Preliminary screening on wound healing potential of *Ocimum tenuiflorum* L. using *in vitro* assays

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**Abstract**

Herbal plants were used for food flavouring and food preservation and for the treatment of some illnesses. *Ocimum tenuiflorum* was one of the ancient herbal plants whereby its medicinal properties have been widely studied such as wound healing. Wound healing involves a sequence of recovery stages and many factors affect the wound healing process. The literature review showed that, leaves part was widely studied by researchers for its wound healing potential. The aim of this study was to screen the wound healing potential of other parts of the plants which are the stems and flowers in comparison to leaves using *in vitro* wound healing assays. Aqueous extraction for all the parts was done using the maceration method. The stems and flowers extracts did not show any promising wound healing effects through cell migration and angiogenesis process but showed significantly (*p*<0.05) higher effect via cell proliferation activity. Conversely, leaves extract have shown positive wound healing potency by significantly enhanced (*p*<0.05) the cell migration and angiogenesis activity. Overall, this experiment proved that the leaves own wound healing potency via cell migration and angiogenesis activity which could be beneficial in wound healing process. Thus, *O. tenuiflorum* leaves extract can be a favorable wound healing treatment yet, further detailed studies are required.

1. **Introduction**

Medicinal plants have been extensively investigated in the development of nutraceutical and herbal supplements because they are rich in bioactive compounds and essential oils (Amit et al., 2013). These plants have been used as traditional medicines to treat many illnesses and health problems (Chowdhury et al., 2017). The demand for these plants likely increases because they have less side effect compared to commercially used pharmaceutical drugs (Palla et al., 2012).

Among various types of medicinal plants, *Ocimum tenuiflorum*, an ancient plant, has been used traditionally to treat numerous illnesses (Pattanayak et al., 2010). This plant is known as the “Queen of Herbs” because it can produce a considerable amount of essential oils consisting of numerous aromatic compounds (Upadhyay et al., 2015). Commonly called as tulsí or holy basil, this plant belongs to the family of Lamiaceae (Cohen, 2014). Jain et al. (2015) indicated that almost all parts, including leaves, stems, flowers, roots, and fruits, of this plant, have medicinal properties. Previous scientific studies confirmed the plant’s medicinal properties, such as antioxidation (Rabeta and Lai, 2013), antidiabetes (Mousavi et al., 2016), anticancer (Lam et al., 2018), hepatoprotection (Lahon and Das, 2011), anti-inflammation (Kumar et al., 2015) and wound healing properties (Francis et al., 2017).

Wound healing is a tissue recovery process that occurs after injuries ensue (Khamlue et al., 2012). This process is complex because of its overlapped phases involving bleeding, inflammatory, proliferative, and remodeling phases (Mohamed Amin et al., 2015). Complete tissue recovery and skin function restoration are important factors in wound healing to regain skin integrity (Sasidharan et al., 2011).

Animal-based products may conflict with religious of wound patients’ beliefs (Eriksson et al., 2013). Researchers and manufacturers should consider the potential of plant-based wound healing product can benefit these patient groups. Therefore, this study was conducted to determine the wound healing potential of
different parts of *O. tenuiflorum* using in vitro assays.

### 2. Materials and methods

#### 2.1 Plant material collection and extraction

*O. tenuiflorum* plants were collected from the botanical garden, Perak. The plant was identified and authenticated by Dr. Rahmad Zakaria at School of Biological Sciences, Universiti Sains Malaysia (USM) (Herbarium number is 11400).

The collected plant materials were separated accordingly to their parts (leaves, stems, and flowers) and were weighed to obtain the initial weight and washed with running tap water. The washed materials were oven dried at 60°C for 24 hrs. Then the weight of the dried sample was recorded, and the materials were ground to fine powder mesh 60. The weight of the powdered material was recorded and stored in dark and air-tight container.

Sample (leaves, stem and flowers) extractions were done separately. The extraction steps were based on the effectiveness in wound healing study by Shetty et al. (2008) with some modification. Approximately 10 g of sample was mixed with 100 mL of distilled water in a conical flask. The mixture was then shaken in an orbital shaker at 160 rpm at 27°C for 24 hrs followed by centrifugation at 3500 rpm for 30 mins to obtain the clear extract. The supernatant was transferred to a beaker and evaporated using the oven at 60°C until semisolid extract was obtained. The obtained extract was stored in an airtight container until use.

#### 2.2 Cell culture maintenance

A human endothelial cell line, EA.hy926 cells (ATCC, Manassas, VA, U.S.A.), were grown in flask containing Dulbecco’s modified Eagle’s medium (DMEM) premixed with 10% fetal bovine serum (FBS), growth supplements, antibiotics consist of L-glutamine, and 1% penicillin-streptomycin. The flask was then incubated in 5% CO₂ at 37°C. Cells at 80-90% confluence were used for seeding and treatment for the experiment (Dao et al., 2016).

