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Apoptosis associated with osteoarthritis inhibited by Shiikuwasha extract via down-regulating JAK2/SATA3 pathway in SW1353 cells

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Abstract
Chondrocyte death relatively contributes to osteoarthritis (OA) pathogenesis. Novel agent for OA is necessary and it might be easy gain from a common citrus fruit. The shiikuwasha is a citrus fruit from Okinawa that has the potential to exert many positive effects on human health. Shiikuwasha extract (SE) displayed antioxidant properties, which were estimated through several widely used assays: DPPH, ABTS and reducing power assay. An OA-similar chondrocyte model was founded by using MIA-stimulated SW1353 cells. SE rescued the cell viability and apoptosis percentage in a dose-dependent manner. The alterations of ROS levels and ΔΨm caused by MIA were also reversed by SE. TNF-α and IL-6 were highly expressed in the culture medium only with MIA, but collagen 2 was less expressed. However, SE decreased the levels of TNF-α and IL-6, and restored collagen 2 level. Toluidine blue O staining revealed that SE blocked proteoglycan loss. The expressions of PARP, caspase 3, BAX, JAK2, p-STAT3 and STAT3 were decreased in the presence of SE, implicating the apoptosis and JAK2/STAT3 pathways participated in the effects of SE on MIA-treated SW1353 cells. Together, these findings demonstrated that SE could modulate MIA-induced apoptosis in SW1353 cells to ameliorate cartilage matrix proteins loss and inflammatory response. SE also could be regarding as bioactive or nutritional supplement for OA.

Keywords: apoptosis; cartilage matrix proteins; osteoarthritis; pro-inflammatory cytokines; shiikuwasha extract

1. Introduction
The death of chondrocytes is usually observed in articular cartilage in osteoarthritis, in which chondrocytes are not functionally responsible for the synthesis and maintenance of the extracellular matrix (ECM) [1]. Moreover, many studies suggest that apoptosis or apoptosis-like programmed cell death primarily induces chondrocyte death in articular cartilage.[1] During OA progression, the apoptosis of chondrocytes can be activated by the Janus kinase 2 (JAK2)/signal transducer and activator of transcription 3 (STAT3) signaling pathway, that demonstrated in vitro and in vivo model [2, 3]. Additionally, damaged chondrocytes can yield harmful substances, such as chemokines, proinflammatory cytokines, reactive oxygen species (ROS), and free radicals [4]. Abnormally elevated proinflammatory cytokines, such as interleukin (IL)-1β, IL-6 and tumor necrosis factor-alpha (TNF-α) [5]. in turn induce cytokine expression, and chondrocytes are then triggered to produce matrix metalloproteinases (MMPs) and aggrecanases, which lead to severe cartilage degradation [1].
OA is a form of degenerative joint pain, causing millions of people disability worldwide. It is characterized by articular cartilage loss, subchondral bone sclerosis and osteophyte formation, and synovial tissue inflammation [6]. Worldwide statistics estimate that OA affected people aged more than 60 years (9.6% of men and 18.0% of women) [7]. OA therapies mainly focus on pain relief rather than reversing the progress of cartilage breakdown. Patients with OA are usually only administered pain-relieving drugs [8]. Thus far, existing OA drugs cannot reverse the damage in affected joints, are limited to relief of OA symptoms, and are accompanied by a high incidence of adverse effects.[9] However, recent studies have shown that dietary antioxidant nutraceuticals result in a positive response to OA-related inflammation [10].
The shiikuwasha (Citrus depressa Hayata) is a citrus fruit grown natively in Taiwan and Okinawa, Japan, and used to make jam, or a yellow juice.[11] Shiikuwasha has abundant ascorbic acid, along with essential oils, minerals, and flavonoids. The shiikuwasha fruit, especially in peel, is rich of flavonoids. Flavonoids are the major bioactive compounds in citrus fruits, including flavanones (like naringenin and hesperetin), flavone glycosides (like naringin and hesperidin), and polymethoxylated flavones (PMFs) [12, 13]. The chemical analysis characterized shiikuwasha as containing high levels of PMFs, and that is used to detect whether shiikuwasha juice is being adulterated.[14] Nobiletin and tangeretin are two most common PMFs in citrus peel [12, 15]. Citrus PMFs possess a number of biologically important properties, such as antioxidation, anti-inflammation, neuroprotection, anticarcinogenesis, antimicrobial and antiobesity properties [16, 17].
OA in vitro studies of primary human chondrocytes have some restrictions such as dedifferentiation, insufficient number of chondrocytes, lack of normal chondrocyte morphology, differences of biological and functional features between donors and preparations [18, 19]. Therefore, a promising substitute for a chondrocytic experiment is to apply chondrosarcoma-derived cell lines which possess satisfactory proliferative activity and consistent response to phenotype stimulation.
Our previous in vivo research had investigated shiikuwasha extract (SE) possessed chondroprotective effects against OA development [20]. Hence, we want to further identify the

mechanism of SE in in vitro model. The human chondrosarcoma cell line SW1353 has been studied worldwide as an in vitro OA model, and the proinflammatory cytokine-induced protease expression within SW1353 is similar to that within human primary chondrocytes [21]. To induce serious chondrocyte cell death and conduct OA progression in cell model and animal model, monosodium iodoacetate (MIA), an inhibitor of glyceraldehyde-3-phosphate dehydrogenase, is widely used to destabilize mitochondria and decrease ΔΨm (mitochondrial membrane potential) in chondrocytes in vivo and in vitro [22, 23]. In this study, we firstly examined the anti-apoptotic function of SE to protect chondrocytes from death in a SW1353 cell model of MIA-induced OA via regulating apoptosis and JAk2/STST3 pathway.

