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Schisandrin C: An active compound from the fruit of *Schisandra chinensis* in anti-inflammation and anti-oxidation

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ABSTRACT

Inflammatory responses are involved in various diseases, such as insulin resistance, atherosclerosis, and hypogonadism. This study investigates the effects of *Schisandra chinensis* extract (SCE) on anti-inflammation and molecular mechanisms in LPS-induced macrophages. RAW 264.7 macrophage cells were treated with LPS for 24 hr, followed by SCE, schisandrin C (Sch C) (1, 10, and 100 μM), and gomisin N (GN) (1, 10, and 100 μM) for 24 hr. Gene expression levels of pro-inflammatory cytokines were measured by qPCR. Protein expression of NLPR3 inflammasome was examined by western blot analysis. The expression levels of pro-inflammatory cytokines, including IL-1β, IL-6, and TNFα, were significantly reduced after SCE treatment. Sch C significantly inhibits these pro-inflammatory cytokines, while GN suppresses only IL6. Furthermore, Sch C significantly prevented the activation of NLRP3 inflammasome complexes such as NLRP3 and caspase-1. Sch C is the major active compound of SCE on anti-inflammation through attenuation of NLRP3 inflammasome.

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Introduction

Inflammatory responses are now widely accepted to contribute to a number of disorders in humans, including insulin resistance and atherosclerosis, which are highly prevalent globally (1,2). The inflammation process is quite complex, and it is initiated by several mediators, including molecules that range from bacteria to chemicals, resulting in cellular trauma or death (3,4). Evidence now suggests that reactive oxygen species (ROS) might play an even more significant role in regulating a series of intracellular signaling pathways to stimulate inflammatory responses (5). Furthermore, ROS-stimulated nod-like receptor protein-3 (NLRP3) inflammasome is one of the well-known inflammations activating signaling pathways through pro-inflammatory cytokines such as IL-1β and perpetuating the development of insulin resistance and atherosclerosis (6).

Recent studies have shown that inflammatory responses are critical mediators of insulin resistance and atherosclerosis, with a progressive infiltration of macrophages into obese adipose tissue and atherosclerotic plaques, respectively (7,8). The macrophages in obese adipose tissue and lesions produce a wide range of mediators to the ROS and pro-inflammatory cytokines, such as IL-1β, IL-6, and TNFα. Interestingly, the NLRP3 inflammasome is stimulated by ROS and subsequent pro-inflammatory cytokine, IL-1β secretion in macrophages (9). A two-step mechanism consisting of priming and activation signals controls the formation of the NLRP3 inflammasome (10,11). First, nuclear factor kappa B (NF-κB) is needed to stimulate pre-IL-1β generation (12). Second, NLRP3 complexes together with adaptor protein apoptosis-associated speck-like protein (ASC) upon activation, which in turn binds to pro-caspase-1 through the interaction of the PYD-PYD and CARD-CARD domains, respectively. Consequently, caspase-1 undergoes proximity-induced self-cleavage and activation, leading to the maturation and secretion of IL-1β (13,14).

Schisandra chinensis (*S. chinensis*), which belongs to the Magnoliaceae family, is an important medicinal plant. The fruits of *S. chinensis* have been used as a sedative, a tonic, an anti-aging drug, and an anti-tussive in Northeast Asian countries, including China, Japan, and Korea, for a long time (15). Composition studies discovered that fruit and the stem and root of *S. chinensis* contained active biological ingredients. These biologically active compounds mainly include lignans, polysaccharides, essential oils, and organic acids (16). Among the active compounds in *S. chinensis*, lignans have received special attention from many researchers due to their role in antioxidant and anti-inflammation effects (17-19). Indeed, numerous studies indicated that lignans decreased the phosphorylation of ERK, p38, and JNK, by which one of the major inflammation-stimulating transcription factors, NF-κB was indirectly inhibited (20-22). Also, the dual anti-inflammation and antioxidant roles of schisandrin C regulated the translocation of NF-κB and nuclear factor erythroid 2-related factor-2 (Nrf-2) to the nucleus, followed by inhibition of the mitogen-activated protein kinase (MAPK) pathway (23). Although there is emerging literature on the anti-oxidative

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and anti-inflammation effects of lignans, it still unclear if lignans affect NLRP3-inflammasome leading to inflammation in the macrophage. The purpose of the current study is to determine the anti-inflammation molecular mechanisms of lignans from *S. chinensis* through NLRP3-inflammation attenuation in LPS-induced macrophages.

