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Original Article

In vitro evaluation of wound healing potential of sulfated galactans from red alga *Gracilaria fisheri* in fibroblast cells

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Abstract

Red alga Gracilaria species have been reported various to exhibit significant pharmacological activities. In this study, sulfated galactans (SG) from *G. fisheri* was investigated for its potential activity in wound healing using *in vitro* approach. L929 fibroblast cells were treated with different concentrations of SG and wound healing activity and collagen content determined. Expression of the specific mRNA transcripts and translated proteins was assessed. In a scratch wound healing culture, SG enhanced the rate of migration of the fibroblast cells, accompanied by increased expression of integrin β1, p-FAK, p-NF-κB, E-cadherin proteins, and the active form of MMP-2. In addition, SG increased expression of COL1A1 mRNA and protein and stimulated collagen production. In conclusion, this study demonstrates that SG from *G. fisheri* promotes would healing of fibroblasts, in part, through integrin-FAK and NF-κB-MMP signaling pathways. This suggests that SG could be a promising compound for further investigation of its potential on wound treatment.

Keywords: sulfated galactans, red alga, fibroblast, wound healing, collagen production

1. Introduction

Fibroblasts secrete molecules that give rise to the structural organization of the extracellular matrix (ECM) such as collagens, proteoglycans and fibronectin, glycoproteins that are able to coordinate the constituents that contribute to the mechanical properties of the tissue. During aging, the interaction between fibroblast and ECM becomes disrupted resulting in loss of fibroblasts and fibroblast activity, which leads to a phenotype of aging marked by a decrease in ECM protein synthesis, dysfunction of matrix-degrading metalloproteinases (MMPs) and tissue remodeling (Phillip, Aifuwa, Walston, & Wirtz, 2015). In the wound healing process fibroblasts play a key role through the deposition of ECM components, wound contraction and remodeling of new ECM. In aging, the fibroblasts significantly decrease their cell migration, delaying re-epithelialization, and collagen remodeling, thus impairing the process of wound healing (Wall *et al*., 2008). Therefore, reducing the negative impact of senescent fibroblasts may serve as a therapeutic strategy to improve health during aging.

Several strategies to reduce the age-related impairment of healing have been intensively studied. Synthetic compounds that prevent or lower the progression of senescence have been shown to be effective, but come with unwanted side effects and are toxic to healthy cells (Malavolta

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et al., 2018). In this regard, bioactive compounds from plants and foods that offer interference with cellular senescence with minimal side effects are highly sought after. Sulfated polysaccharides (SPs) from marine algae are widely used in nutraceutical or functional food products, cosmetics and pharmaceutical applications, and they cause less toxicity (Holdt & Kraan, 2011; Zhang, Li, & Kim, 2012). For decades, A number of studies have reported that SPs possess potential for treatment of cancer, inflammatory diseases, and cardiovascular diseases (Manlusoc *et al*., 2019). Moreover, it has been shown that algal SPs from *Panida tetrastromatica*, *P*. *boergesenii* (Kordjazi *et al*., 2013), and *P*. *gymnospora* (Baliano *et al*., 2016) are capable of improving wound healing by promoting fibroblast migration and collagen formation both *in vitro* and in animal models. Recently, we isolated SPs, namely sulfated galactans (SG), from the red alga *Gracilaria fisheri*, and showed that it exhibits several beneficial properties including immune stimulant (Rudtanatip, Boonsri, Praiboon, & Wongprasert, 2019), anti-tumor (Sae-Lao, Tohtong, Bates, & Wongprasert, 2017), and anti-melanogenesis (Pratoomthai, Songtavisin, Gangnonngiw, & Wongprasert, 2018). In this study we further investigated the effects of SG on wound healing and collagen production in fibroblast cells.

2. Materials and Methods

2.1 Sulfated galactans (SG)

SG was extracted from red alga *G*. *fisheri* as previously described (Wongprasert Rudtanatip, & Praiboon, 2014). The structure of SG consists of a linear backbone of alternating 3-linked β-D-galactopyranose (G) and 4-linked 3,6-anhydro- α-L-galactopyranose (LA) or α-L-galactose 6-sulfate (L6S) units. Stock solution of SG (10 mg/mL) was prepared by dissolving SG in 3D water, heated at 37ºC, and kept at -20ºC until used.

