Original Article

Wound-healing potential of grandiflorenic acid isolated from *Wedelia trilobata* (L.) leaves

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Abstract

The ethyl acetate fraction from ethanolic extract of *Wedelia trilobata* (L.) leaves displayed wound healing properties. The ethyl acetate fraction was further subjected to bioassay-guided fractionation which afforded isolation of grandiflorenic acid which requires further investigation to prove its wound healing potential. The grandiflorenic acid from leaves of *Wedelia trilobata* was assessed for its possible activity on BJ human fibroblast and HaCaT keratinocytes proliferation, and effect on *in vitro* scratch assay, collagen content, TGF-β2 levels, and nitric oxide, TNF-α and IL-1β determination using Raw 264.7 cells. Grandiflorenic acid (2.5μg/mL) produced percentage viability of BJ human fibroblast, and HaCaT keratinocytes 116, and 106% respectively. Grandiflorenic acid (2.5 μg/mL) induced a 100% migration rate in the *in vitro* scratch assay and the collagen content was increased to 171.2 μg/mL compared to the control (61.1μg/mL) with BJ human fibroblast. Grandiflorenic acid (2.5μg/mL) neither produced any significant increase in TGF-β2 *levels* of HaCaT keratinocytes cells nor induced migration of HaCaT cells in the *in vitro* scratch assay. The present study provides scientific evidence that grandiflorenic acid has potential wound healing activity due to combination of fibroblast stimulation and inhibiting prolonging inflammatory phase of wound healing evident by reduced levels of inflammatory cytokines from macrophage Raw 264.7 cells.

Keywords: *Wedelia trilobata*, grandiflorenic acid, BJ human fibroblast, Raw cells 264.7, Keratinocytes HaCaT

1. Introduction

Studies on the traditional use of medicines are recognized as a way to learn about potential future medicines. Researchers have identified 122 compounds used in mainstream medicine that were derived from "ethnomedical" plant sources and 80% of these compounds were used in the same or closely related manner as for their traditional ethnomedical use (Fabricant and Farnsworth 2001). Plants have evolved an ability to synthesize chemical compounds to defend themselves against attack from a wide variety of predators such as insects and fungi. By chance, some of these compounds, whilst being toxic to plant predators, turn out to have beneficial effects when used to treat human diseases. Such secondary metabolites are highly varied in structure, usually aromatic in nature. Most of them are phenols or their oxygen-substituted derivatives (Lai and Roy, 2004; Tapsell *et al.*, 2006).

Plants have an immense potential for the management and treatment of wounds. A large number of plants have been used, by tribal and folklore, in many countries for the treatment of wounds and burns. The presence of various health sustaining constituents in plants has attracted scientists to examine these plants with a view to determine potential wound-healing properties (Nayak and Pinto-Pereira, 2006). The medicinal value of these plants lies in their bio-

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active phytochemical constituents that produce definite physiological actions on the human body (Akinmoladun *et al.*, 2007). These constituents include various members of chemical families like alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, and phenolic compounds (Edeoga *et al.*, 2005). *Wedelia trilobata* (L.) Hitchc (Asteraceae) commonly called creeping daisy, is a creeping herb, native to the tropics of Central America and has naturalized in many wet tropical areas like West Indies, Hawaii, India, Burma, China, Japan, Ceylon, especially at low altitude (USDA-GRIN, 2008; Shanmugam *et al.*, 2011). The leaves or aerial parts of this plant are used in traditional medicine in Caribbean and Central America for backache, muscle cramp, rheumatism, stubborn wounds, sores, swelling, and arthritic painful joints (Arvigo and Balik, 1993; Coe and Anderson, 1996). The ethyl acetate fraction from ethanolic extract of *W. trilobata* leaves displayed antibacterial and fibroblast stimulatory activities thereby suggesting potential wound-healing properties (Balekar *et al.*, 2012a). The ethyl acetate fraction was further subjected to bioassay-guided fractionation which afforded isolation of grandiflorenic acid (ent-kaura-9(11), 16-dien-19-oic acid) which showed antibacterial, stimulation of fibroblast growth and protective effect against hydrogen peroxide induced injury (Balekar *et al.*, 2012b). These activities could play some role in its effect on tissue repair. Therefore, the aim of the present study was to evaluate activity of grandiflorenic acid using *in vitro* cell culture models of dermal fibroblasts, epidermal keratinocytes and macrophage that would influence management of wound infections.

2. Materials and Methods

2.1 Cell lines, chemicals and biochemicals

The BJ human skin fibroblast cells were obtained from ATCC CRL-2522, Rockville, MD, USA, Raw 264.7, macrophage cells were procured from ATCC CRL-2278, Rockville, MD, USA, and human keratinocytes HaCaT cells from CLS-Cell lines services, Eppelheim, Germany.

All the solvents, chemicals and biochemicals used were of analytical grade.

2.2 The grandiflorenic acid (ent-kaura-9(11), 16-dien-19-oic acid) was isolated from leaves of *W. trilobata* (Balekar *et al.*, 2012b), and was used into this investigation without further re-purification.

