-359.
482 Campisi, J., 2005. Suppressing cancer: the importance of being senescent. Science 309, 886-

887

Capell, B.C., Drake, A.M., Zhu, J., Shah, P.P., Dou, Z., Dorsey, J., Simola, D.F., Donahue, G., Sammons, M., Rai, T.S., Natale, C., Ridky, T.W., Adams, P.D., Berger, S.L., 2016. MLL1 is essential for the senescence-associated secretory phenotype. Genes Dev. 30, 321-336.

Coppé, J.P., Desprez, P.Y., Krtolica, A., Campisi, J., 2010. The senescence-associated secretory phenotype: the dark side of tumor suppression. Annu. Rev. Pathol. 5, 99-118.

Coppé, J.P., Patil, C.K., Rodier, F., Sun, Y., Muñoz, D.P., Goldstein, J., Nelson, P.S., Desprez, P.Y., Campisi, J., 2008. Senescence-associated secretory phenotypes reveal cell-nonautonomous functions of oncogenic RAS and the p53 tumor suppressor. PLoS Biol. 6, 2853-2868.

Diekman, B.O., Sessions, G.A., Collins, J.A., Knecht, A.K., Strum, S.L., Mitin, N.K., Carlson, C.S., Loeser, R.F., Sharpless, N.E., 2018. Expression of p16INK 4a is a biomarker of chondrocyte aging but does not cause osteoarthritis. Aging Cell 17, e12771.

Flanagan, K.C., Alspach, E., Pazolli, E., Parajuli, S., Ren, Q., Arthur, L.L., Tapia, R., Stewart, S.A., 2017. c-Myb and C/EBPβ regulate OPN and other senescence-associated secretory phenotype factors. Oncotarget 9, 21-36.

Freund, A., Patil, C.K., Campisi, J., 2011. p38MAPK is a novel DNA damage response-independent regulator of the senescence-associated secretory phenotype. EMBO J. 30, 1536-1548.

Hickson, L.J., Langhi Prata, L.G.P., Bobart, S.A., Evans, T.K., Giorgadze, N., Hashmi, S.K., Herrmann, S.M., Jensen, M.D., Jia, Q., Jordan, K.L., Kellogg, T.A., Khosla, S., Koerber, D.M., Lagnado, A.B., Lawson, D.K., LeBrasseur, N.K., Lerman, L.O., McDonald, K.M., McKenzie, T.J., Passos, J.F., Pignolo, R.J., Pirtskhalava, T., Saadiq, I.M., Schaefer, K.K.,

Textor, S.C., Victorelli, S.G., Volkman, T.L., Xue, A., Wentworth, M.A., Wissler Gerdes, E.O., Zhu, Y., Tchkonia, T., Kirkland, J.L., 2019. Senolytics decrease senescent cells in humans: Preliminary report from a clinical trial of Dasatinib plus Quercetin in individuals with diabetic kidney disease. EBioMedicine 47, 446-456.

Itahana, K., Itahana, Y., Dimri, G.P., 2013. Colorimetric detection of senescence-associated β galactosidase. Methods Mol. Biol. 965, 143-156.

Jeon, O.H., Kim, C., Laberge, R.M., Demaria, M., Rathod, S., Vasserot, A.P., Chung, J.W., Kim, D.H., Poon, Y., David, N., Baker, D.J., van Deursen, J.M., Campisi, J., Elisseeff, J.H., 2017. Local clearance of senescent cells attenuates the development of post-traumatic osteoarthritis and creates a pro-regenerative environment. Nat. Med. 23, 775-781.

Kang, C., Xu, Q., Martin, T.D., Li, M.Z., Demaria, M., Aron, L., Lu, T., Yankner, B.A., Campisi, J., Elledge, S.J., 2015. The DNA damage response induces inflammation and senescence by inhibiting autophagy of GATA4. Science 349, aaa5612.

Kim, S.R., Jiang, K., Ogrodnik, M., Chen, X., Zhu, X.Y., Lohmeier, H., Ahmed, L., Tang, H., Tchkonia, T., Hickson, L.J., Kirkland, J.L., Lerman, L.O., 2019. Increased renal cellular senescence in murine high-fat diet: effect of the senolytic drug quercetin. Transl. Res. 213, 112-123.