2.2 L929 fibroblast cells culture

L929 cells were purchased from American Type Culture Collection (ATCC). The cells were cultured in MEM (Gibco Invitrogen, USA) supplemented with 10% (v/v) FBS, 2.2 g/L sodium bicarbonate ($NaHCO_3$), and 1% (v/v) antibiotic-antimycotic (Gibco Invitrogen, USA). Cells were grown in a 75-cm^3 T flask under humidified air at 37°C, 5% CO_2.

2.3 Cell viability assay

Cytotoxicity of SG was evaluated on fibroblast L929 cells using the 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium bromide (MTT) assay (Pratoomthai *et al*., 2018). Cells were seeded at a density of $1x10^5$ cells $well^{-1}$ in a 96-well plate overnight. The cells were treated with vehicle buffer or various concentrations of SG (10, 50, 100, 500, and 1000 µg/mL) for 24 and 48h. The 100 µL of MTT solution (Sigma, USA) was added to each well and the plate incubated in the dark at 37ºC for 48 h. The solution was removed and 100 µL of DMSO (Merck, Germany) was added to lyse the cells. The formazan product was measured using a Versamax microplate reader (SoftMax® Pro 4.8 analysis software, Molecular Devices, USA) and absorption at OD 490 nm. The viability of SG treated cells was expressed as a percentage of control group.

2.4 Scratch wound healing assay

To investigate the wound-healing property of SG, fibroblast culture in a wound condition was performed. The scratch wound healing assay determined the migration activity of the L929 fibroblasts which is essential for tissue repair. L929 cells were seeded ($1x10^6$ cells/well) in 6-well plates and cultured for 24 h. The cell monolayer was scratched with a sterile pipette tip to create the wound and was followed by treatment with different concentrations of SG (10, 50, 100, and 500 µg/mL). The scratch wound closure was examined and photographed at 0, 12 and 24 h after scratching under an inverted phase contrast microscope (Olympus, Germany). The percentage of distance wound closure was measured, using the data from time 0 (*T*0), the wound area (*Tt:* 12 and 24 h) by the following formula*:*

Percentage of distance wound closure = [(wound area at *T*0 – wound area at *Tt*) / wound area at *T*0]x100

2.5 Gelatin zymography analysis

The gelatinase activity of the scratch wound culture was determined by gelatin zymography. Culture medium was collected after treatment for 24 h, lyophilized, and resolubilized protein concentration was determined. The sample (20 µg) was mixed with sample buffer without dithiothreitol (DTT), and loaded onto 10% SDS-PAGE gel containing 2% gelatin. After electrophoresis the gel was washed in renaturing buffer containing 2.5% (w/v) TritonX-100, then incubated in buffer containing 0.05 M Tris buffer, pH 7.5 containing 200 mM NaCl, 5 mM $CaCl_2$ and 0.05% NaN_3 at 37°C for 18 h. The gel was stained with 0.5% Coomassie brilliant blue R-250 (Bio-Rad, USA) in 5% methanol and 10% acetic acid for 2 h and de-stained with de-staining solution containing 30% methanol and 10% acetic acid for 2 h at room temperature. Protein renaturation restored gelatinase activity, and the gelatin in the gel was digested to give clear bands against the blue background. The area of the clear bands was determined using ImageJ and the gelatinase activity was expressed as fold of control.

2.6 Collagen production

L929 cells were cultured in 6-well plates at a density of $2x10^5$ cells/well with MEM medium for 24 h. Cells were treated with vehicle buffer or various concentrations of SG (10, 50, 100, and 500 µg/mL) for 24 h. The culture medium was collected for determination of the collagen production by the Sirius red staining assay as previously described (Keira, Ferreira, Gragnani, Duarte, & Barbosa, 2004). Briefly, culture medium was precipitated with 25% saturated ammonium sulfate under constant agitation at 4°C for 24 h. The solution was centrifuged at 40000 g, 4°C for 30 min and the pellet was dissolved in 2 mL of 0.5 M acetic acid. 100 µL aliquots were precipitated in 1 mL of 50 µM Sirius Red solution in 0.5 M acetic acid at room temperature for 30 min, followed by centrifugation at 30000 g for 30 min. The

pellet was re-suspended in 1 mL of 0.1N KOH, and vortexed gently for 10 min. The absorbance of 100 µL sample aliquots was read at 540 nm using a Versamax microplate reader (SoftMax® Pro 4.8 analysis software, Molecular Devices, USA). The collagen content of each sample was quantified using a predetermined standard curve of Type I rat tail collagen.

