RESEARCH ARTICLE

Andrographis paniculata Ethanol Extract Alleviates High Glucose-induced Senescence of Human Umbilical Vein Endothelial Cells via the Regulation of mTOR and SIRT1 Pathways

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Abstract

BACKGROUND: Chronic exposure of high glucose (HG) in endothelial cell induces senescence which may contribute to the development and progression of age-related diseases including insulin resistance. *Andrographis paniculata* improves insulin resistance in recent *in vitro* and *in vivo* studies. Anti-inflammatory and antioxidant properties of *A. paniculata* may be the new therapeutic approach to inhibiting premature senescence. However, the senolytic effect of *A. paniculata* on endothelial cells has not been investigated comprehensively. This study was conducted to evaluate the effect of *A. paniculata* extract on HG-induced endothelial cell senescence and the underlying mechanisms.

METHODS: Human umbilical vein endothelial cells (HUVECs) were treated with 33 mM HG and 7.5 µg/mL *A. paniculata* extract for 48 hours. The expressions of p16, p21, interleukin (IL)-6, IL-8, insulin receptor substrate (IRS)-1, mammalian target of rapamycin, and sirtuin 1 (SIRT1) were measured by performing real-time quantitative polymerase chain reaction (RT-qPCR). The senescence-associated-β-galactosidase (SA-β-gal) staining was performed to observe the positive-stained senescent cells, while the cell surface expression of IL-1α was examined with flow cytometry method.

RESULTS: *A. paniculata* extract reversed senescence in HUVECs under HG conditions by reducing mRNA expressions of p16 and p21, the number of SA-β-gal-positive-stained cells, and the expression of IL-1α on cell surface, which decreased the activation of IL-6 and IL-8. In addition, *A. paniculata* extract decreased the mRNA expression of mTOR and increased the mRNA expressions of IRS-1 as well as SIRT1.

CONCLUSION: *A. paniculata* extract ameliorated senescence and improved insulin sensitivity by regulating the mTOR, SIRT1, and IRS-1 mRNA expressions on HG-treated HUVECs.

KEYWORDS: *Andrographis paniculata*, endothelial cell, senescence, high glucose, nutrient-sensing pathways

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Introduction

Cellular senescence is defined by a permanent cessation of cell cycle. Stresses such as DNA damage, detrimental mutations, telomere shortening, metabolic dysfunction, and inflammation, activate p53/p21 and/or pRB/p16 tumor suppressor pathways which lead to permanent cell cycle arrest.(1) Endothelial cell senescence shows its central role in the development and progression of degenerative diseases or age-related diseases including insulin resistance.(2) The common shared mechanism is a condition called chronic inflammation or inflammaging through accumulation of senescence-associated secretory phenotype (SASP). (3) SASP refers to a number of molecules known as pro-inflammatory cytokines, chemokines, growth factors, and matrix metalloproteinases. SASP found specifically in endothelial cells is interleukin (IL)-1α, which acts as a master regulator of other SASP activations such as IL-6 and IL-8. (3) Increased in senescence-associated β-galactosidase (SA-β-gal), which is a lysosomal hydrolase enzyme activated at pH 6.0, is one of the traits of senescence. Although each marker shows promising, none of them are specific for senescence. Therefore, combining cytoplasmic (SA-β-gal), nuclear (p16 and p21), and SASP markers is encouraged to detect senescence.(1,4) Dysregulated nutrient-sensing pathways is one of the mechanisms of cellular senescence which includes the signaling of mammalian target of rapamycin (mTOR) and sirtuin 1 (SIRT1), as well as insulin and insulin-like growth factor (IGF)-1 signaling. (5) Although, insulin receptor substrate (IRS)-1 is not specifically one of the nutrient-sensing pathways, it plays a crucial role in insulin and IGF-1 signaling pathway, which is closely related to nutrient-sensing, glucose metabolism, and insulin resistance.(6) As individuals age, senescent cells accumulate in many tissues and organs and function as a mediator of inflammation. Suppression of endothelial cell senescence reverses phenotypic changes of senescence in several models which becomes the next potential therapy for age-related diseases including insulin resistance.(2,7)

Senolytics are pharmaceutical agents that eliminate senescent cells. Dasatinib, which is a drug for treating myeloid leukemia, and quercetin, a natural flavonoid molecule presents in foods, are among the most extensively studied senolytic drugs.(8) Dasatinib and quercetin were demonstrated to be able to eliminate senescent cells by inducing apoptosis in fibroblasts and extend lifespan in mice. (9) Dasatinib and quercetin has been investigated in elderly individuals with type 2 diabetes mellitus and chronic kidney disease and showed anti-senescence effect in humans.(10) Despite the promising potential of senolytic drugs, there are several challenges and limitations that need to be addressed including potential side effects, limited specificity, and high costs.(11) These limitations highlight the need for exploring alternative strategies, such as herbal medicines.(12) Thus, it is necessary to explore herbal plants which may potentially exhibit anti-senescence.

