Brief Report

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Anti-senescence effects of 4-methoxychalcone and 4-bromo-4'-methoxychalcone on human endothelial cells

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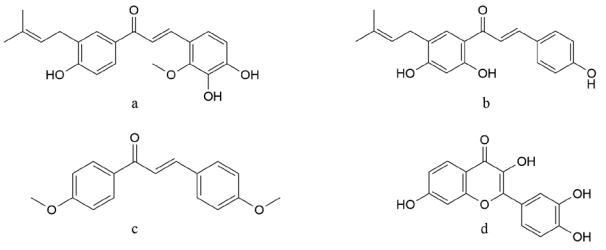
SUMMARY Senolytics are drugs that specifically target senescent cells. Flavonoids such as quercetin and fisetin possess selective senolytic activities. This study aims to investigate if chalcones exhibit anti-senescence activities. Anti-senescence effect of 11 chalcone derivatives on the replicative senescence human aortic endothelial cells (HAEC) and human fetal lung fibroblasts (IMR90) was evaluated. Compound 2 (4-methoxychalcone) and compound 4 (4-bromo-4'-methoxychalcone) demonstrated increased cytotoxicity in senescent HAEC compared to young HAEC, with significant differences on IC_{50} values. Their anti-senescence effects on HAEC exceeded fisetin. Higher selectivity of compound 4 toward HAEC over IMR90 could be attributed to 4-methoxy (4-OMe) substitution at ring A (R1). Chalcone derivatives have potentials as senolytics in mitigating replicative senescence, warranting further research and development on chalcones as anti-senescent agent.

Keywords Chalcones, cellular senescence, endothelial cells, senolytic, senotherapeutics

1. Introduction

Chalcones is a class of plant-derived polyphenolic compounds that belongs to the flavonoid family. These compounds can be found in various natural sources (*1,2*). Chalcones are alternatively known as benzyl acetophenone and come in two isomeric forms: trans and cis. The trans isomer exhibits greater thermodynamic stability (*3*). Structurally, chalcones are characterised by α,β-unsaturated ketones containing two aromatic benzene rings (referred to as rings A and B) with varying arrangements of substituents. These two rings are connected by an aliphatic three carbon series (*4*). Extensive research has explored the potential biological activities of natural, synthetic, and chalcone-derived compounds in preclinical studies. Notably, hydroxyl chalcones and bis-chalcones have shown promising *in-vitro* and *in-vivo* antidiabetic effects (*5,6*). Additionally, chalcones and their derivatives have been found to inhibit nuclear factor kappa-light-chain-enhancer of activated B (NF-κB), suppress cyclooxygenase and inducible nitric oxide synthase (iNOS) activity, and possess anti-inflammatory properties (*7,8*). Moreover, the antioxidant activity of chalcones increases when one or two hydroxyl (OH) groups are substituted on ring B, with the order of potency being 2-OH < 3-OH << 4-OH << 3,4-di-OH (*9*). In addition, the chalcone derivatives also possess potent antiproliferative and cytotoxic effects against several cancer cell lines, highlighting their potential as anticancer agents (*10,11*). Chalcones and their derivatives have also exhibited antimicrobial (*12*), antiparasitic (*11*), and neuroprotective activities (*13*). The diverse range of biological activities demonstrated by chalcones underscores their significance in pharmacological research.

To date, research exploring the potential of chalcones as anti-senescence or senolytic agents has been limited. For instance, licochalcone D (Scheme 1a), a chalcone derivative found in licorice root (*Glycyrrhiza echinata*), has demonstrated a reduction in oxidative-stress-induced senescence through the activation of 5' AMP-activated protein kinase (AMPK) and autophagy pathways (*14-16*). Similarly, another chalcone derivative, bavachalcone (Scheme 1b) has been shown to suppress senescence in human endothelial cells and downregulate mRNA expression of inhibitor of cyclin-dependent kinase 4 (INK4) family member p16 (p16INK4a) and interleukin (IL)-1α by modulating retinoid acid-related orphan receptor alpha (ROR-α) (*15*). Additionally, 4,4′-dimethoxychalcone (Scheme



Scheme 1. Reported anti-senescence flavonoids. (**A**) licochalcone D, (**B**) bavachalcone, (**C**) 4,4'-dimethoxychalcone, (**D**) fisetin.