Materials and Methods

Chemicals and reagents

Schisandrol A (CFN 99012), schisandrol B (CFN 98990), schisandrin A (CFN 99922), gomisin N (GN) (CFN 90125), and schisandrin C (Sch C) (CFN 99708) were purchased from ChemFaces (Wuhan, China). Dimethyl sulfoxide (276855) was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Preparation of ethanol extract

Fruits of *S. chinensis* were obtained from an herbal medicinal market (Munkyung, Korea). The voucher specimen (accession number KS-SC-1) was deposited at the Herbarium of the College of Pharmacy, Kyungsung University. Fifty grams of dried *S. chinensis* fruits were ground with a blender (SMX-M41KP, Shinil, Korea) and extracted two times with 60% aqueous ethanol (200 mL) for 3 hours under reflux. The vacuum rotary evaporator (EYELA N-1000, Rikakikai Co., Tokyo, Japan) was used to concentrate *S. chinensis* extract (SCE) and the concentrated SCE was lyophilized with a freezing dryer (FD5512, Ilshin, Korea). SCE was stored in a refrigerator at 4°C.

ROS scavenging assay

The reactive oxygen species (ROS) scavenging activity was quantitatively determined using the H_2DCFDA (Thermo Fisher Scientific, MA, USA). SCE was dissolved in DMSO at a final concentration of 10, 100, and 1000 μg/mL. For an individual compound of SCE with the highest ROS scavenging activity, GN and Sch C were used at a final concentration of 1, 10, and 100 μM. 10 μL of samples were mixed in a 96-well with 3-morpholinosydnonimine hydrochloride (SIN-1; Sigma-Aldrich, MO, USA), esterase, and H_2DCFDA allowed to react for 30 min in the dark. Trolox (Sigma-Aldrich) was used as a positive control. The fluorescence was measured using a Varioskan™ LUX spectrophotometer (Thermo Fisher Scientific) at 485 and 535 nm, excitation and emission, respectively. The percentage of ROS scavenging activity was calculated, and the significance of differences was determined compared to SIN-1.

Cell culture and treatment

RAW 264.7 cells were purchased from Korea Cell Line Bank (Seoul, Korea). Cells were cultured with 1 g/L D-glucose DMEM supplemented with 10% FBS and 10,000 units of penicillin-streptomycin and incubated at 37°C with 5% CO_2 condition. Subculture was performed after reaching 80% confluence, and cells were detached by incubation in 0.05% trypsin-EDTA. For use in experiments, cells were seeded into 6-well plates, 12-well plates, and 96-well plates and cultured to 70-80% confluence. After being seeded in plates, cells were exposed to LPS (0.1 μg/mL) for 6 hr to induce inflammation.

Measurement of cell viability

The cell viability was evaluated by MTT assay using the EZ-Cytox Cell viability assay kit, which included the WST (water-soluble tetrazolium salt). RAW 264.7 cells were plated in a 96-well plate at a density of 3.5 x 10^4 cells/well and incubated for 24 hr. Then, cells were treated with SCE (62.5, 125, 250, and 500 μg/mL), GN (0.1, 1, 10, and 100 μM), and Sch C (0.1, 1, 10, and 100 μM) for 24 hr. EZ-Cytox was added by a fixed amount (10 μL) to each well and incubated for 2 hr. Then absorbance was measured using a Varioskan™ LUX Multimode Microplate Reader at 450 nm.

