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RESEARCH ARTICLE

Evaluation of *Swietenia mahagoni* Jacq seed extracts in promoting wound healing properties

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Abstract

Swietenia mahagoni or known as tunjuk langit is a widely known plant to possess good properties in treating diseases as well as a wound treatment. The purpose of this work was to examine the wound healing ability of the seed extracts in term of its ability to promote cell proliferation and migration. The extracts from two extraction methods, i.e. supercritical carbon dioxide and Soxhlet, were evaluate using cytotoxicity and scratch assays on human skin fibroblast cells. The findings showed that the extraction yield using supercritical fluid extraction was lower than Soxhlet method with 48.9% yield recovery. In addition, the seed extracts were able to stimulate cell growth and migratory effect. This information can be used as a basis to performed subsequent study to report wound healing activity of this plant material.

Keywords: *Swietenia mahagoni*, cell proliferation, cell migration, extraction methods, wound healing

INTRODUCTION

Wound healing is a process involving inflammation as well as the formation and remodeling of new tissue (Guartner *et al.*, 2008; Fronza *et al.*, 2009). The first phase is re-epithelization, where the processes such as migration of keratinocytes of the injured epidermis and hair follicles followed by proliferation of these cells at the wound edge, are observed at the beginning of new tissue formation. Keratinocytes redifferentiation is then occurred to restore the barrier function. Additionally, fibroblast is important in the repair of the injured dermis. These cells proliferation are expanding and migrate into the wound area, synthesize new extracellular matrix (ECM), as well as express thick actin bundles as myofibroblasts (Schafer and Werner, 2007; Guartner *et al.*, 2008; Fronza *et al.*, 2009).

The use of plants and preparations thereof to accelerate the wound healing process was reported years ago (Reuters *et al.*, 2009; Schmidt *et al.*, 2009). Their use is often based on traditional, without any scientific evidence of efficacy and little knowledge about putative active compounds or their mode of actions. As wound healing is a complex biological process, several *in vitro* and *in vivo* assays are presented. Among these, Liang *et al.* (2007) stated that the scratch assay has been proven as a valuable and inexpensive tool to obtain first insights into how plant preparations or their isolated compounds can positively influence the formation of new tissue.

When wounded or scratched, cell monolayers respond 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 the cell. When performing a scratch assay, an artificial gap, a so-called "scratch" is created in a cell monolayer with a sharp object such as a syringe needle or pipette tip. The assay is performed on individual coverslips or in a multi-well plate. The monolayers recovery and wound healing occur in a process that can be monitored over time. The wound heals in a patterned fashion-cells polarize toward the wound, initiate protrusion, migrate, and close the wound. The progress of these events can be observed by manually imaging samples fixed at different time points, or by time-lapse microscopy (Liang *et al.*, 2007; Fronza *et al.*, 2009).

Swietenia mahagoni (Linn.) Jacq. is a plant mainly grows in tropical areas of Asia, for example, Malaysia, India, Indonesia and southern mainland China. The seeds have been used as folk medicine for hypertension, diabetes, and malaria treatments, while the decoction of its bark has been applied as a febrifuge (Chen *et al.*, 2007). The therapeutic effects associated with the seeds are mainly caused by the biologically active ingredients, fatty acids and etranortriterpenoids (Bacsal *et al.*, 1997). There are reports of *S. mahagoni* seeds having anti-inflammatory, antimutagenicity, and antitumour activities (Guevara *et al.*, 1996). The plant extracts have been accounted to possess antibacterial and antifungal activities. Limonoid obtained from *S. mahagoni* has antifungal activity and diabetes therapy (Ardahe *et al.*, 2010). The seeds of *S. mahagoni* are good agricultural product and have been found potentially rich in fat (64.9%) (Ali *et al.*, 2011).

