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Phytochemical Assessment, Antioxidant Activity, and *in vitro* Wound Healing Potential of *Polygonum minus* Huds

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Abstract

The increasing prevalence of chronic wounds, associated with aging, obesity, and diabetes, is compounded by bacterial resistance and adverse effects associated with commercial wound care products. Therefore, treatment modalities to accelerate the healing process are constantly being sought. This study aimed to investigate the phytochemical composition, antioxidant, and wound healing potential of the aqueous extract derived from the stems and leaves of *Polygonum minus*. The extract was subjected to phytochemical evaluation to assess the diversity of secondary metabolites. The total phenolic content (TPC) and total flavonoid content (TFC) were measured using the Folin-Ciocalteu colorimetric and aluminium chloride methods, respectively. The antioxidant activity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging and ferric-reducing antioxidant power (FRAP) assays. The wound healing effects were evaluated using proliferation and migration assays on human epidermal keratinocyte (HaCaT) cells. The phytochemical evaluation of the aqueous extract revealed the presence of flavonoids, terpenoids, alkaloids, saponins, tannins, steroids, and cardiac glycosides. Furthermore, the extract exhibited high TPC (137.74 ± 0.75 µg/mL GAE) and TFC (177.08 ± 3.16 µg/mL QE) values, as well as radical scavenging activity at 79.50% in the DPPH assay and a FRAP value of 1485.67 ± 0.05 µM/g Fe^{2+}. At the lowest concentration of 7.81 µg/mL, the extract significantly stimulated cell proliferation and migration within 24 hours of treatment. The stimulation of cell migration was comparable with that of allantoin, which was used as a positive control. This study indicated that the *P. minus* aqueous extract contains a high concentration of phenolic compounds, which could contribute significantly to its antioxidant activity and promote the proliferation and migration of keratinocytes. The findings suggest that the extract may merit further investigation for potential applications in topical therapy related to wound healing.

Keywords: *Antioxidant, in vitro wound healing, migration, phytochemical, Polygonum minus, proliferation*

1. Introduction

Cutaneous wound healing starts immediately after an injury to preserve tissue integrity and provide a protective barrier against potential external stimuli or infections. This process requires the well-orchestrated integration of complex biological and molecular activities of cell migration, cell proliferation and deposition of extracellular matrixes (Wang et al., 2023). The process of wound healing involves several overlapping stages which include hemostasis, inflammation, proliferation, and remodelling (Criollo-Mendoza et al., 2023). Keratinocytes are key players in the process of re-epithelialization during the proliferative phase. During the epithelialization stage, the epithelial cells proliferate and migrate from the wound borders to close the wound. The restoration of the compromised epidermis is typically achieved through two primary mechanisms involving the activation of epidermal keratinocytes at the wound margin and the proliferation of epidermal keratinocytes (Dam et al., 2023).

The phases of wound healing normally progress in an orderly, and timely manner to restore the anatomical and functional integrity of the skin. However, chronic wounds lose the ability to inherently self-repair due to several contributing factors, such as aging, stress, infection, neuropathy, vascular insufficiency, wound infection, and excessive pressure at the site (He et al., 2021). This predicament is further exacerbated by the increasing prevalence of an aging population, rising rates of obesity, and the widespread incidence of diabetes (Patel et al., 2019). Although various pharmaceutical preparations are available, the current wound care products are generally expensive, which impose a substantial financial burden to the patient and the healthcare system (Tricco et al., 2015). The situation is also compounded by the rising cases of bacterial resistance and adverse effects associated with the commercial wound care products (Ceilley, 2017). Therefore, there is a need to find alternative therapies that could promote wound healing, reduce treatment cost, and minimize the possibility of severe wound complications (Lordani et al., 2018). Many plants and various preparations thereof have been traditionally used to treat several types of wounds due to their therapeutic activities, availability, affordability, and relatively low cost (Albahri et al., 2023). These phytomedicines have immense potential in the function of wound healing due to the presence of various active constituents, which have been proven to enhance healing, reduce scar formation with minimal unwanted side effects (Maver et al., 2015).