2.7 Western blotting

Cellular proteins in the scratch wound culture were extracted with lysis buffer containing 100x protease inhibitor solution, separated by electrophoresis on 10% SDS-PAGE gels, and transferred to nitrocellulose membranes (Merck, Germany). Each membrane was incubated with a specific primary antibody, including anti-collagen type 1α1, anti-p-NF-κB, anti-integrin β1, anti-p-FAK, and anti-E-cadherin, followed by the corresponding secondary antibody conjugated with HRP. Immunoreactive proteins were detected using a Chemiluminescence ECL Western blotting detection kit (Cell signaling Technology, USA). The expression of protein was quantified relative to β-actin using Image Studio™ Lite software (Li-COR Biotechnology, USA).

2.8 RNA isolation, cDNA transcription, and qRT-PCR analysis

Total cellular RNA was extracted with 200 µL of Tri-reagent extraction kits (Sigma Aldrich, USA) and qualified using a Nano Drop 2000 spectrophotometer (Thermo Scientific, USA), at the absorbance ratio of 260/280 nm. Qualified RNA (1 µg) was used for cDNA synthesis using the Thermo Scientific RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) at 42°C for 1 h. Subsequently, gene expression was amplified by PCR using 1 or 2 µL of cDNA with specific primers. A real-time PCR reaction mixture, 10 µL, consisted of 1 µL of DNA polymerase, 5 µL of SYBR green, and 3 µL of DEPC, 0.5 µL for each forward and reverse primer, and the conditions were 95°C for 30 s followed by 40 cycles of 95°C for 10 s. The sequence of the primers is as follows*:* COL1A1, 5'-GAG AGG TGA ACA AGG TCC CG-3'(forward) and 3'-AAA CCT CTC TCG CCT CTT GC-5'(reverse); COL1A2, 5'-CCC AGA GTG GAA CAG CGA TT-3'(forward) and 3'-ATG AGT TCT TCG CTG GGG TG-5' (reverse); MMP-2, 5'-AAC GGT CGG GAA TAC AGC AG-3' (forward) and 3'-GTA AAC AAG GCT TCA TGG GGG-5' (reverse); MMP-9, 5'-CAG CCG ACT TTT GTG GTC TTC-3' (forward) and 3'-ATA GCG GTA CAA GTA TGC CTC TG-5' (reverse); GAPDH, 5'-GGT GAA GGT CGG TGT GAA-3' (forward) and 3'-CTC GCT CCT GGA AGA TGG TG-5' (reverse). The gene expression was quantified by Bio-Rad CFX Maestro Software (Bio-Rad, USA). The mRNA expression was normalized to the housekeeping gene GAPDH and was presented as fold of control group.

2.9 Statistical analysis

All data were presented as mean ± the standard error of the mean (mean ± SEM) and analyzed by one-way ANOVA followed by Turkey's multiple comparison tests or pair t-test in GraphPad Prism program version 5 (GraphPad software, USA). The statistically significant difference between treatments was considered with *p*-values less than 0.05.

3. Results and Discussion

3.1 SG showed no cytotoxicity on fibroblast L929 cell culture

We demonstrated that the SG ranging from 10 - 1000 µg/mL showed no cytotoxicity on the fibroblast L929 cells for 24 and 48 h (Figure 1). This is consistent with previous studies that reported SPs from marine algae showed very little or no toxicity in normal cells. For instance, the SPs from *Codium tomentosum* (Navya & Khora, 2017) and *Padina tetratromatica* show no cytotoxic effect on L929 fibroblast cells (Jose, Raghavankutty, & Kurup, 2019). Crude polysaccharide from *Caulerpa cupressoides* has no cytotoxicity on murine macrophages (Barbosa et al, 2019). Moreover, SPs from *G. fisheri* show no toxicity in artemia (Rudtanatip *et al*., 2019). Our results indicate that SG was free from cytotoxicity effects on fibroblast L929 cells and is a safe compound for further wound healing study.