1c) has been observed to prolong the lifespan of yeast, worms, and flies, slow down senescence in human cell cultures, and protect mice from prolonged myocardial ischemia (*14*). Among these reported anti-senescence chalcone, licochalcone been widely used in traditional Chinese medicine more specifically, Mongolian medicine as an important raw material. However, the scarcity of studies in this domain underscores the need for further research to elucidate the potential of chalcones in anti-senescence and senolytic interventions.

Cellular senescence refers to a state of irreversible arrest of cell proliferation in response to external and internal stimuli, including oxidative stress, mitochondrial dysfunction, oncogene expression, shortening of telomeres and disrupted autophagy (*17*). Senescent cells are apoptosis resistant, remain metabolically active and possess distinct morphological characteristics, express senescence markers such as senescence-associated β-galactosidase (SA β-gal), p16INK4a, p21, and assume the senescence-associated secretory phenotype (SASP). The secretome of SASP causes chronic low-grade inflammation that disrupts tissue homeostasis, amplifies immune activation and reinforces paracrine senescence. Accumulation of senescent cells contribute to ageing and ageing-related diseases (ARDs) such as cardiovascular, neurodegenerative and musculoskeletal diseases (*18*). Senescent cells develop apoptosis resistance by upregulating the senescence cell anti-apoptotic pathways (SCAPs). Recent approaches to eliminate senescent cells are aimed at inactivating these pro-survival pathways in the senescent cells, leading to the development of drugs termed senolytics. Extensive research has shown that elimination of senescent cells in animal models using senolytics attenuates functional impairment of tissues and organs caused by cellular senescence (*19,20*). The use of dasatinib with quercetin (D+Q) alleviated the age-related systolic dysfunction and improved cardiac vascular function (*21*). To date, only several senolytic compounds such as D+Q, navitoclax and fisetin (Scheme 1d) have been evaluated in preclinical and clinical trials (*22*), warranting more studies to identify effective and safe senolytics. While chalcones have been studied for various biological activities, thus far, there are very few studies evaluating their senolytic potentials. Hence, the aim of the study was to synthesise chalcone derivatives and evaluate their potentials in eliminating senescent endothelial cells.

2. Materials and Methods

2.1. Synthesis of chalcones

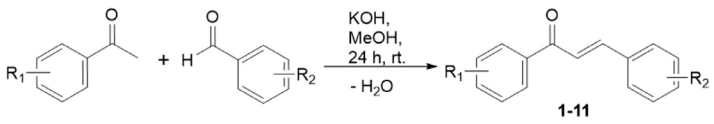
All chemicals (reagent grade) used for compound synthesis were purchased from Sigma-Aldrich Sdn. Bhd. (Selangor, Malaysia). The synthesis of chalcones was performed *via* a Claisen-Schmidt condensation with substituted benzaldehydes (1.0 mmol) and acetophenones (1.0 mmol). The reaction was performed under basic conditions in the presence of aqueous potassium hydroxide (4.0 mmol) and 50 mL methanol and stirred at room temperature for 24 hours to synthesise α,β-unsaturated biaryl systems (Scheme 2). The reaction was then monitored by thin-layer chromatography (TLC) using ethyl acetate/ petroleum ether (1:6 or 1:2 v/v) as the solvent system. TLC was run on Merck silica gel GF254 (Sigma-Aldrich Sdn. Bhd., Selangor, Malaysia). Upon completion of the reaction, the crude product was filtered off and recrystallized from a mixture of dichloromethane and ethanol or purified by column chromatography over silica gel eluting with a mixture of petroleum ether and ethyl acetate to give the pure product. The synthesis generated 11 chalcone candidates (Figure 1). Compound characterisation was performed using proton nuclear magnetic resonance (NMR) spectroscopic method and compared to the published data (*23-29*) (Supplementary data). ^{1}H-NMR spectra were recorded on a Bruker AVN400 spectrometer (Selangor, Malaysia).

