THE WOUND HEALING ACTIVITY OF MIKANIA MICRANTHA ETHANOLIC LEAF EXTRACT

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ABSTRACT

\textit{Mikania micrantha} Kunth is tropical forest known as “selaput tunggui” in Malaysia. Leaves of \textit{M. micrantha} are widely used as poultice for wound healing. The aim of this study is to investigate the ability of \textit{M. micrantha} ethanol extract (MELE) in accelerating the wound healing process. Cell viability and scratch assay were carried out in BJ fibroblast cells treated with various concentrations of the extract. Trolox (100 µM) was used as positive control. Results on the MTT assay showed low cytotoxic effect after 24, 48 and 72 h incubation (IC\textsubscript{50} > 150 µg/ml). Wound healing process was significantly (P \leq 0.005) accelerated after treatment with MELE (7 and 15 µg/ml). In conclusion, MELE has great potential in accelerating wound healing process. Further studies will be carried out to identify the active compounds response in wounded tissues and its molecular mechanism.

Keywords: \textit{Mikania micrantha}; wound healing; cell proliferation; scratch wound assay.

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1. INTRODUCTION

1.1. Wound Healing

Wound is defined as any failure or ruptured of cellular and anatomic or working continuity of living tissues [1]. Healing of wounds is a biological process that involves natural restoration of tissue injury and often terminated by scar formation [2]. The process of wound healing involves multiple cell population, the extracellular matrix and the action of soluble mediators such as growth factors and cytokines. Healing of wound involves physiological response to the injury by replacing the damaged tissue by connective tissue, which then forms a scar to restore tissue integrity. The mechanisms of wound healing process are coagulation and haemostasis, inflammation, proliferation and wound remodeling with scar tissue formation [3].

Medicinal plants have been used for centuries as medicines to treat many disorders, mainly due to the therapeutic constituents of the plants. The potential medicinal value of some plant extracts in promoting wound healing comes from the ability of the extract to regenerate tissue at the wound sites in the shortest time possible with minimal pain and discomfort and fine scar with high tensile strength [4].

1.2. Mikania Micrantha

*M. micrantha* possesses several pharmacological properties and can be used to prevent and cure several diseases such as diarrhea, diabetes and stroke [5]. Traditionally, *M. micrantha* is used for wound dressing and healing of sores [6]. This plant has been considered as one of the “world worst” invader in agriculture especially in cacao, oil palm and rubber plantation in Malaysia. It is a fast growing, perennial creeping weed found widespread in this region [7]. However, many pharmaceutical laboratories acknowledged plants from the Mikania family as one of the bestselling natural product in the world [8].

This plant contains high antioxidant properties such as sesquiterpene lactones and phenolic compounds [9]. These compounds can scavenge free radicals, which in excessive may lead to oxidative stress that are responsible for a number of human disorders. Tannins and flavonoids have been reported to activate collagen synthesis and increase the number of granulation tissue, thus increase the wound healing rate. Extracts from seed and leaves of *M. micrantha* have the ability to inhibit the mouse ear inflammation in response to topical application of
12-O-tetradecanoylphorbol-13-acetate (TPA) and significant antibacterial and anti-inflammatory properties [10]. This plant has also been proven to possess as antistress properties [11].

On that note, a wide range of biological activities has been done on *M. micrantha* and isolation of the potential compounds were conducted. However, no scientific investigation was conducted on the wound healing effect from *M. micrantha* and its dermal toxicity. Therefore, work was carried out to explore the potency and acceleration rate of wound healing activity from *M. micrantha* ethanolic leaf extract (MELE), *in vitro* using fibroblast cells.

2. METHODOLOGY

In this study, *Mikania micrantha* ethanolic extract were investigated to determine its potential as a wound healing agent tested on fibroblast cells. The effect of the extract on wound healing was determined and compared with trolox, a water soluble vitamin E analogue (standard reference). Schematic overview of the methodology design of this study is represented in Fig. 1.