Wound healing process is a dynamic reparative process of three sequential phases including inflammation, proliferation and remodeling. Inflammation phase is essential for the recruitment of the immune cells to defend the body from invading pathogens and remove dead tissue. In the proliferation phase, the wound surface recovers through re-epithelialization, collagen synthesis, extracellular matrix (ECM) formation, and restoration of the vascular network. In

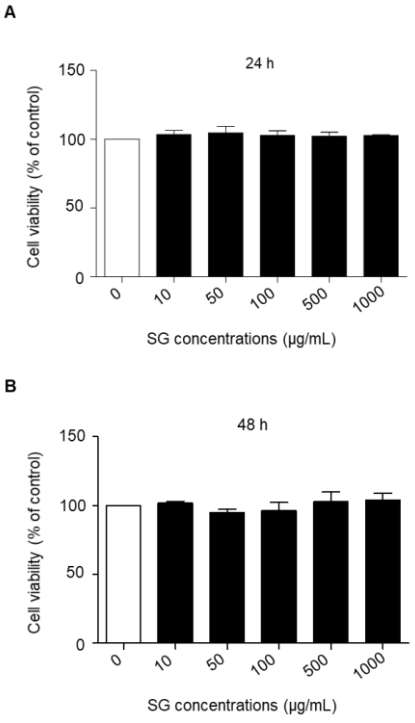

Figure 1. Percentage of viable L929 cells after treatment with SG for (A) 24h and (B) 48h compared to untreated control. Values are mean ± SEM of three independent experiments.

the re-modelling phase, regenerative processes are downregulated and replaced by re-organization of the connective tissue and the initiation of the contractile response (Baron, Glatz & Proksch 2020). During wound healing process, fibroblasts play an important role from the late inflammatory phase until the complete epithelization by secreting growth factors, cytokines and collagens (Bainbridge, 2018). In addition, fibroblast migration into the wound site where they synthesize and secrete the deposition of extracellular matrix (ECM) components is crucial for initiating the proliferative phase of wound repair (Wong, McGrath & Navasaria, 2007). This study was initiated to evaluate a potential for sulfated galactans (SG) from *G. fisheri* to enhance wound closure and collagen production in fibroblast cell culture.

3.2 SG accelerated wound closure

To investigate the wound-healing property of SG, scratch wound assay was performed in fibroblast L929 cell culture followed by SG treatment. This assay is associated with the fibroblast proliferation and migration in wound healing process (Liu *et al*.,2013; Schafer & Werner, 2007). Wound closure was photographed after scratch wound and SG treatment for 0, 12, and 24 h. The results revealed that in the control group wound gap gradually closed while SG treatment significantly accelerated wound closure in a dose- and time-dependent manner (Figure 2A). The wound closure rates at 24 h for SG at 10, 50, 100 and 500 µg/mL were 83.4 ± 3.4%, 85.7 ± 2.5%, 90.4 ± 2.8% and 92.5 ± 3.4%, respectively (Figure 2B). The wound closure rate for control was 67.1 ± 2.0%. The scratch wound test demonstrated that SG significantly improved the migration rate of fibroblast L929 cells. Our results agree with the previous studies reporting that the SPs extracted from *Spirulina platensis* increased migrating activity of dermal fibroblasts (Syarina, Karthivashan, Abas, Arulselvan, & Fakurazi, 2015). Furthermore, SPs from *Bletilla striata* and *Sanguisorba officinalis L*. have been shown to improve wound healing in mice (Zhang *et al*., 2019; Zhang, Chen, & Cen, 2018).

3.3 SG increased MMPs activities and expression of cell migratory associated proteins in fibroblast scratch wound model

During cell migration MMPs, in particular MMP-2 and MMP-9, are highly associated with ECM degradation as a requirement for cellular migration and assumed matrix remodeling (Parks, 1999). In this study, the MMPs enzyme activity was measured in the medium collected from the fibroblast scratch wound cultures treated with or without SG for 24 h. The results revealed that L929 cells had high activity of pro- and active forms of MMP-2 but not MMP-9. SG treatments (10, 50, 100, and 500 µg/mL) increased the activity of active MMP-2 in a dose dependent manner (Figure 3A, B) but still maintained a constant level of pro-form MMP-2 similar to control (Figure 3A, C). This led to an increase in the ratio of active/pro-forms of MMP-2 in all SG treated groups (Figure 3A, D). The enzyme activity results were consistent with qRT-PCR analyses in that, at the basal level, L929 cells expressed a much higher level of MMP-2 than MMP-9 (Figure 4A). This agrees with previous reports that