The extraction methods are also playing an important role in evaluating the effectiveness of the plant extract. The traditional procedures for plant extractions include hydrodistillation and organic solvent extraction (percolation, maceration or Soxhlet methods). However, there are drawbacks with these methods such as time and labour consuming operation, and involves large volumes of hazardous solvents. Nevertheless, increasing solvent acquisition and disposal costs; and regulatory restrictions have triggered the interest in alternative extraction methods with less organic solvent consumption (Yamini *et al.*, 2008). Therefore, it is highly desired to develop alternative extraction procedures with better efficiency and selectivity.

Consequently, supercritical fluid extraction (SFE) is extensively studied separation of active compounds from herbs and other plants as it is environmentally responsible and efficient extraction technique for solid materials. Lang and Wai (2001) have reviewed several advantages of SFE which are: (1) Shorter extraction time when using supercritical fluids, since they have relatively higher diffusivity and lower viscosity, (2) Continuous fluid flowing though samples could provide quantitative or complete extraction) SFE is usually performed at low temperature, (4) The solvent power can be a ted by varying the pressure and temperature, (5) Separation of solutes dissolved in supercritical fluid can be easily done by depressurization, (6) Only a little quantity of sample is needed, (7) Zero or little amount of organic solvent is needed, which is environmentally responsible. In addition, SFE provides more quality extract compare to conventional extraction methods as discussed b asmida *et al.* (2015).

The objective of the present study was to evaluate the ability of *Swietenia mahagoni* seed extracts to promote cells proliferation into and migration to the wounded monolayer which provide a basis on wound heali roperties of the plant material. Human skin fibroblasts (HSF1184) were used, and platelet derived growth factor (PDGF) served as positive control. Two extraction methods were applied in order to comp he effectiveness of wound closure rate of the extracts which were supercritical carbon dioxide (SC-CO_2) and Soxhlet extraction with hexane as solvent.

EXPERIMENTAL

Plant material preparation

S. mahagoni seeds were collected from Indonesia. The seeds were rinsed with tap water to remove any foreign particles and dirt prior to drying. Then, the cleaned seeds were cut into small pieces and dried in an oven at temperature of 50°C for one week to remove its moisture. The seeds were ground by a blender (Panasonic) with average particle sizes varied from 0.25 to 0.75 mm.

Chemicals

Supercritical carbon dioxide (SC-CO_2) extraction

The SC-CO_2 extraction equipment used in is study was similar to those of Mohd Nasir *et al.* (2017). The ground sample of 5 g was placed into an extractor vessel. The extracts were collected into a glass vial placed in the separator at ambient temperature and pressure. A w rate of CO2 was 2 mL/min. The investigated values of pressure, temperature, and particle size were varied m 20 to 30 MPa, 40 to 60°C, and 0.25 to 0.75 mm, respectively. After each extraction, the obtained extract was placed into glass vials, sealed and stored at 4°C to prevent any possible degradation.

Soxhlet extraction

The extraction of *Swietenia mahagoni* seed was carried out as described by Markom *et al.* (2007) with slight modifications. The extraction of *Swietenia mahagoni* was implemented by using Soxhlet extraction technique. To prepare the extract, 5 g of powdered *S. mahagoni* seed were weight and placed in a Whatmann 25 mm x 100 mm cellulose thimble while 150 mL of hexane (100%) were placed at the bottom of the apparatus. The extraction process was done for 6 hours at temperature 65°C. Then, the extraction yield was put in the rotary evaporator (BUCHI rotavapor, R-114) at 40°C for 2 hours to remove the solvent. The extracts were then placed in room temperature condition before weighing gravimetrically to calculate the yields. The samples were stored at 4°C in refrigerator for further analysis.