Analysis of intracellular ROS levels

Intracellular ROS scavenging analysis in RAW 264.7 cells was performed to examine whether SCE and major components inhibited LPS-induced ROS levels. RAW 264.7 cells were plated in a 96-well plate at a density of 2 x 10^5 cells/well and incubated to adhere for 24 hr. After 24 hr, the media was removed, and cells were treated with SCE (1, 10, and 100 μg/mL), GN (0.1, 1, and 10 μM), and Sch C (0.1, 1, and 10 μM) for 24 hr. Then, 0.1 μg/mL LPS was treated for 4 hr. 100 μM DCFDA was allowed to react for 1 hr in the dark. After 1 hr, the media was removed and washed with PBS. The fluorescence was measured using a Varioskan™ LUX spectrophotometer (Thermo Fisher Scientific) at 485 and 530 nm, excitation, and emission, respectively. The percentage of ROS scavenging activity was calculated, and the significance of differences was determined compared to LPS-treated cells.

Gene expression analysis

Quantitative PCR (qPCR) was performed in the expression of the pro-inflammatory cytokine gene to investigate the anti-inflammatory effects of SCE, GN, and Sch C. The cells were plated in a 12-well plate at a density of 3.5 x 10^4 cells/well and incubated for 24 hr. After 24 hr, cells were treated with SCE (10 and 100 μg/mL), GN (1 and 10 μM), and Sch C (1 and 10 μM) for 24 hr. To stimulate the inflammatory responses, 0.1 μg/mL LPS was treated for 6 hr. Total RNA was extracted with a Total RNA Extraction kit (SJ BioScience, Daejeon, Korea), including DNase I (SJ BioScience, Daejeon, Korea). RNA concentration was quantified using nanodrop (MicroDigital Co., Ltd., Gyeonggi-do, Korea). qPCR was performed using Sybr Green PCR Master Mix (SJ BioScience, Daejeon, Korea), using primer sequences provided in blow; *m-18S* forward 5'-GTAACCCGTTGAACCCCATT-3', reverse 5'-CCATCCAATCGGTAGTAGCG-3'; *m-1L1β* forward 5'-GCAACTGTTCCTGAACTCAACT-3', reverse 5'-ATCTTTTGGGGTCCGTCAACT-3'; *m-IL6* forward 5'-GCAGCATCACCTTCGCTTAGA-3', reverse 5'-CAGATATTGGCATGGGAGCAAG-3'; *m-TNFα* forward 5'-CAGGCGGTGCCTATGTCTC-3', reverse 5'-CGATCACCCCGAAGTTCAGTAG-3'. All reactions were carried out at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. All samples were normalized to 18S. The relative gene expression levels were calculated using the $2^{-(\Delta\Delta Ct)}$ method.

Western blot

Whole-cell lysates were homogenized with lysis buffer (50 mM phosphate, 1 mM DTPA, and 50 mM NEM, pH 7.4) and incubated for 30 min on ice. SDS-PAGE

were performed in an equal amount as 40 µg of total protein with western blot analysis performed as described under. Briefly, the protein was separated by SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were blocked for 30 min at room temperature with membrane-blocking solution (Translab, Daejeon, Korea) and incubated with primary antibodies, including caspase-1 (A0964, Abclonal, MA, USA) and NLRP3 (A12694, Abclonal, MA, USA) overnight, followed by secondary antibodies (AS014, Abclonal, 1:10000). Membranes were washed with TBS-T buffer three times before detected using an Azure 600 Western Blot Imager (Azure Biosystems, Dublin, CA, USA). The quantification of proteins was calculated using Image J.

Statistical analysis

Statistical analysis of data was carried out using GraphPad Prism (version 5.0, GraphPad Software, Inc., San Diego, CA, USA). Results were presented as mean ± the standard error of the mean (SEM). One-way ANOVA followed by Dunnett's multiple comparisons test was performed to compare between groups. Statistically significant was considered $P < 0.05$.

