In vitro stimulatory effect of grandiflorenic acid isolated from *Wedelia trilobata* (L.) leaves on L929 fibroblast cells

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Abstract:

*Wedelia trilobata* (L.) has been used in traditional medicine in the Caribbean and Central America for stubborn wounds, sores, swelling and arthritic painful joints. The aim of the study was to evaluate the activity of grandiflorenic acid obtained from leaves of *W. trilobata* on fibroblast cells which are responsible for wound healing. The grandiflorenic acid was assessed for its possible activity on fibroblasts cells by measuring collagen content, lactate dehydrogenase (LDH) activity after an oxidative stress induced by hydrogen peroxide, and an *in vitro* scratch assay. The grandiflorenic acid (2.5 µg/mL) increased collagen content of fibroblast L929 to 95.2 µg/mL as compared to the control (23.8 µg/mL). Cells treated with hydrogen peroxide exhibited 71% of LDH release whereas cells treated grandiflorenic acid (2.5 and 1.25 µg/mL) and then treated with hydrogen peroxide showed less percent release of LDH (25 and 28%) indicating protection of cell membrane integrity. The grandiflorenic acid (2.5 µg/mL) induced a 98.9% migration rate in the *in vitro* scratch assay on day 1, while control showed 57.9% migration rate on day 1. The present study provides scientific evidence that grandiflorenic acid has stimulatory effect on L929 fibroblast cells indicating its potential for wound healing activity.

Keywords: *Wedelia trilobata*; Grandiflorenic acid; Fibroblast L929; Collagen
**Introduction**

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 activated scientists to examine these plants with a view to determine potential wound healing properties [1]. The medicinal value of these plants lies in their bioactive phytochemical constituents that produce definite physiological actions on the human body [2]. These constituents include various members of chemical families like alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, and phenolic compounds [3].

*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 elevation [4, 5]. 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 [6, 7].

*W. trilobata* leaves were utilized to clarify its traditional use by a scientific investigation. The ethyl acetate fraction from ethanolic extract of *W. trilobata* leaves displayed antibacterial and fibroblast stimulatory activities thereby suggesting potential wound healing properties [8]. 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 [9]. These activities could play some role in its effect on tissue repair. Fibroblast first appears in the wound on the third day after injury. In the wound they proliferate profusely and produce the matrix proteins hyaluronan, fibronectin, proteoglycans and type 1 and 3 procollagen. By the end of the first week, abundant extracellular matrix accumulates which further supports cell migration and is essential for the repair process. Collagens are important components in all phases of wound healing. They are synthesized by fibroblasts and impart integrity and strength to all tissues and play a key role in proliferative phases of repair. Collagens act as a foundation for the intracellular matrix formation within the wound. Fibroblast is an important factor in wound healing and repair. Our previous studies on fibroblast revealed increase in growth and proliferation [10]. The present study was to evaluate some more activity of grandiflorenic acid on dermal fibroblasts that would influence measures in wound management and obviate wound infections.

**Materials and methods**

**Cell lines, chemicals and biochemicals**

The mouse fibroblast cell line L929 (Chinese Academy of Preventive Medical Sciences, Beijing, China), was cultured in Dulbecco’s Modified Eagle Medium (DMEM, Gibco®, Grand Island, NY, USA) containing 10% fetal bovine serum (FBS, Gibco®, Grand Island, NY, USA) and antibiotics (100 U penicillin and 100 U/mL streptomycin, Gibco®, Grand Island, NY, USA) under 5% CO₂ at 37°C. The media was changed every second day. When the cells reached confluence, they were harvested using 0.05% trypsin-EDTA (Gibco®, Grand Island, NY, USA), and fresh culture medium was added for producing single cell suspensions for further incubation. All the solvents, chemicals and biochemicals used were of analytical grade.