Kumar, M., Seeger, W., Voswinckel, R., 2014. Senescence-associated secretory phenotype and its possible role in chronic obstructive pulmonary disease. Am. J. Respir. Cell Mol. Biol. 51, 323-333.

Kumar, R., Sharma, A., Kumari, A., Gulati, A., Padwad, Y., Sharma, R., 2019. Epigallocatechin gallate suppresses premature senescence of preadipocytes by inhibition

of PI3K/Akt/mTOR pathway and induces senescent cell death by regulation of Bax/Bcl-2 pathway. Biogerontology 20, 171-189.

Laberge, R.M., Zhou, L., Sarantos, M.R., Rodier, F., Freund, A., de Keizer, P.L., Liu, S., Demaria, M., Cong, Y.S., Kapahi, P., Desprez, P.Y., Hughes, R.E., Campisi, J., 2012. Glucocorticoids suppress selected components of the senescence-associated secretory phenotype. Aging Cell 11, 569-578.

Lee, K.S., Lee, B.S., Semnani, S., Avanesian, A., Um, C.Y., Jeon, H.J., Seong, K.M., Yu, K., Min, K.J., Jafari, M., 2010. Curcumin extends life span, improves health span, and modulates the expression of age-associated aging genes in *Drosophila melanogaster*. Rejuvenation Res. 13, 561-570.

Lim, H., Park, H., Kim, H.P., 2015. Effects of flavonoids on senescence-associated secretory phenotype formation from bleomycin-induced senescence in BJ fibroblasts. Biochem. Pharmacol. 96, 337-348.

Liu, G., Ma, J., Chen, Y., Tian, Q., Shen, Y., Wang, X., Chen, B., Yao, S., 2009. Investigation of flavonoid profile of *Scutellaria bacalensis* Georgi by high performance liquid chromatography with diode array detection and electrospray ion trap mass spectrometry. J. Chromatogr. A. 1216, 4809-4814.

Liu, J.Y., Souroullas, G.P., Diekman, B.O., Krishnamurthy, J., Hall, B.M., Sorrentino, J.A., Parker, J.S., Sessions, G.A., Gudkov, A.V., Sharpless, N.E., 2019. Cells exhibiting strong p16 INK4a promoter activation *in vivo* display features of senescence. Proc. Natl. Acad. Sci. U S A. 116, 2603-2611.

Matsuo, S., Yamazaki, S., Takeshige, K., Muta, T., 2007. Crucial roles of binding sites for NF-kappaB and C/EBPs in IkappaB-zeta-mediated transcriptional activation. Biochem. J.

405, 605-615.

Menicacci, B., Laurenzana, A., Chillà, A., Margheri, F., Peppicelli, S., Tanganelli, E., Fibbi, G., Giovannelli, L., Del Rosso, M., Mocali, A., 2017. Chronic Resveratrol Treatment Inhibits MRC5 Fibroblast SASP-Related Protumoral Effects on Melanoma Cells. J. Gerontol. A Biol. Sci. Med. Sci. 72, 1187-1195.

Mosmann, T., 1983. Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunol. Methods 65, 55-63.

Onat, A., Can, G., 2014. Enhanced proinflammatory state and autoimmune activation: a breakthrough to understanding chronic diseases. Curr. Pharm. Des. 20, 575-584.

Perrott, K.M., Wiley, C.D., Desprez, P.Y., Campisi, J., 2017. Apigenin suppresses the senescence-associated secretory phenotype and paracrine effects on breast cancer cells. Geroscience 39, 161-173.

Reid, J.J., Linden, M.A., Peelor, F.F., Miller, R.A., Hamilton, K.L., Miller, B.F., 2020. Brain Protein Synthesis Rates in the UM-HET3 Mouse Following Treatment With Rapamycin or Rapamycin With Metformin. J. Gerontol. A Biol. Sci. Med. Sci. 75, 40-49.