Andrographis paniculata is a plant that found widely across Southeast Asia and has been consumed traditionally for treating diabetes.(13) Based on previous studies, *A. paniculata* extract and its bioactive compounds, such as andrographolide and 14-deoxy-11,12-didehydroandrographolide (14DAP), showed an anti-senescence effect on keratinocytes and human dermal fibroblasts by showing their antioxidant and anti-inflammatory by reducing IL-6 and tumor necrosis factor (TNF)-α production.(14,15) Additionally, andrographolide has shown its potential on EA.hy926 endothelial-like cells induced senescence by H_2O_2 via its anti-inflammatory and antioxidant properties by activating the PI3K/Akt-endothelial nitric oxide synthase (eNOS) signaling, reducing the levels of IL-6 and TNF-α, reducing reactive oxygen species (ROS) generation, and promoting cellular glutathione (GSH) contents.(16) In *in vivo* studies, andrographolide improved brain senescence in Octodon degus and AβPPswe/PS-1 mice by stimulating the Wnt/β-catenin signaling pathway, as well as reducing Aβ aggregation, Tau phosphorylation, and IL-6 expression. (17,18) A clinical trial of *A. paniculata* extract improved insulin resistance through increasing glucagon-like peptide (GLP)-1 concentration.(19) However, the mechanism of *A. paniculata* extract and its bioactive compounds on endothelial cell senescence has not been investigated, including the effects on cell cycle arrest markers such as p16 and p21, SA-β-gal activity, and SASP markers such as IL-1α, IL-6, and IL-8. Additionally, the effects on nutrient-sensing markers such as mTOR and SIRT1, as well as on insulin signaling through IRS-1, remain unclear. Thus, this research was conducted to analyze the effect of *A. paniculata* ethanol extract along with its mechanism on endothelial cell senescence induced by high glucose (HG).

Methods

A. paniculata Ethanol Extract

A. paniculata ethanol extract was donated by PT. Konimex (Jakarta, Indonesia), and produced following the

manufacturer's procedures. The *A. paniculata* simplicia was extracted using the maceration method, employing 90% ethanol as the solvent. The simplicia required a total of 10 L of ethanol for 1 kg of simplicia. The extract was subsequently evaporated under vacuum at a maximum temperature of 60°C until it reached its maximum thickness, as determined by the total solid end-point parameter, resulting in the formation of a thick extract.(20)

Cell Culture

The human umbilical vein endothelial cells (HUVECs) were cultured in EGM-2 medium (CC-4147 and CC-3156) in a 5% CO_2 incubator at 37°C for 48 hours. Low passage HUVECs between the sixth and nineth passages were used in our study.(21) To assess the effect of *A. paniculata* extract on HG-induced senescence of HUVECs, the cells were seeded in 6-well plates for flow cytometry, 12-well plates for RNA detection, 24-well plate for SA-β-gal staining, and 96-well plate for MTT assay. The protocol of this study was approved by Health Research Ethics Committee University of Indonesia and Cipto Mangunkusumo Hospital, Indonesia (No. KET/77/UN2.F1/ETIK/PPM.00.02/2023).

Cell Viability Assay

Cell viability was measured by the MTT assay. HUVECs were seeded into 96-well plate at a density of 5000 cells/well. Cells were treated with 33 mM HG plus various concentrations of *A. paniculata* extract (1 µg/mL; 2.5 µg/mL; 5 µg/mL; 7.5 µg/mL; 10 µg/mL; 12.5 µg/mL; 15 µg/mL; 20 µg/mL; 22.5 µg/mL; 25 µg/mL), 33 mM HG, 5 mM normoglucose (NG), and 50 µM metformin plus HG medium for 48 hours. Then, 10 µM of 3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyl tetrazolium bromide (MTT) solution (Cat. No. BS186-1g; Biosharp Life Sciences, Beijing, China) was added for 4 hours, followed by 100 µL of 10% sodium dodecyl sulfate (SDS) (Cat. No. 28312; Thermo Fisher Scientific, Waltham, MA, USA) and 0.01M hydrochloric acid/HCl (Cat. No. T8032; Merck, Darmstadt, Germany) and incubated overnight. The absorbance at 595 nm was measured using microplate reader (Multiskan™ GO, Thermo Fisher Scientific).

Senescence Induction and Experimental Groups

Initial experiment was performed by MTT assay in a 96-well plate to identify the therapeutic dose of *A. paniculata* extract in HUVECs treated by HG. For the next experiments, the treatment groups were divided into 5 mM NG, 33 mM HG, 50 µM metformin plus HG, and *A. paniculata* extract at a dose based on the IC_{50} value. After 60-70% confluency, HUVECs were treated with 33 mM HG, 5 mM NG, 50 µM metformin with HG, or *A. paniculata* extract for 48 hours. (22,23) The concentrations and period of HG, NG, and metformin treatment in this study were based on previous studies.(24-27)