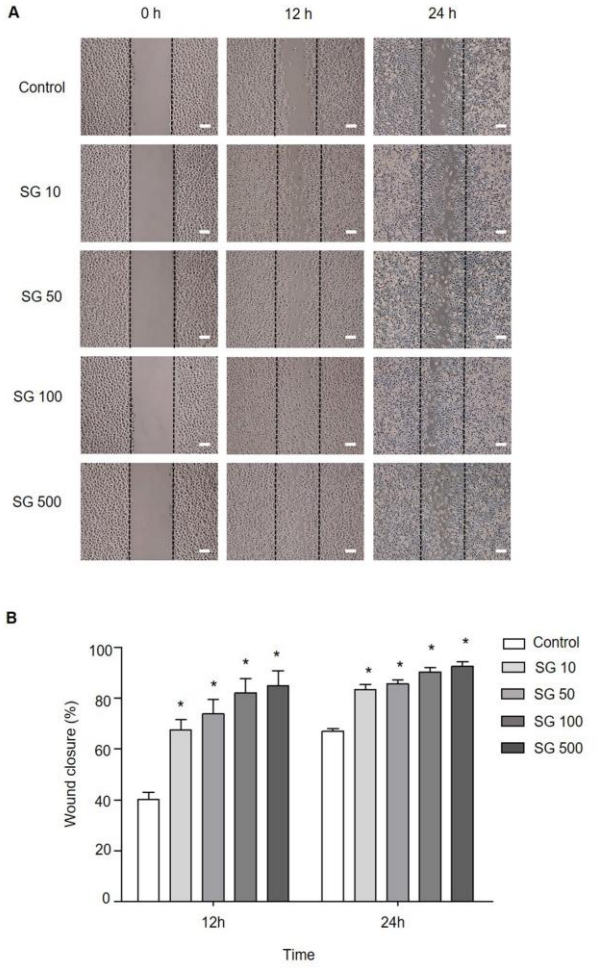

Figure 2. The effect of SG on migration of L929 cells by scratch wound healing assay. (A) Phase contrast micrographs showing the distance of scratch wound in different SG treatment groups. (B) Distance of wound closure expressed as percentage of control in each time point. Cells with scratched wound were treated with SG (10, 50, 100, and 500 µg/mL). Photographs were recorded at 0, 12 and 24 h after scratching. Values are mean ± SEM of three independent experiments. * indicates a significant difference from control, $p < 0.05$. Scale bar = 50 µm

MMP-2 is the most abundant of the MMPs in fibroblasts (Linder *et al*., 2012). In the wound repair process, MMP-2 is mainly secreted by fibroblasts (Toriseva & Kähäri, 2009). In this study, we then determined only the expression of MMP-2. Semi-quantitative analyses showed that SG treatments (10, 50, and 100 µg/mL) increased mRNA transcript of MMP-2 about 2.5-fold, 4.5-fold, and 8.5-fold of control, respectively. SG at 500 µg/mL dramatically increased the mRNA transcript of MMP-2 to about 23.5-fold of control (Figure 4B, C). These results supported the zymography analyses in which the high levels of pro-form MMP-2 with SG treatment remained the same as control while active MMP-2 was increased over control. It is suggested that in the scratch wound culture condition SG stimulated fibroblast cell migration that was associated with increased expression of MMP-2 gene and in the meantime increased the active form of MMP-2.

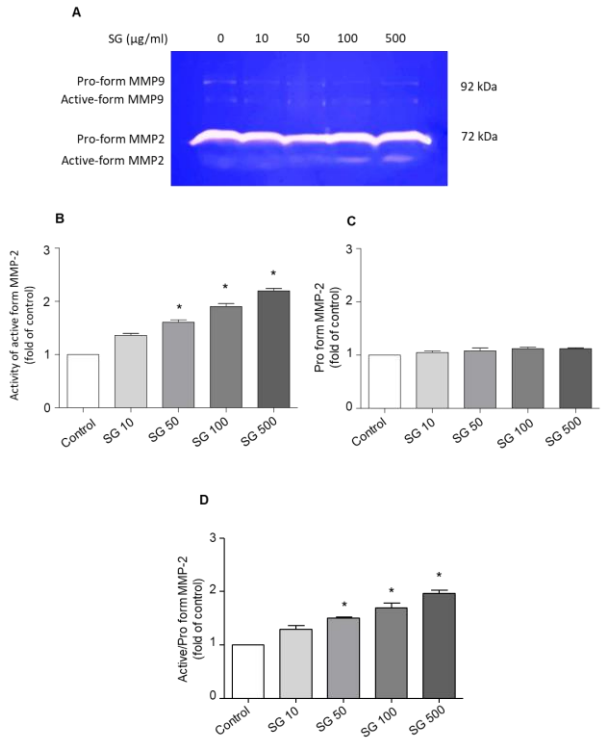

Figure 3. (A) Gelatinolytic activities assessed by zymography in scratch wound L929 cells following treatment with SG. Gelatinolytic bands indicate that the active-form of MMP-2 was increased in SG treated groups compared to control but maintained a constant level of pro-form MMP-2 similar to control. The histograms show the semi-quantitative analysis of the band intensities of (B) active-form MMP-2, (C) pro-form MMP-2 and (D) the ratio of active/pro-form MMP-2 relative to control. The ratio of active/pro-form MMP-2 was increased in SG treated groups. Values are mean ± SEM of three independent experiments; ***** indicates a significant difference from control, $p < 0.05$.