Expression of yield

The extraction yield was calculated using the following equation where m_o and m_1 were mass of sample and extract in grams (g), respectively.

$$Percentage\ of\ extraction\ yield\ (\%) = \frac{m_1}{m_o} \times 100 \quad (1)$$

Cell proliferation assay

The method described by Ranzato *et al.* (2011) was used. The proliferation activity of human skin fibroblast (HSF1184) cells after treated with crude extracts was determined methylthiazol tetrazolium (MTT) colorimetric assay. All cells were cultured in minimum essential medium (MEM) + 10% fetal bovine serum (FB + 1% pen Strype (PS) under 5% CO_2 humidified incubator. The cells were seeded at a density of 2 x 10^5 s/well in 96-well plate and incubated for 24 hr prior to treatment. The test samples were prepared by dissolving *S. mahagoni* extract in MEM to yield the al concentration of 10, 1, 0.1, 0.01, 0.001 and 0.0001 mg/mL. The medium was replaced after 24 hours with 200 µL of MEM conta ng 10% FBS and 1% PS; and serial dilution of plant extracts. After incubation, the cells were washed with phosphate buffered saline (PBS), 20 µL freshly prepared MTT solution (5 mg/mL) was added into each well and cells were incubated at 37°C for 5 hours. The MTT solution was then removed and replaced with 200 µL DMSO to a dissolution of the purple MTT formazan crystal. The absorbance was measured at 540 nm using ELISA plate reader.

Cell migration assay

The wound closure of the HSF1184 was evaluated using scratch wound assay which measures the expansion of a cell population on surfaces modified from Fronza *et al.* (2009). The cells were cultured o 12-well plate at a concentration of 3 x 10^5 cells until confluent. Then, a linear wound was made on the monolayer with a sterile 200 µL pipette tip. Any cellul debris was removed by washing the cells with PBS. MEM (control), platelet derived growth factor (po ve control), the crude extracts (0.01 mg/mL) was add o the cells and incubated for 24 hours at 37°C. Two representatives images from each well of the scratched areas under each condition were photographed at 0, 12 and 24 hours to measure the wound closure. The dat re analysed with NIH ImageJ software (Ranzato *et al.*, 2011). Wound closure was determined by the difference in wound width between 0 hour and 24 hours.

Statistical analysis

D were expressed as the mean ± S.E.M. Results were submitted to the one-way analysis of variance ANOVA, followed by the post hoc Tukey's test, using the SPSS 16.0 software. Values of $p < 0.05$ were considered statistically significant.

RESULTS AND DISCUSSION

Extracted oil yield of *S. mahagoni* seeds

The extraction of *S. mahag* eeds was done using two different extraction methods which were supercritical carbon dioxide (SC-CO_2) and Soxhlet extraction. The comparison of both extraction methods was shown in Fig. 1. Generally, the figure shows that Soxhlet extraction gave the highest percentage oil yield (41.08% ± 0.98) compared to SC-CO_2 extraction which was 20.07% ± 0.48. However, taking into account the extraction time, SC-CO_2 offer better extraction process since Soxhlet extraction required 6 hours of extraction time while SC-CO_2 extraction process only takes 3 hours to obtain the highest extracted oil yield.

The higher oil yield obtained using Soxhlet extraction compared to the SC-CO_2 extraction method is due to the high temperature and long extraction time applied during the extraction procedure. High temperatures can increase the volatility of the solute hence enhancing the solute solubi n hexane. Besides, longer extraction time increase the c ct time between the solute and solvent molecules resulting in high extracted oil yield. The drawback of this properties is that the method will extract all of the solutes, including impurities within the particle. On the other hand, SC-CO_2 extraction technique offers better selectivity ability and it was proven by only 48.9% yield recovery compared to Soxhlet method.

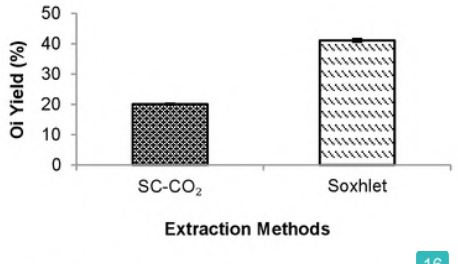

Fig. 1 Percentage of *S. mahagoni* seed oil extract using $SC\text{-}CO_2$ and Soxhlet extraction

The comparison between these two extraction methods had been done previously as the researchers proved the effectiveness of $SC\text{-}CO_2$ extraction to obtain more pure substances. For example, Zhao and Zhang (2014) stated that the Soxhlet had the highest yield, followed by SFE and hydrosilation. They explained that the nature of Soxhlet in which it extracts high molecular weight molecule is one of the reasons for the obtained result. A similar result also published by Liza *et al.* (2012) where the flavonoid content was detected in significant quantity when extracted using $SC\text{-}CO_2$ method.