SA-β-gal Staining

The procedure for SA-β-gal staining was carried out as follows. HUVECs were seeded into 24-well plate. After being treated for 48 hours, the cells were washed twice with cold phosphate-buffered saline (PBS) and fixed with a 4% paraformaldehyde (PFA) solution and incubated for 5 minutes at room temperature, and then exposed to a SA-β-gal staining solution consisting of 40 mM citric acid (Cat. No. 100244; Merck)/sodium phosphate (Cat. No. S3264; Merck) solution, 5 mM potassium ferrocyanide (Cat. No. 05362; Loba Chemie PVT.LTD, Mumbai, India), 5 mM potassium ferricyanide (Cat. No. 05359; Loba Chemie PVT.LTD), 150 mM sodium chloride (Cat. No. GRM853-500G; Himedia, Mumbai, India), 2 mM MgCl2 (Cat. No. MB040-100G; Himedia), and 1 mg/mL X-gal (Cat. No. B1690; Thermo Fisher Scientific) at pH 6.0. The incubation process took place at a temperature of 37°C. During the initial 6-hour period, the cells were observed at 1 hour intervals, followed by 4-6 hours intervals. The positive-stained senescent cells were dyed with a blue-green colour observed under an inverted bright-field microscope (Nikon Diaphot Inverted Tissue Culture Microscope, Nikon, Tokyo, Japan). The reaction was terminated by washing with pure water. The cells were captured on 5 randomly fields using a 100x total magnification for each well, and the positive-stained senescent cells were counted using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and divided by the total number of cells, resulting in a percentage of senescent cells for each group.

Detection of IL-1α on Cell Surface using Flow Cytometry

HUVECs were seeded at 30,000 cells/well in 6-well plates and treated for 48 hours according to the experimental groups mentioned above. The cells were washed with stain buffer (FBS) (BD Pharmingen™, Cat. No. 554656; BD Bioscience, Franklin Lakes, NJ, USA) and stained with fluorescein isothiocyanate (FITC)-labelled monoclonal antibody of IL-1α (Cat. No. 11-7118-82; Thermo Fisher Scientific) and incubated for 30 minutes at 4°C in the dark. Then, the cells were analyzed on BD FACSCanto™ II (BD Biosciences) and FlowJo software (Tree Star, Ashland, OR, USA) was used to determine the percentage of positive-labelled senescent cells.

Real-time Quantitative Polymerase Chain Reaction (RT-qPCR) Analysis

Total RNA was isolated from HUVECs using Direct-zol RNA Miniprep Plus (Cat. No. R2073; Zymo Research, Irvine, CA, USA). The total RNA was reverse transcribed into cDNA using ReverTra Ace™ qPCR RT Master Mix with gDNA Remover (Cat. No. FSQ-301; Toyobo, Osaka, Japan) and qPCR (MiniOpticon™ Real-Time PCR System, Bio-Rad, Hercules, CA USA) was performed using the THUNDERBIRD™ SYBR® qPCR Mix (Cat No. QPS-201; Toyobo). The qPCR cycle program was set initially at 95°C for 2 minutes followed by 40 cycles of denaturation at 95°C for 5 s, annealing at 60°C for 10 s, and extension at 72°C for 20 s. The mRNA expression of each gene was normalized to that of β-actin. The relative expression of each gene was obtained by the $2^{-\Delta\Delta Ct}$ method. The primers specific for genes involved in cell cycle arrest (p16 and p21), SASP markers (IL-6 and IL-8), nutrient-sensing (mTOR and SIRT1), and insulin signaling (IRS-1) were listed in Table 1.

Table 1. Primer sequences used for RT-qPCR.

Gene		Sequence
p16	Forward	CACCAGAGGCAGTAACCATGCCCGC
	Reverse	GTAGGACCTTCGGTGACTGATGATC
p21	Forward	GGAAGACCATGTGGACCTGTCACTG
	Reverse	AGATCAGCCGGCGTTTGGAGTGGTA
IL-6	Forward	GAAGCTGCAGGCACAGAACCAGTGGC
	Reverse	CTGACCAGAAGAAGGAATGCCCATT
IL-8	Forward	TCTGCAGCTCTGTGTGAAGGTGCAG
	Reverse	GTGTGGTCCACTCTCAATCACTCTC
mTOR	Forward	TTTAGCGGTCATGTCAATGG
	Reverse	CATCAGGTTGGATGGGTGT
SIRT1	Forward	TGCTGGCCTAATAGAGTGGCA
	Reverse	CTCAGCGCCATGGAAAATGT
IRS-1	Forward	AGTCTGTCGTCCAGTAGCACCA
	Reverse	ACTGGAGCCATACTCATCCGAG
β-actin	Forward	TCGCCTTTGCCGATCCG
	Reverse	ATGATCTGGGTCATCTTCTCG

Statistical Analysis

All experiments were replicated with minimum of three biological replicants. All results were presented as the mean±standard error of mean (SEM) and analyzed using GraphPad Prism 9.5 (GraphPad Software Inc., San Diego, CA, USA). Comparisons between two or more groups were performed using One-way ANOVA followed by Tukey's post hoc test or Fisher's test. A value of $p<0.05$ was considered statistically significant.

Results

Effect of *A. paniculata* Ethanol Extract on Viability of HUVECs Treated with HG

Preliminary study showed that the concentrations of *A. paniculata* extract at 25 µg/mL and higher were toxic to HUVECs. In this study, therefore, HUVECs were treated with HG and different concentrations of *A. paniculata* extract (1, 2.5, 5, 7.5, 10, 12.5, 15, 20, 22.5, and 25 µg/mL) as shown in Figure 1A. The viability of HUVECs treated with HG and *A. paniculata* extract for 48 hours was found to be decreased in a dose-dependent manner. HUVECs treated with HG and *A. paniculata* extract at 7.5 µg/mL could inhibit the viability of half population of the cells (IC_{50} value) (Figure 1B). Based on the IC_{50} value, 7.5 µg/mL of *A. paniculata* extract was used for future experiments. In addition, HUVECs with HG treatment tended to decrease the viability of HUVECs, as compared to HUVECs treated with NG. Interestingly, metformin with HG treatment on HUVECs were found to significantly reduce the viability of HUVECs ($p<0.05$) in comparison with HUVECs treated with HG (Figure 1C).

