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## Asian Pacific Journal of Tropical Biomedicine

journal homepage: [www.elsevier.com/locate/apjtb](http://www.elsevier.com/locate/apjtb)Original article <http://dx.doi.org/10.1016/j.apjtb.2015.10.009>

## The effects of glycyrrhizic acid and glabridin in the regulation of CXCL5 inflammation gene on acceleration of wound healing

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## ARTICLE INFO

## Article history:

Received 24 Aug 2015

Received in revised form 17 Sep 2015

Accepted 10 Oct 2015

Available online 10 Dec 2015

## Keywords:

Wound healing

Glycyrrhizic acid

Glabridin

CXCL5

Inflammation

Cell proliferation

Cell migration

## ABSTRACT

**Objective:** To evaluate the anti-inflammatory property of both glycyrrhizic acid (GA) and glabridin in reducing inflammation to accelerate wound regeneration on 3T3-L1 and NIH-3T3 fibroblast cell lines.

**Methods:** Cell proliferation and viability assay (MTT assay), scratch wound healing assays, and quantitative real-time PCR were conducted to investigate the effects on cell proliferation, cell migration, and expression of CXC chemokine ligand 5 inflammation gene respectively.

**Results:** Results showed that at a low concentration of  $1 \times 10^{-8}$  mol/L, glabridin down regulated cell proliferation in NIH-3T3 significantly, suggesting its involvement in ERK1/2 signaling pathway. GA and glabridin significantly accelerated cell migration through wound healing in both 3T3-L1 and NIH-3T3 and significantly down regulated the expression of CXC chemokine ligand 5 in 3T3-L1 at concentration  $1 \times 10^{-8}$  mol/L, indicating the possible involvement of nuclear factor- $\kappa$ B and cyclooxygenase 2 transcriptions modulation.

**Conclusions:** Both GA and glabridin can serve as potential treatment for chronic inflammatory disease, and glabridin as an oncogenic inhibitor due to its anti-proliferative property.

## 1. Introduction

Wound healing is a complex biological event due to the interplay of various tissue structures and many resident and infiltrating cell types that serve as both inflammatory and immunological effector cells. It consists of four phases: hemostasis, inflammation (0–3 days), migration and proliferation (3–12 days), and tissue remodeling (maturation) (3–6 months) that influence the anabolic and catabolic reactions of tissue repair. Profound study especially on chemokines which involved in the specific trafficking and recruitment of leukocytes during inflammation phase is essential to illustrate the dynamic correlation between inflammation, cell proliferation and migration [1].

Leukocyte subsets such as neutrophils which act as immunological effector cells migrate immediately to the wound surface to form a dense barrier against infecting pathogens, which often associated intimately with the onset of acute inflammation.

However, prolonged inflammation will lead to undesirable pathogenesis [2]. Chemokines of the CXC family, particularly CXC chemokine ligand 5 (CXCL5) has been reported as key regulatory links between inflammation and angiogenesis corresponding to CXC chemokine receptor 2 (CXCR2) [3,4]. Major efforts have focused in finding efficient and novel therapeutic agents. The use of antiseptics and antibiotics are the current wound healing treatments that work in preventing and ceasing of infection but do not directly involve in supporting the wound healing process.

Prior study showed that liquorice (*Glycyrrhiza glabra*) was a potent healing agent in open skin wounds in rabbits [5]. These plant-derived compounds: glycyrrhizic acid (GA) and glabridin from liquorice extract contain anti-oxidant and anti-inflammatory properties that could be potential therapeutic candidate to accelerate wound healing. It is hypothesized that anti-inflammatory compounds could down regulate

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Peer review under responsibility of Hainan Medical University. The journal implements double-blind peer review practiced by specially invited international editorial board members.

Foundation Project: Support by the Exploratory Research Grants Scheme (ERGS/1/2012/STG08/TAYLOR/03/2), Ministry of Higher Education, Malaysia.

inflammation phases during wound healing and thus, improve wound closure. Present study aimed to evaluate the effects of GA and glabridin on wound healing by accessing the cell proliferation, cell migration and CXCL5 gene expression in 3T3-L1 and NIH-3T3 mouse fibroblast cell lines.