2. Materials and Methods

2.1. Preparation of SE

The SE was supplied by ARKRAY Inc. (Kyoto, Japan), where it was manufactured from Okinawan shiikuwasha fruit. The general production process was descripted below. The shiikuwasha fruit was extracted by hydrated ethanol or pressed to juice. The extract was filtered to remove precipitates, and concentrated by removing water and ethanol. Furthermore, solvent partitioning with ethyl acetate was applied to gain higher content of PMF. Then, the mixture was dried to form a powder. Finally, the content of PMF analyzed by HPLC and as shown in Certificate of Analysis provide by ARKRAY Inc. is 75.7%. Residual portion is other polyphenols and other ingredients.

2.2. Assay for total phenolic acid and flavonoids

The phenolic content was analyzed through the method reported by Singleton and Rossi with modifications.[24] Gallic acid was used as a standard phenolic compound. The SE powder was dissolved by water and mixed Folin and Ciocalteu's phenol reagent, and sodium carbonate solution (7.5%) was added to mixture and stood for 90 min. Then the absorbance was determined at 760 nm. The results were obtained from a calibration curve of gallic acid and expressed in milligram quercetin equivalents per gram of dry weight of extract (mg GAE/g DW).

The flavonoid content was analyzed through the method reported by Jia *et al*. [25] The SE powder was dissolved by water and mixed with Al $(NO_3)_3$ and CH_3COOK. The mixture stood in a dark room for 40 min, and the absorbance was determined at 415 nm using a spectrophotometer. The results were obtained from a calibration curve of quercetin and expressed in milligram quercetin equivalents per gram of dry weight of extract (mg QE/g DW).

2.3. Antioxidant Activity

For the determination of free radical scavenging of SE, the 1-diphenyl-2-picryl-hydrazyl (DPPH) assay was used.[26] In brief, Sample extracts SE (20 mg/mL) and standard antioxidant Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid, 20 mg/mL) were prepared in methanol and serially half diluted from 20 to 0.625 mg/mL. A fresh solution of DPPH (0.1 mM) was prepared in methanol. Next, 1.5 mL of each SE concentration and Trolox in methanol were added to 0.5 mL of DPPH solution. These mixtures were mixed well and incubated in the dark for 30 min. The absorbance was then measured at 517 nm. Trolox was used as the positive control. The inhibition of DPPH radical scavenging was calculated by the following formula: inhibition (%) = (1 − absorbance of sample/absorbance of control) × 100%.

The 2,2'-azobis(3-ethylbenz-thiazoline-6-sulfonic acid (ABTS) radical scavenging activity of SE was assessed using the spectroscopic method [27]. First, 250 μL of deionized water, 250 μL of 500 μM H_2O_2, 250 μL of 1000 μM ABTS, and 250 μL of 8.8 U/mL peroxidase were mixed well. After the mixture stood for 10 min, a solution of stable blue-green $ABTS^+$ radicals was formed. Sample extracts SE (2 mg/mL) and standard antioxidant Trolox (2 mg/mL) were prepared in dimethyl sulfoxide (DMSO) and serially half diluted from 2 to 0.0625 mg/mL. Next, 250 μL of differently concentrated SE and Trolox solutions were added to the $ABTS^+$ radical solution that had stood for 10 min. The absorbance was then measured at 734 nm. The inhibition of $ABTS^+$ radical scavenging was calculated by the following formula: inhibition (%) = (1 − absorbance of sample/absorbance of control) × 100%

For the determination of SE's reducing ability, the reduction of $[Fe(CN)_6]^{3-}$ to $[Fe(CN)_6]^{4-}$ was used.[28] Sample extracts SE (20 mg/mL) and standard antioxidant Trolox (20 mg/mL) were prepared in DMSO and serially half diluted from 20 to 0.625 mg/mL. Next, 1.25 mL of differently concentrated SE and Trolox were added to 1.25 mL of phosphate buffer (0.2 M, pH 7.4) and 1.25 mL of potassium ferricyanide $[K_3Fe(CN)_6]$ (1%). Then, the solution was incubated at 50°C for 20 min. After the incubation period, 1.25 mL of trichloroacetic acid (TCA) was added (10%). Finally, 0.5 mL of $FeCl_3$ (0.1%) was transferred to this mixture, and the absorbance value was measured at 700 nm in a spectrophotometer.