2.2. Cell lines

HAECs were purchased from ScienCell Research Laboratories (California, USA). HAECs were maintained at 37°C in humidified atmosphere of 5% CO_2 in air and cultured in complete endothelial cell medium (ECM; ScienCell Research Laboratories, California, USA) supplemented with 5% v/v fetal bovine serum (FBS, iDNA, Singapore), 1% v/v endothelial cell growth supplement (ECGS, ScienCell Research Laboratories, California, USA) and 1% v/v penicillin-streptomycin (Gibco, Massachusetts, USA). IMR90 human fetal lung fibroblast was purchased from ATCC (Virginia, USA) and were cultured in Minimum Essential Medium (MEM, Sigma Aldrich, Missouri, USA) supplemented with 10% FBS at 37°C in humidified atmosphere of 5% CO_2 in air.

2.3. Establishment of replicative senescence cell culture

Replicative senescence (RS) culture of HAEC was



Chalcones	R_1	R_2
1	2-OH, 4-OMe, 6-OMe	4-Br
2	Phenyl	4-OMe
3	Phenyl	Naphthyl
4	4-OMe	4-Br
5	4-OMe	4-Me
6	2-OMe, 4-OMe, 6-OMe	4-Br
7	2-OMe, 4-OMe	Phenyl
8	Phenyl	2-NO_2
9	2-OH	Phenyl
10	Phenyl	4-Br
11	2-OH, 4-OMe	Phenyl

Scheme 2. Chalcone synthesis with various acetophenones (R_1) and benzaldehydes (R_2).

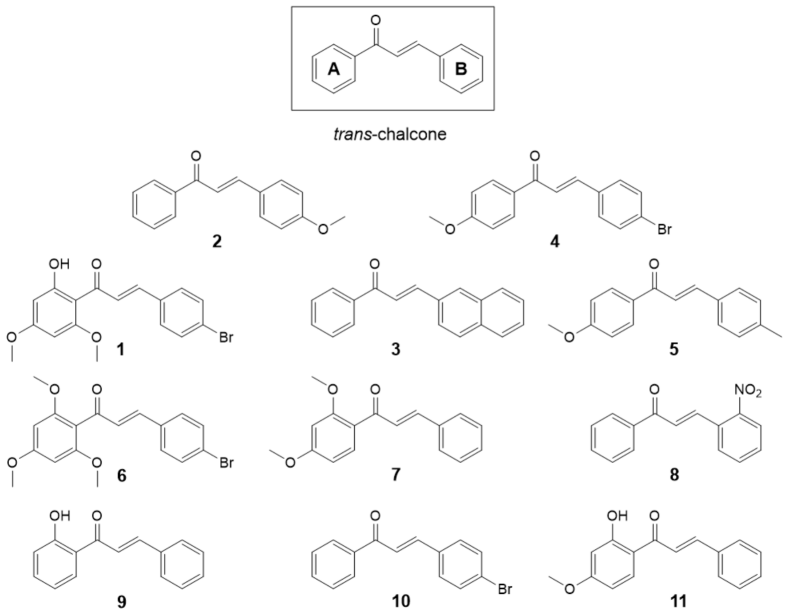


Figure 1. Eleven chalcone candidates synthesized in the present study.