2.3 *In vitro* assays relevant to wound healing

2.3.1 Cytotoxicity assay and soluble collagen production using BJ human skin fibroblast cells

Cytotoxicity was evaluated by the method previously reported by Balekar *et al.* (2012a). The BJ human skin fibroblast cells at a concentration of 2×10^4 cells/ mL were seeded into a 96-well plate in complete MEM containing 10% FBS

and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), under 5% CO_2 at 37ºC. After 24 h, the culture medium was replaced by fresh medium. The stock solution of grandiflorenic acid, (100 mg/mL in DMSO), filtered through a 0.22 µm membrane filter was further diluted to 1 mg/mL with phosphate buffered saline (PBS) and further dilutions were made in DMEM to achieve a final concentration in each well of 10-0.08 µg/mL of grandiflorenic acid. The cells were treated with DMEM medium containing dimethyl sulfoxide (0.1%). Cells without grandiflorenic acid served as negative controls and Proteoglycan-IPC (Ichimaru Pharcos Co. Ltd., Gifu, Japan) (10, 5 and 2.5 µg/mL) solution of proteoglycan extracted from nasal cartilage of *Oncorhynus keta* (Salmon) having property like epidermal growth factor was used as a positive control. After incubation for 24 h at 37ºC with 5% CO_2, the supernatants were collected and the cells were then treated with 100 µL of freshly prepared media along with 50 µL of 3- (4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) solution (5 mg/mL) and incubated at 37ºC for 4 h. Then, the medium containing MTT was removed and 100 µL of DMSO was added. The absorbance was determined by a microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at a wavelength of 570 nm. The percentage of cell viability was calculated and compared to a negative control.

The total amount of soluble collagen type I was assayed in the supernatant using the Sircol® collagen assay kit (Bicolor Life Science Assays, Northern Ireland, UK). Briefly, 100 µL of experimental supernatant was mixed with 1 mL of dye solution at room temperature for 30 min. Then the samples were centrifuged at 15,000 g for 10 min to form a pellet of collagen. The supernatant was removed and the produced soluble collagen was dissolved in 1 mL of alkali reagent. The resultant alkali reagent solutions were transferred to a 96-well plate and assayed by a microplate reader (Biohit 830, Biohit®, Helsinki, Finland) at a wavelength of 540 nm. The amount of collagen was calculated based on a standard curve of soluble collagen (bovine skin collagen type I standard from American disease-free animals).

2.3.2 Cytotoxicity assay and determination of TGF-β2 using Keratinocytes cells

Cytotoxicity was evaluated by the method previously reported by Amjad *et al* (2007). The keratinocytes HaCaT cells at a concentration of 2×10^4 cells/ mL were seeded into a 96- well plate in complete DMEM containing 10% FBS and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin), under 5% CO_2 at 37ºC. After 24 h, the culture medium was replaced with fresh medium. The stock solution of grandiflorenic acid (100 mg/mL in DMSO), filtered through a 0.22 µm membrane filter, was further diluted to 1 mg/mL with phosphate buffer saline (PBS) and further dilutions were made in DMEM to achieve a final concentration of 10-0.08 µg/mL of grandiflorenic acid in DMEM. The cells were treated with DMEM medium containing DMSO (0.1%). Cells without grandiflorenic acid served as negative controls. After incuba-

tion for 24 h at 37ºC with 5% CO_2, the supernatants were collected and assayed for human TGF-β2 using ELISA assay kit (R & D Systems, Minneapolis, MN, USA) and the cells were treated with MTT for cytotoxicity determination. The percentage of cell viability was calculated and compared to a negative control.

2.3.3 *In vitro* scratch assay using BJ human skin fibroblast and keratinocytes HaCaT cells

The fibroblast and keratinocytes cells ($5×10^4$ cells/mL) in MEM and DMEM containing 10% FBS were seeded in a 6 well plate. Once the confluent monolayer was formed, a linear scratch was generated in the monolayer with a sterile pipette tip. The cellular debris was removed by washing with phosphate buffer saline (PBS) and replaced with 2 mL of MEM containing grandiflorenic acid (2.5, 1.25 0.625 µg/mL). Proteoglycan-IPC (10 and 5 µg/mL) served as a positive control, and MEM without sample served as a negative control for BJ human fibroblast and grandiflorenic acid (2.5, 1.25, 0.625 µg/mL) and DMEM without sample served as a negative control for keratinocyte HaCaT. Photographs were taken at a 10x magnification using a light microscope (Olympus CK2, Japan) on day 0, then plates were incubated at 37ºC with 5% CO_2 and photographs were taken at days 1 and 2. The images acquired for each sample were further analyzed quantitatively using computing software (ImageJ 1.42q/Java1.6.0_10). By comparing the images from day 0 to 2, the distance of each scratch closure was determined and the percentage migration rate was calculated. In each well two parallel scratches were made and on each scratch six points were considered. Average of left scratch and right scratch were taken separately. Percent migration was calculated for left scratch and then right scratch using

% Migration rate =

$$\frac{\text{Average distance between scratch (Day 0) - Average distance}}{\text{Average distance between scratch (Day 0)}} \times 100$$

Samples were in quadruplicate. Percent rate of migration obtained from all four wells were averaged and recorded (Balekar *et al.*, 2012a).

