"Identification of Camellia chekiangoleosa Hu Extract as a novel senolytic ingredient"

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1. Introduction

Skin aging is characterized by a spectrum of phenotypic alterations, including wrinkle formation, epidermal thinning, dysregulated pigmentation, and compromised barrier function, fundamentally driven by cellular senescence processes[1-6]. The molecular underpinnings of cellular senescence involve six interconnected pathways: 1. Cell cycle arrest, mediated through p16INK4A-dependent inhibition of CDK4/6 kinase activity and elevated senescence-associated β-galactosidase (SA-β-gal) expression[7]; 2. Telomere attrition and genomic instability, exacerbated by ultraviolet (UV)-induced reactive oxygen species (ROS) and telomeric DNA damage[8]; 3. Senescence-associated secretory phenotype (SASP), marked by excessive secretion of pro-inflammatory cytokines (e.g., IL-6) and matrix metalloproteinases (MMPs) that degrade dermal collagen networks[9, 10]; 4. Collagen metabolic imbalance, resulting from TGF-β signaling suppression (reducing synthesis) and MMP-driven extracellular matrix degradation[8, 11]; 5. Oxidative stress, characterized by mitochondrial ROS accumulation and diminished antioxidant enzyme activity[4, 12]; 6. Epigenetic dysregulation, including aberrant DNA methylation and microRNA expression profiles[10].

Accumulation of senescent cells in aged skin has been causally linked to wrinkle pathogenesis[7, 13], positioning senolysis-targeted clearance of senescent cells-as a transformative anti-aging strategy[14, 15]. Emerging evidence highlights immune-mediated senescent cells elimination, exemplified by cytotoxic CD4+T cells (CD4 CTLs) targeting human cytomegalovirus glycoprotein B (HCMV-gB) on senescent fibroblasts via HLA-II-dependent apoptosis[16]. In addition, clearance of senescent cells by genetic approaches or senolytic drugs improves aging phenotypes[17-19]. For instance, the elimination of p16INK4A-positive senescent cells in the transgenic INK-ATTAC mice is beneficial to the improvement of age-associated disease such as cardiac hypertrophy and neurodegeneration[20]; Pharmacological approaches, such as senolytics (e.g., navitoclax[21], fisetin[22], and the dasatinib-quercetin cocktail[23]), have demonstrated efficacy in ameliorating age-related phenotypes. Recent studies further identify plant-derived compounds and extracts, such as gingerenone A[24], Solidago alpestris[25], and Deschampsia antartica[26], as novel senolytic candidates.

While Camellia chekiangoleosa Hu Extract (CCHE) exhibits documented anti-inflammatory properties[27], its potential role in senolysis remains unexplored. In this research, we identify CCHE as a potent senolytic extract through multi-modal mechanistic validation as follows: 1. Reduction in senescent cells viability; 2. Suppression of intracellular SA-β-gal activity; 3. Mitigation of ROS accumulation and ATP depletion; 4. Activation of apoptotic pathways, evidenced by PARP cleavage, elevated Bax/Bcl-2 ratio, and caspase-3 activation; 5. Induction of autophagic flux, indicated by increased LC3b-II/I ratio. These findings establish CCHE as a novel botanical senolytic with translational potential for combating skin aging.

2. Materials and Methods

Cell Culture

Human dermal fibroblasts (HDFs) were cultured in high-glucose DMEM supplemented with 100 µg/mL streptomycin, 100 U/mL penicillin, and 10% FBS (pH 7.2) at 37°C under 5% CO2. At 80-90% confluence, cells were digested with trypsin, centrifuged at 1000 rpm for 5 min, and reseeded at appropriate densities. 8 groups were setted for assays: young group (YG, passage 6-9), young group+CCHE (YGC), old group (OG, passage 15-20), old group+CCHE (OGC), old group+UVB (OGU) and old group+UVB+CCHE (OGUC); 2 groups unshown here: young group+UVB (OGU), young group+UVB+CCHE (OGUC).

UVB Irradiation Model

Cells were irradiated using a solar UV simulator (BIOSUN). Prior to irradiation, culture medium was replaced with PBS. Control groups were shielded with aluminum foil. Complete medium was restored after irradiation.

CCK-8 Assay

Cells (1×10^5/mL) were seeded in 96-well plates. After 24 h, CCK-8 working solution (100 µL/well) was added. Absorbance at 450 nm was measured after 0.5-4 h incubation. Cell viability was calculated as:

Cell viability (%) = 100×(A450, sample-A450,blank)/(A450,control-A450,blank)

SA-β-Gal Staining Assay

Cells were fixed and stained using the Senescence β-Galactosidase Staining Kit. SA-β-gal-positive cells were quantified under microscopy.