2.4. Cell culture

SW1353 cells, which originated from chondrosarcoma cells, were obtained from the Bioresource Collection and Research Center of the Food Industry Research and Development Institute in Hsinchu, Taiwan. The SW1353 cells were maintained in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F-12 (DMEM/F12, Gibco BRL, Grand Island, NY, USA) and incubated at 37°C and 5% CO_2. The complete medium was supplemented with 10% fetal bovine serum (FBS) (HyClone, Auckland, NZ, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco BRL, Grand Island, NY, USA).

2.5. Cell viability analysis

SW1353 cells (10^4 cells) were seeded into each well of 96-well plates and left overnight. The cells were treated with 5-25 μg/mL SE in the presence or absence of 2 μM MIA for 24 h. Finally, 0.5 mg/mL 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was added to the cell medium for 4 h. The living cells were expected to convert yellow tetrazole MTT to purple formazan dissolved in DMSO. The intensity of the formazan was measured at 565 nm using a microplate reader (Multiska GO, Thermo Scientific, Waltham, MA, USA). Additionally, cell morphology following SE treatment was observed using living cell images. After the cells were plated at a density of 5×10^5 cells/60-mm Petri dish and left overnight, they were treated with 5-25 μg/mL SE in the presence of 2 μM MIA for 24 h. Cell morphology was observed using an inverted phase contrast microscope (Olympus, Tokyo, Japan).

2.6. Annexin V-propidium iodide analysis
SW1353 cells (5×10^5 cells) were seeded into 60-mm Petri dishes overnight. The cells were treated with 5-25 μg/mL SE in the presence of 2 μM MIA for 24 h. Apoptosis was detected by using an annexin V-propidium iodide (PI) staining kit (BioVision, Mountain view, CA, USA) according to the manufacturer's instructions and analyzed using a BD FACSAria flow cytometer equipped with BD FACSDiva software (Becton Dickinson, San Jose, CA, USA).

2.7. Measurement of ROS and ΔΨm
Over the period of treatment, cells were harvested and stained with 20 μM 2',7'-dichlorofluorescein diacetate (DCFDA) and 10 μM rhodamine 123 (Rh123) (Sigma, St Louis, MO, USA) at 37°C in the dark for 30 min. Then, intracellular ROS and ΔΨm were analyzed using the flow cytometer.

2.8. ELISA detection of IL-6, TNF-α, MMP13 and collagen 2 production
SW1353 cells (2×10^6 cells/60-mm Petri dish) were treated with various concentrations of SE (5, 15, and 25 μg/mL) in the presence of 2 μM MIA for 24 h. The culture media were collected to quantify the levels of IL-6, TNF-α, MMP13, and collagen 2 by using commercially available enzyme-linked immunosorbent assay (ELISA) kits (Elabscience Biotechnology, Wuhan, China) according to the manufacturer's protocol.

2.9. Toluidine blue O Staining
SW1353 cells (5×10^5 cells) were seeded into each well of a six-well plate and treated with various concentrations of SE (5, 15, and 25 μg/mL) in the presence of 2 μM MIA for 24 h. The cells were rinsed twice with phosphate-buffered saline (PBS), fixed in cool methanol for 10 min, and then stained with 0.5% toluidine blue O for 10 min at room temperature. To quantify the formazan product, the absorbance at 630 nm was measured.

2.10. Western blot
SW1353 cells (2×10^6 cells) were seeded into 100-mm Petri dishes and treated with various concentrations of SE (5, 15, and 25 μg/mL) in the presence of 2 μM MIA for 6 h. The SW1353 cell were lysed in RIPA buffer (Goal Bio, Tao Yuan, Taiwan) containing 1× protease inhibitor cocktail (Goal Bio, Tao Yuan, Taiwan) and its concentration was quantified using a BCA protein assay (Thermo Fisher Scientific Inc., Rockford, IL, USA). The whole cell extract (60 μg) was mixed with 4 × sodium dodecyl sulfate (SDS) sample buffer and boiled for 5 min. The mixture were separated on SDS-polyacrylamide gel and transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). After blocking, the primary antibodies of rabbit anti-poly (ADP-ribose) polymerase (PARP) (GeneTex, Irvine, CA, USA), rabbit anti-caspase 3 (GeneTex, Irvine, CA, USA), rabbit anti- B-cell lymphoma 2 (Bcl-2) (GeneTex, Irvine, CA, USA), rabbit anti-Bcl-2-associated X protein (BAX) (GeneTex, Irvine, CA, USA), rabbit anti-JAK2 (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-p-STAT3 (Cell Signaling Technology, Beverly, MA, USA), rabbit anti-STAT3 (Cell Signaling Technology, Beverly, MA, USA) and rabbit anti-GAPDH (GeneTex, Irvine, CA, USA) were used to probed with their target protein overnight at 4°C. The membrane was then incubated with the secondary antibodies of goat anti-rabbit IgG (GeneTex, Irvine, CA, USA), using enhanced chemiluminescence (ECL) reagents (Millipore, Billerica, MA, USA) and images were captured using advanced imaging system (UVP Chemstudio, Analytik Jena AG, Thuringia, Germany).