Polygonum minus Huds. (syn. *Persicaria minor*) (Polygonaceae) grows in Southeast Asian countries such as Malaysia, Thailand, Indonesia, and Vietnam (George et al., 2014). This aromatic plant, commonly known as kesum or laksa leaf in Malaysia is a popular flavouring ingredient in culinary dishes. It is also consumed raw as a salad due to its potential health benefits, which include improving digestion, promoting skin health, and reducing inflammation and pain (Vikram et al., 2014). Several studies have shown that the leaves of *P. minus* contain natural antioxidants due to the phenolic compounds (Azlim Almey et al., 2010; George et al., 2014; Maizura et al., 2011; Qader et al., 2012; Sumazian et al., 2010). In particular, an aqueous extract from the leaves of *P. minus* was reported to contain flavonoids, such as quercetin, that could contribute to its antioxidant activity as evidenced by its potent radical-scavenging activity (George et al., 2014; Huda-Faujan et al., 2007). Previous studies have demonstrated that several secondary metabolites, mainly phenolic compounds such as quercetin and kaempferol from various plant extracts, have the potential to promote wound healing (Agour et al., 2022; Ebbo et al., 2022; El-Sayed et al., 2016). These compounds have the potential to accelerate skin wound healing when applied topically, primarily by regulating inflammatory markers and mitigating oxidative stress (Yadav et al., 2018). Moreover, research has explored the wound healing potential of other plants within the Polygonum genus, including *P. cuspidatum* and *P. barbatum* (Wu et al., 2012; Nkuete et al., 2015). However, *P. minus* remains unexplored in terms of its wound healing potential. Hence, this study was carried out to evaluate the phytochemical composition, antioxidant activity, and wound healing potential of aqueous extract from the stem and leaves of *P. minus* on the proliferation and migration of human epidermal keratinocytes.

2. Objectives

The objectives of the study are:

1) To qualitatively determine the presence of secondary metabolites in *P. minus* aqueous extract by phytochemical evaluation.

2) To determine the antioxidant capacity, total phenolic, and flavonoid contents of *P. minus* aqueous extract.

3) To evaluate the effect of *P. minus* aqueous extract on cell viability, proliferation, and migration of human keratinocyte (HaCaT) cells.

3. Materials and methods

3.1 Collection and extraction of *P. minus*

Plant material was procured from Biotropics Malaysia Berhad, Malaysia. The sample was authenticated by a taxonomist from the Institute of Bioscience, Universiti Putra Malaysia (UPM) and voucher specimen (SK 2077/12) was deposited in the Herbarium of the Institute of Bioscience, UPM Malaysia. An aqueous extract was obtained from the aerial parts, including the stems and leaves of the plant. Fresh plant material was first subjected to oven-drying until its moisture content dropped to below 10%. The dried stems and leaves were then finely chopped into fragments. The extraction process involved immersing these fragments in water at a ratio of 1:20 and percolating the mixture through two cycles, each lasting 4 hours at 80°C. The resulting liquid was filtered and evaporated. Subsequently, the concentrated liquid was freeze-dried until its moisture content decreased to below 8% w/w. To ensure preservation, the extract was vacuum-sealed in aluminium foil, safeguarding it in a cool, low-humidity environment with no direct exposure to sunlight.

3.2 Phytochemical screening

The aqueous extract of *P. minus* was assessed for the presence of secondary metabolites including flavonoids, terpenoids, alkaloids, saponins, tannins, steroids, and cardiac glycosides according to procedures described by Sharma et al. (2020).

3.3 Estimation of total phenolic content (TPC)

The TPC of the *P. minus* aqueous extract was determined using the Folin-Ciocalteu reagent as described by Sahlan et al. (2018). *P. minus* extract (25 µL) was mixed with 25% Follin reagent (100 µL) and shaken for four minutes at room temperature. Then, sodium carbonate solution (75 µL, 7.5% w/v) was added. The solutions were mixed and allowed to stand in the dark for two hours at room temperature. Absorbance was measured at 765 nm and the analysis was performed in triplicate. The standard curve was calibrated using gallic acid and the results were expressed as µg/mL Gallic Acid Equivalents (GAE).