2.3.4 Cytotoxicity assay, and nitric oxide using RAW 264.7 cells

The cytotoxicity and nitric oxide was determined using method described by Chuealee *et al* (2011). The Raw 264.7 cells in DMEM medium containing 10% FBS, were seeded at an initial concentration of $1×10^4$/ml into 96-well plates and incubated at 37ºC with 5% CO_2. After 24 h, the culture medium was replaced with fresh medium. The stock solution of grandiflorenic acid (100 mg/mL in DMSO), filtered through a 0.22 µm membrane filter, was further diluted to 1 mg/mL with phosphate buffer saline (PBS) and further dilutions were

made in DMEM to achieve a final concentration of 10-0.08 µg/mL of grandiflorenic acid in DMEM. Cells without drug sample served as negative controls. After incubation for 24 h at 37ºC with 5% CO_2, the supernatants were collected and assayed for nitric oxide by the Griess reaction and the cells were treated with MTT for cytotoxicity determination. For nitric oxide, 100 µl Griess reagent (1% N-(1-napthyl)-ethylene-diamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid) was mixed with 50 µl of experimental cell supernatant and absorbance was recorded at 540 nm and quantification was done.

2.3.5 TNF-α and IL-1β determination using RAW 264.7 cells.

The TNF-α and IL-1β was determined using the method described by Chuealee *et al* (2011). The Raw 264.7 cells in DMEM medium containing 10% FBS, were seeded at an initial concentration of $1×10^4$/ml into 96 well plates and incubated at 37ºC with 5% CO_2. After 24 h, the culture medium was replaced with fresh medium. The cells were then treated with grandiflorenic acid (2.5-0.156 µg/mL). Cells with DMEM medium served as negative controls. After incubation for 24 h at 37ºC with 5% CO_2, the supernatants were collected and assayed for TNF-α, and IL-1β using rat ELISA assay kit (R & D systems, MN, USA). The minimal detectable doses of TNF-α, IL-1β were approximately 5.0 pg/ml and 3.0 pg/ml, respectively. All experiments were done in quadruplicate. The TNF-α reaction was quantitatively recorded at 450 nm, based on standard curve of 14-875 pg/ml. The same wavelength was used to record IL-1β levels, based on standard curve of 12.5 - 800 pg/ml. Lipopolysaccharide (LPS) from *E. coli* was used as positive control (15.65-500 ng/ml).

2.3.6 *In vitro* hemolysis assay

Lysis of human red blood cells (RBC) was evaluated as described by Chuealee *et al* (2011). Briefly, erythrocytes (Blood Bank, Department of Pathology, Faculty of Medicine, Songklanagarind Hospital, PSU, Thailand) were isolated from fresh human blood, washed three times with phosphate buffered saline solution (PBS) and centrifuged at 1500g for 5 min. The stock solution of grandiflorenic acid (100 mg/ml in DMSO) was further diluted with PBS to 1 mg/ml. The grandiflorenic acid was then added to the suspended erythrocytes and the suspension diluted with PBS to give final grandiflorenic acid concentrations in the range of 1-8 µg/ml and a final hematocrit of 1%. It was then incubated at 37°C for 0.5, 3, 6 and 24h. The unlysed cells were removed by centrifugation at 3000g for 5 min and the hemoglobin in the supernatant was determined at absorbance 540 nm. The positive control was 1% Triton X-100 (Sigma-Aldrich, Steinheim, Germany) and negative control was phosphate buffer saline. Hemolysis was reported as a percentage of the positive control (100% hemolysis) according to the formula

$$\frac{(\text{Absorbance of sample} - \text{Absorbance of negative control})}{(\text{Absorbance of positive control} - \text{Absorbance of negative control})} \times 100$$

2.4 Statistical analysis of data

Data are expressed as a mean ± S.D. Statistical evaluation was carried out using one-way ANOVA followed by Tukey's test using "Graphpad Instat" version 3.00 for Windows 95, Graphpad software, San Diego California USA. The values of $p<0.05$ were considered to be statistically significant. All experiments were done in quadruplicate.

3. Results

3.1 Chemical elucidation of the isolated compound

The isolated compound was identified as Grandiflorenic acid (ent-kaura-9(11),16-dien-19-oic acid) (Figure 1) by FT-IR, ^{1}H-NMR, ^{13}C NMR spectra (Balekar *et al.*, 2012b).

3.2 Influence of grandiflorenic acid on cell viability and collagen content using L929 fibroblast

The grandiflorenic acid at 10-0.08 μg/mL produced cell viability above 97% (Figure 2). The dose range of grandiflorenic acid (2.5-0.08 μg/mL) produced cell viabilities in the range of 117-100% (Figure 2). The ent-kaura-9(11), 16-dien-19-oic acid was found in *W. trilobata*. Cells treated with 0.1% DMSO showed no toxicity with cell viability to 100%. This indicated that this concentration of DMSO had no significant toxic effects on cells. Proteoglycan (10, 5 and 2.5 μg/mL) used as positive control exhibited percent viability 100, 102 and 101%, respectively. The percent viability with grandiflorenic acid (2.5 μg/mL) was 116%, higher than that with proteoglycan (2.5 μg/mL) which was 101%. The results