**Plant material collection, extraction and isolation**

The leaves of *W. trilobata* were collected from the campus of Prince of Songkla University, Thailand. The whole plant was first authenticated by Dr. Supreeya Yuenyongsawad and a voucher specimen (SKP051232001) was deposited at the herbarium of Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, PSU, Thailand.

The leaves of *W. trilobata* were shade dried, coarsely powdered and defatted with hexane. The defatted marc was subjected to soxhlet extraction with 95% ethanol. The ethanolic extract obtained was subjected to silica gel column and eluted with hexane, ethyl acetate and chloroform: methanol respectively. The ethyl acetate fraction (WEA) was further separated...
using a silica gel column. Five fractions were obtained (WEA-A, B, C, D and E). All fractions were subjected to antibacterial and fibroblast proliferation activity. The fraction WEA-B showed promising activity and thus was purified by recrystallization from cold methanol to afford a white crystalline powder. The isolated compound was identified as grandiflorenic acid (ent-kaura-9(11), 16-dien-19-oic acid) from leaves of *W. trilobata* [9]. The grandiflorenic acid was then further subjected to evaluate its wound healing potential.

**In vitro assays relevant to wound healing**

The grandiflorenic acid at 10-0.08 µg/ml was evaluated for *in vitro* cytotoxicity test using L929 fibroblast. The dose of 2.5 µg/ml and below produced cell viabilities more than 97%. Therefore, for further evaluation of activity of grandiflorenic acid on L929 fibroblast dose range selected was from 2.5 µg/ml and below.

**Determination of soluble collagen production using L929 fibroblast**

The soluble collagen was determined by the method described by Balekar *et al.* (2012a) [8]. Fibroblast L929 cells were seeded at an initial concentration of 2 x 10^4 cells/mL in a 96 well plate in DMEM containing 10% FBS. After 24 h, the culture medium was replaced by fresh medium. The stock solutions of grandiflorenic acid (100 mg/mL) were further diluted to 1 mg/mL with phosphate buffer saline (PBS) and further dilutions were made in culture medium to achieve a final concentration in each well. 2.5-0.625 µg/mL of grandiflorenic acid. Cells without a drug sample served as negative controls and proteoglycan-IPC (Ichimaru Pharmcos Co. LTD., Gifu, Japan) (10 and 100 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 37°C with 5% CO₂, the media in the wells was removed and the cells were treated with 100 µL of complete DMEM containing 0.5 mM of H₂O₂ and incubated at 37°C with 5% CO₂ for 24 h. At the end of the incubation, medium was collected and assayed for LDH release using cytotoxicity detection kit (LDH) from Roche Diagnostics, Mannheim, Germany. All experiments were done in quadruplicate. The LDH was quantitatively recorded at 490 nm.

\[
\text{% Release of LDH} = \left( \frac{A_{\text{exp}} - A_{\text{control}}}{A_{\text{total}} - A_{\text{control}}} \right) \times 100 \quad (1)
\]

Where A_{control} is the absorbance of the negative control group, and A_{exp} is the absorbance of the test group, and A_{total} is the absorbance of the H₂O₂ treated group.

**In vitro scratch assay using L929 fibroblast**

Migration of cells is integral to many physiological processes, such as tissue regeneration, and wound healing. When the cell monolayer is scratched, it responds to the disruption of cell-cell contacts by increasing the
concentration of growth factors and cytokines at the wound edge, thus initiating proliferation and migration of different cell types, such as keratinocytes and fibroblasts [12]. The cells (5 x 10^4 cells/mL) in DMEM containing 10% FBS were seeded in a 6-well plate [8]. Once the confluent monolayer was formed, a linear scratch was generated in the monolayer with a sterile pipette tip. Any cellular debris was removed by washing with phosphate buffer saline (PBS) and replaced with 2 mL of DMEM containing grandiflorenic acid (2.5, 1.25, 0.625 µg/mL) for fibroblast L929, and proteoglycan-IPC (10 and 100 µg/mL) was used as positive control and DMEM without sample served as a control. Photographs were taken at a 10x magnification using a microphotograph (Olympus CK2, Japan) on day 0, then plates were incubated at 37°C with 5% CO2 and photographs were taken at days 1 and 2. The images acquired for each sample were further analyzed quantitatively by using computing software (ImageJ1.42q/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 scratch were made (left and right) and per 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

\[
\% \text{ Migration rate} = 100 \times \frac{\text{Average distance between scratch (Day 0)} - \text{Average distance between scratch (Day 1 or 2)}}{\text{Average distance between scratch (Day 0)}}
\]