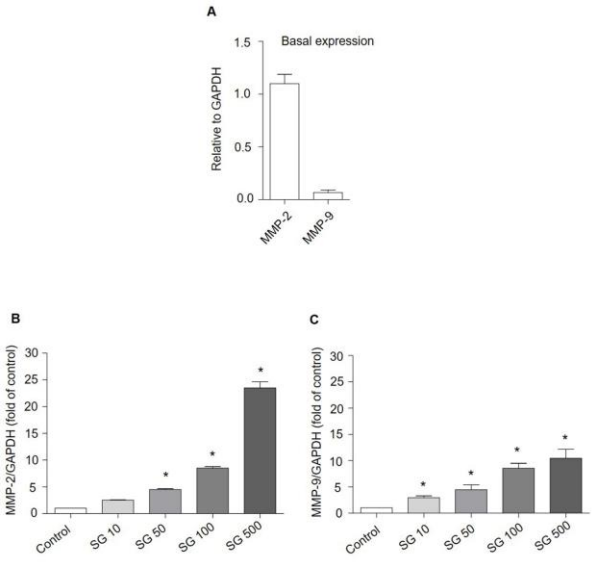

Figure 4. Expression level of MMP-2 and MMP-9 transcripts in L929 cells by qRT-PCR analysis. (A) Basal expression level of MMP-2 and MMP-9 transcripts in L929 cells relative to GAPDH. L929 cells expressed high level of MMP-2 and expressed low level of MMP-9. The mRNA transcription level of (B) MMP-2 and (C) MMP-9 in L929 cells after scratch wound and treated with SG relative to GAPDH, and expressed as fold of control. Scratch wound with SG treatments showed up-regulation of MMP-2 and MMP-9 transcripts. Values are mean ± SEM of three independent experiments. ***** indicates a significant difference from control, $p < 0.05$.

Integrin β1 receptors are the cell surface receptors located in the fibroblast cell membrane and play a significance role in maintaining ECM homeostasis such as supporting cell adhesion, proliferation, migration, and tissue repair (Zeltz & Gullberg, 2016). Migrating cells express high level of integrins, and integrins relay extracellular signals to activate focal adhesion kinase (FAK), thereby exerting transcriptional regulation of migration signaling pathways (Boraschi-Daz, Wang, Mort, & Komarova, 2017; Zhao *et al*., 2016). In this study, we determined the effect of SG on the expression of integrin β1 receptor and p-FAK at 24 h after scratch wound using western blot analysis. The results showed that SG increased the level of integrin β1 (Figure 5A, B) and p-FAK (Figure 5A, C) proteins. Thus, it is reasonable to suggest that SG promotes L929 cell migration during the wound healing process by up-regulating integrin β1 and FAK phosphorylation. Furthermore, SG induced an increase in the epithelial adhesion marker E-cadherin (Figure 5A, D). This has been explained in previous studies in which up-regulation of E-cadherin induced cell migration while maintaining tight cell-cell adhesion, called *"*collective migration*"*, which protects the epithelial barrier from further damage after wounding (Li *et al*., 2012). This suggests that SG induced cell to cell contact as migration increased. The increased level of E-cadherin has also been reported in a wound healing study using the compound DK223 (Ho *et al*., 2014).

Accumulated evidence has demonstrated that activation of NF-κB/MMP signaling pathway is critically important in cell migration and wound healing. Upon stress circumstances, fibroblast cells respond to injury by secretion of certain cytokines, which subsequently autocrine MMPs expression. This signal transduction for MMPs expression has been shown to be regulated via NF-κB pathway (Bigot *et al*., 2012). Our results showed that the expression of p-NF-κB (Figure 5A, E) was increased over control in all SG treated groups. In this study, fibroblast cells were stressed by scratch wound, and treatment with SG increased the release of MMP-2 and p-NF-κB activity. The concomitant increase of MMP-2 and p-NF-κB suggests that SG may up-regulate expression of MMP-2 gene via the activation of NF-κB. These findings appear to be corroborated by a previous study which showed that SPs isolated from the sea cucumber *Stichopus japonicas* activated NF-κB signaling and induced MMP-2 protein expression during neural stem/progenitor cell migration (Cui *et al*., 2016).