showed that collagen type I production in BJ fibroblast cells increased significantly after treatment with grandiflorenic acid at concentrations of 2.5, 1.25 and 0.625 μg/mL ($p<0.01$) as compared to the control. Collagen production by fibroblast cells when treated with proteoglycan (10, 5 and 2.5 μg/mL) was 181.7, 191.2, and 212 μg/mL (Table 1). The grandiflorenic
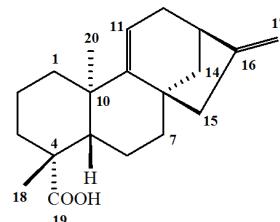
Figure 1. Chemical structure of grandiflorenic acid (ent-kaura-9(11), 16-dien-19-oic acid) from leaves of *W. trilobata*.
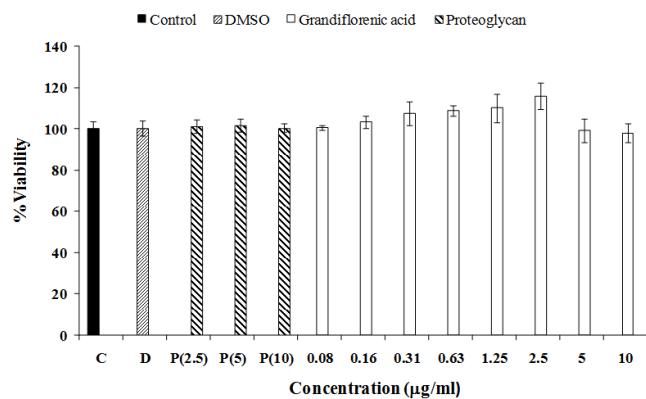
Figure 2. Percentage of BJ human skin fibroblast cells surviving after 24 h incubation at 37°C with 5% CO_2 and treated with grandiflorenic acid and proteoglycan-IPC at various concentrations. Data expressed as mean±s.d., (n= 4). *$p<0.01$ vs negative control group (one-way ANOVA, followed by Tukey's test).

Table 1. Collagen type-I production in the BJ human fibroblast cell line when treated with various concentrations of grandiflorenic acid from the leaves of *W. trilobata*.

Treatment	Conc (μg/mL)	Collagen production (μg/mL)
Negative control	-	61.1 ± 6.7
Grandiflorenic acid (μg/mL)	0.08	58.2 ± 5.7
	0.16	61.8 ± 6.2
	0.31	73.6 ± 6.5
	0.63	125.8 ± 5.6*
	1.25	158.9 ± 5.8*
	2.5	171.2 ± 5.4*
	5	122.2 ± 5.0*
	10	91.1 ± 4.8*
Proteoglycan	2.5	181.7 ± 5.8*
(Positive control)	5	191.2 ± 5.3*
	10	212.0 ± 6.5*

Values are mean ± S.D., (n=4); *$p<0.01$ vs negative control group

acid (2.5 µg/mL) produced collagen 171.2 µg/mL, which was comparable to the collagen produced by proteoglycan (2.5 µg/mL) treatment.

3.3 Influence of grandiflorenic acid on cell viability and TGF-β2 levels using human keratinocytes HaCaT

The grandiflorenic acid (10-0.08 µg/mL) produced cell viability in the range of 89-98%. The dose range of grandiflorenic acid (2.5-0.31 µg/mL) produced cell viabilities in the range of 106-103%. This data indicated better viability of keratinocytes at the concentration range used in this study. The level of TGF-β2 produced by HaCaT keratinocytes after exposure to grandiflorenic acid at a concentration of 10-0.08 µg/mL was 40-43 pg/ml, which was similar to the level produced by control group (43 pg/mL). No significant changes were observed in TGF-β2 levels.

3.4 Effect of grandiflorenic acid on *in vitro* scratch assay using BJ human fibroblast and keratinocytes HaCaT cells

The grandiflorenic acid was evaluated for changing the rate of migration of the BJ fibroblast cells and keratinocytes HaCaT. The cellular proliferation and migration of fibroblasts was studied on day 0, 1 and 2 (Figure 4 and Table 2). The presence of grandiflorenic acid (2.5, 1.25, 0.625 µg/mL) caused an increased number of fibroblasts in the denuded area when comparing either days 1 or 2 to the control (p< 0.01). As shown in Figure 4 an incubation time of two days resulted in the highest number of migrated cells in the denuded area.