3.4 Estimation of total flavonoid content (TFC)

The aluminium chloride colorimetric method, as described by Farasat et al. (2014), was used to determine the TFC of the *P. minus* aqueous extract. *P. minus* extract (20 µL) was mixed with a 10% aluminium chloride solution (20 µL), 1M potassium acetate (20 µL), and distilled water (140 µL). The mixture was shaken for 1 minute prior to incubation for 30 minutes in the dark at room temperature. Absorbance readings were taken at 415 nm and data were analysed in triplicate. The standard curve was calibrated using quercetin and the results were expressed as µg/mL Quercetin Equivalent (QE).

3.5 DPPH free radical scavenging assay

The DPPH free radical scavenging activity of the *P. minus* aqueous extract was conducted according to the method described by Nafi et al. (2019). Briefly, a 1 mM DPPH solution was prepared by diluting DPPH (5 mg) in methanol (100 ml). Trolox was used as a reference standard. Then, different concentrations of *P. minus* aqueous extract and the standard Trolox were prepared (7.81, 15.63, 31.25, 62.50, 125.00, 250.00, 500.00, and 1000.00 µg/mL) using the serial dilution method with dimethyl sulfoxide (DMSO) as the solvent. The standard and sample solutions (25 µL) were added to a 96-well plate. Then, the methanolic DPPH solution (200 µM) was added to each well and mixed uniformly. A negative blank solution was prepared by mixing 1 mM DPPH (200 µL) with DMSO (25 µL). The plate was then incubated in the dark at room temperature for 30 minutes. After incubation, the absorbance (abs) was read at 517 nm using a microplate reader, and the procedure was repeated three times. The DPPH radical scavenging activity was calculated using Equation (1):

$$\text{Inhibition (\%)} = \frac{(\text{Abs of blank} - \text{Abs of sample})}{\text{Abs of blank}} \times 100\% \quad (1)$$

3.6 Ferric reducing antioxidant power assay

The ferric reducing antioxidant power (FRAP) of the *P. minus* aqueous extract was determined using the method described by Hernandez Zarate et al. (2018) with slight modifications. In short, the FRAP reagent

comprising three solutions was prepared; solution 1 (300 µM pH 3.8 acetate buffer) was prepared by diluting sodium acetate ($C_2H_3NaO_2$) (3.12 g) and glacial acetic acid (CH_3COOH) (1.6 mL) in distilled water (1 L). For solution 2, 2,4,6-tripyridyl-S-triazine (TPTZ) (0.031 g) was dissolved in 37% hydrochloric acid (HCl) (10 mL). Concentrated HCl (36.46 mol) (1.46 mL) was added to distilled water (1 L). Solution 3 (20 mM of ferric chloride hexahydrate ($FeCl_3.6H_2O$)), was prepared by dissolving $FeCl_3.6H_2O$ (0.054 g) in distilled water (10 mL). The FRAP reagent was formed by mixing all the three solutions in a 10:1:1 volume ratio (v/v/v) and heated at 37°C for 10 minutes before use. The *P. minus* aqueous extract (30 µL) was added. The resulting mixture was allowed to rest for 30 minutes at 20°C in the dark. The absorbance was read at 593 nm using a microplate reader spectrophotometer. All measurements were conducted in triplicate. The results were calculated using a calibration curve of ferrous sulfate as standard.

3.7 *In vitro* wound healing assay

3.7.1 Cell culture

Human epidermal keratinocyte (HaCaT) cell line was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). HaCaT cells from passage 10-15 were maintained in complete media Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum, 1% penicillin-streptomycin and incubated at 37°C, 5% carbon dioxide (CO_2) humidified incubator. The cells were propagated until the density reached 80–95% confluency before subculture.