Results

Effects of *Schisandra chinensis on* ROS scavenging activity

ROS is a kind of reactive molecule that can easily react with some molecules and is known to activate inflammation. To evaluate the effects of SCE on free radical scavenging activity, H_2DCFDA ROS detection assay was performed. As shown in Fig. 1A, a significant increase in H_2DCFDA fluorescence was observed in SIN-1 treated group. However, SCE remarkably reduced ROS levels in a dose-dependent manner ($P < 0.001$). Our previous HPLC data has shown five major compounds in SCE (24); thus, we evaluated the ability of various active compounds to inhibit free radicals. Among five compounds, GN and Sch C were the highest ROS scavenging active compounds ($P < 0.05$ and $P < 0.001$) (Fig. 1B). Indeed, as shown in Fig. 1C and 1D, GN and Sch C reduced the ROS level in a dose-dependent manner as compared to SIN-1 treated group ($P < 0.01$ and $P < 0.001$). These results indicate that Sch C and GN, the active components of SCE, are antioxidant activity compounds of SCE.

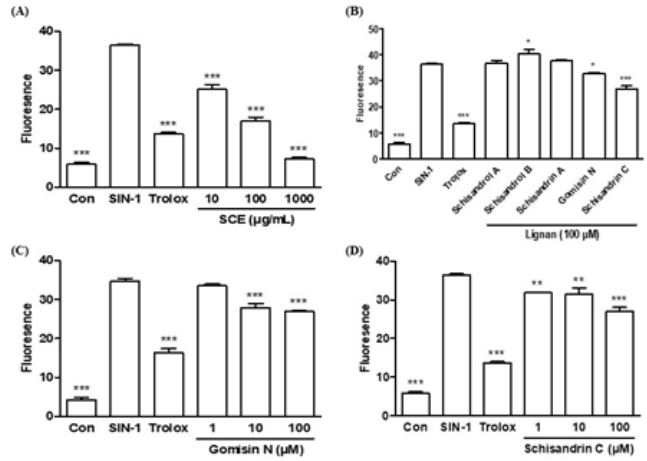

Figure 1. ROS scavenging activity of SCE (A), lignans from SCE (B), GN (C), Sch C (D). The reactive oxygen species (ROS) scavenging activity was quantitatively determined using the H_2DCFDA (n=8). One-factor ANOVA was used to determine the significance of differences: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus SIN-1 treated group.

Cell viability effects of SCE and its active components on RAW 264.7 cell

In order to investigate the impact of SCE and active compounds on cell viability, RAW 264.7 cells were exposed to SCE, GN, and Sch C for 24 hr at various concentrations (SCE; 62.5, 125, 250, and 500 µg/mL, GN, and Sch C; 0.1, 1, 10 and 100 µM). Although no significant cytotoxicity was found in Sch C-treated cells compared to the control group (Fig 2C), a significant reduction the cell viability was shown in SCE (250 and 500 µg/mL, $P < 0.05$ and $P < 0.001$, respectively) and GN (100 µM) ($P < 0.01$) (Fig 2A and B). Therefore, the concentrations of SCE and active compounds (GN and Sch C) were used at concentrations of 10 and 100 µg/mL and 1 and 10 uM individually for further studies.

Effects of SCE and its active components on intracellular ROS levels

To determine the effects of SCE on intracellular ROS levels, we conducted an H_2DCFDA ROS detection assay on RAW 264.7 cells. In the ROS scavenging assay result, GN and Sch C were determined to be active antioxidant compounds of SCE. Thus, we evaluated the ability of GN and Sch C to inhibit the LPS-induced ROS levels. As shown in Fig. 3A, intracellular ROS levels were significantly increased when incubated with LPS. However, SCE decreased the LPS-induced ROS level of RAW 264.7 cells in a dose-dependent manner ($P < 0.001$). Indeed, GN 0.1, 1, and 10 µM reduced LPS-induced ROS levels by 9.58%, 10.79%, and 16.93%, respectively ($P < 0.05$ and $P < 0.001$). Sch C 0.1, 1, and 10 µM were able to reduce the

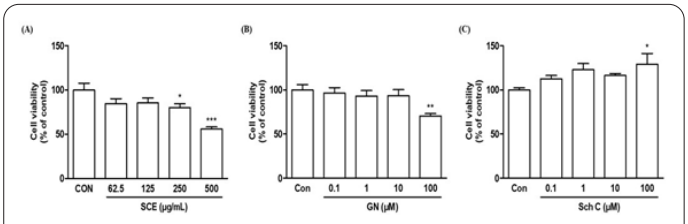

Figure 2. Changes in the viability of RAW 264.7 cells after incubation of SCE, GN, and Sch C. Cell viability of macrophages after exposure to SCE (A), GN (B), and Sch C (C) for 24h (n=8). One-factor ANOVA was used to determine the significance of differences: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus vehicle-treated control cells (Con).