The length between the scratch mark edges with grandiflorenic acid (2.5 µg/mL) was 346.5±13.2 µm (day 0) and there was closure of the gap on day 1. The length between the scratch mark edges with negative control was 336.3±8.5µm on day 0, 166±9.6 µm on day1 and 73.8±4.6 µm on day 2, and with proteoglycan (5 and 10 µg/mL) was 322.5±10 and 345.8±6.6 µm (day 0), and complete closure of gap on day 1. The grandiflorenic acid (2.5 µg/mL) showed a 100% migration rate on day 1 while dose of 1.25 µg/mL showed 72% on day 1 and on day 2 complete closure of gap
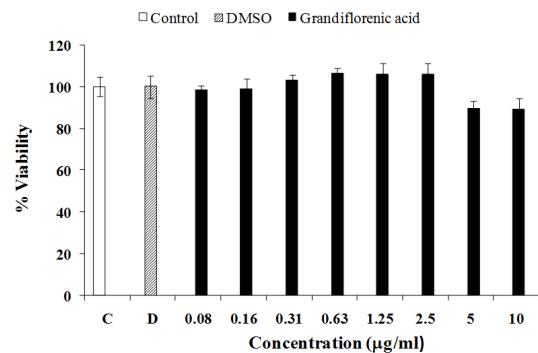
Figure 3. Percentage of keratinocytes HaCaT cells surviving after 24 h incubation at 37 °C with 5% CO_2 and treated with grandiflorenic acid at various concentrations. Data expressed as mean ± s.d., (n= 4).
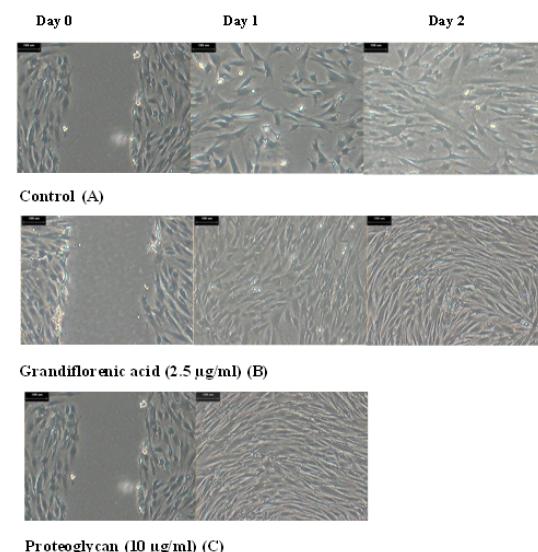
Figure 4. Measurement of cell migration in the *in vitro* scratch assay. A BJ human skin fibroblast layer subjected to scratch and treated with negative control (A), grandiflorenic acid (2.5 µg/mL) (B), and Proteoglycan (10 µg/mL) (C). Images captured at 10x magnification using a light microscope at day 0, 1 and 2 after incubation. The rate of migration was measured by quantifying the total distance that the cells moved from the edge of the scratch towards the center.

Table 2. Effect of the grandiflorenic acid from the leaves of *W. trilobata* on *in vitro* scratch assay using BJ human fibroblast.

Treatment	Dose (µg/mL)	Length between the scratch (µm)			% Migration rate of cells	
		Day 0	Day 1	Day 2	Day 1	Day 2
Negative control	-	336.3±8.5	166±9.6*	73.8±4.6*	50.6±2.9	78.1±1.4
Grandiflorenic acid	0.625	336±10.7	111.3±3.1*	56±7.0*	66.5±0.9*	83.3±2.1*
	1.25	331.8±9.0	95.8±6.5*	CC	71.5±1.9*	100*
	2.5	346.5±13.2	LC	CC	100*	100*
Proteoglycan(Positive control)	5	322.5±10.0	CC	CC	100*	100*
	10	345.8±6.6	CC	CC	100*	100*

Values are mean± S.D., (n=4); *P< 0.01 vs negative control group; LC: loosely closed; CC: complete closure of the scratch

Table 3. Effect of the grandiflorenic acid from the leaves of *W. trilobata* on *in vitro* scratch assay using keratinocytes HaCaT.

Treatment	Dose (µg/mL)	Length between the scratch (µm)			% Migration rate of cells	
		Day 0	Day 1	Day 2	Day 1	Day 2
Negative control	-	374.8±3.6	353.5±6.4*	319.3±9.9*	5.7±1.7	14.8±2.7
Grandiflorenic acid	0.625	374.0±10.3	355.0±7.5*	333.8±7.3*	5.1±2.0	10.8±1.9
	1.25	376.8±11.8	356.8±8.3*	322.3±13.4*	5.3±2.2	14.5±3.6
	2.5	375.0±9.3	356.0±13.2*	301.0±11.8*	5.1±3.5	19.7±3.1

Values are mean ± S.D., (n=4); *p<0.01 vs negative control group

resulting to 100% migration rate. Proteoglycan (5 and 10 µg/mL) showed a 100% migration rate on day 1. Migration rate of grandiflorenic acid and proteoglycan was found to be two times higher than that of the negative control. The grandiflorenic acid and proteoglycan treatment restored the BJ fibroblast cells to a confluent or near confluent state within 24 h, in contrast to the negative control (more than 48 h).

The presence of grandiflorenic acid (5, 2.5, and 1.25 µg/mL) did not cause an increased number of keratinocytes in the denuded area when comparing either days 1 or 2 to the negative control (Table 3). As shown in Figure 5 incubation time of two days did not result in the migration of the cells in the denuded area. The grandiflorenic acid (2.5 µg/mL) showed a 19.7% and control group 14.8% migration rate on day 2. The results indicating no significant activity of grandiflorenic acid on keratinocytes proliferation.

3.5 Effect of grandiflorenic acid on cell viability, and nitric oxide using Raw 264.7 cells

The viability of Raw 264.7 cell lines was estimated after being challenged with grandiflorenic acid (10-0.08 µg/ml). The viability of Raw 264.7 cells was found to be more than 90% (Figure 6) at concentration between 2.5-0.08 µg/ml. Nitric oxide is usually produced during inflammatory conditions such as wound healing by the inducible isoform of the enzyme NO synthase. Production of NO by Raw 264.7 cells exposed to grandiflorenic acid could not be detected in culture supernatants.