2.11. Statistical analysis
Data are presented as the mean ± standard error of the mean (SEM). Each experiment was repeated at least three times. SPSS 15.0 software (SPSS Inc., Chicago, IL, USA) was applied for statistical analysis. Antioxidant activity differences compared with trolox were evaluated through Two-way analysis of variance (Two way ANOVA) followed by Dunnett's test. The differences between control group and SE-treated groups; MIA only group and SE-treated groups were analyzed using One way ANOVA followed by Dunnett's test. A p value of <0.05 was considered statistically significant.

3. Results
3.1. SE demonstrated free radical scavenging potential and antioxidant activities
To analyze the antioxidant activities of SE, we measured the free radical scavenging activity and reducing power of SE. The DPPH free radical scavenging activity of SE compared with standard Trolox is shown in Figure 1A. Standard Trolox demonstrated considerable inhibition of 92%-93% at each concentration (0.625-20 mg/mL). SE increased DPPH free radical scavenging activity with increases in its concentration in the range of 0.625-20 mg/mL and peaked at 91.6% ± 1.0% inhibition at 10 mg/mL (Figure 1A). SE exhibited potent ABTS radical scavenging activity in a concentration-dependent manner (Figure 1B). SE reached inhibition of more than 50% at 1 mg/mL, the same inhibition as Trolox at 2 mg/mL. The reducing power of SE in comparison with the standard Trolox at 700 nm is shown in Figure 1C. The reducing power of SE increased with increasing SE concentration (0.625-20 mg/mL). The content of total phenolics and flavonoids of SE are 31.77 mg GAE/g DW and 34.53 mg QE/g DW respectively (Table 1).

3.2. SE reduced SW1353 cell death in SW1353 exposed to MIA
To examine SE's protective effects against MIA exposure on the cell viability of SW1353 cells, the cells were treated with different concentrations of SE in the presence or absence of 2 μM MIA for 24 h, and then cell viability was assayed. Treatment with only 2 μM MIA for 24 h induced abnormal cellular morphology, which showed the cell shrank following the destruction of cytoskeleton observed using an inverted phase microscope (Figure 2A). However, the addition of 5, 15 and 25 μg/mL SE changed the cell morphology in the presence of 2 μM MIA, and the cell morphology was like healthy adherent cell and almost identical to the control group. As shown in Figure 2B, cell viability declined in MIA only group, whereas significantly increased with 25 μg/mL SE treated group compared with MIA only group. Figure 2C shows that treatment with 5, 15 and 25 μg/mL SE did not affect cell viability in the absence of MIA. Therefore, SE treatment potentially protected against MIA-induced cell death.

3.3. SE reduced apoptosis and ROS production, and increased ΔΨm in SW1353 cell under MIA exposure

To determine whether SE affected the apoptosis induced by MIA, cells were treated with different concentrations of SE in the presence of 2 μM MIA for 24 h and subsequently analyzed through annexin V-PI staining. The annexin V+/PI− cells represented the cells undergoing early apoptosis, and the annexin V+/PI+ cells represented the cells undergoing late apoptosis (Figure 3A). The total apoptosis rate of MIA only group increased compared with control group. In addition, the treatment of 5, 15 and 25 μg/mL SE groups exhibited significantly lower total apoptosis rate (56.3% ± 3.2%, 16.5% ± 1.8%, and 11.4% ± 2.6%) than MIA only group (82.2% ± 3.6%) (Figure 3A). Therefore, SE treatment potentially protected the cells from MIA-induced apoptosis. Figure 3B indicated that, compared with control group, MIA only group induced significantly higher levels of ROS (192.2% ± 5.7%) in the SW1353 cells. However, after treatment with SE in doses of 15 or 25 μg/mL, ROS levels were dramatically decreased (118.0% ± 4.2% and 116.4% ± 6.9%) (Figure 3B). Conversely, as shown in Figure 3C, ΔΨm was lower in MIA only group (83.8% ± 3.4%) compared with control group (100%). However, ΔΨm significantly increased in the 25 μg/mL SE-treated group (97.7% ± 0.7%) compared with MIA only group. Therefore, SE treatment decreased ROS production and elevated ΔΨm, thus exerting protective effects on SW1353 cells under MIA exposure.

3.4. SE modified expression of TNF-α, IL-6, collagen 2 and MMP13, and alleviated proteoglycan loss in SW1353 cells under MIA exposure