Effect of *A. paniculata* Ethanol Extract on the Number of SA-β-gal-stained in HUVECs Treated with HG

To further evaluate the senescence on HUVECs, SA-β-gal staining was performed. The result showed that compared to HUVECs treated with NG, HG treatment in HUVECs significantly increased the number of SA-β-gal-stained cells ($p=0.005$). In comparison with those treated with HG, HUVECs treated with metformin with HG significantly decreased the number of SA-β-gal-stained cells ($p<0.05$). Moreover, the number of SA-β-gal-stained cells in HUVECs treated with 7.5 µg/mL of *A. paniculata* extract compared to HUVECs with HG treatment, were significantly decreased to 12.40% ($p<0.05$) (Figure 2).

Effect of *A. paniculata* Ethanol Extract on Cell Surface IL-1α Expression of HUVECs Treated with HG

The result of IL-1α expression showed that compared to those treated with NG, HUVECs treated with HG showed significant increased in the positive-cells of IL-1α on cell surface ($p<0.001$) (Figure 3). In addition, treatment with metformin plus HG also significantly ($p<0.001$) decreased the number of HUVECs expressing IL-1α on cell surface, as compared to those treated with HG. It was also shown that the positive-cells of IL-1α on cell surface on HUVECs

treated with 7.5 µg/mL of *A. paniculata* extract were significantly decreased (p=0.005), as compared to those treated with HG alone (Figure 3).

Effect of *A. paniculata* Ethanol Extract on the mRNA Expressions of p16, p21, IL-6, IL-8, mTOR, SIRT1, and IRS-1 in HUVECs Treated with HG

Compared to those treated with NG, HUVECs treated with HG showed a tendency to enhance the mRNA expressions of p16, p21, IL-6, IL-8 and mTOR, while the mRNA expressions of SIRT1 and IRS-1 tended to be decreased (Figure 4). Interestingly, treatment of metformin plus HG tended to increase p16, p21, SIRT1, and IRS-1 mRNA expressions, as well as decrease IL-6, IL-8, and mTOR mRNA expressions (Figure 4). Meanwhile, treatment with *A. paniculata* extract at 7.5 µg/mL on HUVECs tended to reduce the mRNA expressions of p16, p21, IL-6, IL-8, and mTOR, compared to HUVECs treated with HG