![Fig.1. The schematic overview of the study](image)

2.1. Plant Extract Preparation

The fresh leaves of *M. micrantha* were collected around Klang Valley area and then washed with tap water, then followed with distilled water. The leaves were left to dry for 5 to 7 days in the incubator at 40°C and finely grind to powder with Waring blender (E8000, USA). Ground sample (100 g) was soaked in 1000 ml of 95% ethanol in a conical flask and
occasional shaken for 3 days at room temperature. Then, filtered using muslin cloth followed by filter paper (Whatman No.1). The filtrates were then evaporated using rotary evaporator at 45°C to ensure complete removal of residual solvent. The ethanolic extract was kept in -20°C until further use.

2.2. Cytotoxicity Activity (MTT Assay)

The BJ human fibroblast cells (ATCC CRL-2522, Rockville, MD, USA) at density 1×10^6 cells/ml seeded in 96-well plate with DMEM complete media for 24 h in an incubator (37°C, 5% CO_2). The cells were treated with 0.1, 1.0, 10, 100 and 1000 µg/ml extract and trolox (100 µM) as positive control. Cell viability was determined based on tetrazolium dye (20 µl) of MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (colourimetric method for determining the number of cell deaths in cytotoxicity assays) was added onto the cells and incubated 4 hr at 37°C. At the end of incubation, DMSO (100 µl) was added to dissolve the formazon. The plates were then read using a microplate reader (Tecan Infinite M200, Switzerland) at a wavelength of 490 nm. The percentage (%) of cell viability was determined using the following equation:

\[ \text{Cell Viability} \% = \{100 - \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \} \times 100 \]

where \( A_{\text{control}} \) = Absorbance of the control (cells) and \( A_{\text{sample}} \) = Absorbance in the presence of sample (treatment compound)

Then, the graph percentage of cell viability versus log concentration (µM) was plotted to determine the IC_{50} (inhibitory concentration at 50%) values of the extract at various time points. IC_{50} values of < 100 µM were considered potentially cytotoxic.

2.3. Scratch Assay

The BJ human fibroblast cells were seeded in 6-well plates, grown until reaching cell confluence of 90-95%. Then, a linear scratch was made 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 DMEM containing MELE (7, 5, 30 and 60 µg/ml). Trolox (100 µM) served as a positive control and DMEM without sample served as a negative control. Cell migration was assessed by microphotography at 0, 18 and 24 h. By comparing the images from 0 to 24 h, the distance of each scratch closure was determined and the percentage migration rate was calculated as previously described [20].
% Migration rate = \frac{\text{Average distance between scratch (0 h)} - \text{Average distance (x)}}{\text{Average distance between scratch (0 h)}} \times 100

2.4. Cell Cycle Analysis

Cell cycle analysis was carried out to distinguish cells in the different phases of the cell cycle by using flow cytometer (BD Facscalibur, UK). Approximately $5 \times 10^5$ cells/ml of BJ human fibroblast cells were plated into 6-well plates. The cells were incubated for 24 h at 37°C in a humidified 5% CO$_2$ atmosphere. Then, the cells were treated with several concentrations of MELE (7, 15, 30 and 60 µg/ml). Trolox (100 µM) acted as a positive control and DMEM without sample acted as a negative control. Then, the cells were incubated further for 24 h. Media in the 6-well plates were removed and the cells were washed with 1 ml of phosphate buffer saline (PBS). Next, the adherent cells were trypsinized with 500 µl of trypsin solution and incubated for 5 min at 37°C in 5% CO$_2$ atmosphere to remove the cells. In the centrifuge tube, the aliquot was put in with 1 ml of media to naturalize trypsin, then centrifuged at 2000 rpm for 5 min. Supernatant were then discarded. The cells were fixed with cold 70% ethanol for 24 h. After that, the cells were rinsed 3 times with PBS and resuspended in 1 ml of permeabilizing solution (Triton 100X (0.25%), sodium azide (0.01%) and RNAsa A (100 µg/µl Sigma-Aldrich) in PBS for 10 min. Next, the cells rinsed once with PBS were resuspended with 1 ml of PBS with propidium iodide (2.5 mg/ml) and incubated for 15 min at 4°C. Cell cycle distribution profiles were analyzed using a flow cytometer (BD FACSCanto, Becton Dickinson) at a slow flow rate and with doublet discrimination. The PI fluorescence signal at FL2-A peak versus counts was used to determine the cell cycle distribution and the data were analyzed using the Modfit software. The percentage of cells in G0/G1, S and G2/M was determined.