established by serially passaging these cells every 3 to 4 days until the cells have ceased to achieve the typical population doubling level (PDL). PDL refers to the total number of times the cell population has doubled during *in vitro* culture. The percentage of senescent HAEC was determined by detecting senescence-associated-β-galactosidase (SA-β-gal) activity through flow cytometry using a fluorogenic substrate, 5-dodecanoylaminofluorescein di-β-D-galactopyranoside (C_{12}FDG) (Invitrogen, Massachusetts, US) to stain the senescent cells. In brief, cells were treated with Bafilomycin A1 (MedChemExpress, New Jersey, USA), a lysosomal inhibitory drug, to increase the internal lysosomal environment to pH 6 for an hour at 37°C in 5% CO_2 incubator. C_{12}FDG (33 μM) was then added to the cell medium and incubated for another 2 hours. Cells were harvested, washed and centrifuged to remove the cell supernatant. The cell pellets were then resuspended in ice cold potassium buffered saline (PBS, Gibco, Massachusetts, USA) and analysed using a flow cytometer. Fluorescence signals in the cells were measured by fluorescein isothiocyanate (FITC) detector channel and acquisition was performed using

FACSDiva software (Becton Dickinson, New Jersey, US) and data were analysed using FlowJo (Ashland, Oregon, US). The percentage of positive SA-β-Gal cells was determined by the number of events within the bright fluorescence quadrant/total number of cells in the parental scatter plot × 100%. Cell cultures that exhibited > 60% SA-β-gal positive cells were considered as replicative senescence (RS) cultures whereas those < 20% SA-β-gal positive cells were considered as young (Y) cultures (Figure 2).

2.4. Treatment of cells with compounds

The cytotoxicity of chalcone derivatives toward Y and RS was determined by measuring real-time growth kinetics of cells using the xCELLigence® Real Time Cell Analyzer (RTCA, Roche Diagnostics, Mannheim, Germany). RTCA measures impedance that increases when adherent cells are attached and have spread across the electrode sensor surface and vice versa, decreases when the cells are dead and detached from the sensor surface. Cells were seeded into the 96-well electronic (E) plate at 5×10^4 cells per well and background impedance readings were recorded. Cells were allowed to attach to the wells for 24 hours. When the cells entered the logarithmic growth phase, they were treated with increasing concentrations (0.78 – 50 µM) of chalcone derivatives for 72 hours. 17-allylamino-17-demethoxygeldanamycin (17-AAG, Sigma Aldrich, Missouri, USA), a heat shock protein 90 (HSP90) inhibitor, which has been shown to target senescent endothelial cells was used as positive control. Fisetin (Sigma Aldrich, St Louis, MO, USA) (Scheme 1d), a flavonoid which has been shown to induce cell death in senescent but not young umbilical vein endothelial cell (HUVECs) and young IMR90 fibroblast (*30*) was also used as positive control compound. The impedance values were expressed as the Cell Index (CI). The growth curves were normalised to the CI of the last measured time point before the addition of treatment or vehicle control. IC_{50} values were extracted individually from the RTCA software and the results were plotted using GraphPad Prism Version 5 (GraphPad Software, California, USA). Selectivity index (SI) was calculated by dividing the average IC_{50} values of the young with senescent or HAEC with IMR90 fibroblast.

3. Results and Discussion

Initially, we compared the cytotoxicity of compounds **1-11** on HAEC. Based on the IC_{50} values (Table 1), compound **10** emerged as the most cytotoxic compound out of the 11. Compounds **1**, **3**, **5**, **6-11** were equally cytotoxic to both young and senescent HAEC. Compound **10** differs from the basic chalcone structure, *trans*-chalcone by bearing a 4-bromo (4-Br) substituent in R_2. The 4-Br substituent in R_2 and an unsubstituted R_1 in Compound **10** resulted in increased cytotoxicity to young and senescent HAEC compared to *trans*-chalcone. Compounds **7**, **9** and **11** retained their cytotoxicity in the young HAEC, albeit with higher IC_{50} values compared to compound **10**. Coincidentally, compounds **7**, **9** and **11** are chalcones bearing unsubstituted R_2 and electron donating groups, OMe and OH on R_1. Thus, an unsubstituted R_2 may have contributed to the cytotoxic activity, regardless of the variation of the substituent at R_1. In comparison to the highly cytotoxic compound **10**, cytotoxicity towards young HAEC was reduced when R_1 was functionalised with 4-OMe substituent in compounds **1**,