Cell proliferation analysis of *S. mahagoni* seed extracts

Cytotoxicity test using (3-(4,5-dimethyl-tiazol-2y) 2,5-diphenyl-tetrazolium bromide (MTT) assay was done to obtain the suitable extracts concentration which gives non-toxic effect towards cell proliferation. MTT assay is useful *in vitro* model for testing cytotoxicity because it can show the ability of the compound to stimulate the proliferation of the broblast. The toxic compounds will affect the basic functions of the cells and the toxicity can be determined by assessing cellular damage. MTT will enter the cell and pas the mitochondria and the ring of MTT will be reduced to a dark purple formazan product which is insoluble in the aqueous solution. The reduction of the MTT can only be implemented by the metabolically active cells, and therefore this reduction can directly measure the viability of the cell. In other words, this screening can assist for the identification of the suitable amount of extracts that can be utilized for next cell culture study.

Fig. 2 shows the relative viability of fibroblast cells after being ted with *S. mahagoni* extracts for 24 hours at different concentrations ranging from 0.0001 to 10 mg/mL for both extraction methods nerally, all extracts showed the same pattern of relative viability at 0.1, 0.01 and 0.001 mg/mL extracts' concentrations. The highest relative cell viability was indicated by linoleic acid (LA), which was the standard chemical marker in this study, at 0.1, 0.01 and 0.001 mg/mL with 145% ± 0.015, 190% ± 0.037 and 165% ± 0.037, respectively, provide an idea that the high quality compound without impurities shows a favourable response to cell proliferation rate. Meanwhile, the $SC\text{-}CO_2$ and Soxhlet extracts sho only a slight difference in cell proliferation between each other at concentrations of 0.1, 0.01 and 0.001 mg/mL. However, the extracts obtained from Soxhlet extraction demonstrates relatively low cell viability compared to others suggesting that the $SC\text{-}CO_2$ extraction from *S. mahagoni* seeds results in a better effect on the rate of cell proliferation.

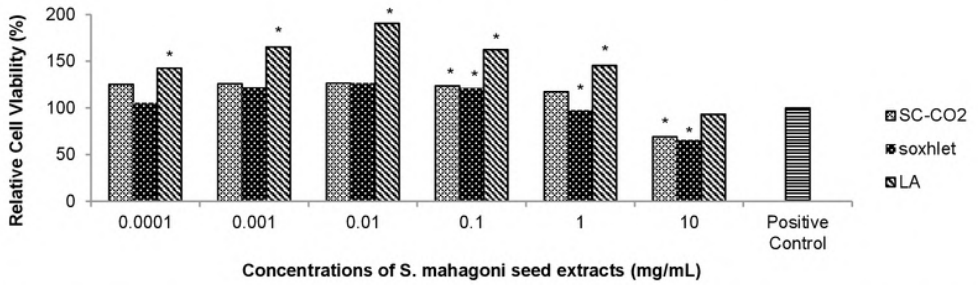

Figure 2 Effect of *S. mahagoni* extracts using $SC\text{-}CO_2$ and Soxhlet extraction on human skin fibroblast (HSF1184) cells evaluated for 24 hours.