3.7.2 Cell viability and proliferation assays

The cell viability and proliferation were determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay according to the method described by Kamarazaman et al. (2022). The extract (1000 - 7.81 µg/mL) was prepared by serial dilution using 0.5% DMSO. Cells were seeded into 96-well plate at $1x10^4$ cells per well and incubated for 24 hours at 37°C. After incubation, the cells were treated with different concentrations of *P. minus* extract or with vehicle (0.5% DMSO) in complete media and further incubated at 37°C for 24 hours. Then, 0.5% MTT solution was added into each well, and the plates were incubated again for 4 hours at 37°C. The produced water-insoluble formazan product was solubilised using DMSO and absorbance was read using a microplate reader at 570 nm. Vehicle-treated cells served as control. The percentage of cell viability was calculated using Equation (2). The procedure was also repeated at 48 and 72 hours.

$$\text{Cell viability (\%)} = \left(\frac{\text{Absorbance of treated cells}}{\text{Absorbance of control cells}}\right) \times 100 \quad (2)$$

3.7.3 Cell migration assay

The effect of the *P. minus* extract on the migration of HaCaT cells was performed according to the procedure described by Kamarazaman et al. (2022). The cells were seeded at $1x10^6$ cells per well into six well plates. After reaching confluency, a scratch was made across the well, using a 200 µL pipette tip. Subsequently, the cells were treated with or without the *P. minus* extract at different concentrations (15.63, 31.25, 62.5, and 125 µg/mL). Allantoin at 10 µg/mL served as positive control. Phase-contrast images were recorded at the time of wounding (0 h), 18, and 24 hours thereafter. Cell migration was quantified as the remaining scratch wound area relative to the initial wound area using ImageJ software.

3.8 Statistical analysis

Each experiment was conducted in triplicate. Values are shown as mean ± standard deviation (SD). Data were analysed by analysis of variance (ANOVA) and Bonferroni test using SPSS software (version 20). Statistical significance was fixed at $P < 0.05$.

4. Results and discussion

4.1 Phytochemical screening

The results of the phytochemical screening are shown in Table 1. Phytochemical screening is a convenient technique to classify the diversity of secondary metabolites present in different medicinal plant species (Singh et al., 2022). This is a reliable technique to provide preliminary information on the metabolites present in plant extracts (Ibrahim et al., 2016). The results of phytochemical analysis of the *P. minus* aqueous extract revealed the presence of all seven metabolic groups consisting of flavonoids, terpenoids, alkaloids, saponins, tannins, steroids, and cardiac glycosides (Table 1). These findings align with a study reported by Imelda et al. (2014) who

identified the presence flavonoids, alkaloids, saponins, tannins, and triterpenoids in an ethanolic extract of *P. minus*, while steroids were notably absent. Similarly, these results are in accordance with a study reported by Kartikasari et al. (2022), which reported the presence of alkaloids, flavonoids, saponins, and tannins in the ethanolic extract of *P. minus*, while observing the absence of steroids and triterpenoids. Additionally, Qader et al. (2012) reported that the ethanolic extract of *P. minus* contained phenolic compounds such as coumaric acid, gallic acid, and rutin. These secondary metabolites perform specific biological functions that enhance the therapeutic activities including antioxidant, anti-inflammatory, antiulcer, and antimicrobial activities (Christapher et al., 2015).

4.2 Estimation of total phenolic content (TPC) and total flavonoid content (TFC)

Table 2 shows the TPC and TFC values of the *P. minus* aqueous extract in comparison to Trolox. The TPC value for the *P. minus* extract was higher at 137.74 ± 0.75 µg/mL GAE compared to Trolox, which recorded TPC value at 63.38 ± 0.32 µg/mL GAE. Similarly, *P. minus* showed a greater TFC value of 177.08 ± 3.16 µg/mL QE, while Trolox had a lower TFC value of 33.00 ± 0.02 µg/mL QE.