3.6 Effect of grandiflorenic acid on TNF-α and IL-1β using Raw 264.7 cells

Figures 7 and 8 represent the amounts of IL-1β and TNF-α released from the Raw 264.7 cells after grandiflorenic acid activation. The level of IL-1β produced by Raw 264.7 cells after exposure to grandiflorenic acid at a concentration of 2.5-0.16 µg/ml was 6.5-7.5 pg/ml, whereas the levels of TNF-α produced by Raw 264.7 cells after exposure to grandiflorenic acid was in range of 50-32 pg/ml. The LPS-activated Raw 264.7 cells produced inflammatory cytokines at significantly higher levels than Raw 264.7 cells activated by grandiflorenic acid (p<0.01). The production of IL-1β and
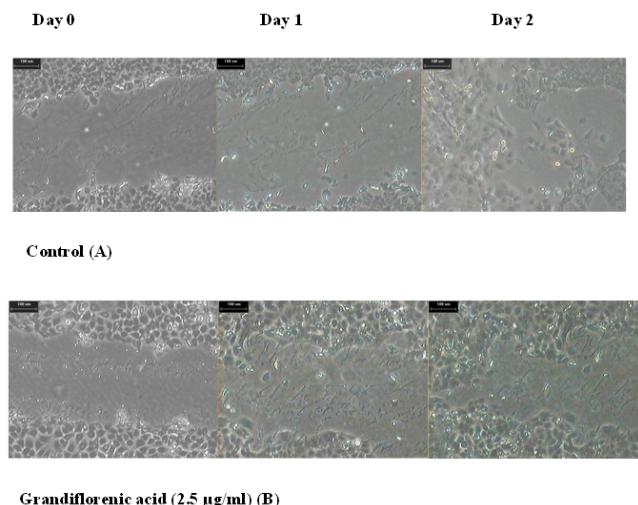
Figure 5. Measurement of cell migration in the *in vitro* scratch assay. The human keratinocytes HaCaT subjected to scratch and treated with negative control (A), and grandiflorenic acid (B). Images captured at 10x magnification using a light microscope at day 0, 1 and 2 after incubation. The rate of migration was measured by quantifying the total distance that the cells moved from the edge of the scratch towards the center.
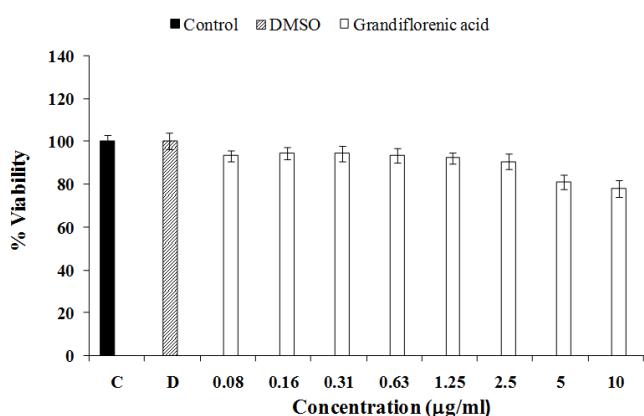
Figure 6. Percentage of Raw 264.7 cells surviving after 24 h incubation at 37°C with 5% CO_2 and treated with grandiflorenic acid at various concentrations. Data expressed as mean ± s.d., (n = 4).
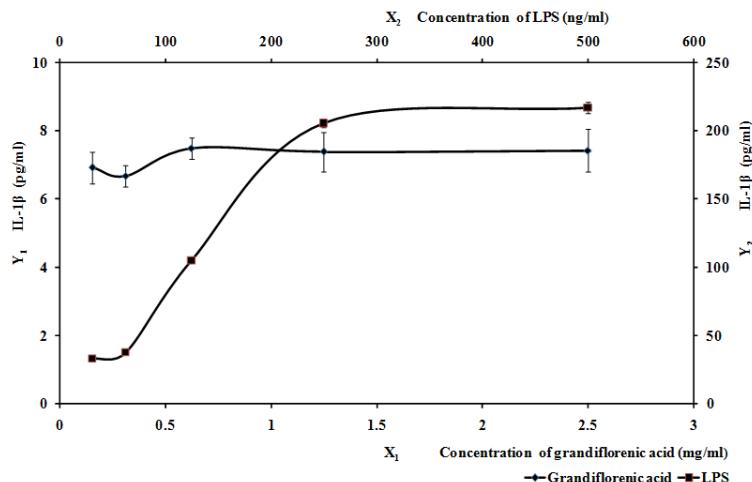
Figure 7. The effect of various concentrations of grandiflorenic acid on IL-1β production in Raw 264.7 cell lines after 24 h of culture.
          Data expressed as mean ± s.d.,(n= 4).
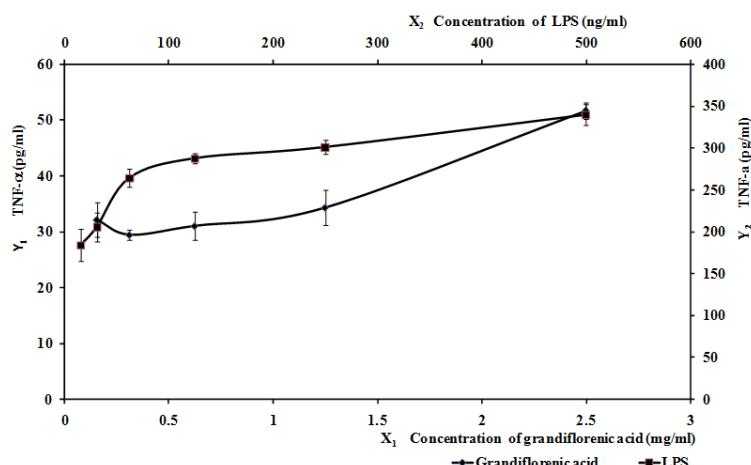
Figure 8. The effect of various concentrations of grandiflorenic acid on TNF-α production in Raw 264.7 cell lines after 24 h of culture.
          Data expressed as mean ± s.d.,(n= 4).