3. RESULTS AND DISCUSSION

3.1. Cytotoxic Effects of Mikania Micrantha Ethanol Extract

The cell viability of fibroblast cells treated with various concentration $M. micrantha$ ethanolic leaf extract (MELE; 0.1, 1.0, 10,100 and 1000 µg/ml) was determined by MTT assay. The result showed no cytotoxic effect on fibroblast cells at the lower concentration up to (200 µg/ml) in Fig 2. After 24, 48 and 72 h of incubation with the extract, IC$_{50}$ values were
determined as tabulated in Table 1.

![Graph showing cell death percentage](image.png)

**Fig.2.** Effects of fibroblast cells treated with MELE concentration at 0.1, 1, 10, 100 and 1000 µg/ml on the percentage of the cell death, evaluated by MTT assay in incubation time at 24 h at 37°C (±5% CO₂). Data were presented as mean±S.E.M

<table>
<thead>
<tr>
<th>Time Incubation (h)</th>
<th>MELE, IC₅₀ (µg/ml)</th>
</tr>
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<tbody>
<tr>
<td>24</td>
<td>233.97 ± 12.74</td>
</tr>
<tr>
<td>48</td>
<td>237.09 ± 10.29</td>
</tr>
<tr>
<td>72</td>
<td>188.00 ± 10.29</td>
</tr>
</tbody>
</table>

Table 1. IC₅₀ values of *M. micrantha* ethanolic leaf extract determined by MTT assay (n = 3)

MELE had almost similar IC₅₀ values for both 24 and 48 h incubation (IC₅₀ =233.97±12.74 and 237.09±10.29 µg/ml, respectively) indicating low cytotoxicity on the fibroblast. However, at 72 h incubation, the IC₅₀ value was 188.00±10.29 µg/ml, lower than the 24 and 48 h incubation which may be due to the sensitivity of the cells towards the active compounds in MELE and the tissue specific response after a much longer exposure [12]. Previous finding reported that the higher the concentration of flavonoids in the extract contributed to the antiproliferation effect on normal and tumor cells [13-14]. This was also supported by a study done by [15] on *Aloe vera* that showed isolated flavonoids at lower concentration were responsible for proliferation activity and increased extracellular matrix proteins.
3.2. Wound Healing on Fibroblast Cells by Scratch Assay

To validate the traditional use of *M. micratha* leaves in accelerating wound healing, scratch assay was conducted on BJ fibroblast cells to measure the gap closure rate at different time points, 0, 18 and 24 h. Static images were captured as previously described. MELE (7, 15, 30 and 60 µg/ml) caused an increased number of fibroblasts in the denuded area compared to the negative control (p < 0.001) (Fig. 3, Fig. 4 and Table 2). The length between the scratch mark edges with MELE (7 µg/mL) was 170.03 ± 9.54 mm (0 h) and there was closure of the gap after 18 h (42.62 ±1.93 mm) and 24 h (11.9 ± 3.27) mm. The length between the scratch mark edges with negative control was 184.64±8.32 mm at 0 h, 83.51±4.42 mm at 18 h and 48.77±4.67 mm at 24 hr, and with trolox (100 µM) was 171.95±2.09 at 0 h, 40.97± 1.93 mm at 18 h and 11.28 ±3.27 mm at 24 h.

As shown in Fig. 2, an incubation time of 24 h, resulted in the highest number of migrated cells in the denuded area. MELE at 7 µg/ml showed the best closure of gap at 24 h compared to 15, 30 and 60 µg/ml of MELE at the same time of incubation. Migration rate of MELE (7 µg/ml) after 24 h was 93%, while MELE at 15, 30 and 60 µg/ml of were 89.34%, 82.69% and 80.59%, respectively. Migration rate of MELE and trolox were found to be higher than that of the negative control. Treatment of MELE and trolox fixed the BJ fibroblast cells to a near confluent state within 24 h, in contrast to the negative control. From our results, the lowest concentration of the extract and trolox showed the highest cell proliferation and migration rate.