A study by Abdullah et al. (2017) found that the TPC values of *P. minus* aqueous extract was at 174.00 µg/mL GAE, which was higher than the TPC value of the *P. minus* in this study. Conversely, Khalid, & Babji (2018) and Christapher et al. (2016) reported lower TPC values for *P. minus* aqueous extracts, at 48.23 µg/mL GAE and 74.25 µg/mL GAE, respectively. Additionally, Abdullah et al. (2017) also recorded a lower TFC value for *P. minus* (43.65 µg/mL QE) than what was observed in this study. This observation is consistent with another study by Christapher et al. (2016), where the TFC value for *P. minus* was measured at 11.95 µg/mL QE. Several flavonoid compounds (quercetin, rutin, and catechin) and phenolic acid compounds (coumaric acid and gallic acid) have been detected in *P. minus* extracts (Hamid et al., 2020; Yahaya et al., 2020). The presence of these metabolites contributes to the biological activities of *P. minus*, including antibacterial, anti-inflammatory, antiviral, and anticancer properties (Ridzuan et al., 2019). The concentration of polyphenolic compounds in *P. minus* can vary depending on factors such as light intensity, carbon dioxide (CO_2) levels, soil water content, and nutrient levels (Mohd Yusof et al., 2021).

4.3 DPPH free radical scavenging assay

Table 3 shows the scavenging activity of DPPH free radicals by the *P. minus* aqueous extract in comparison to Trolox. Based on Table 3, the DPPH free radical scavenging activity of the *P. minus* aqueous extract was at 79.50%, and the DPPH inhibition of Trolox was 99.83%. The DPPH IC_{50} values indicate the concentration of samples required to inhibit 50% of DPPH free radicals. Lower IC_{50} values denoting higher DPPH scavenging activity at lower concentration. The IC_{50} value of *P. minus* was 213.20 ± 50.96 µg/mL. Trolox had the lowest IC_{50} values at 70.52 ± 5.93 µg/mL.

Table 1 Qualitative analysis of the phytochemical assessment of the *P. minus* aqueous extract.

Phytochemical metabolites	Presence
Flavonoids	++
Triterpenoid	+++
Alkaloids	+++
Saponins	+++
Tannins	++
Steroids	+++
Cardiac Glycosides	+++

Key: "+": present; "++": moderately present; "+++": highly present; "- ": absent.

Table 2 TPC and TFC values of *P. minus* and the standard, Trolox.

Sample	TPC value (µg/mL GAE)	TFC value (µg/mL QE)
P. minus	137.74 ± 0.75	177.08 ± 3.16
Trolox	63.38 ± 0.32	33.00 ± 0.02

Note: Results were reported in mean ± standard deviation. "TPC": Total phenolic content; "TFC": Total flavonoid content; "GAE": Gallic acid equivalent; "QE": Quercetin equivalent. Assays were done in triplicate.

Table 3 DPPH free radical scavenging activity and DPPH IC_{50} value of *P. minus* in comparison to Trolox.

Sample	DPPH inhibition (%)	DPPH IC_{50} (µg/mL)
P. minus	79.50	213.20 ± 50.96
Trolox	99.83	70.52 ± 5.93

Note: Results were reported in mean ± standard deviation. Assays were done in triplicate.

Table 4 FRAP values of *P. minus* and the standard, Trolox.

Sample	FRAP values (µM Fe^{2+})
P. minus	1485.67 ± 0.05
Trolox	6584.98 ± 0.07

Note: Results were reported in mean ± standard deviation. Assays were done in triplicate.

In human physiology, free radicals and reactive oxygen species (ROS) are formed as by-products of cellular metabolic processes (Adli et al., 2022). Accumulation of free radicals and ROS can lead to oxidative stress, which can induce the development of cardiovascular diseases, cancers, and neurodegenerative diseases (Yilmaz-Ozden et al., 2021). Antioxidants are a group of compounds that can counter the destructive effects of free radicals and ROS by inhibiting their actions and protecting cells from oxidative stress (Rahim et al., 2019). The DDPH assay in this study was conducted by assessing the free radical scavenging capability of an aqueous *P. minus* extract and the standard, Trolox, to reduce the 1,1-diphenyl-2-picrylhydrazil (DPPH) free radical, as indicated by the colour changes from deep purple to pale yellow (Baluchamy, & Subramaniam, 2023).