It has been reported that inflammatory cytokines are involved in the pathogenesis of OA.29 These cytokines cause their downstream effectors, MMPs, to be synthesized, and MMPs degrade cartilage ingredients.29 To determine whether proinflammatory cytokines or MMPs are involved in the MIA-induced changes or the protective effects of SE in SW1353, the cells were treated with 2 μM MIA and SE at the indicated concentrations (5, 15 and 25 μg/mL) for 24 h; subsequently, the cultured medium was collected and subjected to ELISA. As shown in Figure 4A, 4B, 4C and 4D, MIA increased the levels of IL-6, TNF-α and MMP-13, and decreased the level of collagen 2. However, the SE treatments significantly decreased the levels of IL-6 and TNF-α, and increased the level of collagen 2 compared with MIA only group. Even though MMP-13 level could be decreased by 15 and 25 μg/mL SE treatments, there was no significant differences when compared with MIA only group. Therefore, IL-6, TNF-α, collagen 2, and MMP13 participated in the MIA-induced changes, and the irregular levels of IL-6, TNF-α and collagen 2 were recovered by SE. The cartilage ECM comprises mostly type II collagen and large networks of proteoglycans that have acidic polysaccharides, such as chondroitin sulfate, hyaluronic acid, and aggrecan.30 To determine whether SE alleviates proteoglycan loss induced by MIA, SW1353 cells were treated with different concentrations of SE in the presence of 2 μM MIA for 24 h, and then proteoglycans were stained with toluidine blue O, which reacted with their acidic polysaccharides. Figure 4E revealed that MIA reduced the acidic polysaccharide content, as determined through toluidine blue O staining, and 15 and 25 μg/mL SE significantly increased the acidic polysaccharide content compared with MIA only group. These results indicate that although MIA induced proteoglycan loss, SE treatment led to proteoglycan recovery.

3.5. SE suppressed apoptotic mediators and JAK2/STAT3 signalling pathways in MIA-induced SW1353 cells

To examine the induction of apoptotic pathways following SE treatment in the presence of MIA, the cells were analyzed the protein levels of PARP, caspase 3, Bcl-2 and BAX by Western blot assays. The active forms of caspase 3 and PARP were decreased following treatment with 5-25 μg/ml of SE and 2 μM MIA for 6h in SW1353 cells (Figure 5). Besides, SE increased Bcl-2 protein levels only at the dose of 5 μg/ml SE in the presence of 2 μM MIA; however, the protein levels of BAX protein was decreased by treatment with SE (Figure 5). To examine whether JAK2/STAT3 pathways involved in the chondroprotective effect of SE on against MIA, the protein levels of JAK2, p-STAT3 and STAT3 were analyzed by western blot. Treatment with 5-25 μg/ml SE for 6 h decreased the protein levels of JAK2, p-STAT3 and STAT3 in SW1353 cells stimulated with 2 μM MIA (Figure 5). GAPDH protein was used as an internal control that did not affect after treatment with SE.

4. Discussion

Because many functional ingredients in citrus fruits as antioxidants against many chronic diseases, global citrus consumption has grown quickly. Citrus-fruit-derived PMFs and their metabolites have exhibited considerable protective effects, including anti-inflammatory activities.[29, 30] Hence, we use SE which was particularly concentrated the PMFs content to do the research for against OA progression in cell model. Asikin *et al*. have determined the EC_{50} values for DPPH radical scavenging activity of extracts obtained using steam distillation and a cold-press system were 34.09 and 206.45 mg/mL, respectively.[31] Here, we observed more effective inhibition of SE in the DPPH radical scavenging assay; SE reached more than 50% inhibition at 2.5 mg/mL. Additionally, SE reached greater than 50% inhibition even at 1 mg/mL, as determined with the ABTS assay.

Apoptosis is one of important features of OA progression. Instead of using IL-1β to induce chondrocyte apoptosis, we were more perfect to use MIA due to it could cause serious apoptosis. Jiang *et al*. determined that chondrocytes treated with MIA underwent apoptosis due to mitochondrial abnormalities and ROS production [22]. Consistent with this finding, we determined from observation that MIA-induced apoptosis is based on an increase in annexin V^+/PI^+ cells and a decrease in ΔΨm. Moreover, SE addition was able to inhibit ROS production and apoptosis occurrence efficiently and to reverse ΔΨm changes; the results after these effects were the same as those in the control group without MIA treatment (Figure 3).

It has been reported that MIA can induce transient production of IL-1β, IL-6, TNF-α and prostaglandin E_2 in rat models, as determined by serum concentrations.[32] Our study showed that MIA could increase the production of IL-6 and TNF-α in SW1353 cells (Figure 4). This suggests that chondrocytes might be involved in the increased production of serum proinflammatory mediators in MIA-induced OA models of rodent species. Chondrocytes are the resident cells of articular cartilage and are responsible for the

anabolism and catabolism of the ECM. Cartilage ECM is composed of proteoglycan (mainly aggrecan) and collagen (mainly type II). Chondrocyte death, especially apoptosis, leads to the pathogenesis of OA and the degradation of the ECM.[1] The proinflammatory cytokines IL-1β, IL-6 and TNF-α have been reported to play roles in ECM degradation.[33] These proinflammatory cytokines could drive the production of ECM-degrading enzymes (MMPs and aggrecanases), which act as crucial downstream effectors in the inflammatory signal cascade. Increased MMPs (particularly MMP-1, MMP-3, and MMP-13) and aggrecanase can augment ECM lesions.[33] Previously Ishiwa et. al. reported that citrus bioactive compounds like Tangeretin and nobiletin dose-dependently suppressed the release of matrix metalloproteinase 9/progelatinase B induced by IL-1β in rabbit synovial cells.[34] Consistent with these reports, our data showed that although MIA elevated proinflammatory cytokine and MMP expression, which eventually leads to proteoglycan and collagen loss, in SW1353 cells, treatment with SE reversed the effects caused by MIA (Figure 4).

