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Evaluation of the Potential *In Vitro* effects of *Plantago major* L. on Wound Healing in Human Umbilical Vein Endothelial Cells (HUVEC)

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Abstract

The treatment of skin wounds remains a major concern in the field of medicine, particularly in the case of chronic wounds resulting from various disorders such as diabetes. The utilization of herbs or herbal preparations for the purpose of healing skin wounds presents a therapeutic challenge within the realm of traditional medicine. *Plantago major* L. is known to have bioactive compounds that have wound healing activity such as aucubin. This study aimed to determine the *in vitro* wound healing potential of *Plantago major* L. extract (PLE). The study involved several assays, including phytochemical examination of PLE using TLC, cell viability testing using MTT assay, and wound healing testing using scratch assay on human umbilical vein endothelial cells (HUVEC). The results confirmed the presence of aucubin as one of the compounds in PLE. It was observed that PLE with 125 µg/mL exhibited the highest wound closure percentage at 90.66%. This study shows that PLE possesses wound healing capabilities.

Keywords: Plantago major *L., PLE, cytotoxic assay, wound scratch assay, HUVEC.*

INTRODUCTION

The skin is the first defense barrier of the human body against physical and chemical environmental challenges. Because of its large surface area, the skin is frequently subjected to a wide variety of damages and injuries, which can result in both acute and chronic wounds. Typically, the skin possesses a limited capacity for self-repair. In addition, the ability of the skin to repair itself is restricted when it comes to serious and widespread skin injuries such as deep burns, lacerations, and diabetic foot ulcers (Azari, *et al*., 2022; Criollo-Mendoza, *et al*., 2023).

When skin damage happens, a prompt and synchronized reaction is triggered to

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mend the wound. The process of healing a skin wound is composed of three separate yet interconnected stages, known as the inflammatory phase, proliferative phase, and remodeling phase. This process incorporates various agents, including inflammatory cells, growth factors, and cellular and extracellular components (Takahashi, *et al*., 2021). Following skin injury, many types of cells are attracted to the site of the wound. Inflammatory cells are drawn to the site of infection and eliminate the damaged tissues. To create new tissue, keratinocytes and fibroblasts migrate to the wound area and proliferate. The epithelial cells proliferate and migrate from the edges of the wound during the epithelization stage in order to close it. This underscores the importance of cell migration as a key component of the wound healing process (Chiangnoon, *et al*., 2022).

Over the years, there has been significant interest in the use of natural products or herbal medicines for wound healing due to their ability to activate various mechanisms such as anti-inflammatory, angiogenesis stimulation, fibroblast proliferation, extracellular matrix formation, and immunomodulatory effects (Liao, *et al*., 2023). One herb that has been highly esteemed for its benefits since ancient times is *Plantago major* L. *P. major* is a perennial plant that belongs to the Plantaginaceae family. It was previously predominantly found in Europe and North and Central Asia, but it has now spread extensively over the globe. *In vitro* and *in vivo* tests have shown that the leaves and seeds of *P. major* have analgesic, anti-inflammatory, antioxidant, immunomodulator, antifungal, anticancer, and wound healing properties (Zhakipbekov, *et al*., 2023). The wound healing properties of *P. major* are related to a wide range of bioactive compounds, such as flavonoids, terpenoids, phenolics, iridoid glycosides and polysaccharides. Plantaginin, baicalein, and hispidulin are flavonoids that act as antioxidants and free radical scavengers, promoting wound healing (Adom, *et al*., 2017). Aucubin, a member of the iridoid glycosides, is also known to have antioxidant and anti-inflammation activity that can help wound healing (Zeng, *et al*., 2020).

However, there is currently a lack of clarity in wound healing activity of *P. major* in the human umbilical veins endothelial cells (HUVEC). HUVEC are primary cells isolated from the umbilical cord vein. These cells are commonly used in standardized *in vitro* angiogenesis assays to study how normal endothelial cells behave (Medina-Leyte, *et al*., 2020). When it comes to wound healing, HUVECs are preferred because they enable detailed analysis of endothelial cell migration, an early stage in angiogenesis. Therefore, this study was intended to investigate the migration activity of *P. major* L. extract on the HUVEC.