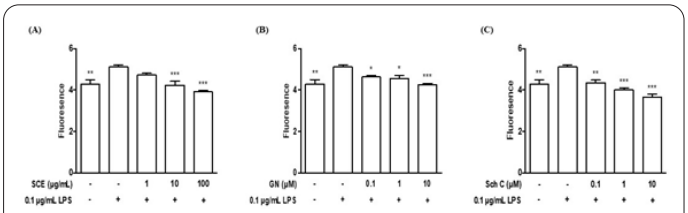

Figure 3. Intracellular ROS level inhibited by SCE (A), GN (B), and Sch C (C). Cell lysates were analyzed to determine whether SCE and major components inhibited LPS-induced ROS levels (n=8). One-factor ANOVA was used to determine the significance of differences: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$ versus LPS treated group.

LPS-induced ROS level in a dose-dependent manner and 4.76%, 21.44%, and 28.56%, respectively ($P < 0.01$ and $P < 0.001$). The antioxidant activity of Sch C was higher than GN. Therefore, Sch C is an active antioxidant compound of SCE.

Effects of SCE and its active components on gene expression

As we have a significant increase in ROS scavenging activity under SCE, GN, and Sch C treatment, we examined whether they can down-regulate LPS-induced pro-inflammatory cytokines gene expression levels, including IL-1β, IL-6, and TNFα in LPS-exposed RAW 264.7 cells. Interestingly, IL-1β and IL-6 were significantly decreased in SCE-exposed RAW 264.7 cells in a dose-dependent manner ($P < 0.001$) (Fig. 4A and B). But SCE did not alter the TNFα expression levels (Fig. 4C). Consistent with the IL-1β and IL-6 gene expression under SCE treatment and free radicals scavenging activity, Sch C significantly down-regulated IL-1β, IL-6, and TNFα in a dose-dependent manner ($P < 0.001$) (Fig. 4G-I). GN decreased IL-6 and TNFα ($P < 0.05$ for IL-6 and $P < 0.001$ for TNFα), but did not alter the IL-1β expression levels (Fig. 4D-F). Therefore, these results indicate that SCE and its active components have potential anti-inflammatory effects through enhancing antioxidant activity.

Effects of SCE and its active components on inflammasome

NLRP3 inflammasome is a well-known inflammation promoter by overproduction of pro-inflammatory cytokines, especially IL-1β. Given that Sch C suppressed IL-1β, IL-6, and TNFα gene expression levels, we were persuaded that the NLRP3 inflammasome might have been attenuated by Sch C to inhibit inflammation in the macrophage. Exposure to Sch C of LPS-triggered RAW 264.7 cell for 24 hr significantly reduced the components of the NLRP3 inflammasome, NLRP3 ($P < 0.05$), and caspase-1 ($P < 0.01$) (Fig. 5B). However, NLRP3 inflammasome was not altered when exposed to GN (Fig. 5A). These results indicated GN and Sch C could reduce the inflammation by reducing the expression of the inflammasome-related enzyme.