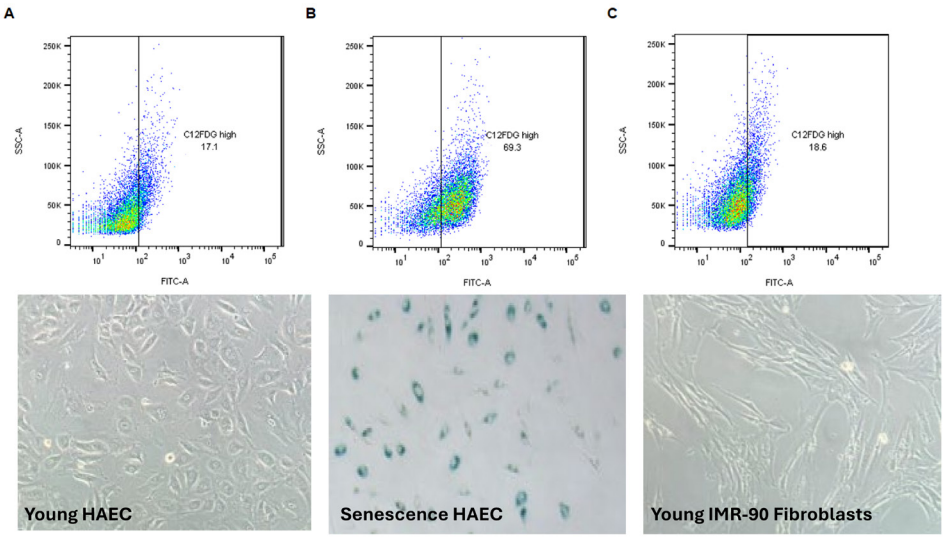


Figure 2. Representative figures of SA-β-Gal staining of endothelial and fibroblast cells. (**A**) Young HAEC, (**B**) RS HAEC and (**C**) Young IMR90. C_{12}FDG quadrants contain the population of senescent cells. Bottom panel shows representative cell morphology of young HAEC, RS HAEC and young IMR90 fibroblast. Senescent cells are stained in blue.

Table 1. Potential anti-replicative senescence activity against young and senescence HAEC

Compound	HAEC (mean ± SD; *n* = 3)		SI (Young/RS)	
	Young	RS		
2	11.50 ± 1.06	3.42 ± 0.53	3.4	Potential anti-RS compounds for HAEC
4	12.17 ± 1.09	5.23 ± 0.32	2.3	
17-AAG	114.00 ± 2.06	40.35 ± 1.29	2.8	
1	8.08 ± 0.91	7.38 ± 0.87	1.1	Less potential anti-RS compounds for HAEC
3	10.29 ± 0.01	19.78 ± 1.30	0.5	
5	12.12 ± 1.08	13.53 ± 1.13	0.9	
6	11.43 ± 1.06	10.25 ± 1.01	1.1	
7	4.39 ± 0.64	2.56 ± 0.41	1.7	
8	4.16 ± 0.62	2.34 ± 0.37	1.7	
9	4.63 ± 0.69	2.27 ± 0.14	2.0	
10	1.38 ± 0.14	1.65 ± 0.22	0.8	
11	7.37 ± 0.87	4.95 ± 0.70	1.5	
trans-chalcone	3.53 ± 0.55	3.71 ± 0.57	1.0	
fisetin	32.94 ± 1.39	24.32 ± 1.52	1.4	