The same was observed in a study done by [14] that reported *Achillea eriophora* extract at the lowest concentration had the best proliferation and migration stimulatory effect on wounded human fibroblasts [16]. Another study also reported that 25 µg/ml of *Aloe vera* L. had a higher migration rate compared to 50 and 75 µg/ml of the extract [17]. Thus, indicating that phytochemicals in the plant extracts such as phenolics, flavonoids or glycoproteins have antioxidant and antimicrobial properties that play an important role in enhancing the proliferation and extracellular matrix of human dermal fibroblast to accelerate wound closure [18].
Fig. 3. Effect of MELE on BJ fibroblasts cells on scratch gap closure. The gap was assessed by microphotography Dino capture 2.0 at 0, 18 and 24 h after treatment with 7, 15, 30 and 60 μg/ml of MELE and trolox (100 μM). Data are represented as mean ± SEM in triplicates, one-way ANOVA followed by post-hoc Benferroni Test compared to control group.
Fig. 4. Microphotography using Dino capture 2.0 of BJ human skin fibroblast layer subjected to scratch assay after treatment with 7, 15, 30 and 60 µg/ml of MELE and trolox (100 µM) at 0, 18 and 24 h

Table 2. Effect of migration rate of various concentrations of MELE on BJ fibroblasts. Values are expressed as mean±SEM (n = 3)

<table>
<thead>
<tr>
<th>Types of Treatment</th>
<th>Observation Time</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>18 h</td>
</tr>
<tr>
<td>untreated</td>
<td>48.32±4.42</td>
</tr>
<tr>
<td>100µM trolox</td>
<td>76.28±4.94*</td>
</tr>
<tr>
<td>7µg/ml</td>
<td>74.93±1.93*</td>
</tr>
<tr>
<td>15µg/ml</td>
<td>70.25±1.18*</td>
</tr>
<tr>
<td>30µg/ml</td>
<td>65.94±6.22</td>
</tr>
<tr>
<td>60µg/ml</td>
<td>59.59±5.06</td>
</tr>
</tbody>
</table>

The P values are significantly different at P < 0.001 one-way ANOVA followed by post-hoc Benfferoni Test compared to control group.

3.3. Effects of Mikania Micrantha Ethanol Extract on BJ Fibroblast Cells by Cell Cycle

The effect of MELE on BJ fibroblast cells via cell cycle analysis was determined. As shown in Fig. 5, there was no significant difference observed compared to control after 24 h.
Interestingly, our results showed that treatment with 7 µg/ml (S phase; 40.62%±0.38, G2/M; 13.53 ±0.38), 15 µg/ml (S phase ;40.71% ±0.36 and G2/M phase; 13.69% ±0.48) and 30 µg/ml (S phase; 40.83% ± 0.36, G2/M phase 12.54% ± 0.36 respectively) of MELE did not show any significant difference compared to control. However, treatment with 60 µg/ml of MELE (S phase; 36.41% ± 0.27, G2/M phase 10.88 % ± 0.11) was significantly different compared to trolox (P = 0.002). The result suggested that MELE do not affect the cell cycle distribution and continuously increase in DNA synthesis and mitotic division in fibroblast cells. According to [16], most cell cycle arrest at G2/M is due to the cytotoxic properties contained in the plant. Hence, suggesting MELE to be non-toxic against fibroblast cells. In [19] also found that Drypetes klainnei stem bark extract at the concentration of 6 and 12 µg/ml, induced DNA synthesis and mitotic activity in 3T3 cell during cell migration compared to 25, 50 and 100 µg/ml which demonstrated the presence of apoptotic cells.

**Fig.5.** Cell cycle analysis of BJ fibroblast cells after 24 h treatment with 7, 15, 30 and 60 µg/ml of *M. micrantha* ethanolic leaf extract, Trolox 100 (µM). (a) Representative histograms. (b) Quantification of cells in specific cell cycle compartments. Data are represented as mean ± SEM in triplicates, one-way ANOVA followed by post-hoc Benfieroni Test, P < 0.05 compared to control group.
4. CONCLUSION

From the results of our study, *Mikania micrantha* ethanolic leaves extract (MELE) has great potential to be developed as a nutraceutical agent for accelerating wound healing that supports its traditional use. This would stimulate further research into identifying the active compounds, which induce the coordinated cell migration and proliferation response in wounded tissues. Hence, promoting a better perception of this fundamental homeostatic process.

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6. REFERENCES


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