MATERIALS AND METHODS

Ethical Concern

The ethical clearance for all the experiments was granted by the Medical and Health Research Ethics Committee (MHREC), Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (UGM) (reference number KE/FK/1932/EC/2023).

Determination of Aucubin in the *Plantago major* Leaf Extract (PLE)

Plantago major leaf extract, referred to as PLE, was obtained from initial research (Hertiani, *et al*., 2023). The determination of aucubin in PLE was conducted using the high performance thin layer chromatography (HPTLC) method, following the previously published procedure with minor adjustments (Taskova, *et al*., 2002). PLE with a concentration of 10,000 μg/mL as diluted in methanol (Merck, Darmstadt, Germany), while aucubin standard (purity ≥98%, Catalog no. 55561-5MG-F) obtained from Sigma-Aldrich (St. Louis, Missouri, USA), with a concentration of 100 μg/mL was diluted in

WFI. The samples were applied as spots on the stationary phase. The stationary phase consisted of a silica gel plate 60 F254 (Merck) measuring 10 cm in length and 3 cm in width. The mobile phase used was n-butanol (Merck), methanol (Merck), and distilled water with ratio 70:5:10. The development process utilized 5 mL of mobile phase in an twin chamber (Camag, Sonnenmattstrasse, Muttenz Swizertland) that had been pre-equilibrated with the mobile phase for 20 minutes at room temperature. Following the development process, the plates were dried using warm air and then sprayed with anisaldehyde sulfuric acid. The results were observed using visible light, UV light at 254 nm, and 366 nm.

Cell Culture

The HUVEC (Catalog no. SCCE001, Merck®) were cultured in Molecular, Cellular, and Developmental Biology 131 (MCDB131) medium obtained in ready to use condition from Dermama Biotechnology Laboratory, Indonesia. The cell was incubated in a 5% carbon dioxide (CO_2) humidifier incubator (Thermoscientific Series 8000 DH, USA) at 37°C. A new medium was substituted every 48 h. Cells were considered suitable for seeding and treatment only after they reached a convergence level of 70-80%.

Sample Preparation

The sample solvent employed was dimethyl sulfoxide (Sigma-Aldrich). PLE was prepared as stock solutions with concentrations of 100,000 µg/mL. PLE were diluted using the medium culture into serial concentrations: 250; 125; 62.5; 31.25; and 15.62 µg/mL.

Cell Viability Assay

Cell viability of the extract was determined using MTT assay. This assay was conducted using the methodology described by (Zakaria, *et al*., 2023) with some adjustments. HUVECs were cultured in 96-well plate (Corning, New York, USA) at a density of 1×10^4 cells per well. The cells were then incubated for 24 h. After 24 h, the culture media was replaced with the samples and cultured for 24 h. Subsequently, the samples were removed and a 10% solution of MTT (Bio Basic, Markham, Canada) was added. The mixture was then allowed to incubate for 4 h. Following this, the sample was examined using an inverted microscope (Leica DM IL Inverted Phase Contrast, Germany) to determine the presence of formazan, a purple sediment. If formazan was detected, 100 µl of stopper solution (100 µl SDS 10% in 0.01 N HCl) was added to dissolve the purple sediment. The 96-well plate were read using a microplate reader (Bio-Rad iMarkTR, USA) to measure the absorbance at 570 nm. The absorbance data acquired were analyzed using Microsoft Excel 2019 to compute the average value and % cell viability using the following formula :

$$\text{Cell viability } \% = \frac{\text{absorbance of test sample} - \text{absorbance of blank}}{\text{absorbance of negative control} - \text{absorbance of blank}} \text{ x } 100\%$$

The data significance against the control was analyzed using one-way ANOVA with GraphPad Prism 8.