HAEC: Human aortic endothelial cell; RS: replicative senescence

4 and **6**. Further reduction in cytotoxicity, as observed with IC_{50} values > 10 µg/mL were observed when other functional groups (4-OMe, Naphthyl and 4-methyl (4-Me)) were added to the phenyl moiety of R_2 (compounds **2, 3** and **5**). However, an exception was noted in compounds **8** which had 2-nitro (2-NO_2) on the phenyl moiety of R_2. It remained as cytotoxic as compounds **7**, **9** and **11**, suggesting an unfavourable cytotoxicity by 2-NO_2 substituent at R_2. It can be summarised that substitutions with electron withdrawing groups such as Br and NO_2 have resulted in greater cytotoxicity in normal cell line such as endothelial cells, contrary to the findings of Bai *et al*. (*31*) which concluded that chalcone bearing electron withdrawing groups exhibited lower cytotoxicity toward cancer cell lines compared to chalcones with electron donating groups (*31*). Fisetin, which is known to have a favourable safety profile showed the least cytotoxicity towards both young and senescent HAEC, with the highest IC_{50} values compared to those of the chalcone derivatives.

Comparison of young to replicative senescence cells, established by consecutive passing of the young passage cells till proliferation has ceased, is an established *in vitro* model for screening compounds that preferentially inhibits senescent cells over young such as quercetin and others (*32,33*). In the present study, SI was calculated to determine the anti-senescence potential of the compounds. High SI values indicate greater cytotoxicity toward senescent HAEC compared to young cells. Among the 11 compounds tested, compounds **2** and **4** showed the highest SI values (Table 1), indicating their higher anti-senescence potentials compared to the other chalcone derivatives and fisetin, showing the similar effects as the positive control, 17-AAG. It appears that the addition of 4-OMe to the phenyl moiety of R_2 of compound **2** may have contributed to the increased selectivity for senescent over young HAEC. Modifications on R_1 and R_2 to synthesise compound **4** (4-OMe and 4-Br, respectively) have slightly reduced the selectivity for the senescent HAEC. Similarly, substitution of 4-Br with 4-Me at R_2 (compound **5)** has led to a decrease in the SI compared to compound **4**. On the other hand, the appearance of 4-OMe on R_1 in compound **4** has greatly reduced the cytotoxicity toward young and senescent HAEC compared to compound **10**. In comparison with compound **4**, addition of OH or OMe at the 1,6-positions of R_1, respectively, to generate compounds **1** and **6**, did not increase the selectivity for senescent HAEC. Thus, the addition of 4-OMe on the phenyl moiety of R_2 may offer advantages in targeting senescent cells while minimising toxicity to young endothelial cells.

The cell selectivity of the compounds was evaluated in young HAEC and IMR90 fibroblasts (Table 2). A higher SI value indicates increased selectivity toward endothelial cells. Compounds **1**, **3** and **5** were highly cytotoxic to HAEC but not to IMR90, indicating the selectivity of these compounds to endothelial cells. By comparing the SI of compounds **1** and **4** (SI 8.7 and 28) with **6** and **10** (SI 4 and 3.1), respectively, the substitution of 4-Br at R_2 plays no role in increasing the selectivity for HAEC. Rather, modifications on R_1 at at 2-position or 2-6-position with -OMe or -OH (compounds **1**, **6**, **7**, **9** and **10**) may have contributed to increased toxicity to HAEC. Likewise, incorporating 2-NO_2 substitutions on the phenyl moiety of R_2 (compound **8**), as well as OH substitutions on the phenyl moiety of R_1 (compounds **9** and **11**), did not result in enhanced specificity for HAEC. Interestingly, the candidate anti-senescence compounds, **2** and **4** demonstrated selectivity for endothelial cells, as observed for fisetin and 17-AAG. Compound **4**, in particular, showed the highest selectivity for endothelial cells, with SI 8.7. This suggests that a single 4-OMe

Table 2. Potential anti-replicative senescence activity against young HAEC and young IMR90 cell lines