## 2. Materials and methods

### 2.1. Cell culture and maintenance

NIH-3T3 mouse fibroblast and 3T3-L1 pre-adipocyte cell lines were adopted due to their angiogenesis nature. They were cultured and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma–Aldrich, USA) supplemented with 10% fetal calf serum. Phosphate-buffered saline (PBS; Sigma–Aldrich, USA) was used to rinse and wash the cells prior to trypsinization with trypsin (Sigma–Aldrich, USA) to detach the cells during sub-culturing, passaging and seeding.

### 2.2. Cell proliferation and viability assay (MTT assay)

Both NIH-3T3 and 3T3-L1 cell lines were harvested at 80%–90% confluence and seeded into 96 well plates at density  $1.5 \times 10^4$  cells. Twenty-four hours post seeding, 150  $\mu$ L of culture medium was replaced with equal volume of GA and glabridin respectively at concentrations ranging from  $1 \times 10^{-4}$  mol/L to  $1 \times 10^{-10}$  mol/L. After 24 h of incubation, MTT solution (10  $\mu$ L of 5  $\mu$ g/mL MTT in sterile PBS) was added and subsequently incubated for another 3.5 h. The supernatant was decanted and 100  $\mu$ L of dimethyl sulfoxide was added to each well. The absorbance at 595 nm was measured using a micro plate reader. The assay was carried out in triplicates [6]. Results were analyzed statistically using statistical software, IBM SPSS Statistics version 20 via independent *t*-test with control: DMEM medium with 0.1% absolute ethanol. Results were marked significant at  $P < 0.05$  and  $P < 0.01$ .

### 2.3. Scratch wound healing assay

NIH-3T3 and 3T3-L1 fibroblast cell lines were seeded into 6 well plates at density of  $2.0 \times 10^5$  cells. Pipette tip (size: 10–200  $\mu$ L) was used to create the wound after 24 h of cell seeding. Each well was first washed with sterile PBS and subsequently 2 mL per well of GA and glabridin were added separately into each well with concentrations ranging from  $1 \times 10^{-5}$  mol/L to  $1 \times 10^{-9}$  mol/L with DMEM medium with 0.1% absolute ethanol as negative control. Images of wound closure were captured and the measurement of each wound gap was taken at a treatment interval of 6, 12 and 24 h in five replicates. The percentage of wound closure was calculated based on the formula below [7]:

$$\left[ \frac{\text{Initial wound size at 0 treatment hour} - \text{Specific interval of wound size}}{\text{Initial wound size}} \right] \times 100\%$$

Results obtained were analyzed statistically using statistical software, IBM SPSS Statistics version 20 via independent *t*-test and marked significant at  $P < 0.05$ .

### 2.4. RT-qPCR quantification of total CXCL5 gene expression

#### 2.4.1. Seeding and treatment of cell culture

Both NIH-3T3 and 3T3-L1 cell lines were seeded at density of  $1 \times 10^6$  into culture dishes. After 24 h of incubation, cells were then washed with sterile PBS and treated with 3 mL volume of GA and glabridin at concentration that induced the best migration rate. DMEM medium with 0.1% absolute ethanol was added as control and treatments were done in triplicates.

#### 2.4.2. Total mRNA extraction from cell lines

Total mRNA extraction for both control and treated cell lines were done using FavorPrep™ Blood/Cultured Cell Total RNA Mini Kit. The purity and quantity of total RNA extracted was checked using Beckman Coulter NanoDrop Spectrophotometer (USA) at wavelength 280 nm and 260 nm. The integrity of the total RNA extracted was also being assessed by denaturation at 72 °C for 10 min followed by visualization using 2% agarose gel electrophoresis run at 80 V for approximately 40 min.