The Bcl-2 family regulates proapoptosis and antiapoptosis signaling. This family contains both antiapoptotic members (Bcl-2) and proapoptotic members (BAX).[35] Furthermore, the activation of caspase 3 and the cleavage of PARP are after the up-regulation of the expression of proapoptotic Bcl-2 family members.[36] Our data indicated that MIA stimulated an increase in the protein levels of PARP, cleaved-caspase 3 and BAX, and a decrease in the protein level of Bcl-2. But SE did totally opposite effects in the present of MIA (Figure 5). JAK/STAT pathway is described as to be related in the production of MMP-13 in IL-1β-induced SW1353 cells.[35] Here, we showed that MIA induced MMP-13 and activated JAK2/STAT3 signaling in SW1353 cells. However, SE reversed the effects of MIA on MMP-13 induction and JAK2/STAT3 signaling activation (Figure 4D and 5). In our previous in vivo research also exhibited consistent result that SE decreased caspase 3 and p-JAK2 expression in the chondrocytes of rat OA joints.[20] In the same time, SE displayed the improvement of blood lipid profile and cytokine TNF-α to regulate the inflammatory response. Additionally, SE treatment could effectively inhibit cartilage destruction, chondrocyte disappearance and proteoglycan depletion in rat OA joints.[20]

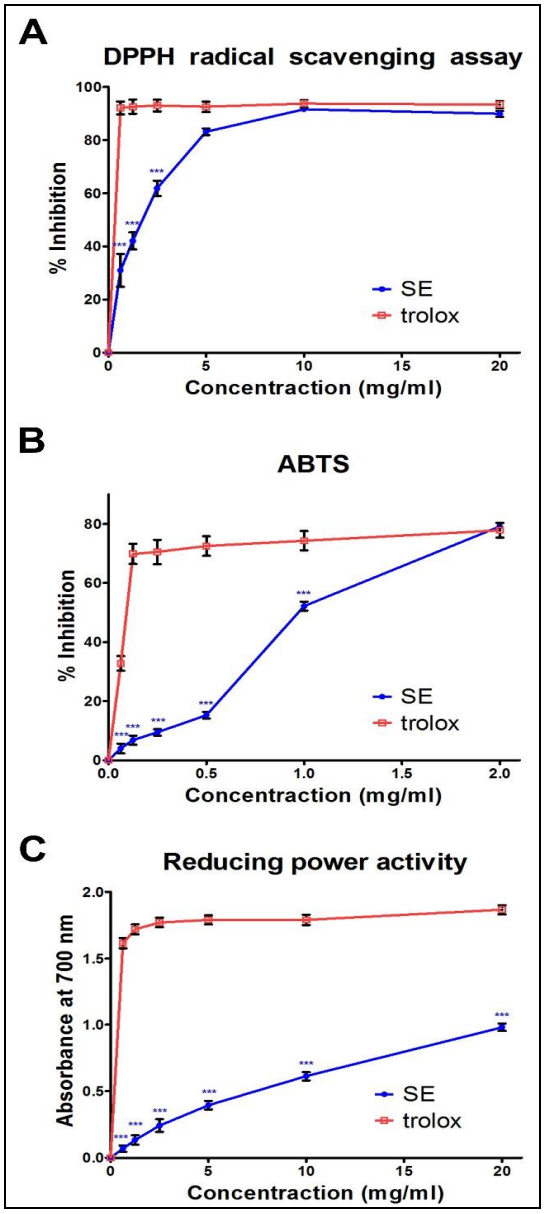

Fig 1: Free radical scavenging activity of the positive control, Trolox (0.625-20 mg/mL), and SE (0.625-20 mg/mL), as determined through (A) DPPH assay, (B) ABTS assay, and (C) reducing power assay. Values represent the mean ± SEM from three separate experiments. ***$p < 0.001$ indicates a significant difference between Trolox and SE samples.