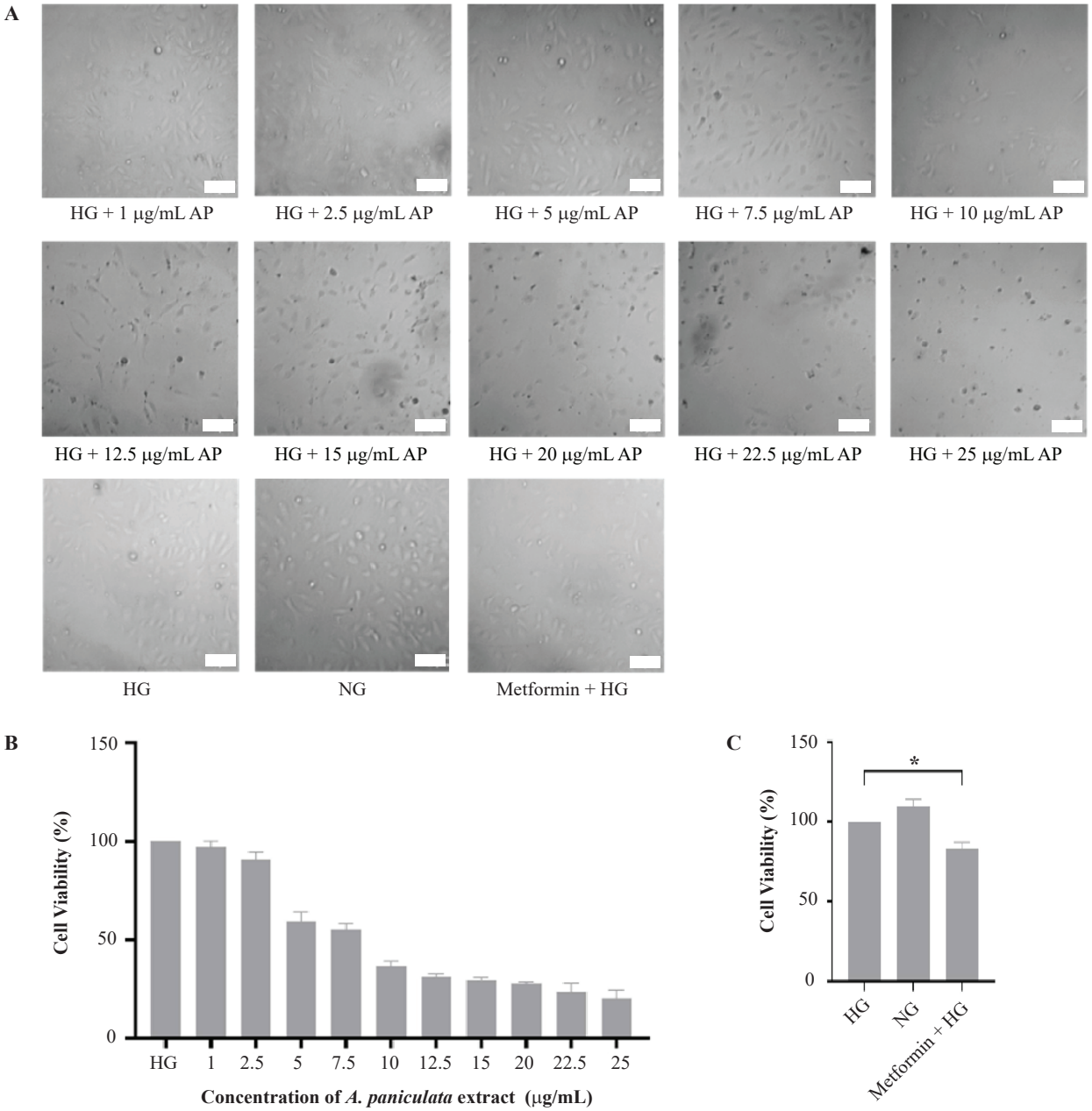

Figure 1. Viability of HUVECs following treatment with various concentration of *A paniculata*. A: HUVECs treated with HG and *A. paniculata* extract, HG only, NG only, and metformin with HG under the inverted microscope with 40x total magnification (White bar = 10 µm). B: The effect of *A. paniculata* extract from 1 to 25 µg/mL on cell viability in HG-exposed HUVECs. C: The effect of NG, HG, and metformin with HG stimulation on cell viability in HG-exposed HUVECs. Each bar represents the mean percentage number of viable cells±SEM of minimum three samples. *p<0.05. AP: *Andrographis paniculata.*

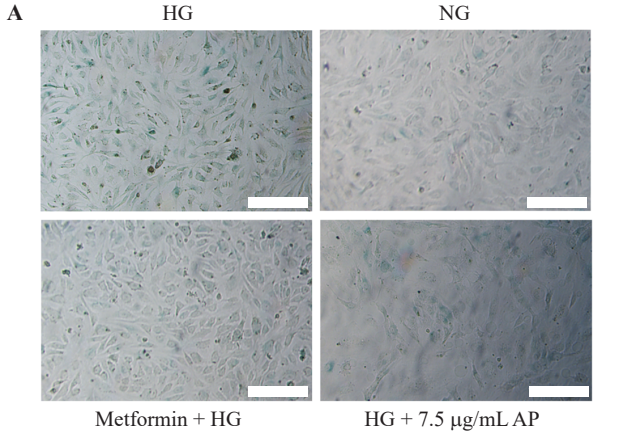

Figure 2. The effect of *A. paniculata* extract on the number of SA-β-gal-stained cells in HUVECs. Each group was performed in triplicate. The cells were captured in 5 random fields from each sample. A: Representative figures of each group with 100x total magnification (White bar = 10 µm). B: The mean of the number of SA-β-gal-stained cells±SEM. **p*<0.05; ***p*=0.005. AP: *Andrographis paniculata.*

(Figure 4A-4E). However, the statistical analysis did not reveal significance. In contrast, the mRNA expressions of SIRT1 and IRS-1 were significantly ($p<0.001$) increased in HUVECs treated with 7.5 µg/mL of *A. paniculata* extract as compared to those treated with HG (Figure 4F-4G). The results suggest that 7.5 µg/mL of *A. paniculata* extract regulated the mRNA expression of mTOR and SIRT1 as well as IRS-1 in HG-induced senescence in HUVECs.

Discussion

Senolytic drugs eliminate senescent cells and are potentially effective in preventing and treating chronic diseases.(8) This present study revealed that *A. paniculata* extract showed a senolytic effect on HUVECs treated with HG via reducing cell viability in a dose-dependent manner. The ethanol extract of *A. paniculata* improved senescence in HUVECs treated with HG through attenuating cell cycle arrest, SA-β-gal, and SASP, as well as regulating the mTOR, SIRT1, and IRS-1 pathways, which provides a new direction for preventing the progression of insulin resistance caused by HG stimulation.

As illustrated in Figure 1, cell viability was decreased to 50% at the concentration of 7.5 µg/mL *A. paniculata* extract. Therefore, all subsequent experiments were performed with 7.5 µg/mL of *A. paniculata* extract. Previous study showed that the IC_{50} value of *A. paniculata*