#### 2.4.3. cDNA synthesis

The extracted RNA samples were incubated with RNase-Free DNase (Promega RQ1 RNase-free DNase) reaction mixture at 37 °C for 30 min. A volume of 1  $\mu$ L of RQ1 DNase Stop Solution was added to stop the reaction followed by 10 min incubation at 65 °C. The treated total RNA was then used to synthesize cDNA using qPCRBIO cDNA Synthesis Kit (PCR Biosystems, United Kingdom). The RNA samples were incubated at 42 °C for 30 min followed by 85 °C for 10 min to denature the RTase.

#### 2.4.4. Primer design for mouse total CXCL5 and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene

Gene specific forward and reverse primers were designed for mouse total CXCL5 and GAPDH gene (Table 1) by Primer3 BioTools and cross-checked via National Centre for Biotechnology Information website using the standard nucleotide Basic Local Alignment Searching Tool program.

Expression of the respective genes was quantified using the optimized RT-qPCR parameters and protocols (Table 2) by 2  $\times$  qPCRBIO SyGreen Mix Lo-ROX (PCR Biosystems, United Kingdom) in 10  $\mu$ L reaction mixtures. All RT-qPCR and analysis were carried out on Eppendorf Mastercycler® ep realplex PCR thermal cyler (Germany).

The comparison of mouse total CXCL5 expression between control, GA-treated and glabridin-treated cell samples were performed using the comparative CT ( $\Delta\Delta$ CT) method [8]. The

**Table 1**

Characteristics of primers for total CXCL5 and GAPDH amplification.

Primer	Sequence (5'→3')	Length (bp)	T <sub>m</sub> (°C)	Amplicon size (bp)
Mouse CXCL5 forward primer	TTCCTCAGTCATAGCCGCAA	20	56.3	168
Mouse CXCL5 reverse primer	GGATCCAGACAGACCTCCTTC	21	56.6	168
Mouse GAPDH forward primer	GTTTCTCGTCCCGTAGACA	20	56.4	200
Mouse GAPDH reverse primer	CCTTGACTGTGCCGTTGAAT	20	55.7	200

**Table 2**

Optimized RT-qPCR thermal cycling protocols for mouse total CXCL5 and GAPDH amplification.

Steps	Temperature (°C)	Time	Remarks
Polymerase activation	95	2 min	
Denaturation	95	5 s	35 cycles
Annealing/Extension	57	25 s	
Melt analysis			

amplified PCR products were also visualized using 2% agarose gel electrophoresis run at 80 V for approximately 40 min. Statistical analysis for CXCL5 expression was performed using the relative expression software tool MCS Beta 2006.

### 3. Results

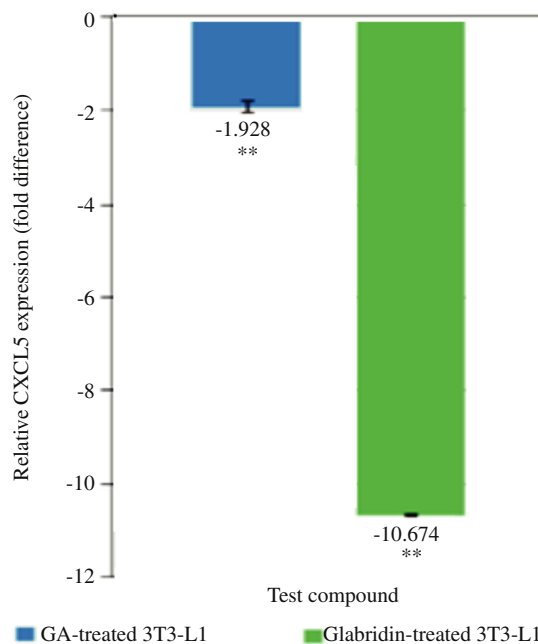
#### 3.1. Expression of mouse total CXCL5

Down regulation of mouse total CXCL5 inflammation gene was observed in Figure 1 by GA and glabridin in 3T3-L1 with a fold difference of  $-1.928 \pm 0.128$  ( $P < 0.01$ ) and  $-10.674 \pm 0.025$  ( $P < 0.01$ ) respectively, and in NIH-3T3 (Figure 2) with a fold difference of  $-1.300 \pm 0.146$  and  $-1.623 \pm 0.148$  respectively. This down regulatory phenomena by the anti-inflammation property of both GA and glabridin could be due to involvement of nuclear factor- $\kappa$ B (NF- $\kappa$ B) and cyclooxygenase 2 (COX-2) transcriptions modulation.