Robles, S.J., Adami, G.R., 1998. Agents that cause DNA double strand breaks lead to p16INK4a enrichment and the premature senescence of normal fibroblasts. Oncogene 16, 1113-1123.

Rodriguez-Mateos, A., Vauzour, D., Krueger, C.G., Shanmuganayagam, D., Reed, J., Calani, L., Mena, P., Del Rio, D., Crozier, A., 2014. Bioavailability, bioactivity and impact on health of dietary flavonoids and related compounds: an update. Arch. Toxicol. 88, 1803-1853.

Salminen, A., Kauppinen, A., Kaarniranta, K., 2012. Emerging role of NF-κB signaling in the

induction of senescence-associated secretory phenotype (SASP). Cell. Signal. 24, 835-845.

Singh, T., Newman, A.B., 2011. Inflammatory markers in population studies of aging. Ageing Res. Rev. 10, 319-329.

Storer, M., Mas, A., Robert-Moreno, A., Pecoraro, M., Ortells, M.C., Di Giacomo, V., Yosef, R., Pilpel, N., Krizhanovsky, V., Sharpe, J., Keyes, W.M., 2013. Senescence is a developmental mechanism that contributes to embryonic growth and patterning. Cell 155, 1119-1130.

Sundar, I.K., Rashid, K., Gerloff, J., Li, D., Rahman, I., 2018. Genetic Ablation of $p16^{INK4a}$ Does Not Protect against Cellular Senescence in Mouse Models of Chronic Obstructive Pulmonary Disease/Emphysema. Am. J. Respir. Cell Mol. Biol. 59, 189-199.

Wang, P., Han, L., Shen, H., Wang, P., Lv, C., Zhao, G., Niu, J., Xue, L., Wang, Q.J., Tong, T., Chen, J., 2014. Protein kinase D1 is essential for Ras-induced senescence and tumor suppression by regulating senescence-associated inflammation. Proc. Natl. Acad. Sci. U S A 111, 7683-7688.

Xu, M., Pirtskhalava, T., Farr, J.N., Weigand, B.M., Palmer, A.K., Weivoda, M.M., Inman, C.L., Ogrodnik, M.B., Hachfeld, C.M., Fraser, D.G., Onken, J.L., Johnson, K.O., Verzosa, G.C., Langhi, L.G.P., Weigl, M., Giorgadze, N., LeBrasseur, N.K., Miller, J.D., Jurk, D., Singh, R.J., Allison, D.B., Ejima, K., Hubbard, G.B., Ikeno, Y., Cubro, H., Garovic, V.D., Hou, X., Weroha, S.J., Robbins, P.D., Niedernhofer, L.J., Khosla, S., Tchkonia, T., Kirkland, J.L., 2018. Senolytics improve physical function and increase lifespan in old age. Nat. Med. 24, 1246-1256.

Yousefzadeh, M.J., Zhu, Y., McGowan, S.J., Angelini, L., Fuhrmann-Stroissnigg, H., Xu, M.,

598 Ling, Y.Y., Melos, K.I., Pirtskhalava, T., Inman, C.L., McGuckian, C., Wade, E.A., Kato,
599 J.I., Grassi, D., Wentworth, M., Burd, C.E., Arriaga, E.A., Ladiges, W.L., Tchkonia, T.,
600 Kirkland, J.L., Robbins, P.D., Niedernhofer, L.J., 2018. Fisetin is a senotherapeutic that
601 extends health and lifespan. EBioMedicine 36, 18-28.

602 Figure legends

603

604 Fig. 1. The chemical structures of 17 flavonoid derivatives isolated from *S. baicalensis* used
605 in this study (A) and synthesis of 2',5,7-trihydroxyflavone (**8**) (B).

606

607 Fig. 2. Effects of the ethanol extract of Scutellaria radix on the expression of IL-6, IL-8, IκBζ,
608 and p21 and BrdU uptake in bleomycin-treated BJ cells.