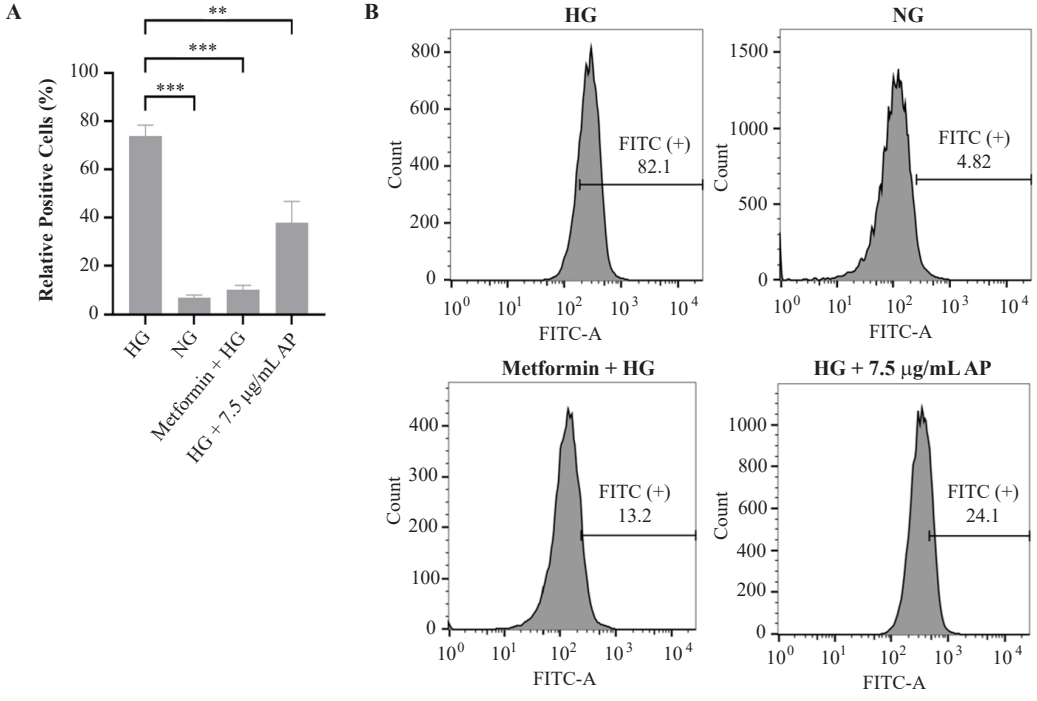

Figure 3. The effect of *A. paniculata* extract on the expression of IL-1α on cell surface on HUVECs. A: The mean relative IL-1α expression±SEM in triplicate. B: Representative FITC analysis results of IL-1α expression on HUVECs in each group. ***p*=0.005; ****p*<0.001. AP: *Andrographis paniculata.* FITC: Fluorescein Isothiocyanate.

extract on HCT 116 cells (human colorectal carcinoma), HepG2 cells (hepatocellular carcinoma), and A549 cells (human lung cancer) were 45 µg/mL, 60 µg/mL, and 40 µg/mL, respectively.(28) The cytotoxic compounds found in *A. paniculata* are andrographic acid methyl ester and 6-Epi-8-O-acetyl-harpagide from aerial parts and several compounds from diterpenoid lactones group including andrograpanin from whole part, deoxyandrographiside from aerial parts, 14-deoxy-12-methoxyandrographolide from whole part, 14-deoxy-11-methoxyandrographolide from aerial parts, 12-S-hydroxyandrographolide from aerial parts, and 14-deoxy-15-isopropylidine-11,12-didehydroandrographolide. Another cytotoxic compounds of *A. paniculata* were found from flavones group including 5-hydroxy-7,8,2'-trimethoxflavone from aerial parts, echioidinin from whole part, 2',5-dihydroxy-7,8-dimethoxyflavone-2'-O-β-D-glucopyranoside from aerial parts, andrographidine C from aerial parts, and 5,6,4-trihydroxy-7-methoxyflavone-5-O-β-D-glucoside from aerial parts.(29) In line with this study, prior research reported that *A. paniculata* extract containing 50% of andrographolide exhibited cytotoxic effect on human triple-negative breast adenocarcinoma MDA MB-231, human triple-negative breast carcinoma MDA MB-453, and normal human fibroblast (FN1) cells with IC_{50} values of 7.67, 6.3, and 9.8 mg/mL, respectively.(30)

To identify the senescence progression, the mRNA expressions of p16, p21, IL-6, IL-8, IL-1α expression on cell surface, and the SA-β-gal-stained cells were examined. The concentration of *A. paniculata* ethanol extract at 7.5 µg/mL exhibited senolytic effect which was indicated by a decrease of p16, p21, IL-6, and IL-8 mRNA expressions (Figure 4A-4D), as well as a decrease in the number of SA-β-gal-stained cells (Figure 2) and IL-1α expression (Figure 3). To support this finding, *A. paniculata* extract showed anti-aging on HEpSCs through upregulation of vascular endothelial growth factor (VEGF) production.(31) Further study is required to isolate the specific phytochemical constituents within this extract that contribute synergistically or antagonistically to its anti-senescence effect.