#### 2.3 Cell proliferation assay

The cells were seeded into each well of a 96-well plate in triplicate at a density of 5×10⁴ in 100 µL medium per well. The plate was then incubated for 24 hrs at 37°C in 5% CO₂. The medium was preincubated with concentrations of 12.5 µg/mL and 200 µg/mL of extracts treatment for 24 hrs. Then, the cell morphology images were taken using microscope following by a 10 µL of 5 mg/mL of 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyl Tetrazolium Bromide (MTT) reagent was added to each well and then incubated for 3-4 hrs. Finally, the medium was discarded, and the purple formazan was dissolved by adding 150 µL of dimethyl sulfoxide (DMSO) to all the wells including control (without treatment). The plate was gently swirled to mix well and incubated for 30 mins in dark area at room temperature. The cells proliferations were observed using TECON microplate reader at 570 nm with reference of 620 nm. Graph of absorbance against the number of cells was plotted to determine the cells proliferation as per standard methods (Muhammad et al., 2013).

#### 2.4 Cell migration assay

The cells were seeded into each well of a 6-well plate in triplicate and incubated at 37°C in 5% CO₂ until a confluent cell monolayer. Next, the media was pipetted out and discarded followed by, a small area was scratched using sterile 200 µL pipette tip, and the cells were then rinsed with phosphate buffer solutions (PBS) to remove the loosened debris of the cells. Two concentrations of extracts dissolved in media (12.5 µg/mL and 200 µg/mL) were added to the wells and the plate was incubated at 37°C in 5% CO₂. The distance between two layers of the scratched cells was inspected and captured using a digital camera attached to microscope and computer system at 0, 6, 12, and until the treatment wells cells closed. The distance of cells migration was analyzed using ImageJ software (Muhammad et al., 2013).

#### 2.5 Rat aorta ring assay

##### 2.5.1 Animal preparation

Three adult male Sprague-Dawley (SD) rats weighing around 200-230 g, age 8-10 weeks old were used in this experiment. All the rats were kept under 12 hrs light and dark cycle and allowed for free access to food and water. The experiment was carried out in accordance with Universiti Sains Malaysia (USM) animal ethics committee guidelines and approval. The ethical approval number is USM/IACUC/2018/ (111) (915).

##### 2.5.2 Preparation of assay media

Two parts of media were prepared according to Aisha et al. (2012). Part A media consists of 3 mg/mL of fibrinogen, 5 µg/mL of aprotinin and 1% L-glutamine dissolved in M199 serum-free growth media while Part B media consists of 20% FBS, 1% L-glutamine, 0.1% 6-aminoacaproic acid, 1% amphotericin and 0.6% gentamicin dissolved in M199 serum-free growth media. Thrombin was separately prepared at concentration 50 NIH U/I.
2.5.3 Isolation, seeding and treatments of rat aorta rings

The assay was done according to the method described by Samad et al. (2018) with slight modifications. Firstly, the rat was anesthetized using ketamine and xylazine intraperitoneally 40-100 mg/kg and 5-13 mg/kg BW, respectively. Then, a rat dissection was done to excise the aorta and the fibro-adipose tissue was carefully removed. The aorta was then rinsed and flushed with freshly prepared and filtered media. About 1 mm thickness, the aorta was sliced and seeded into each well of a 48-well plate (Coster Corning, USA) containing 500 μL of the part A media. The rings were adjusted to the center of each well and solidify using 10 μL of thrombin. The plate was incubated at 37°C under 5% CO₂ for at least 3 hrs. Then, the part B media which was premixed with the plant extracts with concentrations of 12.5 and 200 μg/mL, respectively was added to each well in triplicates. DMSO was used as a negative control. On the 4th day, the top layer of media was discarded and replaced with freshly prepared part B media premixed with the extract and incubated again. On day 5th, the sprouting blood vessels formed from aortic rings were observed and captured using a digital camera attached to microscope and computer system and data were analyzed using ImageJ software.

2.6 Statistical analysis

Data were expressed as mean ± standard error mean (SEM). Results were analyzed statistically using one-way ANOVA followed by Tukey post hoc test and p<0.05 is considered statistically significant.

3. Results

3.1 Cell proliferation assay

Figure 1 shows the cell proliferation effect of the EA.hy926 cell was significantly higher (p<0.05) in stems and flowers extracts at the concentrations of 12.5 μg/mL and 200 μg/mL, respectively when compared to negative control. Figure 2 shows that the proliferation effect of EA.hy926 cells after treated with different parts of O. tenuiflorum extracts. It can be seen that the number of cells was higher in 12.5 μg/mL treated stem extract when compared to the negative control group.