SA-β-gal positivity (%) =Positive cells/Total cells×100

Immunofluorescence Assay

Cells on coverslips were fixed with 4% PFA, permeabilized with 0.15% Triton X-100, blocked with 10% FBS, and incubated with primary antibodies (4°C, overnight) followed by fluorescent secondary antibodies.

Morphological Observation

Cell morphology was analyzed using an ECLIPSE Ts2 inverted microscope (Nikon, Japan) equipped with phase-contrast optics.

Western Blotting Analysis

Cells were lysed on ice using RIPA lysis buffer supplemented with protease and phosphatase inhibitors (Beyotime, P0013B). After 20 min of lysis, the lysates were centrifuged at 14,000 ×

g for 10 min at 4°C. The supernatant was collected for protein quantification using a BCA assay kit (Beyotime, P0012S), followed by denaturation at 95°C for 5 min in Laemmli buffer. Samples were stored at −80°C until use.

Proteins were separated by SDS-PAGE (10% gel) according to molecular weight and subsequently transferred onto PVDF membranes (Millipore, IPVH00010) using a semi-dry transfer system (Bio-Rad). Membranes were blocked with 5% (w/v) non-fat milk in TBST (20 mM Tris-HCl, pH 7.6; 150 mM NaCl; 0.1% Tween 20) for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C. After three washes with TBST (10 min each), membranes were incubated with HRP-conjugated secondary antibodies (1:5000, Beyotime) for 1 h at room temperature. Protein bands were visualized using an ECL detection system (Beyotime, P0018S) and quantified with Image Lab™ Software (Version 6.1, Bio-Rad).

Quantitative Real-Time PCR (qRT-PCR)

Total RNA was extracted using RNAiso Trizol reagent (Invitrogen) and reverse-transcribed into cDNA with the MCE Reverse Transcription Kit (MedChemExpress). qPCR reactions were performed on a CFX Connect Real-Time PCR Detection System (Bio-Rad, USA) using SYBR Green I chemistry (SYBR qPCR Mix, GenStar).

Gene expression was normalized to GAPDH using the comparative ΔΔCt method. Primer sequences are listed in table 1:

Table 1 Primer sequences

Gene	Forward primer	Reverse primer
COL1A2	ACTGTAAGAAAGGGCCCAGC	AGCAAAGTTCCCACCGAGAC
FNB2	CTGAAGGCGGGTTTCTAGCG'	CAAATCGGGACAATGCACTGG'
ADAMTS2	CTCCTCAGCGTTTGTGGTG	CATCCAGCAGGCAGTCATAGG
MMP-9	GATCATTCCTCAGTGCCGGA'	GACCATAGAGGTGCCGGATG
MMP-3	GATCCTGCTTTGTCCTTTGATGCTGT	CTGAGGGATTTGCGCCAAAAGTG
GAPDH	ATTCCATGGCACCGTCAAGG	TCGCCCCACTTGATTTTGGA

ROS Detection

Cells were loaded with 10 μM DCFH-DA (Invitrogen) diluted in serum-free culture medium for 30 min at 37°C. Following three washes with PBS (pH 7.4), intracellular ROS levels were determined by measuring DCF fluorescence intensity using a fluorescence microscope.

ATP Level assay

ATP concentrations were determined using the ATP Assay Kit (Beyotime, S0026) through a luciferase-based bioluminescence system. Values were normalized to total protein content measured by BCA assay.

Reagents and Antibodies

The reagents and antibodies mentioned in this research are listed in the following two tables as follows：

Table 2. Reagents

Reagents	Source
DMEM medium	Gibco (Grand Island, NY, USA)
Senescence β-Galactosidase Staining Kit	Cell Signaling Technology (Boston, MA, USA)
RNAiso Trizol Reagent	Invitrogen (Grand Island, NY, USA)
RT-PCR Kit	MedChem Express (MCE, NJ, USA)
SYBR qPCR Mix	GenStar(Beijing, China)
Fetal bovine serum (FBS)	Gibco (Grand Island, NY, USA)
DCFH-DA	Invitrogen (Grand Island, NY, USA
CCK-8 Kit	Dojindo（Kumamoto, Japan)
20X TBS Buffer	Sangon Biotech (Shanghai, China)）
Tris-Glycine-SDS Buffer (powder)	Sangon Biotech (Shanghai, China)
SDS-PAGE Loading Buffer (5×)	Beyotime Biotechnology (Shanghai, China)
ECL Chemiluminescent Kit	Beyotime Biotechnology (Shanghai, China)
BCA Protein Assay Kit	Beyotime Biotechnology (Shanghai, China)
RIPA Lysis Buffer	Beyotime Biotechnology (Shanghai, China)
ATP Assay Kit	Beyotime Biotechnology (Shanghai, China）
Tri-color Prestained Protein Marker	Epizyme (Shanghai, China）
Trypsin	Solarbio (Beijing, China)