4.4 Ferric reducing antioxidant power (FRAP) assay.

Table 4 shows the FRAP values of *P. minus* aqueous extract in comparison to Trolox. The FRAP values of *P. minus* was 1485.67 ± 0.05 µM Fe^{2+}, meanwhile the FRAP value of Trolox was 6584.98 ± 0.07 µM Fe^{2+}.

The FRAP assay measures the ability of samples to reduce ferric ions (Fe^{3+}) to ferrous ion (Fe^{2+}), where the reduction reaction can be observed by the colour changes from colourless to blue (Hamid et al., 2020). Based on a study by Abdullah et al. (2017), the FRAP value of *P. minus* aqueous extract was 898.33 µM Fe^{2+}, which was lower than the FRAP value of *P. minus* observed in this study. The finding was in line with a study conducted by Ghazali et al. (2014), where the FRAP value of *P. minus* aqueous extract measured at 842.61 µM Fe^{2+}.

The metabolites in *P. minus* leaves are responsible for the antioxidant activity of *P. minus* extract (Yahaya et al., 2020). There was a direct correlation between TPC and TFC with antioxidant activities (Adli et al., 2022). The higher the concentration of phenolic acids and flavonoids in the *P. minus* extract, the higher its free radical scavenging capability (Christapher et al., 2016). While the exact mechanisms governing the interaction between these phytochemical compounds and free radicals remain poorly understood, Abdullah et al. (2017) suggested that these compounds function as electron donors, reducing the free radicals and thereby neutralizing them into harmless molecules.

Furthermore, it is noteworthy that the polarity of the solvent used for extracting phytochemicals from *P. minus* can significantly influence its biological activities. Christapher et al. (2016) demonstrated that the methanol extract exhibited the highest TPC, whereas the aqueous extract displayed the highest TFC. Likewise, the antioxidant analyses demonstrated that the methanol extract possessed a higher antioxidant

capacity compared to the aqueous extract (Abdullah et al., 2017). Organic solvents such as methanol are less polar compared to water, which means they can effectively extract a wider range of polar and non-polar antioxidant compounds from plant materials (Chaves et al., 2020). This might include a broader spectrum of phenolic compounds, flavonoids, and other antioxidants that contribute to the overall antioxidant capacity (Abdullah et al., 2017).

4.5 *In vitro* wound healing assay

Figure 1 illustrates the viability of HaCaT cells following a 24-hours treatment with varying concentrations of the *P. minus* aqueous extract. The results indicate that the *P. minus* aqueous extract did not induce any cytotoxic effects on HaCaT cells at all tested concentrations, with the percentage of cell viability consistently exceeding 80%. In line with the findings of Roy et al. (2023) and Che Zain et al. (2020), extracts are considered non-toxic when cell viability remains above 80%. Previous research supports these findings, demonstrating the non-toxic nature of *P. minus* aqueous extract on both normal endothelial (EA.hy926) and cancer (HCT116, HT29, and HeLa) cells (Christapher et al., 2016). Similarly, a study by Wahab et al. (2015) reported that *P. minus* methanol extract did not exhibit cytotoxicity toward Vero cells with an LC_{50} value of 875 mg/L. As none of the tested concentrations displayed cytotoxic effects, the same concentrations of the extract were chosen for the subsequent proliferation assay.