Samples were in quadruplicates. Percent rate of migration obtained from all four wells were averaged and recorded.

**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 quadruplicates.

**Results and Discussion**

**Chemical elucidation of the isolated compound**

The isolated compound was identified as grandiflorenic acid (ent-kaura-9(11), 16-dien-19-oic acid) (Fig. 1) by FT-IR, ^1^H-NMR, ^13^C NMR spectra [9].

**Soluble collagen content from fibroblast L929 preteated with grandiflorenic acid**

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 [13]. 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 [14]. The results showed that collagen type I production in L929 cells increased significantly after treatment.

![Figure 1](https://example.com/figure1.png) Chemical structure of grandiflorenic acid (ent-kaura-9(11), 16-dien-19-oic acid) from leaves of *W. trilobata*
with grandiflorenic acid at concentrations 2.5 and 1.25 µg/mL (p < 0.01) as compared to the control. Collagen production by fibroblast cells when treated with proteoglycan (100 and 10 µg/mL) was 101 and 33.6 µg/mL (Table 1). The production of collagen with grandiflorenic acid 2.5 µg/mL was 95.2 µg/mL and was comparable to the collagen produced by proteoglycan (100 µg/mL) treatment. It is evident from the data that grandiflorenic acid (2.5 µg/mL) could stimulate collagen production from fibroblast cells resulting in increasing tensile strength during tissue repair.

**Effect of grandiflorenic acid on LDH activity**

Hydrogen peroxide, one of the major reactive oxygen species, is produced at a high rate as a product of normal aerobic metabolic pathway to cope with oxidative stress and reflected from an increase in LDH activity. The LDH activity of cells subjected to oxidative stress increased significantly when compared to normal one which indicates loss of membrane integrity [15]. Fig. 2 depicts the percentage of LDH activity released into the medium in normal cells, cells treated with 0.5 mM hydrogen peroxide treated cells as compared to untreated control and cells pre-incubated with grandiflorenic acid (2.5-0.08 µg/mL). The percentage LDH release from L929 fibroblast cells pretreated with grandiflorenic acid (2.5-0.08 µg/mL) after H2O2 treatment was found to be 25-72%. One-way analysis of variance revealed that there was an overall significant difference in percent LDH release between the control, H2O2 treated, and grandiflorenic acid treated fibroblasts (p < 0.01). Subsequent multiple comparisons by the Tukey’s test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Conc. (µg/mL)</th>
<th>Collagen production (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-</td>
<td>23.8 ± 1.5</td>
</tr>
<tr>
<td>Grandiflorenic acid</td>
<td>2.5</td>
<td>95.2 ± 2.8*</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>45.2 ± 1.9*</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>15.3 ± 1.1</td>
</tr>
<tr>
<td>Proteoglycan</td>
<td>100</td>
<td>101.1 ± 2.1*</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>33.6 ± 2.5*</td>
</tr>
</tbody>
</table>

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

Table 1 Collagen type-I production in the fibroblast cell line L929 when treated with various concentration of grandiflorenic acid from the leaves of *W. trilobata*.