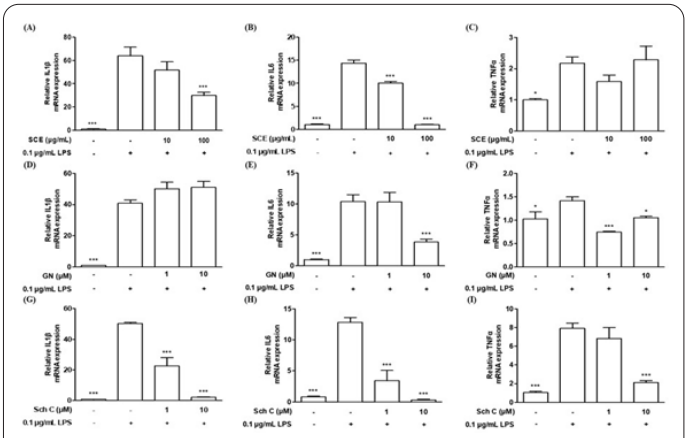

Figure 4. The effect of SCE, GN, and Sch C on IL-1β, IL-6, and TNFα production in RAW 264.7 cells by concentration. Cell lysates were analyzed for pro-inflammatory cytokine levels by Q-PCR (n=6). SCE reduced the secretion of IL-1β (A) and IL-6 (B), respectively. GN and Sch C reduced the secretion of IL-1β (G), IL-6 (E and H), and TNFα (F and I). One-factor ANOVA was used to determine the significance of differences: *$P < 0.05$; ***$P < 0.001$ versus LPS-treated cells.

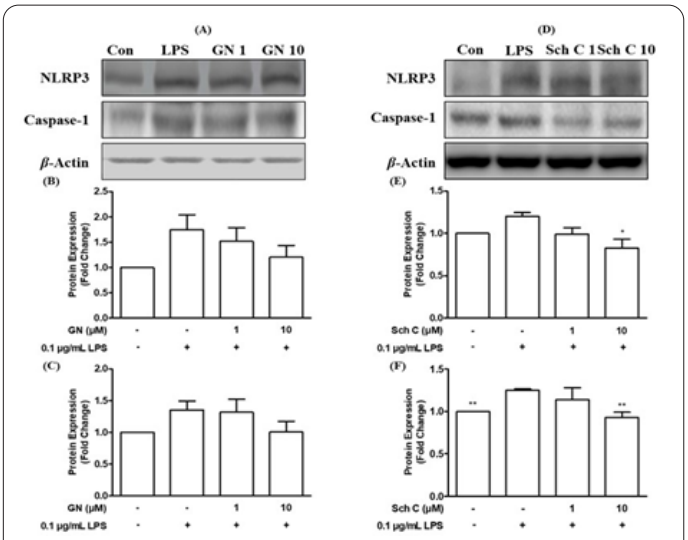

Figure 5. The effect of GN and Sch C on NLRP3 inflammasome inactivation in LPS-induced RAW 264.7 cells. Cell lysates were analyzed for levels of NLRP3 inflammasome complex, NLRP3, and caspase-1 by western blot analysis (n=5). β-actin expression was used as a loading control. Western blot images represent five different experiments of GN (A) and Sch C (D) exposure groups in RAW 264.7 cells. Quantification results of NLRP3 (B and E) and caspase-1 (C and F). One-factor ANOVA was used to determine the significance of differences: *$P < 0.05$; **$P < 0.01$ versus LPS-treated cells.

Discussion

Oxidative stress refers to the excessive production of ROS in the cells and tissues, and the antioxidant defense system cannot be able to neutralize them (25,26). An imbalance in this protective mechanism can trigger inflammatory responses through the production of cytokines, consequently raising the risk of chronic inflammatory diseases (27). Therefore, emerging studies aimed to evaluate natural compounds' protective effects against oxidative stress and inflammatory responses (28,29). Lignans are among herbal's most interesting molecules and are ascribed to several biological activities such as antioxidant and anti-inflammation (28,29). One of the major active compounds of SCE, Sch C, demonstrated its effectiveness against biologically generated inflammatory responses through scavenging free radicals in LPS-induced macrophages (30). Sch C's and other lignans' inhibitory activities were linked to attenuating MAPK, ERK, and JNK signaling pathways (30). Although several kinds of research have shown the effects of SCE, Sch C, or GN on oxidative stress and inflammatory response, there has not been any prior evidence of the molecular mechanisms of NLRP3 inflammasome inhibition.