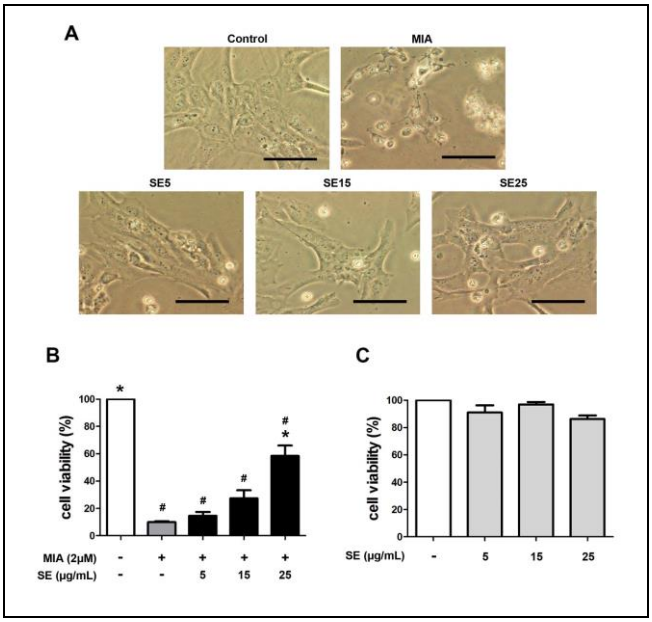

Fig 2: SE recovered cell viability in a concentration-dependent manner in SW1353 cells and caused no toxicity to the cells. SW1353 cells were treated with 5-25 μg/mL SE in the presence or absence of 2 μM MIA for 24 h. At the end of treatment, (A) the cell morphology was observed through an inverted microscope. (×200 magnification). Scale bars = 100 μm. (B and C) Cell viability was measured by MTT assay. Values represent the mean ± SEM from three separate experiments. #p < 0.05 indicated significant differences when compared with control group.*p < 0.05 indicated significant differences when compared with MIA only group. MIA, monosodium Iodoacetate; SE, shiikuwasha extract; SE5, 5 μg/mL SE; SE15, 15 μg/mL SE; SE25, 25 μg/mL SE.

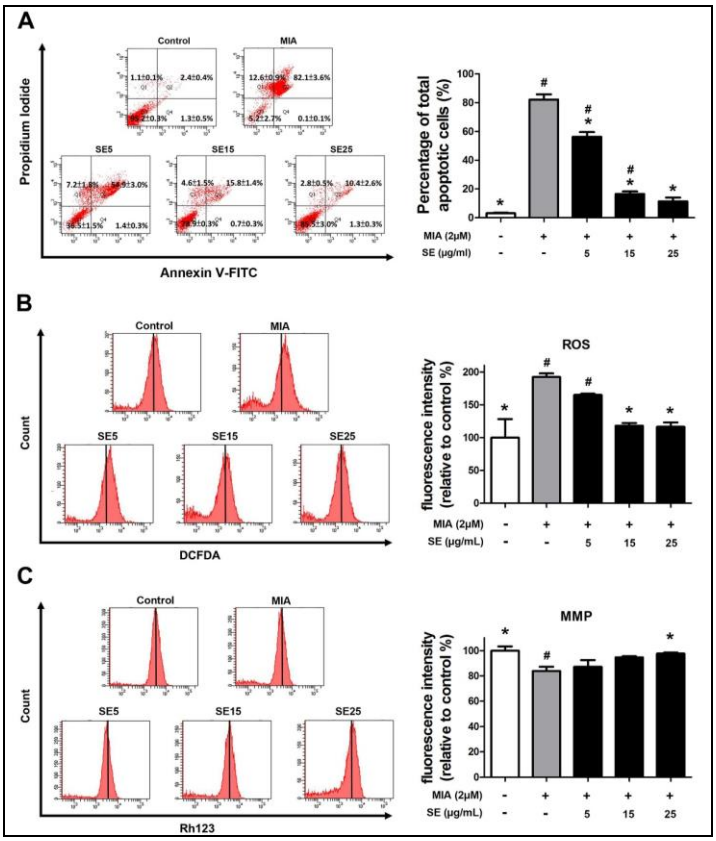

Fig 3: SE reduced apoptosis and ROS production, and stabilized ΔΨm in SW1353 cells with induction of MIA. SW1353 cells were treated with 5–25 μg/mL SE in the presence of 2 μM MIA for 24 h. (A) These cells were stained by annexin V–PI to determine apoptosis using flow cytometry analysis. The cells undergoing early apoptosis are appear as annexin V+/PI− cells (lower right quadrant region in the dot plot), whereas late apoptosis cells are annexin V+/PI+ (lower right quadrant region in the dot plot) cells. The dot plots represented one of tree experiments. The total percentage of apoptotic cells, expressed as the sum of late and early apoptotic cells. (B) The production of ROS was examined using DCFDA staining and analyzed by flow cytometry. (C) ΔΨm was measured using Rh123 fluorescence staining and analyzed by flow cytometry. Values represent the mean ± SEM from three separate experiments. #p < 0.05 indicated significant differences when compared with control group. *p < 0.05 indicated significant differences when compared with MIA only group. MIA, monosodium Iodoacetate; SE, shiikuwasha extract; SE5, 5 μg/mL SE; SE15, 15 μg/mL SE; SE25, 25 μg/mL SE; FITC, fluorescein isothiocyanate; DCFDA, 2',7' –dichlorofluorescin diacetate; ROS, reactive oxygen species; Rh123, rhodamine 123; MMP, mitochondrial membrane potential.