In the proliferation assay, an increase in cell viability was observed at all tested concentrations after 48 hours of treatment, as depicted in Figure 2. Notably, the proliferative effect of the extract was particularly significant ($p<0.05$) at concentrations of 15.63 and 7.82 µg/mL, leading to cell proliferation rates of 132.68 ± 0.08% and 145.65 ± 0.09%, respectively, compared to the control. However, following the 72-hours of treatment, the proliferative effect of the extract was observed within the concentrations of 62.5 - 7.81 µg/mL, with statistically significant results ($p<0.05$) noted at concentrations of 31.25 - 7.81 µg/mL (ranging from 140.27 - 153.08%) in comparison to the control. In contrast, at concentrations of 125, 250, 500, and 1000 µg/mL, the extract inhibited cell proliferation and exhibited cytotoxic effect on HaCaT cells after 72 hours. Consequently, concentrations within the range of 62.5 - 7.81 µg/mL were selected to assess the cell migratory activity of the extract.

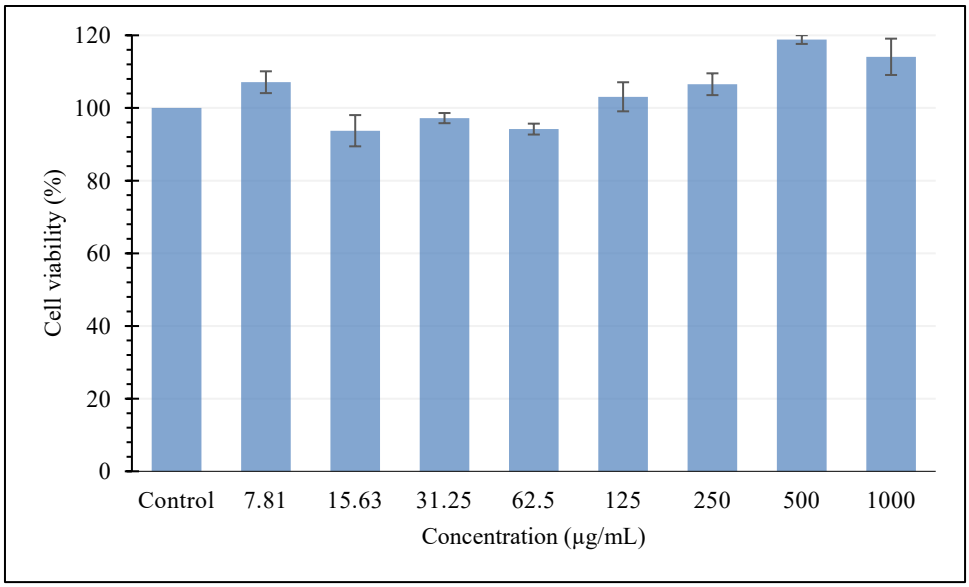

Figure 1 Effect of the *P. minus* aqueous extract on the viability of HaCaT cells after 24 hours exposure to various concentrations of the *P. minus* aqueous extract. Control group represents cells treated with 0.5% DMSO. Experiments were performed in triplicate and data are presented as mean ± SD.