Table 3. Antibodies

Antibodies	Cat. No.	Source
PARP	13371-1-AP	Proteintech, China
Bax	50599-2-Ig	Proteintech, China
Cleaved Caspase-3	9661	Cell Signaling Technology (Boston, MA, USA)
Bcl-2	HA721235	HUABIO ,China
PCNA	2586	Cell Signaling Technology (Boston, MA, USA)
LAMP2	12987-1-AP	Proteintech, China

P62	18420-1-AP	Proteintech, China
LC3A/B	12741	Cell Signaling Technology (Boston, MA, USA)
β-actin	AC028	Abclonal, China
Goat anti-mouse IgG-HRP	A0216	Beyotime Biotechnology (Shanghai, China）
goat anti-rabbit IgG-HRP	A0208	Beyotime Biotechnology (Shanghai, China）

Statistical analysis

Data processing and visualization were performed using GraphPad Prism v9.0. Continuous variables are expressed as mean ± SD. Comparative analyses between groups were conducted via one-way ANOVA with Tukey's correction for multiple testing. Statistical significance was assigned when $P < 0.05$ is taken as statistically significant.

3. Results

CCHE shows senolytic activity in UVB-induced model

Two type cells from passage 6-9(young group) and 15-20(old group) human dermal fibroblasts (HDF) were used for assessment of aging status. These cells were screened using the CCK-8 cell viability and SA-β-Gal activity assays. As shown in figure 1a and 1b, the proliferation ability of cells in the young group was significantly stronger than that in the old group, but there was no big difference in SA-β-Gal activity staining. In order to achieve a more significant effect of senescence staining, the cells in old group exposure to UVB. Interestingly, treatment with CCHE decreased cell viability in OGU and OG but had no effects on YG (shown in figure 1c). The viability of 0.05% CCHE in OGUC compared with OGU decreased by 13.6% and 16.7% in OGC compared with OG. In addition, treatment with CCHE reduced the intracellular SA-β-galactosidase activity from 47.8% (OGU) to 18.6% (OGUC). These results showed that CCHE displayed senolytic activity.
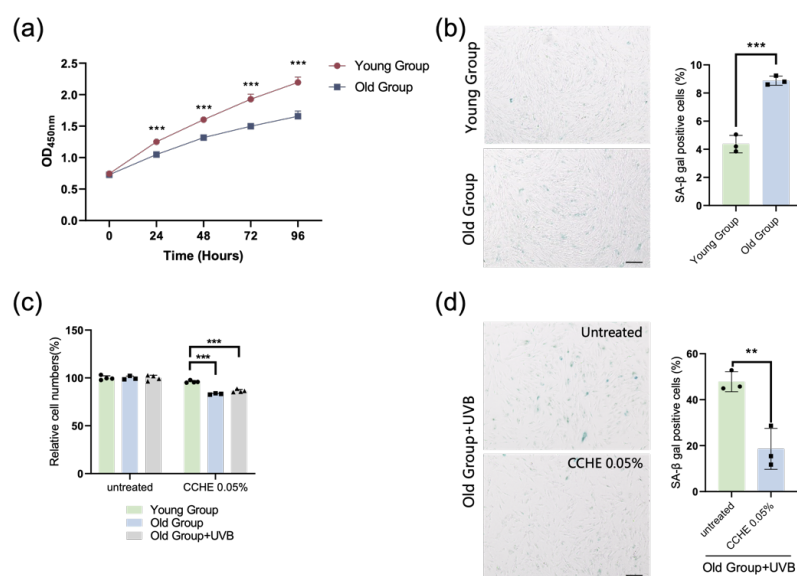
Figure 1. The effect of selective removal senescent cell treated with CCHE. (a) Comparison of proliferative capacity between Young Group (YG) and Old Group (OG) at 0h, 24h, 48h, 72h and 96h. (b) Staining and quantification of SA-β-Gal activity. (c) Cell viability statistics in three group treated or untreated with CCHE. (d) SA-β-Gal activity staining and quantification of OGU treated or untreated with CCHE. Scale bars = 200μm.