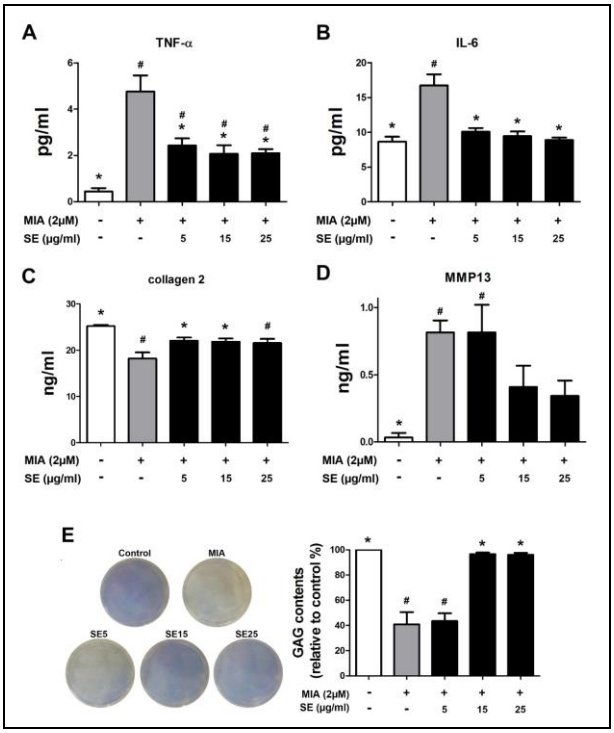

Fig 4: SE normalized levels of TNF-α, IL-6, collagen 2 and MMP13, and reversed the proteoglycan loss in MIA-stimulated SW1353 cells. SW1353 cells were incubated with SE (5, 15, or 25 μg/mL) and MIA (2 μM) for 24 h. Supernatants were harvested at the end of experiment. (A) TNF-α, (B) IL-6, (C) collagen 2, and (D) MMP13 levels were measured by ELISA. (E) The acidic polysaccharides contained in the proteoglycan were measured by toluidine blue O staining. Quantitative analysis relied on colorimetric assay. Values represent the mean ± SEM from three separate experiments. #$p < 0.05$ indicated significant differences when compared with control group.*$p < 0.05$ indicated significant differences when compared with MIA only group. MIA, monosodium Iodoacetate; SE, shiikuwasha extract; SE5, 5 μg/mL SE; SE15, 15 μg/mL SE; SE25, 25 μg/mL SE; TNF-α, tumor necrosis factor; IL-6, Interleukin 6; MMP13, matrix metallopeptidase 13; GAG, glycosaminoglycans.

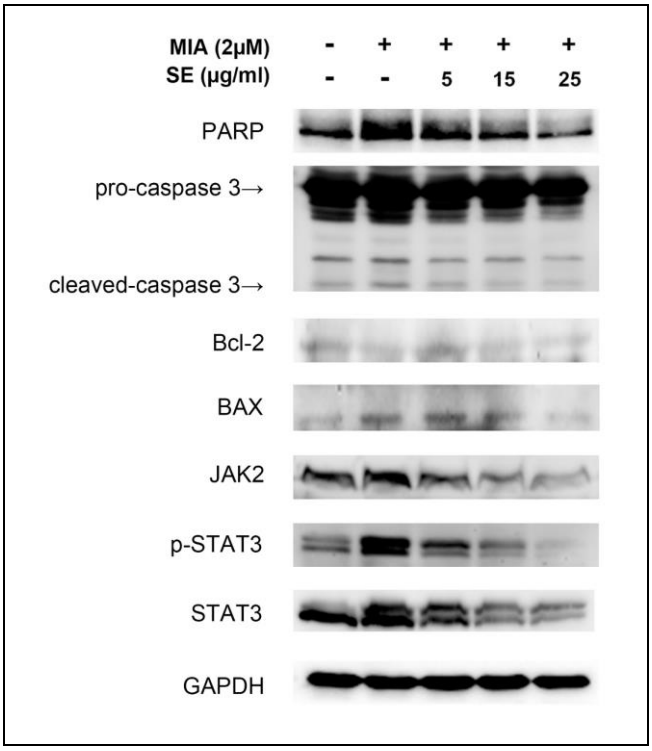

Fig 5: SE decreased the protein expressions of apoptotic mediators and JAK2/STAT3 signalling pathways. SW1353 cells cultured in 100-mm Petri dishes were incubated with 5-25 μg/mL SE in the presence of 2 μM MIA for 6 h. The whole cell extracts were subjected to Western blot analysis using anti-PARP, anti-caspase 3, anti-Bcl-2, anti-BAX, anti-JAK2, anti-p-STAT3, anti-STAT3 and anti-GAPDH antibodies. GAPDH protein was considered as the internal control. The images were represented from one of three independent experiments with similar results. MIA, monosodium Iodoacetate; SE, shiikuwasha extract; PARP; poly (ADP-ribose) polymerase; Bcl-2, B-cell lymphoma 2; BAX, Bcl-2-associated X protein; JAK2, Janus kinase 2; p- STAT3, phospho-signal transducer and activator of transcription 3.

Table 1: Contents of total phenolics and flavonoids of SE.

	Total phenolic content (mg GAE/g DW)	Total flavonoid content (mg QE/g DW)
shiikuwasha extract	31.77±3.68	34.53±0.46

Values represent the mean ± SEM from three separate experiments.

5. Conclusion

The present study demonstrated that MIA exposure induces cell death, apoptosis and ROS production; decrease in ΔΨm, collagen 2 and proteoglycan; release of IL-6 and TNF-α; up-regulation of JAK2/STAT3 pathway. All of these effects can be reversed through treatment with SE. This suggests that SE, which blocked cell death and apoptosis in OA mimic cell model, is a potential, beneficial and novel supplement for OA.

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7. References

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