3.2 Cell migration assay

Figure 3 shows the cell migration effect of the EA.hy926 cell was significantly (p<0.05) enhanced up to 90% in leaves extract treated group at the concentration of 12.5 μg/mL within 12 hrs when compared to negative control group. Figure 4a that the leaves extract of O. tenuiflorum treated cells migrated and closes the scratch area almost 100% within 12 hrs at 12.5 μg/mL concentration when compared to negative control and other treatment groups. Figure 4b shows that the leaves extract of O. tenuiflorum treated cell migrated and closes the scratch area faster than stem and flower extracts treated group. The cell migrated faster in leaves extract and closes the wound about 90% when compared to negative control group within 12 hrs when treated at 200 μg/mL of concentration.
3.3 Rat aorta ring assay

Figure 5 shows the proangiogenic effect of the rat aorta ring was significantly increased ($p<0.05$) when treated with leaves extract at the concentration of 12.5 µg/mL. Figure 6a shows that the growth of blood vessels (angiogenesis) on day 5 was enhanced in *O. tenuiflorum* leaves extract treated group when compared to negative control and other treatment groups at the concentration of 12.5 µg/mL. Figure 6b showed that the growth of blood vessels (angiogenesis) was reduced when compared to negative control group on day 5 after treated with 200 µg/mL of different parts *O. tenuiflorum* extracts.

4. Discussion

Wound healing is a natural process of tissue or skin upon injury. It consists of four stages which are the bleeding, inflammation, proliferation, and remodelling stages (Lakshmi *et al.*, 2011). When the skin is cut or injured, bleeding may occur. Once the skin bleeds, the inflammatory phase subsequently starts. In this phase, blood vessels become constricted, leading to the release of inflammatory factors. In the proliferative phase, granulation tissue forms, and this process involves fibroblasts and angiogenesis. In the remodeling phase or the final part of wound healing, a collagen fiber is reformulated and improved, thereby enhancing the tensile strength of the wound (Panda *et al.*, 2011). Given that wound healing comprises different phases, other important factors, such as cell proliferation, cell migration, and angiogenesis, should be considered (Landén *et al.*, 2016). Shah *et al.* (2012) stated that cell proliferation and migration are necessary during wound epithelialization, which is the last part of the proliferative phase. For efficient wound healing, sufficient oxygen and nutrients should be transported regularly. This mechanism happens with the help of blood vessels; oftentimes, pre-existing blood vessels tend to rupture due to injuries, and angiogenesis or the formation of new blood vessels is needed to ease wound healing (Lali and Metcalfe, 2014).

Extracts from different parts of *O. tenuiflorum* were subjected to three different in vitro assays. Their wound healing potential was evaluated on the basis of their cell
proliferation, cell migration, and proangiogenic effects. Two of these assays focused on cell migration and angiogenesis and demonstrated that the leaf extract showed a prominent wound healing potential. This finding explained why most literature surveys have shown that leaves are the most commonly studied part of *O. tenuiflorum* (Goel et al., 2010; Gautam and Goel, 2013; Francis et al., 2017).

A previous study done by Asha et al. (2011), indicated that the potential wound healing properties of *O. tenuiflorum* aqueous leaf extract were probably due to the presence of bioactive compounds such as flavonoids, which act as an antioxidant by scavenging free radicals. Considering that the delay of wound healing is associated with many factors, such as nutrition, diabetes, infections, and aging, oxidative stress is believed to be one of them. Oxidative stress causes cell damage, which impedes wound epithelialization and interrupts the process (Tümen et al., 2018).

Among various nutrients, carbohydrates and proteins are the most needed in this process. Carbohydrate, which is the main source of energy production, produces cellular adenosine triphosphate that aids in wound healing through angiogenesis and inhibits the depletion of protein molecules (Guo and Dipietro, 2010). This depletion leads to prolonged wound healing by dragging the inflammatory phase; interfering the synthesis of fibroplasia, collagen, and proteoglycan; and preventing wound restoration (Mackay and Miller, 2003). Hence, nutritional composition is another possible reason for the potential wound healing properties of *O. tenuiflorum* leaves, which contain higher amounts of carbohydrates and proteins than stems do (Koche et al., 2011).

5. Conclusion

Overall, all parts (leaves, stems, flowers) of *O. tenuiflorum* plant owned potential wound healing properties. However, only the leaves extract showed promising results in two assays which is satisfactory for the study. Future studies are needed to further elucidate the possible mechanism lies behind the potential wound healing properties of the plant.

Conflict of Interest

The authors declare no conflict of interest.

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References