Incubation with CCHE identifies mitochondrial functional properties in senolytic assay

The effects of CCHE on mitochondrial function that was associated with senolysis were tested (shown in figure 2a and 2b). Treatment with CCHE reduced the ROS level in OGUC and ATP content decreased by 24.7% compared with OGU. However, incubation with CCHE in YG had no significant change on ATP content. These results indicated that Oxygen free radicals and energy metabolism were affected in senescent cells treated with CCHE.
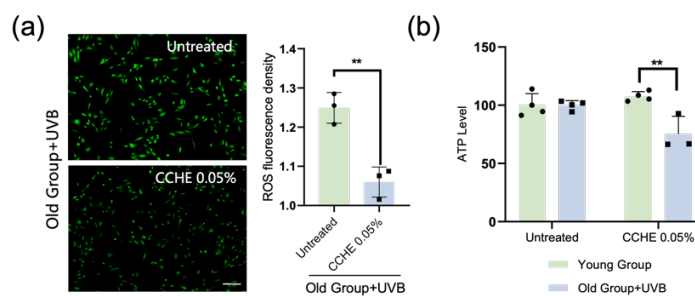

Figure 2. The effect of mitochondrial function treated or untreated with CCHE in OGU. (a) Staining and quantification of ROS. (b) Quantification of ATP content between YG and OGU treated or untreated with CCHE. Scale bars = 200μm.

CCHE induces apoptosis in senescent cells through autophagic cell death

To explore the mechanism behind the clearance of senescent cells, the apoptosis pathway and autophagy pathway were screened by Western blot, included Cleaved Caspase-3(CC3), Bax, Bcl-2 and Lc-3 proteins (figure 3a). Interestingly, CCHE induced an increase in the ratio of Lc3 II/I and Bax/Bcl-2, the level of the pro-apoptotic protein CC3, LAMP2, PARP but decrease in p62 and PCNA in senescent cells after being treated for 24h. Meanwhile, apoptosis and autophagy were further confirmed by the fluorescence staining of apoptotic proteins and the bright field of autophagic vesicles in senescent cells (shown in figure 3b and 3c). These results indicated that CCHE may induce senescent-cell death through autophagy and caspase-3.
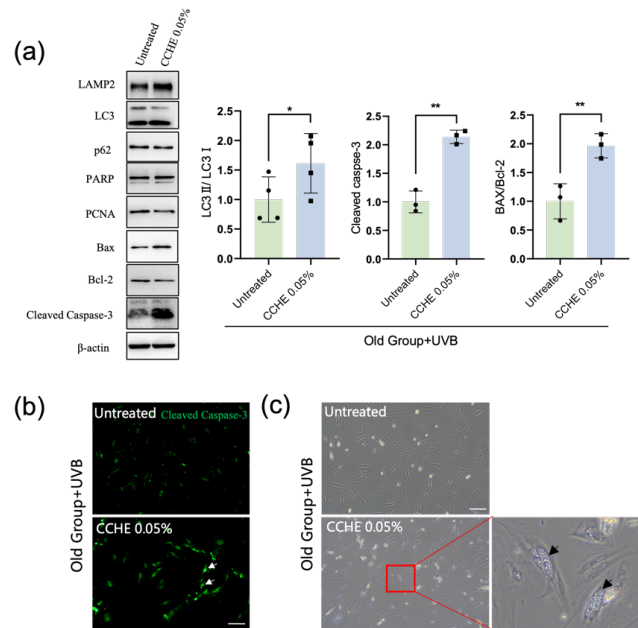
Figure 3. Screening of molecular mechanisms of senolytic activity in OGU treated or untreated with CCHE. (a) Western blot and quantification of cell autophagy and apoptosis related proteins. (b) Staining of cleaved caspase-3 in OGU treated or untreated with CCHE. (c) Bright field of autophagic vesicles in OGU treated or untreated with CCHE. Scale bars = 200μm.