#### 3.2. Scratch wound healing assay

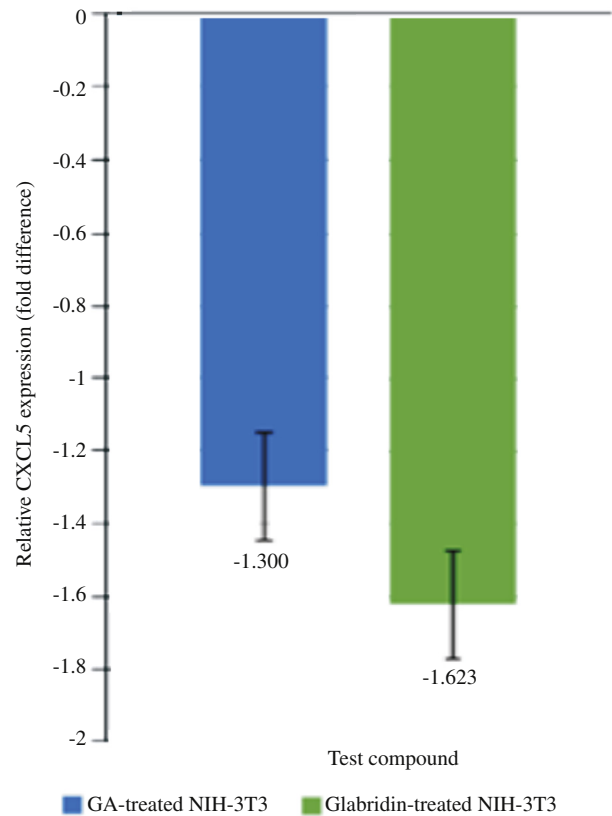
##### 3.2.1. Cell migration within time interval

Cell migration was observed and recorded in the form of percentage of wound closure at interval of time. Figures 3 and 4 show a series compilation of wound healing progression of control, GA-treated and glabridin-treated 3T3-L1 and NIH-3T3 cell lines at the



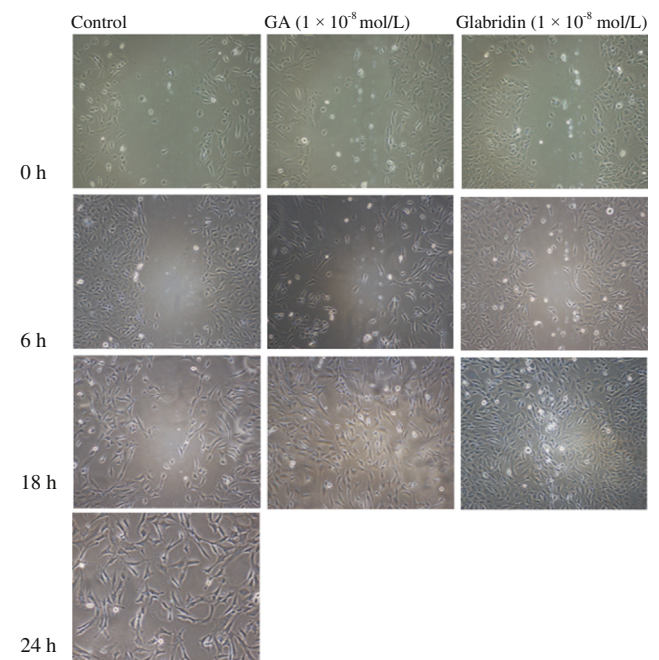
**Figure 1.** Relative expression for CXCL5 in 3T3-L1 cell line using GAPDH as the endogenous reference, GA-treated and glabridin-treated groups as targets and control group as calibrator.