Compound	Young HAEC (mean ± SD; *n* = 3)	Young IMR90 (mean ± SD; *n* = 3)	SI (Young IMR90/Young HAEC)	
2	11.50 ± 1.06	24.32 ± 1.39	2.1	Potential anti-RS compounds selective for HAEC
4	12.17 ± 1.09	105.60 ± 2.02	8.7	
17-AAG	114.00 ± 2.06	Not converged	-	
1	8.08 ± 0.91	226.00 ± 2.25	28.0	Compounds with no/minimal cytotoxicity on IMR90
3	10.29 ± 0.01	177.20 ± 2.35	17.2	
5	12.12 ± 1.08	Not converged	-	
6	11.43 ± 1.06	46.01 ± 1.66	4.0	
7	4.39 ± 0.64	8.59 ± 0.93	2.0	Compounds with cytotoxicity on both HAEC and IMR90
8	4.16 ± 0.62	4.24 ± 0.63	1.0	
9	4.63 ± 0.69	6.73 ± 0.83	1.5	
10	1.38 ± 0.14	4.27 ± 0.63	3.1	
11	7.37 ± 0.87	8.08 ± 0.91	1.1	
trans-chalcone	3.53 ± 0.55	6.99 ± 0.84	2.0	
fisetin	32.94 ± 1.39	75.88 ± 1.88	2.3	Compound with least cytotoxicity on both HAEC and IMR90

HAEC: Human aortic endothelial cell; RS: replicative senescence

substitution at R_1, unlike the two substitutions on compound **6** could have likely conferred the specificity towards HAEC. Fisetin has been previously reported to reduce the viability of HUVEC to a greater extent than IMR90 fibroblast (*30*), consistent with our observation.

This study, to the best of our knowledge, revealed that 4-methoxychalcone (compound **2**) and its derivative, 4-bromo-4-methoxychalcone (compound **4**) possess anti-senescence potentials, particularly on endothelial cells. Endothelial senescence contributes to endothelial dysfunction giving rise to cardiovascular diseases (*34,35*). Compound **2** is a well characterised compound and has been extensively researched, whereas compound **4** is less well characterised for its biological activities. Previous studies have shown that 4-methoxychalcone (compound **2**) and its derivatives exhibit a varying degree of cytotoxicity to hepatocarcinoma, HepG2 cells (*31*) where 4-methoxychalcone enhances the chemosensitivity to cisplatin in lung carcinoma, A529 cells by inhibiting the anti-oxidative NF-E2-related factor 2/antioxidant responsive element (Nrf2/ARE) signalling pathway (*36*). In non-cancerous cells, 4-methoxychalcone possess melanogenesis inhibitory effect in B16F10 cell line at doses 3.125 μM to 12.5 μM but has phototoxic and ecotoxic potentials, with EC_{50} of 3.57 μg/mL and EC_{50} of 0.0047 mg/L, respectively (*37*). It also enhances adipocyte differentiation at 5 μM dose by activating the transcription factor, peroxisome proliferator-activated receptor gamma (PPARγ) (*38*). Compounds that can eliminate senescent cells in a tissue microenvironment can potentially attenuate ageing-related pathologies and development of cardiovascular, neurodegenerative and musculoskeletal diseases. Hence, further studies are required to extensively characterise the anti-senescent effects and identify the mechanisms that underpin the senolytic effects of these compounds to fully harness their potentials.

In conclusion, we have demonstrated that compound **2** and compound **4** in comparison to other compounds, exhibited higher anti-senescence potentials as evidenced by a large difference of IC_{50} value between young and senescent HAEC, as well as HAEC and IMR90 fibroblast. 4-OMe substitution on the phenyl moiety of R_1, particularly in compound **4**, conferred specificity towards senescent HAEC while minimising toxicity to young endothelial cells and fibroblasts. Substituting 4-Br at R_2 or incorporating other substitutions did not significantly enhance selectivity. Hence, compound 4 can be investigated in future studies as a potential senolytic agent to eliminate senescent endothelial cells within the vasculature.

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Conflict of Interest: The authors have no conflicts of interest to disclose.

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