![Image of Figure 2](image-url)
indicated that percent LDH release was significantly lower (p < 0.01) after treatment with hydrogen peroxide when compared with the control and grandiflorenic acid treated fibroblasts. The control group showed very small amount of LDH release (4%). It was observed that concentrations of 2.5, and 1.25 µg/mL showed percentage LDH release of 25 and 28% whereas at lower doses (0.63-0.31 µg/mL) the percentage LDH release was over 35%. The lowest dose of grandiflorenic acid (0.08 µg/mL) showed maximum release of LDH i.e. 72% which was similar to the percentage LDH release from cells treated with H₂O₂ (71%). Cells treated with grandiflorenic acid at 2.5 and 1.25 µg/mL showed low percent release of LDH indicating protection of cell membrane integrity.

**Effect of grandiflorenic acid on in vitro scratch assay using fibroblast L929 cells**

The spreading and migration capabilities of fibroblast L929 cells was 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 [12]. 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. Proteoglycans are glycoproteins that have a core protein with one or more covalently attached glycosaminoglycan (GAG) chains such as chondroitin sulfate and dermattan sulfate. They are widely distributed in skin and cartilage as a major component of extracellular matrix [16]. 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 [17]. Since the role of proteoglycan is similar to epidermal growth factor, it was hence taken as a positive control. The grandiflorenic acid was evaluated for changing the rate of migration of the fibroblast L929 cells. The cellular proliferation and migration of fibroblasts was studied on day 0, 1 and 2. (Fig. 3 and Table 2). The presence of grandiflorenic acid (2.5, 1.25, 0.625 µg/mL) caused an increase in number of fibroblasts in the denuded area when comparing either days 1 or 2 to the control (p < 0.01). As shown in Fig. 3, 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 214.4 ± 4.8 µm (day 0), and 2.1 ± 0.2 µm (day 1) respectively. The control was 212.4 ± 4.3 µm (day 0), 80.7 ± 4.1 µm (day 1) and 32.7 ± 1.1 µm (day 2), respectively and proteoglycan (10 µg/mL) was 212 ± 3.7 µm (day 0), and complete closure of gap on day 1, respectively. The grandiflorenic acid (2.5 µg/mL) showed a 98.9% migration rate on day 1 and 100% closure on day 2. Proteoglycan (100 and 10 µg/mL) showed a 100% migration rate on day 1. Migration rate of grandiflorenic acid and proteoglycan was found to be 1.7 times higher than that of the control. The grandiflorenic acid and proteoglycan treatment restored the fibroblast L929 cells to a confluent or near confluent state within 24 h, in contrast to the control (more than 48 h).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (µg/mL)</th>
<th>Length between the scratch (µm)</th>
<th>% Migration rate of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Day 0</td>
<td>Day 1</td>
</tr>
<tr>
<td>Control</td>
<td>-</td>
<td>212.4 ± 4.3</td>
<td>80.7 ± 4.1</td>
</tr>
<tr>
<td>Grandiflorenic</td>
<td>2.5</td>
<td>214.4 ± 4.8</td>
<td>2.1 ± 0.2°</td>
</tr>
<tr>
<td>acid</td>
<td>1.25</td>
<td>215.3 ± 5.7</td>
<td>10.9 ± 1.9°</td>
</tr>
<tr>
<td></td>
<td>0.625</td>
<td>210.7 ± 5.7</td>
<td>23.9 ± 1.8°</td>
</tr>
<tr>
<td>Proteoglycan</td>
<td>100</td>
<td>206.6 ± 4.4</td>
<td>CC</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>212 ± 3.7</td>
<td>CC</td>
</tr>
</tbody>
</table>

Values are mean ± S.D. (n=4); *p < 0.01 vs control group; CC: complete closure of the scratch.
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 [18, 19]. 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.

**Conclusion**

Fibroblasts play a key role in tissue healing by producing the majority of extracellular matrix components, favouring granulation tissue formation, and stimulating re-epithelialization. Grandiflorenic acid from *W. trilobata* (L.) leaves has indicated role in wound healing and repair through the stimulation of fibroblast which was evident by collagen production, LDH activity and in vitro scratch assay.

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Declaration of interest

The authors report no conflicts of interest.

References