609 A, Effects on IL-6 and IL-8 protein production and BrdU uptake by the ethanol extract
610 (100, 50, and 20 μg/ml) for the last 24 h after senescence completion. B, Effects on the gene
611 expression of IL-6, IL-8, IκBζ, and p21 by the ethanol extract (100 and 50 μg/ml) for the last
612 24 h after senescence completion. C, Effects of the ethanol extract (20, 10, and 5 μg/ml) on
613 IL-6 and IL-8 production and BrdU uptake by concomitant treatment with bleomycin during
614 cellular senescence. D, Effects of the ethanol extract (20, 10, and 5 μg/ml) on the gene
615 expression of IL-6 IL-8, IκBζ, and p21 by concomitant treatment with bleomycin, Bl:
616 bleomycin, EXT: ethanol extract. $*P < 0.05$, $**P < 0.01$, significantly different from the
617 bleomycin-treated control group ($n = 3$).

618

619 Fig. 3. Effects on IL-6/IL-8 production and BrdU uptake by flavonoid derivatives isolated
620 from the ethanol extract of Scutellaria radix.

621 A, Effects on IL-6 production by 17 compounds for the last 24 h after senescence
622 completion. B, Effects of 17 compounds on IL-6 production when simultaneously treated
623 with bleomycin during cellular senescence. C, Effects on IL-8 production and BrdU uptake
624 by 5 compounds (compound **1**, **5**, **8**, **10**, and **11**, 10 μM), having strong activity, for the last 24

h after senescence completion. D, Effects of 5 compounds on IL-8 production and BrdU uptake when simultaneously treated with bleomycin. Bl: bleomycin. *P < 0.05, **P < 0.01, significantly different from the bleomycin-treated control group (n = 3).

Fig. 4. Effects of 5 flavonoid derivatives on the gene expression of SASP and cellular senescence itself when simultaneously treated with bleomycin during cellular senescence.

A, Effects of flavonoids (compound **1**, **5**, **8**, **10**, and **11**, 10 μM) on the gene expression of SASP (IL-6, IL-8, IL-1α, IL-1β, GM-CSF, CXCL1, MCP-2, and MMP-3). B, Effects on the gene expression of IκBζ and p21. C, Effects of compound **8** and **11** on SA-β-gal activity, ×100, Bl: bleomycin, A: apigenin. *P < 0.05, **P < 0.01, significantly different from the bleomycin-treated control group (n = 3).

Fig. 5. Effects of compound **8** and **11** on cellular signaling molecules and C/EBPβ knockdown on SASP expression in bleomycin-induced cellular senescence.

Compound **8** and **11** (5 -10 μM) were simultaneously treated with bleomycin (50 μg/ml) and incubated further for 6 days. A, Effects of compound **8** and **11** on protein expression of IRAK1, IκBα, p-STAT3, pRb, and p21. B, Effects of compound **8** and **11** on protein expression of IκBζ and C/EBPβ in nuclear fraction. C, Effects of compound **8** and **11** on the immunofluorescence staining of IκBζ and C/EBPβ protein. *P < 0.05, **P < 0.01, significantly different from the bleomycin-treated control group (n = 3). D, Effects of transfection of C/EBPβ siRNA (25 nM) on the gene expression of SASP (IL-6, IL-8, IL-1α, IL-1β, GM-CSF, CXCL1, MCP-2, and MMP-3), IκBζ and p21. Each blot is a representative for three separate experiments. Bl: Bleomycin, A: Apigenin, DAPI: 4',6'-diamino-2-

phenylindole, N: non-targeting siRNA, C: C/EBPβ siRNA. *P < 0.05, **P < 0.01, significantly different from that with non-targeting siRNA control group (n = 3).

Fig. 6. Inhibitory activity of compound **8** on the gene expression of SASP molecules, IκBζ, and C/EBPβ in aged rats.

Compound **8** (in 0.3% CMC) was orally administered into young rats (5-month-old, 5 m) and aged rats (21-month-old, 21 m) at 2 - 4 mg/kg/day for 10 days. A, Effects on SASP genes (IL-6, IL-1α, IL-1β, GM-CSF, and CXCL1) in the kidneys. B, Effects on IκBζ and C/EBPβ genes in the kidneys. ^{+}P < 0.1, *P < 0.05, **P < 0.01, significantly different from that 0.3% CMC-treated aged (21 m) control group (n = 5).