\*\* :  $P < 0.01$ .

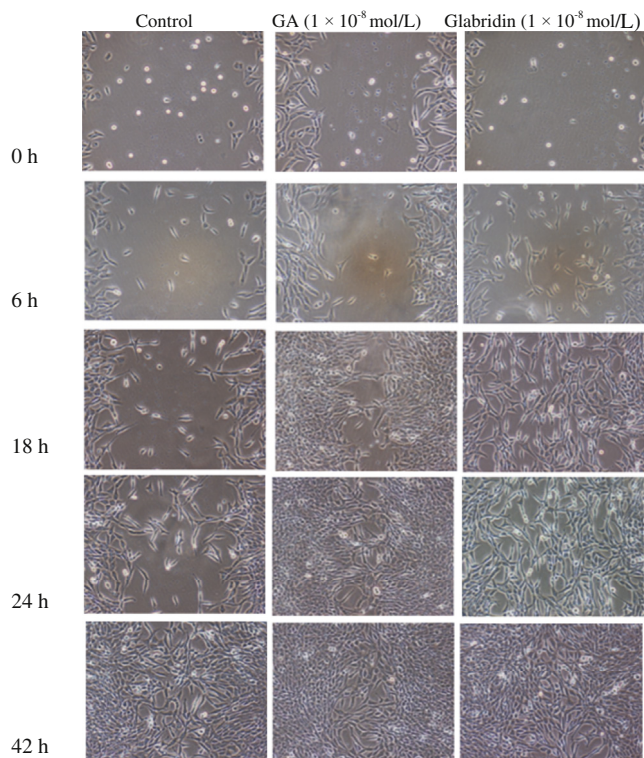


**Figure 2.** Relative expression for CXCL5 in NIH-3T3 cell line using GAPDH as the endogenous reference, GA-treated and glabridin-treated groups as targets and control group as calibrator.

effective concentration (highest cell migration) respectively. Complete wound closure was observed at 24 h for controls of 3T3-L1 cell line and at 42 h for controls of NIH-3T3 cell line.



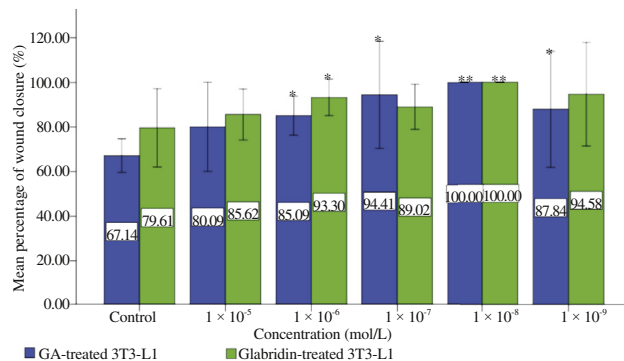
**Figure 3.** Wound closure images (10 $\times$  magnification) of GA-treated and glabridin-treated 3T3-L1 cell line at effective concentration in comparison with control.



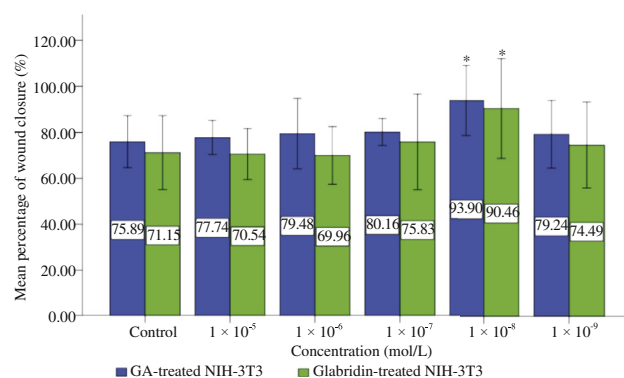
**Figure 4.** Wound closure images (10× magnification) of GA-treated and glabridin-treated NIH-3T3 cell line at effective concentration in comparison with control.