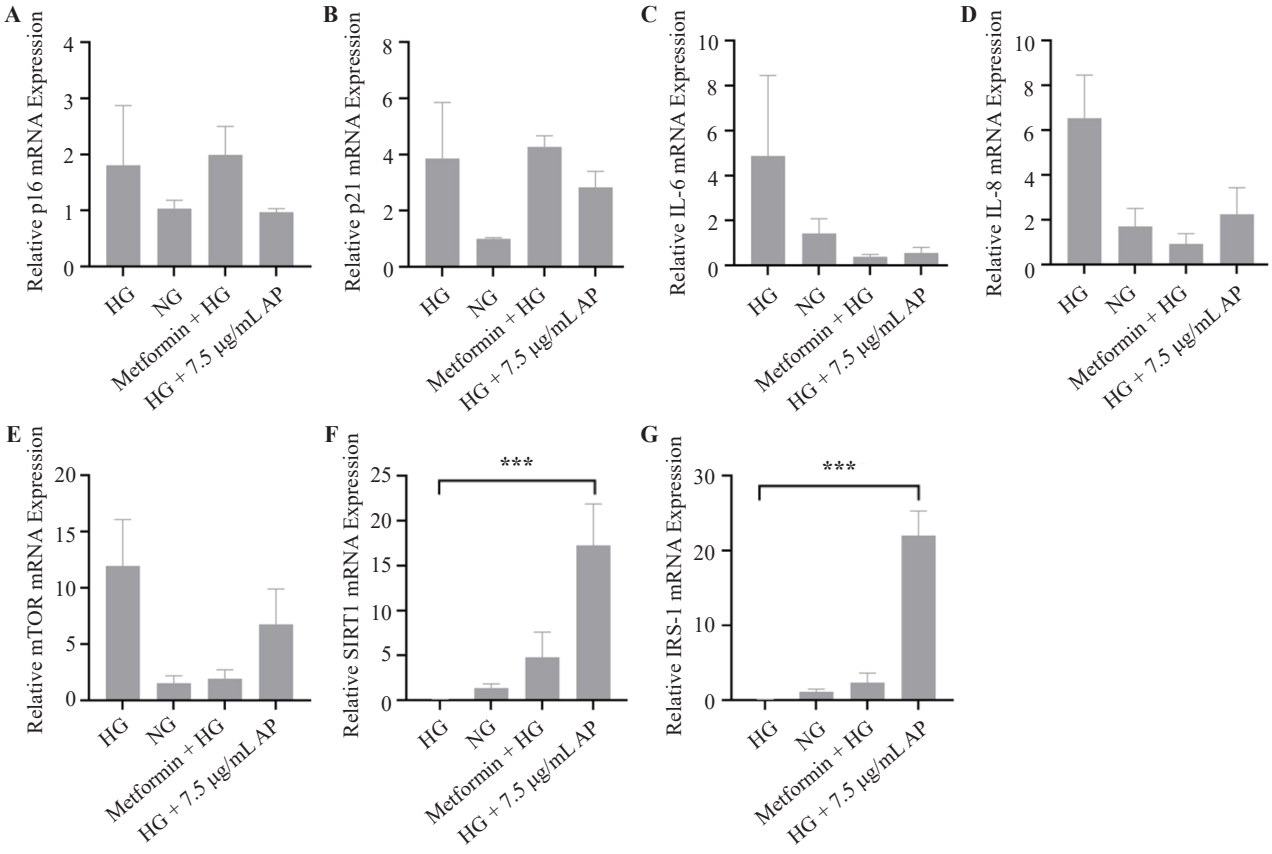

Figure 4. The effect of *A. paniculata* extract on the mRNA expressions of p16, p21, IL-6, IL-8, mTOR, SIRT1, and IRS-1 on HUVECs. A: p16 mRNA expression. B: p21 mRNA expression. C: IL-6 mRNA expression. D: IL-8 mRNA expression. E: mTOR mRNA expression. F: SIRT1 mRNA expression. G: IRS-1 mRNA expression. Each bar represents the mean relative mRNA expression±SEM of four samples. ***p<0.001. AP: *Andrographis paniculata.*

To further analyze the mechanism of senescence and its association with insulin signaling, the mRNA expressions of mTOR and SIRT1, as well as the mRNA expression of IRS-1 in insulin signaling, were analyzed. Compared to HUVECs with HG treatment only, the downregulated of mTOR mRNA expression (Figure 4E) and upregulated of SIRT1 (Figure 4F) mRNA expression were investigated on HUVECs treated with 7.5 µg/mL of *A. paniculata* extract indicating that the regulation of mTOR and SIRT1 underlies the mechanism of anti-senescence in HUVECs with HG stimulation. Furthermore, the mRNA expression of IRS-1 in HUVECs treated with 7.5 µg/mL of *A. paniculata* extract was upregulated, compared to HUVECs with HG treatment (Figure 4G), indicating that insulin resistance in HUVECs treated with *A. paniculata* extract may be attenuated by improving senescence caused by HG through restoring the insulin signaling. In line with our study, it has been demonstrated that *A. paniculata* extract improved insulin resistance in high-fat-diet-induced obesity in mice via IRS-1/Akt/AS160/GLUT4 pathway.(32)

Another approach to exhibit the anti-senescence effect of *A. paniculata* is through its anti-inflammatory properties, which counteract the SASP production in HG-induced senescence in HUVECs. A study reported that the compounds of *A. paniculata* from its aerial parts, which exhibited anti-inflammatory effects, were andrographatoside, andrographolide, neoandrographolide, andrograpanin, 14-deoxyandrographolide, isoandrographolide, quercetin, caffeic acid, ferulic acid, and protocatechuic acid.(29) In addition, previous study reported that *A. paniculata* extract decreased glucose and insulin level in circulation and improved insulin resistance through IRS-1/Akt/AS160/glucose transporter type 4 (GLUT4) pathway in mice treated with high-fat-diet.(32) In line with this study, previous studies demonstrated that *A. paniculata* extract showed anti-inflammatory effects by inhibiting TNF-α and Caspase-3 expressions, as well as decreasing the ICAM-1 and E-selectin expressions in rats.(33,34) Additionally, andrographolide, as one of the main bioactive compounds of *A. paniculata*, showed anti-inflammatory property in HUVECs treated with HG via reducing the production of IL-1β, IL-6, and TNFα. Andrographolide also improved endothelial injury via PI3K/Akt/eNOS pathway.(35) Additionally, andrographolide also showed antioxidant effect on human endothelial cell line EAhy926.