TNF-α by Raw 264.7 cells exposed to grandiflorenic acid could be detected at a very low level, while very high levels of IL-1β and TNF-α were generated from Raw 264.7 cells exposed to LPS.

3.7 Effect of grandiflorenic acid on hemolysis

In vitro hemolytic activity on human erythrocytes was determined using grandiflorenic acid at concentrations of 1, 2, 4, 6 and 8 μg/ml. None of the doses of grandiflorenic acid possessed hemolytic activity against human RBC upto 24 h.

4. Discussion

In vitro cytotoxicity tests are based on the idea that toxic chemicals affect basic cellular functions that are common to all cells, and that the toxicity can be measured by assessing cellular damage. Early screening of compounds for toxicity can help for identification as to whether they can be further utilized for evaluating biological activity (Barile *et al.*, 1994). The grandiflorenic acid did not produce any cyto-toxicity when treated with BJ human fibroblast, keratinocytes HaCaT and Raw 264.7 macrophage cells. Previous reports have suggested that terpenoids stimulated the growth of fibroblasts (Adetutu *et al.*, 2011). The results obtained from ent-kaura-9(11), 16-dien-19-oic acid (grandiflorenic acid), a diterpene from *W. trilobata*, also support the scientific findings.

The fibroblast is the connective tissue cell responsible for collagen deposition that is needed to repair the tissue injury. In normal tissues collagen provides strength, integrity and structure. When tissues are disrupted following injury, collagen is needed to repair the defect and restore anatomic

structure and function (Diegelmann and Evans, 2004). Early in the proliferation phase fibroblast activity is limited to cellular replication and migration. Around the third day after wounding the growing mass of fibroblast cells begin to synthesize and secrete measurable amount of collagen. Collagen levels rise continually for approximately three weeks. The amount of collagen secreted during this period determines the tensile strength of the wound. An increase in collagen production is an important factor for wound healing. Type-I collagen is the main collagen of bone, skin, tendon and newly healed wounds (Aramwit *et al.*, 2009a). Proteoglycans are glycoproteins that have a core protein with one or more covalently attached glycosaminoglycan (GAG) chains such as chondroitin sulfate and dermatan sulfate. They are widely distributed in skin and cartilage as a major component of extracellular matrix (Perrimon and Bernfield, 2001). They play a vital role in cell proliferation, migration and adhesion. Most of the growth factors and cytokines that are involved in wound healing are immobilized at the cell surface and in extracellular matrix through proteoglycan binding (Pratibha and Gupta, 2000).

In our study it was observed that the grandiflorenic acid above 10 μg/mL was cytotoxic to the fibroblast cells. The dose 10 and 5 μg/mL showed good viability and increase in collagen content, which was more than that of negative control. Further reduction of dose of grandiflorenic acid (2.5 and 1.25 μg/mL) the viability was increased (109 and 112%) with significant increase in collagen content. Further reduction of dose of grandiflorenic acid showed good viability but with reduced collagen content although that reduction was not greater than that of negative control, It could be concluded that dose of 2.5 and 1.25 are optimum dose with good viability and increased collagen content. The grandiflorenic acid and proteoglycan showed an increase in collagen production as compared to negative control group. The production of collagen with grandiflorenic acid was comparable to that produced by proteoglycan.

Wounds that alter the epithelia lining the body and internal organs represent a potential threat to the integrity of the organism. A rapid re-epithelialization provides a barrier from the external environment and prevents pathogenic and toxic insults. In the skin, re-epithelialization is accomplished by the migration of epidermal cells, the keratinocytes, into the wounds. It is defined as the reconstitution of an organized, stratified, and squamous epithelium that permanently covers the wound defect and restores functions. The regeneration of a functional epidermis depends on the reconstitution of the dermoepidermal junction (DEJ), which anchors the epidermis to the dermis, and on the terminal differentiation of keratinocytes into a protective cornified layer (Laplante *et al.*, 2001). Transforming growth factor-β (TGF-β) is a family of multipotent growth factors involved in the regulation of cell proliferation, adhesion, migration, differentiation, and extracellular matrix deposition, and is therefore intimately involved in regulating wound healing and fibrotic scar formation (Leask and Abraham, 2004). The three mammalian isoforms,

TGF-β 1, 2, and 3, are structurally almost identical, and yet they play an important role in a number of biological processes. This is evident in healing as manipulation of the levels of the three TGF-βs affect the extent of scar tissue formation. By reducing the levels of TGF-β1 and 2 with neutralizing antibodies, or addition of TGF-β3, suppression of fibrosis and scar formation occurs in healing wounds (O'Kane and Fergusson, 1997). Amjad *et al* (2007) reported keratinocytes did not release detectable levels of TGF-β1 until 40-hour incubation, and even then the levels were very low (40 pg/mL). In contrast, keratinocytes released large amounts of TGF-β2 into the cultured medium, which increased progressively reaching > 500 pg/mL by 50 hours, but did not release detectable levels of TGF-β3. TGF-β is an important regulator of the extracellular matrix (ECM), stimulating fibroplasia and collagen deposition, inhibiting ECM degrading proteases and upregulating the synthesis of protease inhibitors. Since all these processes are integral to wound healing, the role of TGF-β in wound healing and regulation of their activity is of major clinical significance (O'Kane and Fergusson, 1997). However, grandiflorenic acid did not show stimulatory activity on HaCaT keratinocytes and on levels of TGF-β2. It may act directly on fibroblast for its wound-healing activity.