Wound Scratch Assay

The effects of PLE on the migration of HUVECs was examined using the scratch assay, which replicates cell migration during wound healing process in living organisms. The wound scratch assay was conducted using the methodology described by (Zakaria, *et al*., 2023) with some adjustments. The HUVECs were seeded in 24-well plates (Iwaki, Fukushima Prefecture, Japan) at a density of 1×10^5 cells per well and incubated in a 5% carbon dioxide (CO_2) humidifier incubator at 37°C. The confluent monolayers were meticulously scraped using sterile P200 pipette tips to create tiny, evenly sized, and spaced linear scratches. The cellular debris was removed by washing the cells with Dulbecco's Phosphate Buffered Saline (DPBS) (Elabscience Biotechnology, USA), followed by the addition

of PLE at different concentrations (7.81, 31.25, and 125 µg/mL). Wound closures were monitored and photographed immediately using a motorized inverted microscope at ×4 magnification. The photos taken 24 h after incubation of HUVECs were examined using Image-J software. The percentages of the closed region before and after the treatment were compared. A total of two replication of the scratch assay were conducted. The wound healing percentages were evaluated by measuring the reduction in scar area at a certain time point.

$$\text{Wound healing (\%)} = \frac{(\text{scratch area at 0h} - \text{scratch area at 24 h})}{\text{scratch area at 0 h}} \times 100\%$$

The data significance against the control was analyzed using one-way ANOVA followed by a Dunnet test with GraphPad Prism 8 to compare the means of the treatment group with the control group.

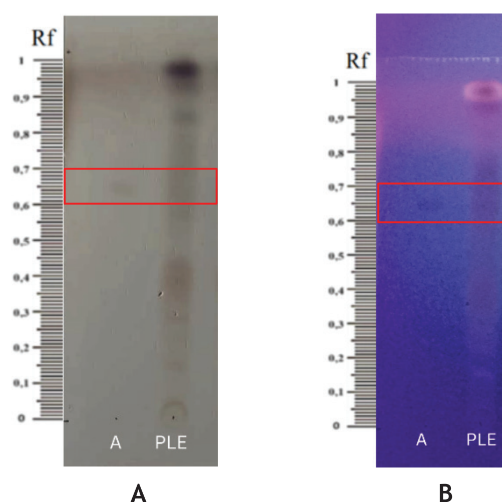

Figure 1. HPTLC profile of PLE with mobile phase butanol:metanol:air (70:5:10) v/v after being sprayed with anisaldehyde sulfuric acid. A. HPTLC plate under visible light, B. HPTLC plate under UV366nm. Wherein A (aucubin standard) and PLE (*P. major* extract).

RESULTS

Determination of Aucubin in the *Plantago major* Leaf Extract (PLE)

Aucubin in PLE were verified through thin-layer chromatography (HPTLC) methods. The spots produced during the elution of the PLE were compared to the aucubin standard. The TLC data shown in Figure 1 indicates the presence of aucubin compound in the PLE. A spot in the PLE profiling appeared adjacent to the aucubin standard spot, displaying a similar color under the visible light after being sprayed with anisaldehyde sulfuric acid. The retention factor (Rf) of aucubin standard and PLE was 0.67.

Effect of *P. major* Extract (PLE) on HUVEC Cell Viability

Aucubin, at a concentration of 34.63 µg/mL, was used as a positive control because it is known to have a wound-healing effect (He, *et al.*, 2023). The cells were subjected to various concentrations ranging from 15.625 µg/mL to 250 µg/mL of PLE and aucubin standard for a duration of 24 h, and the cytotoxic effect of the extract was assessed. There is a significant decrease (p<0.0001) in the percentage of cell viability of HUVEC in the positive control group with 71.10%±2.79 and PLE at 250 µg/mL with 87.94%±2.48. These findings suggest that PLE with a concentration 15.625-125 µg/mL does not have cytotoxic effects on