In this study, metformin treatment in HUVECs under HG condition resulted in a decrease in cell viability. Consistent with our findings, another study reported that the proliferation of HUVECs by MTT assay was decreased in metformin treatment group. However, metformin showed paradoxical effects in different types of cells.(36,37) To the best of our knowledge, the mechanisms and the regulatory components for the anti-proliferation or pro-proliferation properties of metformin in HUVECs, remain unclear and further study is necessary. This study also revealed that metformin increased the mRNA expressions of p16 and p21 in HUVECs treated with both metformin and HG. Similar with this result, previous studies have shown that metformin promoted cell cycle arrest by increasing the p21 mRNA expression and decreasing the Cyclin D1 mRNA expression in HUVECs (36), as well as upregulating the expression of p53 in hepatoma cells (38). Metformin also impaired growth of endometrial cancer cells by inhibiting the cell cycle, and inducing autophagy, along with apoptosis. However, further studies are necessary to thoroughly explore the effects and mechanisms of metformin in HUVECs.(39)

In conclusion *A. paniculata* extract has shown its ability to ameliorate senescence through various mechanisms,

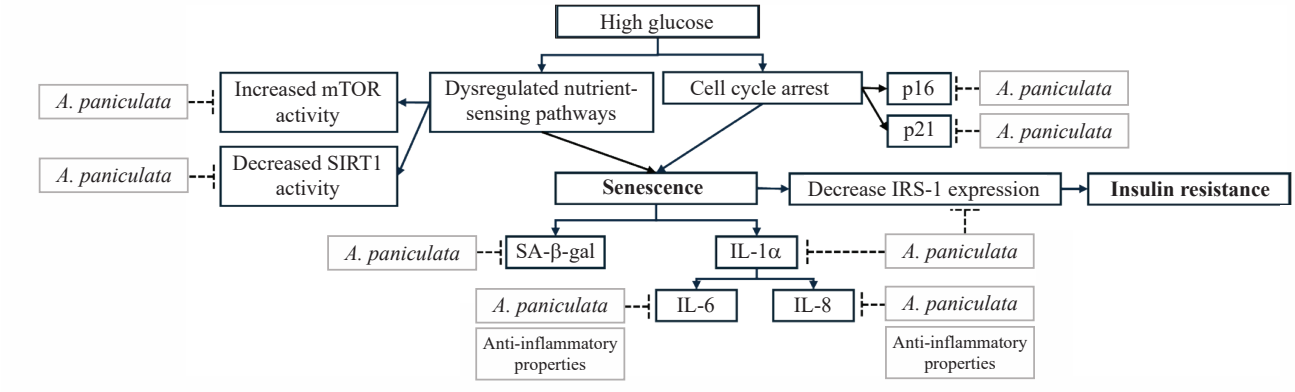

Figure 5. Proposed mechanism of *A. paniculata* ethanol extract effect on HG-induced senescence in HUVECs as the potential senolytic agent.

including SIRT1 activator and mTOR inhibitor, as reflected by a decrease in mRNA expressions of p16, p21, IL-6, and IL-8, as well as IL-1α cell surface expression and SA-β-gal. *A. paniculata* extract also improved insulin resistance through the mRNA expression of IRS-1. Thus, the proposed mechanism of *A. paniculata* extract on endothelial cell senescence was illustrated in Figure 5. This study focuses on assessing the anti-senescence effect of *A. paniculata* extract on HUVECs treated with HG; however, future research is needed to investigate the association between different organs, not just restricted to endothelial cells. Further studies are required to examine the anti-senescence effect of *A. paniculata* extract in animals (*in vivo*), specifically its effect on vascular and its relationship with other tissues and organs related to insulin resistance such as adipocyte tissues, skeletal muscles, and liver. Determining the specific compounds of *A. paniculata* extract, which potentially show the anti-senescence effect in the premature senescent endothelial cells, is necessary to be explored in more depth.

Conclusion

A. paniculata extract at 7.5 µg/mL attenuated HG-induced senescence on HUVECs by decreasing the mRNA expression of mTOR and increasing the mRNA expressions of IRS-1 and SIRT1. This study not only examined the cellular senescence markers of *A. paniculata* extract, but also provides evidence of its correlation with improving insulin resistance through senescence.

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Authors Contribution

NGK, WA, and AJB participated in the conceptualization, planning, and data collection of the study. NGK and MRF conducted the analysis of the experimental data. RDA, NSH, and IS assisted NGK to analyze and interpret the results. NGK, WA, and AJB drafted the manuscript and prepared all the figures. All authors participated in the critical review and modification of the manuscript.

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