The spreading and migration capabilities of BJ human fibroblast and keratinocytes HaCaT cells were assessed using a scratch wound assay which measures the expansion of a cell population on surfaces. One of the major advantages of this method is that it mimics to some extent migration of cells in vivo (Liang *et al.*, 2007). To estimate the wound re-epithelialization potential of a test substance and to have a quality control for the assay, a positive control (standard) is required. A number of growth factors and cytokines have been reported to affect fibroblast and keratinocytes motility directly or indirectly. Since the role of proteoglycan is similar to epidermal growth factor, it was taken as a positive control.

Altogether, results obtained showed that the scratch assay is a convenient and inexpensive tool to evaluate wound-healing activity. The scratch assay covers the second phase of wound healing characterized by a proliferation and migration of either keratinocytes or fibroblasts (Schafer and Werner, 2007; Gurtner *et al.*, 2008). Although the scratch assay cannot substitute for *in vivo* studies as a final proof for promoting wound healing, this study confirms its usefulness for gaining an insight into the potential of a compound to repair injured dermis. Fibroblasts, which are known to be involved in granulation and collagen metabolism, are stimulated by the grandiflorenic acid resulting in proliferation and migration within the wound site. The grandiflorenic acid seems not to act through keratinocytes stimulation as evident from the data showing no proliferation and migration within the wound site.

Nitric oxide is usually produced during inflammatory conditions such as wound healing by the inducible isoform of the enzyme NO synthase. Normally, large quantities of NO are generated during cell inflammation and have detrimental

effects on various cellular functions that are linked to cGMP. However, lower concentrations of NO can be potentially beneficial. In the presence of low concentrations of NO, fibroblast collagen synthesis together with total protein synthesis is enhanced, and this increase is independent of the collagenase pathway (Aramwit *et al.*, 2009b). Production of NO by Raw 264.7 cells exposed to grandiflorenic acid could not be detected in culture supernatants.

The Raw 264.7 cells are phagocytic cells playing important roles in defense. They undergo increased oxidative metabolism and release inflammatory cytokines (IL-1β or TNF-α) following their activation by phagocytosis (Shah *et al.*, 2002). These cytokines are involved in a variety of immunological functions as well as interactions with variety of target cells. It is widely accepted that IL-1, which is constitutively expressed in keratinocytes and accumulates in all epidermal layers, is a very important inflammatory mediator in the skin, and it is believed to be the main switch that initiates the inflammatory response. TNF-α is another proinflammatory cytokine that induces the expression of cutaneous and endothelial adhesion molecules to develop skin irritation and inflammatory responses. It is stored in epidermal mast cells and is also expressed by keratinocytes in response to irritation. The expression of TNF-α is transiently induced after treatment with various irritants and is independent of release of IL-1. Both IL-1 and TNF-α are first response cytokines, which initiate a cascade of events involving other second response cytokines, ultimately leading to inflammation. Previous studies have reported that IL-1 β and TNF-α at concentration less than 100 ng/ml are not toxic to keratinocytes (Aramwit *et al.*, 2009b). The grandiflorenic acid did not cause the Raw 264.7 cells to produce toxic cytokines and NO at a level that would cascade to other inflammatory mediators thereby reducing prolongation of inflammatory phase of wound healing.

Hemolytic assay was performed because compounds possessing wound-healing activity may not be useful in pharmacological preparations for open wounds if they possess hemolytic effect. Results showed no evidence of hemolysis with grandiflorenic acid upto 8 μg/ml. It showed wound-healing activity at dose below 3 μg/ml and this dose did not show any hemolysis. Thus, it should be possible to use grandiflorenic acid for open and chronic wounds.

5. Conclusion

The present study provides scientific evidence that grandiflorenic acid has potential wound-healing activity due to a combination of fibroblast and macrophage stimulating activity. It can be used as a monotherapeutic wound healing agent or may be combined with other wound-healing agent for synergistic effects.

Conflicts of interest

The authors report no conflicts of interest.

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Abbreviations:

DMEM, Dulbecco's Modified Eagle Medium;
DMSO, dimethyl sulfoxide;
EDTA, ethylene diamine tetraacetic acid disodium salt;
FBS, fetal bovine serum;
IL-1β, interleukin-1β;
LPS, lipopolysaccharide;
MEM, Minimum Essential Medium Eagle;
MTT, 3- (4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide;
NO, nitric oxide;
PBS, phosphate buffer saline;
ROS, reactive oxygen species;
SD, standard deviation;
TGF-β2, transforming growth factor- β2;
TNF-α, tumor necrosis factor-α.