### 3.2.2. Cell migration at different concentrations of test compounds

Both GA and glabridin stimulated effective cell migration at lower concentration of  $1 \times 10^{-8}$  mol/L. Complete wound closure of  $(100.00 \pm 0.00)\%$  ( $P < 0.01$ ) was observed in 3T3-L1 treated with GA and glabridin at 18 h respectively (Figures 3 and 5), whereas in NIH-3T3, wound closure of  $(93.90 \pm 3.53)\%$  ( $P < 0.05$ ) and  $(90.46 \pm 5.05)\%$  ( $P < 0.05$ ), were observed at 24 h for GA-treated and at 18 h for glabridin-treated NIH-3T3 respectively (Figures 4 and 6).



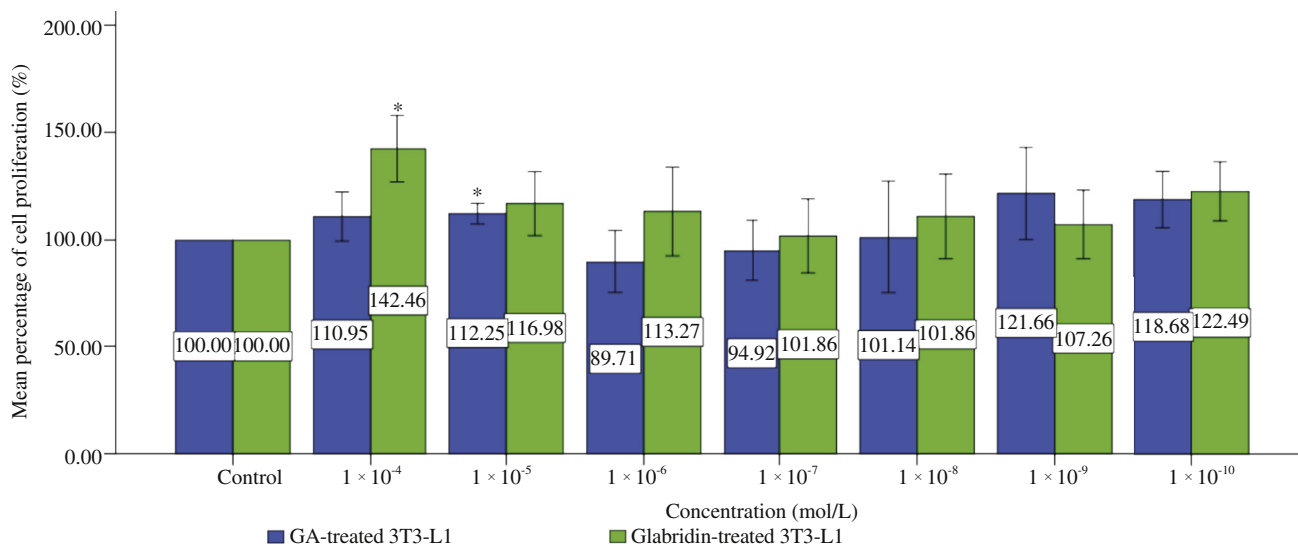
**Figure 5.** Comparison of mean percentage of wound closure against different concentrations of GA and glabridin on 3T3-L1 cell line at 18 h. \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ .