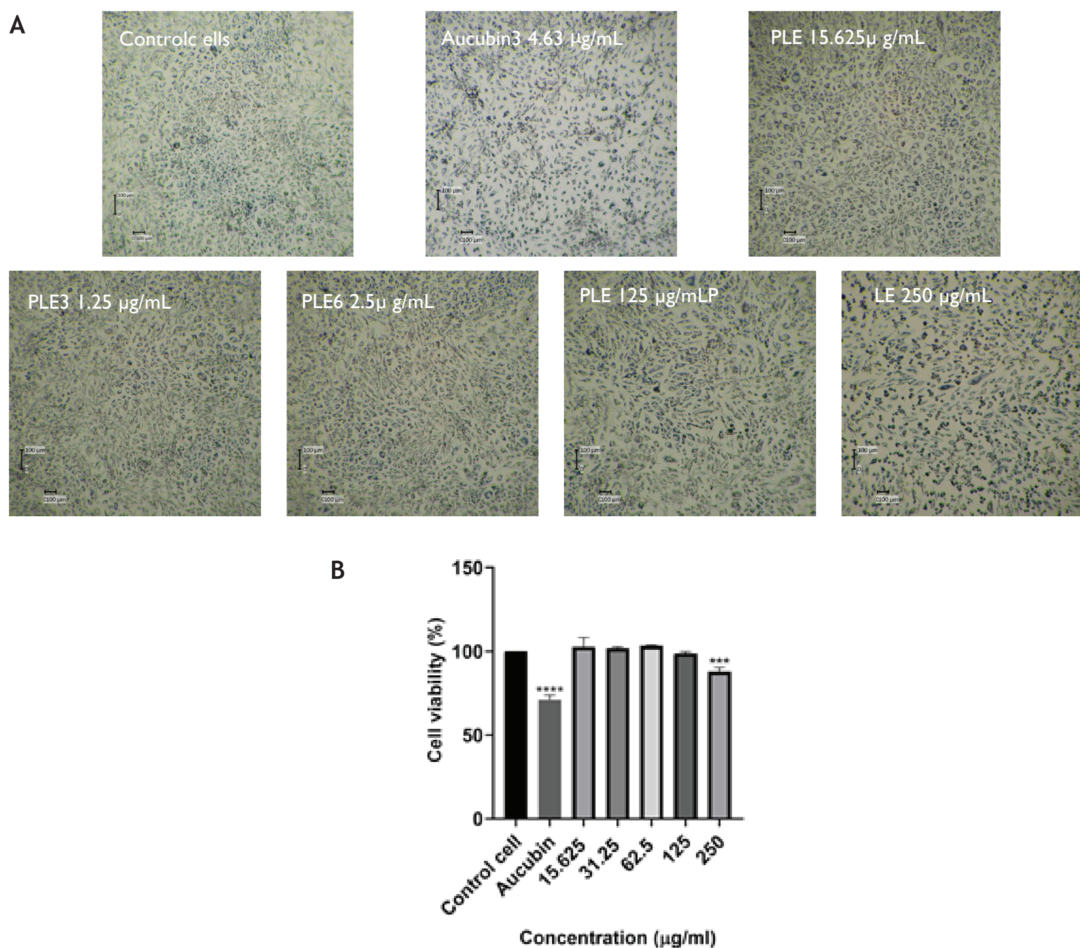

Figure 2. Effect of PLE on HUVEC cell viability, (A) The microscope images of HUVEC under different concentrations of extracts in MTT assay for 24 h. Scale bar: 100 µm Images were taken and measured with a confocal microscope with × 4 magnification equipped with a digital camera. (B) The effects of *P. major* extract at different concentrations on the cell viability of the HUVEC after 24 h. Data are expressed as the mean percentage ±SD (n=3). ****p*<0.0005; *****p*<0.0001 versus the control group.

cells and can be further evaluated for its potential therapeutic capabilities. Then, PLE with 7.81, 31.25, and 125 µg/mL concentrations were chosen for wound scratch assay. The morphological changes of HUVEC and the percentage of cell viability of PLE on HUVEC are depicted in Figures 2.

Wound Scratch Assay

Aucubin, at a concentration of 34.63 µg/mL, was used as a positive control because it is known to have a wound-healing effect (He, *et al*., 2023). Following a 24 h exposure to PLE, it was observed that all cells treated with PLE, aucubin standard of 34.63 µg/mL, as well as untreated cells, exhibited migration towards the gap. The wound closure percentage was determined by calculating the decrease in gap area relative to the original gap area. The results showed that concentration 125 µg/ml displayed the highest wound closure percentage at 90.66%, while the aucubin standard exhibited wound closure percentage at 82.27% (Figure 3).