3.4 SG induced collagen production and modulated expression of collagen genes

We also examined whether SG could induce collagen production in fibroblast cells. Our results revealed

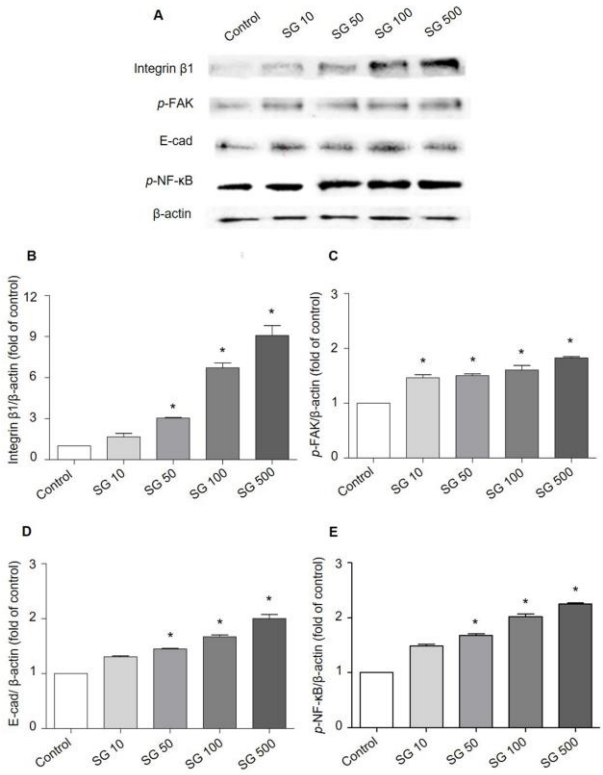

Figure 5. (A) Western blot analysis showing the expression of integrin β1, p-FAK, E-cadherin, and p-NF- κB proteins in L929 cells after scratch wound and treated with SG. The histograms show the semi-quantitative analysis of the band intensities of (B) integrin β1, (C) p-FAK, (D) E-cadherin and (E) p-NF-κB proteins relative to β-actin, and are expressed as fold of control. Results are presented as mean ± SEM of three independent experiments. ***** indicates a significant difference from control, $p < 0.05$.

that L929 cells treated with SG for 6 h at concentrations of 50, 100, and 500 μg⁄mL significantly increased soluble collagen production compared to control. For 24 h treatment, SG at 10, 50, 100, and 500 μg⁄mL showed a significant increase in collagen production (Figure 6). qRT-PCR analyses revealed that cells treated with SG for 24 h at 10-500 μg⁄mL showed a significant up-regulation of the COL1A1 mRNA transcript (Figure 7A) while COL1A2 gene was upregulated in cells treated with SG at 100 and 500 μg⁄mL (Figure 7B). Western blot analyses showed that cells treated with SG for 24 h at 50, 100, and 500 μg⁄mL significantly increased level of COL1A1 protein (Figure 7C). Our results are consistent with the previous studies of other SPs. For instance, SPs from algae *Hizikia fusiforme* (Wang *et al*., 2018), *Padina tetrastromatica* and *P. boergesenii* (Kordjazi *et al*., 2013) and *Fucus vesiculosus* (Song *et al*., 2014) were shown to promote collagen synthesis and ECM reconstruction. Here, we demonstrated that SG enhances collagen production via increased expression of COL1A1.

4. Conclusions

Our findings demonstrate that SG from *G. fisheri* exhibits *in vitro* wound healing property in fibroblasts under

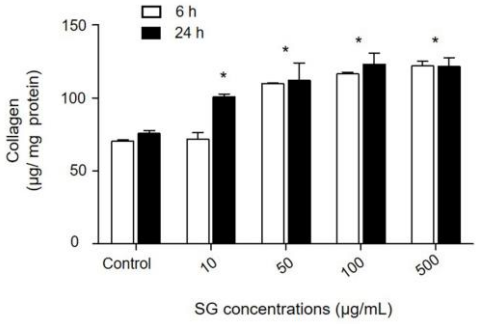

Figure 6. Soluble collagen concentration in the culture medium of L929 cells treated with the indicated concentrations of SG determined by Sirius red staining. Collagen concentration is expressed as μg⁄ mg protein. Values are mean ± SEM of three independent experiments. ***** indicates a significant difference compared to control group at the respective time point, $p < 0.05$.