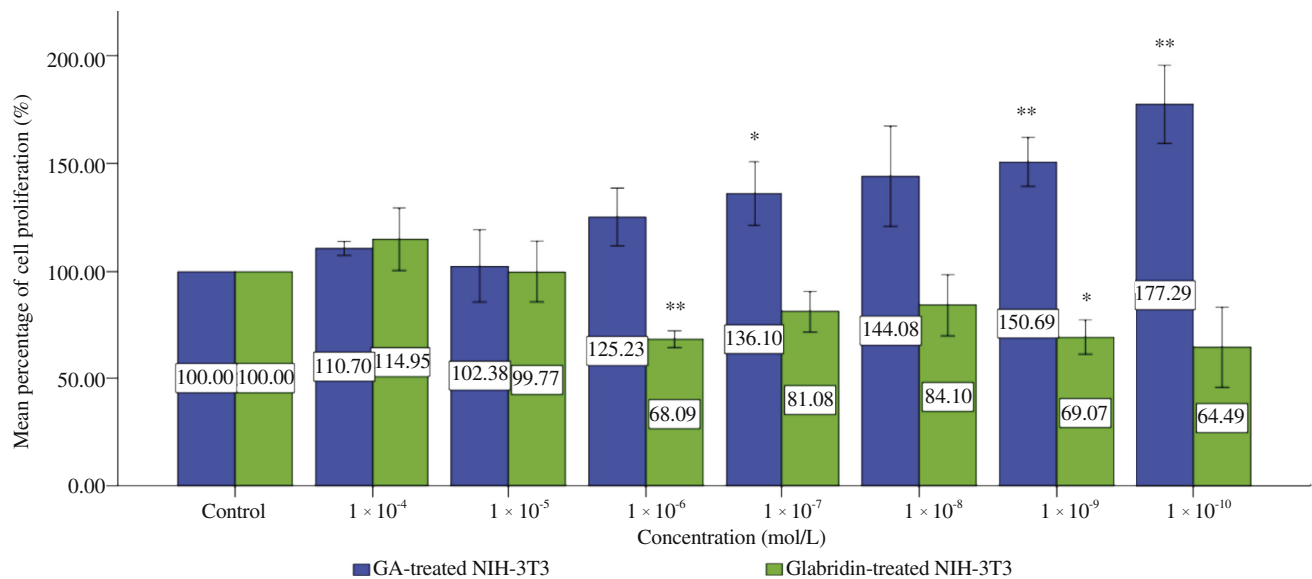
**Figure 6.** Comparison of mean percentage of wound closure against different concentrations of GA and glabridin on NIH-3T3 cell line at 24 h. \*:  $P < 0.05$ .

### 3.3. MTT assay

Both GA and glabridin exhibited a similar trend in 3T3-L1 cell line in which, both increased cell proliferation in higher drug concentrations and decreased cell proliferation at lower drug concentrations (Figure 7). However, conflicting effects were exhibited by GA and glabridin on cell proliferation in NIH-3T3 cell line. Lower concentration, specifically concentration  $1 \times 10^{-8}$  mol/L, which was also the effective concentration for increased cell migration and decreased inflammation, of glabridin showed down regulation of cell proliferation on NIH-3T3 cell lines with mean percentage of  $(84.10 \pm 5.63)\%$ , compared to highest



**Figure 7.** Comparison of mean percentage of cell proliferation against different concentrations of GA and glabridin on 3T3-L1 cell line respectively. \*:  $P < 0.05$ .



**Figure 8.** Comparison of mean percentage of cell proliferation against different concentrations of GA and glabridin on NIH-3T3 cell line respectively. \*:  $P < 0.05$ ; \*\*:  $P < 0.01$ .

concentration  $1 \times 10^{-4}$  mol/L, which up regulated cell proliferation with mean percentage of  $(114.95 \pm 5.63)\%$  (Figure 8).

## 4. Discussion

### 4.1. Expression of total CXCL5 inflammation gene

#### 4.1.1. Down-regulation of NF- $\kappa$ B inflammation pathway by inhibition of reactive oxygen species generation by neutrophils

CXCL5 is produced upon stimulation of cells with inflammatory cytokines. According to Akamatsu *et al.*, the proposed anti-inflammatory action of GA is by inhibiting the generation of reactive oxygen species by neutrophils, the most potent inflammatory mediator at the site of inflammation [9]. Yokota *et al.* indicated that the anti-inflammatory action of glabridin was by its two hydroxyl groups that inhibit superoxide anion productions and cyclooxygenase activities [10]. Therefore, it can be postulated that both GA and glabridin down regulate the NF- $\kappa$ B transcription factor via inhibition of oxidative stimulus, which down regulates the activation of I $\kappa$ B kinase, an enzyme complex that is involved in the upstream NF- $\kappa$ B signal transduction cascade that propagates the cellular response to inflammation. When inflammatory cytokines are down regulated, production of CXCL5 is also down regulated.