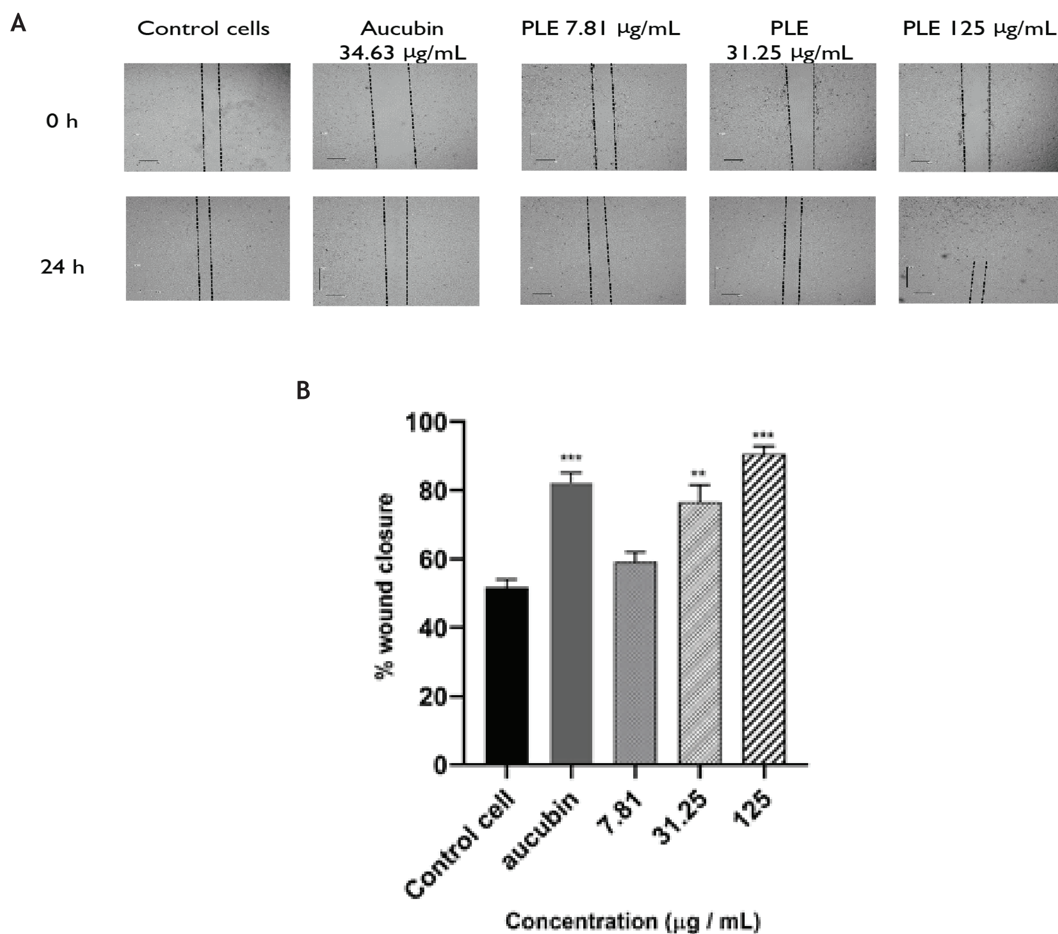

Figure 3. Wound healing activity of PLE on HUVEC (A) Microscopic images of HUVEC treated with PLE in the scratch assay after 24 h incubation. Images were taken and measured with a confocal microscope with ×4 magnification equipped with a digital camera. (B) The effects of PLE at different concentrations on the cell migration of the HUVEC after 24 h. Data are expressed as the mean percentage ±SD (n=3). ***p*<0.005; ****p*<0.0005 versus the control group.