Our previous study shows five lignans in *S. chinensis*; schisandrol A, schisandrol B, schisandrin A, GN, and Sch C. Further, the contents of GN and Sch C were determined 1.42% and 0.3%, respectively, in SCE. Among those five lignans from *S. chinensis,* schisandrol A and GN suppressed oxidative stress in H_2O_2-induced TM3 Leydig cells (24). In this study, we used a classical inflammatory cell model, LPS-induced RAW 264.7 cells, to examine the effects of SCE on anti-oxidation through ROS scavenging assay. Consistent with our previous DPPH and ABTS

study, SCE is able to scavenge ROS (24). And of the five lignans, schisandrol A and GN are the higher active compounds in DPPH and ABTS. Interestingly, however, it was revealed that Sch C and GN are the higher active compounds in ROS scavenging assay. Indeed, Sch C and GN suppressed ROS levels dose-dependently without affecting cell viability. This might be happening because of a different principle of each assay. Therefore, for further study, we have selected Sch C and GN to investigate oxidative stress and inflammatory responses in more detail.

Oxidative stress is involved in the inflammatory responses, including stimulation of pro-inflammatory cytokines generation (31,32). ROS activates macrophages to enhance the transcription factors NF-κB translocation from cytosol to the nucleus leading to the transcription of gene encodings such as IL-1β, IL-6, and TNFα (33). Interestingly, SCE inhibits the production of pro-inflammatory cytokines, IL-1β, IL-6, and TNFα in LPS-induced RAW 264.7 macrophage. One of the higher active-compound of *S. chinensis*, schisandrin C, also significantly prevents IL-1β, IL-6, and TNFα at a concentration of 1 and 10 μM that does not show cytotoxicity. GN, however, only attenuates IL-6 at the concentration of 10 μM. These results indicate that Sch C rather than GN is the main component of *S. chinensis* in anti-inflammatory responses through amelioration of ROS production.

Interestingly enough, of the three cytokines, Sch C most targets IL-1β. Compelling evidence shows that the NLRP3 inflammasome has been proven essential in IL-1β production and secretion (6,12,34). Our study, for the first time, shows that consistent with pro-inflammatory cytokine results, Sch C but not GN inhibits the NLRP3 inflammasome complex, including NLRP3 and caspase-1, indicating that one of the potential roles of Sch C from *S. chinensis* might target NLRP3 inflammasome. The previous study has been shown to play a role in ROS to trigger the NLRP3 inflammasome (35). In this study, Sch C could suppress the ROS level, which might be a cause of alteration of NLRP3 inflammasome activation. So, we speculate that Sch C inhibits inflammatory responses by attenuating ROS level and NLRP3 inflammasome. Although both gomisin N and Sch C exhibit comparable efficacy in inhibiting the NLRP3 complex, it is important to note that Sch C demonstrates a remarkable capacity to suppress the release of IL-1β, which is activated by the NLRP3 inflammasome. Consequently, we have concluded that Sch C has a significant role among the compounds derived from *Schisandra chinensis*.

In conclusion, our study demonstrated that Sch C could alter the ROS level and NLRP3 inflammasome for the first time, improving inflammatory responses. These findings provide new insight into the active compound and molecular mechanisms underlying the alteration of inflammation. Indeed, the mediators, including IL-1β, IL-6, and TNFα, produced by macrophages within obese adipose tissue, play a pivotal role in influencing insulin resistance and atherosclerosis (36,37). There are many reports (38-49) about the effect of medicinal plants in the treatment of various diseases. The inhibitory effects observed with Sch C suggest that it has the potential to alleviate insulin resistance and atherosclerosis by reducing these mediators within obese adipose tissue. This implies that Sch C could be a potential agent in preventing the development of insulin resistance and atherosclerosis by repressing the production of these mediators in the obesity.

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Author contributions

Conceptualization: *MHP, HKK*; Methodology: *MHP, HKK*; Formal analysis: *TWK, SH MSP*; Investigation: *TWK, SH MSP*; Writing - original draft: *TWK, SH MSP, MHP, HKK*; Funding Acquisition: *MHP, HKK*; Supervision: *MHP, HKK*

Conflict of interest

The authors have declared no conflicts of interest.

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