#### 4.1.2. Down-regulation of COX-2 inflammation pathway

The down-regulatory effect observed can also be explained via the mechanism of COX-2 inflammation pathway. The down regulation of NF- $\kappa$ B transcription factor further down regulates COX-2 expression, which is of similar mechanism to non-steroidal anti-inflammatory drugs (NSAIDs) that are widely used for the treatment of pain, fever, and inflammation. NSAIDs act via inhibition of COX-2 enzyme at the site of inflammation, which catalyzes the conversion of arachidonic acid to prostaglandins. However, NSAIDs therapy produces harmful side effects such as gastric toxicity due to non-regulated inhibition of COX-1 [11]. Therefore, the sensitivity and specificity of anti-inflammatory action for both GA and glabridin should be further explored.

### 4.2. GA and glabridin induced higher cell migration in both 3T3-L1 and NIH-3T3 cell line

The stimulated cell migration effect could be one of the response by the down regulation of inflammation during wound healing. The effect can be further supported by the study done by Dong *et al.*, [12]. Down regulation of CXCL5 inflammation gene down regulates the activation of CXCR2 receptor, which further down regulates the activation of epidermal growth factor receptor that is responsible for the activation of PI3K/Akt pathway, followed by NF- $\kappa$ B and COX-2 pathway, which resulted in down regulation of key inflammatory regulator in chronic infections, prostaglandins E2. Hence, it can be suggested that down regulation of inflammatory phase accelerates the healing process to the third phase, cell migration to allow faster wound regeneration. However, the exact mechanism is yet to be determined due to the involvement of various growth factors and wound healing phases.

### 4.3. GA and glabridin regulated cell proliferation in both 3T3-L1 and NIH-3T3 cell line

The mitogen-activated protein kinase family constitutes of extracellular signal-regulated kinase, stress-activated protein kinases, and Jun amino-terminal kinases, all of which can be activated by upstream regulators for example Ras and Raf-1, that phosphorylate downstream proteins in the respective cascades to regulate expression of various genes corresponding to cell proliferation and inflammation [13]. Results were in agreement with the study conducted by Zhou *et al.*, which showed that the down regulation of CXCL5 inflammation gene which corresponds to CXCR2 chemokine receptor, down regulates phosphorylation of extracellular signal-regulated kinase pathway that mediates cell proliferation [14]. Thus, a decrease in inflammation will subsequently reduce cell proliferation as activation of both inflammation pathway, PI3K/Akt and cell proliferation pathway, extracellular signal-regulated kinases are modulated by chemokines for example CXCL5. Both GA and glabridin interfere with cell proliferation and inflammation via similar pathways. However, glabridin exhibited better down regulatory

effect on inflammation compared to GA in both 3T3-L1 and NIH-3T3 cell lines. This could be due to glabridin interfering directly on COX-2, compared to GA that acts upon the upstream NF- $\kappa$ B protein complex.

In conclusion, both GA and glabridin exhibited promising potential in wound healing. Future work should explore on other beneficial properties of both GA and glabridin and transform them into regenerative cell therapy for treating chronic inflammations. The anti-proliferative property of glabridin can be proposed as a chemotherapeutic agent for treating cancer associated fibroblasts.

### Conflict of interest statement

We declare that we have no conflict of interest.

### Acknowledgments

We acknowledge the financial support from the Exploratory Research Grants Scheme (ERGS/1/2012/STG08/TAYLOR/03/2), Ministry of Higher Education, Malaysia. This work was supported by Taylor's University through its PhD Scholarship Programme.

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