Referring to Ranzato *et al.* (2009) and Wang *et al.* (2011), relative cell viability higher than control is considered to have a growth promoting activity, while lower values than control show a toxic effect. Based on the data obtained from this analysis, it can be concluded that all extract's concentrations ranging from 0.1 to 0.001 mg/mL did not show any toxicity effects on HSF1184, even promoting fibroblast proliferation as the cell's survival rate was higher than the control. The concentrations dose higher than that is not suitable to be applied might be due to its high antioxidant properties which will provide harmful effect towards the fibroblast cells (Mbata, 2014). Previously, Ghosh *et al.* (2009) found t wo doses of methanolic extract of *S. mahagoni* seeds (50 and 100 mg/kg) did not show any significant toxic effect up to 1.2 g/kg of rat weight within 24 hours. According to other studies conducted using brine shrimp lethality assay, LD_{50} oral acute toxicity for *S. mahagoni* methanolic extracts exceed 2,500 mg/kg suggests that the extract of this plant was relatively nontoxic. The results of the study coincide with the fact that this plant can be used as traditional medicine (Sahgal *et al.*, 2010).

Although *in vitro* cytotoxicity studies conducted on monolayer culture (fibroblasts) cannot be directly linked to *in vivo* assays, this finding provides an important basis for subsequent studies on specific bioactive compounds and mechanisms that act as proliferation agent. Besides, the data of this study can be used as a quick assessment of the cytotoxic effects of *S. mahagoni* seed extract on fibroblast cells. Therefore, based on these result (Figure 2), the concentration of 0.1, 0.01, and 0.001 mg/mL were selected as the appropriate concentrations to be applied in the next assay which was scratch assay to evaluate the ability of the extracts in promoting cell migration.

Cell migration analysis of *S. mahagoni* seed extracts

Scratch assay have been previously used to study the characteristics of proliferation and migration of various cell types after being treated with extracts from different species such as *Astragalus*, *Calendula*, *Hemigraphic*, *Matricaria* and *Simmondsia* (Fronza *et al.*, 2009; Edwin and Nair, 2011 ; Ranzato *et al.*, 2011; Sevimli-Gur *et al.*, 2011). The reported optimum incubation period after the sctraching and cell treatment for both fibroblasts and keratinocytes with plant extracts is 12-72 hours, depending on the type of cells, extracts and scratches. The application of plant extracts with an optimum concentration of specific compounds can reduce or decrease the rates of cell proliferation and migration. High extract concentrations of legume genus *Astragalus* (10 mg/mL) and jojoba (*Simmondsia chinensis*) seed (wax, 5% v/v) are less favorable for proliferation and migration of cells than less concentrated extracts (1 mg/mL; 1%, v/v) (Ranzato *et al.*, 2011; Sevimli-Gur *et al.*, 2011). To confirm the healing properties of *S. Mahagoni* seed extracts discussed in the previous section, the ability of this extract to encourage fibroblast migration has been studied.

In this study, migration of fibroblast cells was studied after 24 hours of wounded cells and treated with two different *S. mahagoni* extracts. Fig. 3 shows the effects of different *S. mahagoni* samples based on the methods and concentration of extracts towards fibroblast cells. The chart illustrates that SC-CO_2 extract at concentration of 0.001 mg/mL and Soxhlet extract at 0.1 and 0.01 mg/mL showed significant effect on cell migration compared to negative control (MEM + 10% FBS + 1% antibiotic mixture) ($p < 0.05$), with the values of 85.306% ± 0.966, 93.496% ± 3.314, and 83.904% ± 2.901, respectively, and not statistically different compared to LA and PDGF-BB (positive control). However, there is no significant difference noticed on the trend of wound closure percentage when treated with *S. mahagoni* extracts from both extraction methods, applying that the extracts of both methods can promote cell migration.