CCHE improves the extracellular matrix of senescent cells

In order to investigate the effects on extracellular matrix (ECM) of senescent cells, these genes including FNB2, ADAMTS2, COL1A2, COL3A1, MMP3 and MMP9 were tested by Q-PCR. After treating for 24h, CCHE increased the mRNA levels of collagen genes and genes involved in promoting collagen production. Conversely, MMP3 and MMP9 decreased the mRNA expression (shown in figure 4a). These results suggest that CCHE can not only selectively eliminate senescent cells, but also reconstitute the extracellular matrix of senescent cells.
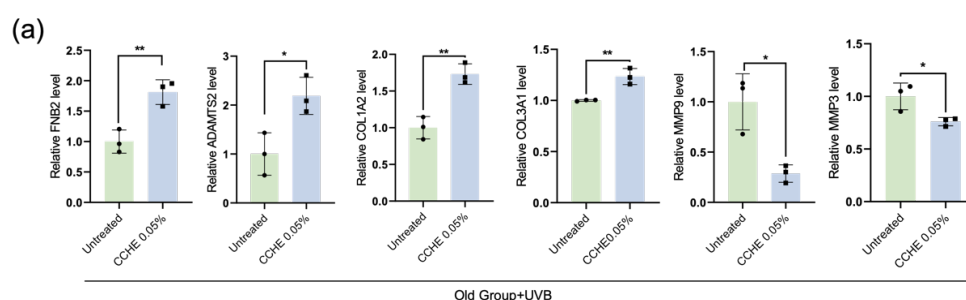

Figure 4. Remodeling of the extracellular matrix in OGU treated or untreated with CCHE. (a) mRNA expression assay for ECM related genes.

4. Discussion

Cellular senescence is a state of permanent cell cycle arrest triggered by diverse stressors. The accumulation of senescent cells during aging drives immunosenescence and chronic inflammation through senescence-associated secretory phenotype (SASP), which releases pro-inflammatory mediators including IL-6 and IL-8. These factors compromise skin barrier

integrity and accelerate aging processes through oxidative stress, mitochondrial dysfunction, and subsequent collagen degradation[28, 29]. Current anti-aging strategies focus on developing senolytics to selectively eliminate senescent cells. In this context, we investigated the senolytic potential of Camellia chekiangoleosa Hu extract (CCHE), demonstrating its ability to reduce senescent cell viability, suppress reactive oxygen species (ROS), and elevate cleaved caspase-3 levels.

The dual role of autophagy in cell fate determination warrants careful consideration. While macroautophagy sustains cell survival under nutrient deprivation via cytoplasmic recycling, it may also collaborate with caspase-dependent apoptosis (Type II programmed cell death). Experimental evidence reveals context-dependent outcomes: autophagy-related gene (Atg) knockout delays cell death under specific stressors yet enhances apoptosis in other scenarios. Notably, spatiotemporal correlations between autophagic flux and caspase activation observed in Drosophila salivary gland remodeling, neurodegeneration, and chemotherapy-resistant cancers suggest coordinated regulatory mechanisms[30]. In this study, our findings reveal CCHE-induced activation of both apoptosis and autophagy pathways in senescent cells, proposing a synergistic mechanism for senescence clearance.

Our previous study showed that Camellia chekiangoleosa Hu Extract (CCHE) could inhibit the secretion of inflammatory factors in LPS-induced inflammation model[27]. In the present study, we investigated matrix remodeling and SASP factor secretion by CCHE in senescent cells. SASP activation includes a range of chemokines, proinflammatory cytokines, growth factors, and matrix remodeling enzymes that affect its microenvironment[29]. CCHE-treated senescent cells showed decreased secretion of MMP3 and MMP9, and increased expression of FNB2, ADAMTS2, COL1A2, COL3A1 genes involved in extracellular matrix remodeling. Other pro-inflammatory factors, such as IL-1 and IL-8, need to be further verified to support the anti-inflammatory effect of CCHE on inhibiting the secretion of SASP while targeting senescent cells.

This study identifies CCHE as a novel botanical agent with senolytic capacities: eliminating senescent cells through apoptosis-autophagy coordination. To advance therapeutic development, further two critical steps are warranted: 1. Mechanistic dissection of CCHE's senolytic pathway using autophagy inhibitors (e.g., chloroquine) and apoptosis antagonists; 2. Bioactivity-guided fractionation to isolate active constituents.

5. Conclusion

Our experimental findings demonstrate that Camellia chekiangoleosa Hu extract (CCHE) activates an autophagy-related pathway and triggers an apoptotic cascade, specifically targeting senescent cells while sparing their non-senescent counterparts. These distinctive pharmacological properties, particularly the preferential elimination of senescent cells (senolysis), strongly suggest that CCHE may serve as a novel senotherapeutic agent with promising potential in advanced skincare formulations targeting cellular senescence-related aging.

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