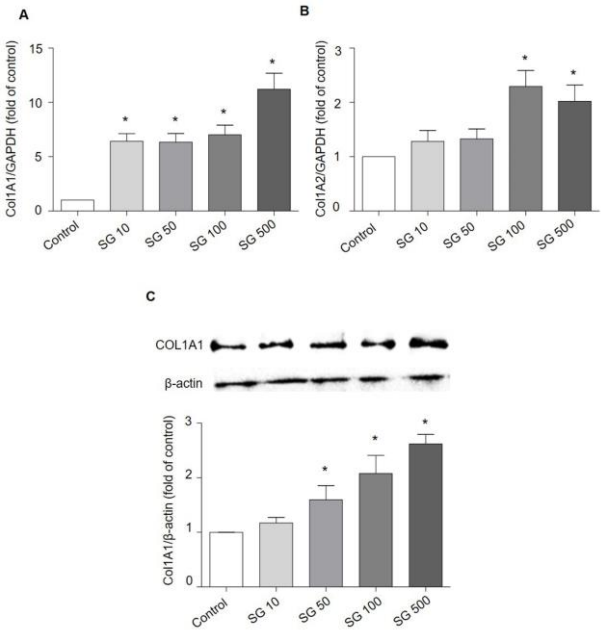

Figure 7. The mRNA transcription level of (A) COL1A1 and (B) COL1A2 in L929 cells treated with SG (10, 50, 100, and 500 μg⁄mL) relative to GAPDH. (C) Western blot analysis showing the expression of COL1A1 protein in L929 cells after treatment with SG for 24 h. The histograms show the semi-quantitative analysis of the band intensities of COL1A1 relative to β-actin. Results are expressed as fold of control. Values are mean ± SEM of three independent experiments. ***** indicates a significant difference from control, $p < 0.05$.

environmental stress by promoting fibroblast migration and collagen production, which are critical for tissue repair. The proposed underlying molecular mechanism of SG induced accelerated wound closure in fibroblast L929 cells is shown in Figure 8. We propose that SG enhances fibroblast cells migration by increased activity and expressions of MMP-2 and E-cadherin. SG mediated increased MMP-2 up-regulation involves activation of integrin β1 on cell surface that subsequently activating FAK/NF-κB/MMP signaling. These

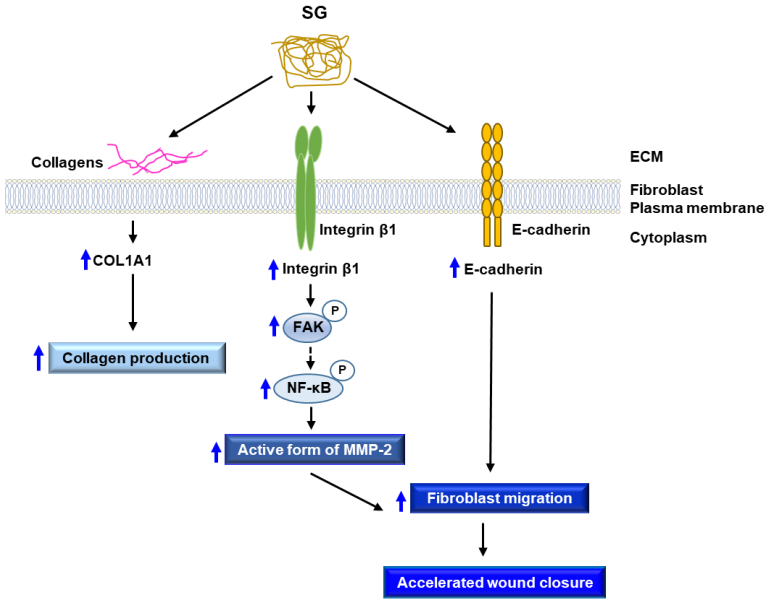

Figure 8. A schematic diagram showing the proposed underlying molecular mechanism of SG induced accelerated wound closure in fibroblast L929 cells. SG possibly enhances fibroblast cells migration by upregulating MMP-2 through activating integrin β1 on fibroblast cell membrane, which subsequently activating phosphorylation of FAK and NF-κB/MMP signaling. Furthermore, SG also induces increases in expression of E-cadherin and collagen production.

findings of SG make it a potential compound for further *in vitro* and *in vivo* studies in future applications for wound treatment or tissue regeneration.

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