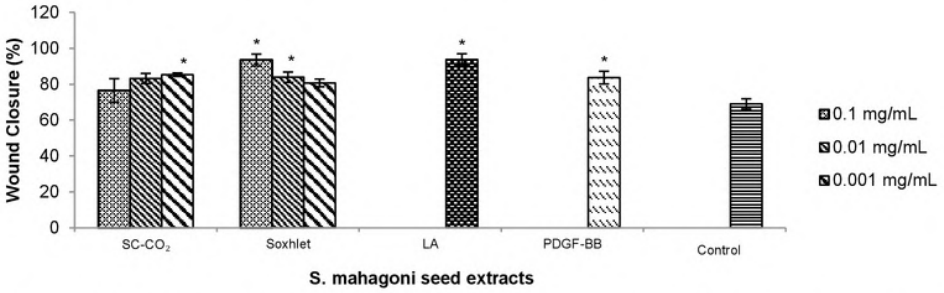

Fig. 3 Cell migration after wounded and treatment using different *S. mahagoni* seed extracts at concentration 0.1, 0.01 and 0.001 mg/mL, standard marker, positive and negative controls for 24 hours.

The extracts from *S. mahagoni* seed, LA and PDGF repaired the cells to the > 90% confluent level within 24 hours, in contrast with negative control (Fig. 4).

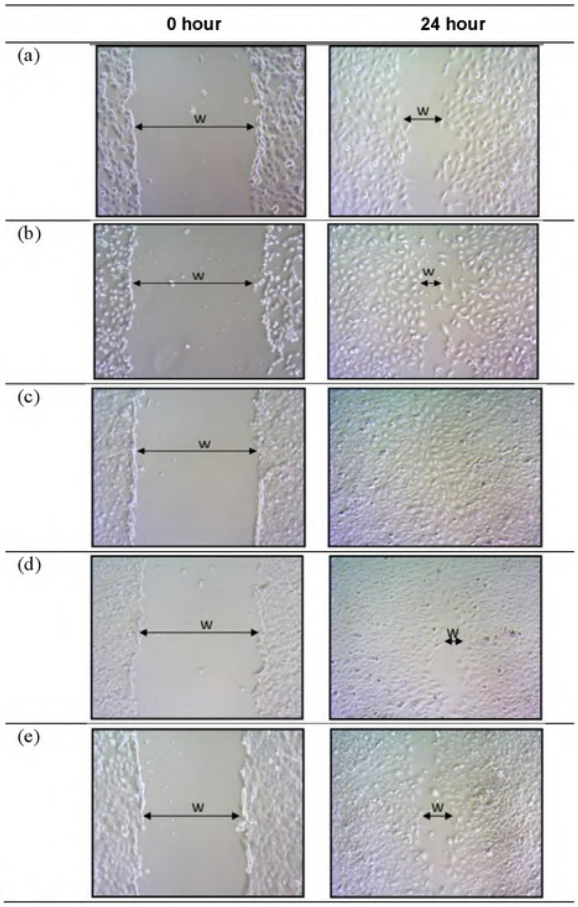

Fig. 4 Cell migration of (a) negative control, (b) positive control, (c) Linoleic acid (LA), (d) SC-CO_2 extract of *S. mahagoni*, (e) Soxhlet extract of *S. mahagoni* at 0 and 24 hours.

The extract at a concentration of 0.1 mg/mL showed a higher percentage of wound closure than positive control (2 mg/mL of PDGF). This finding explains that *S. mahagoni* seed extracts can promote similar wound healing activity as PDGF at selected concentrations. The ability of the extracts in accelerating wound closure process might be due to the linoleic acid content which acts as an essential tissue remedy as it promotes chemotactic and angiogenesis by maintaining moisture in the environment and speeding up the tissue granulation process (Ferreira *et al*., 2012). Linoleic acid also plays an important role of chemotaxis for macrophages, and it is very crucial in the expression of components for the fibrinolysis system (controlling the production of collagenase). Ferreira *et al.* (2012) reported high concentrations of linoleic acid (65%) was one of the factors that contribute to positive results in wound healing.

CONCLUSION

The ability of *Swietenia mahagoni* seed extracts in possessing wound healing properties was accessed in term of cell proliferation and migration using cytotoxicity and scratch assays, respectively. The findings suggest that all extracts can stimulate fibroblast growth in which could play a vital role in its effect on tissue repair. These assessments can be used as a basis to report wound healing activity of plant material and subsequent study should be made especially *in vivo* assay to determine the suitable dosage for human consumption.

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