DISCUSSION

Aucubin is known as the main iridoid glycoside present in *Plantago major* and considered as chemotaxonomic markers for Plantago spp (Khalaf, *et al*., 2018). The presence of aucubin in the PLE was verified in this study using TLC methods, as shown in Figure 1. Numerous studies have reported the wound healing effects of the aucubin. Based on study by Shim, *et al*., (2007), a 0.1% aucubin solution reduces inflammation and stimulates re-epithelialization and collagen matrix synthesis in oral mucosal wound healing. In a study by Kartini, *et al*., (2018), it was found that applying a gel containing 20 µg and 40 µg aucubin led to complete wound closure (100%) in hyperglycemic rats, compared to 83% in the negative control group. The application of aucubin gels also resulted in a significant reduction in wound healing time (11.7 days) compared to the negative control group (24.4 days) (Kartini, *et al*., 2018). This suggests that the

wound healing activity of PLE is attributed to their aucubin content. Existing literature indicates a lack of information regarding the wound healing activity of PLE using endothelial cells. Therefore, in this study, we evaluated the wound healing properties of PLE by *in vitro* scratch assay using HUVEC.

Firstly, we performed a viability assay, which aimed to screen for toxicity in various biological substances. This preliminary assessment is instrumental in evaluating its biological therapeutic significance. Evaluating the viability impact of the plant extract on cells or an *in vivo* model is crucial since certain plant metabolites may exhibit toxic effects on cells due to their intermolecular interactions within the cellular environment. The viability test results (Figure 2) showed that the highest concentration (250 µg/mL) and aucubin significantly reduced the percentage of cell viability, indicating a toxic effect on HUVEC. Meanwhile, the 15.62-125 µg/mL concentration was not toxic, with cell viability remaining above 90%. These results align with previous studies showing that Lithospermi Radix and Mitragyna speciosa extracts are not toxic at low concentrations to HUVEC cells (Park, *et al*., 2018; Zakaria, *et al*., 2023). Therefore, PLE with 7.81, 31.25, and 125 µg/mL concentrations were selected for use in the cell migration test (scratch assay).

Endothelial cells are a key component in the wound healing process, especially in the context of angiogenesis, which is the formation of new blood vessels from existing ones. The use of endothelial cells in migration assays in wound healing aims to assess their ability to migrate to the wound area, which is an important step in the tissue regeneration process (Gothai, *et al*., 2016). Thus, the effects of PLE on HUVEC migration were tested using *in vitro* wound healing with the scratch assay method. Previous study by Zubair, *et al*., (2012) revealed that both water and ethanol-based extract of *P. major* with a concentration of 1.0 mg/mL (on dry weight basis) increased the migration of the oral epithelial cells compared to the negative control. Based on research by He, *et al*., (2023), aucubin at concentrations of 5, 10, and 20 µM can increase the migration ability of HUVEC cells injured with su5416 after 12 h. The wound healing assay results (Figure 3) showed that the highest concentration of PLE (125 µg/mL) had better wound healing activity than the positive control aucubin.

In scratch wound healing assays, a starvation medium (low serum medium) or antiproliferative agents like mitomycin-C are often used to distinguish between cell migration and proliferation. In this study, HUVEC cells were grown without serum supplementation, making using a starvation medium unnecessary. The wound closure observed is attributed to cell migration rather than proliferation, as the cell viability assay showed no significant increase in viability, indicating that the extract did not induce cell proliferation. Therefore, the wound closure in this study is because of PLE's ability to induce HUVEC cell migration.

Based on our result, the wound healing ability of PLE is not only influenced by aucubin. Polyphenols such as plantamajosid and polysaccharides are known to play a role in wound healing because they have antioxidant and anti-inflammatory activities (Zubair, *et al*., 2012). In addition, the antioxidant activity of flavonoid compounds such as plantaginin, baicalein, and hispidulin played a role in wound healing (Adom, *et al*., 2017). A more detailed phytochemical analysis of PLE and further testing of individual compounds in future studies help clarify their specific roles in the healing process. Also, investigating key angiogenesis-related signaling pathways, such as VEGF, FGF, or MMPs, will provide valuable insights into how PLE influences endothelial cells. Overall, this study confirms that PLE enhances wound healing by stimulating the movement of endothelial cells.

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CONCLUSION

In conclusion, *Plantago major* L. extract from the maceration extraction method contained aucubin. *Plantago major* L. extract with a 15.625-125 µg/mL concentration does not have cytotoxic effects. *Plantago major* L. extract 125 µg/mL showed the highest wound closure percentage. These results further demonstrate that *Plantago major* L. possesses wound healing capabilities and could be a viable source for extracting natural chemicals promoting wound healing.

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