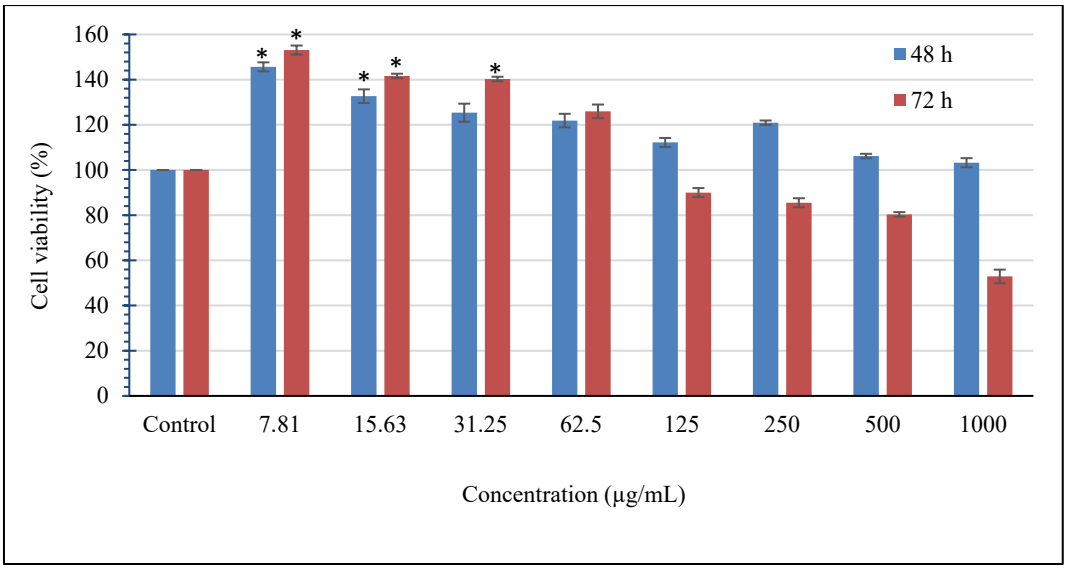

Figure 2 Effect of *P. minus* aqueous extract on the proliferation of HaCaT cells at 48 and 72 hours. Control group represents cells treated with 0.5% DMSO. The data are presented as mean ± SD of three independent experiments. Values of $*P < 0.05$ were considered statistically different as compared to control.

Keratinocyte cell migration plays a vital role in the process of re-epithelialization following various types of skin injuries, including superficial wounds and deep burns (Abate et al., 2021; Long et al., 2018). In this study, the migration rate of HaCaT cells was assessed using a scratch assay, which tracks the progression of wound closure over time. Specifically, the changes in wound gap were measured at three-time intervals (0, 18 and 24 hours) after creating the scratch. The percentage of cell migration was calculated based on the reduction in gap area compared to the initial gap area (Kamarazaman et al., 2022). As illustrated in Figure 3, after 24 hours of treatment, cells treated at lower concentrations (15.63 and 7.81 µg/mL) exhibited a significant ($p<0.05$) increase in the percentage of wound closure, measuring 35.34 ± 3.02% and 45.63 ± 4.00%, respectively, compared to the control group (26.64 ± 2.00%). Interestingly, there was no significant difference ($p>0.05$) in the percentage of wound closure between the group treated with allantoin (47.43 ± 2.09%), serving as the positive control, and the group treated with the *P. minus* aqueous extract at 7.81 µg/mL (45.63 ± 4.00%). The visual representations of the cells are shown in Figure 4.

The present study demonstrates that *P. minus* aqueous extract promotes wound closure by stimulating keratinocyte cell migration in the scratch assay, a pivotal event in the proliferative phase of the wound healing process. Upon tissue injury, ROS are released, and an excess of ROS can lead to oxidative stress, negatively impacting cell proliferation and inhibiting healthy cell turnover (Alexander et al., 2019). Therefore, antioxidant enzymes play a crucial role in defending cells against ROS. Additionally, it has been reported that certain plant extracts with antioxidant activity can enhance the process of cutaneous wound healing (Addis et al., 2020; Suntar et al., 2012). The present study confirms previous findings regarding the antioxidant activity of *P. minus* aqueous extracts as evidenced by both DPPH and FRAP assays (Abdullah et al., 2017; George et al., 2014; Hassim et al., 2015). *P. minus* extract is rich in polyphenol compounds, which are associated with various biological properties, including antioxidant activity. This activity is instrumental in wound healing, as it helps prevent and protect against oxidative damage caused by free radicals (Matos, 2009). Furthermore, studies have shown that flavonoids can promote proliferation and survival of human keratinocyte through stimulating the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase/protein kinase B (PI3K/Akt) signaling cascades, which are important molecular pathways involved in re-epithelialization (Zulkefli et al., 2023; He et al., 2020).

Additionally, the present study revealed that the aqueous extract of *P. minus* enhances the migration of HaCaT cells in a reverse dose-dependent manner. The percentage of cell

migration was significantly increased at lower concentrations, comparable to the effect of allantoin, a known promoter of cell proliferation and migration (Forero-Doria et al., 2020). The results were similar to previous reports indicating that extracts at higher concentrations do not necessarily yield more desirable effect (Kamarazaman et al., 2022; Abdel Latif et al., 2019; Che Zain et al, 2020).

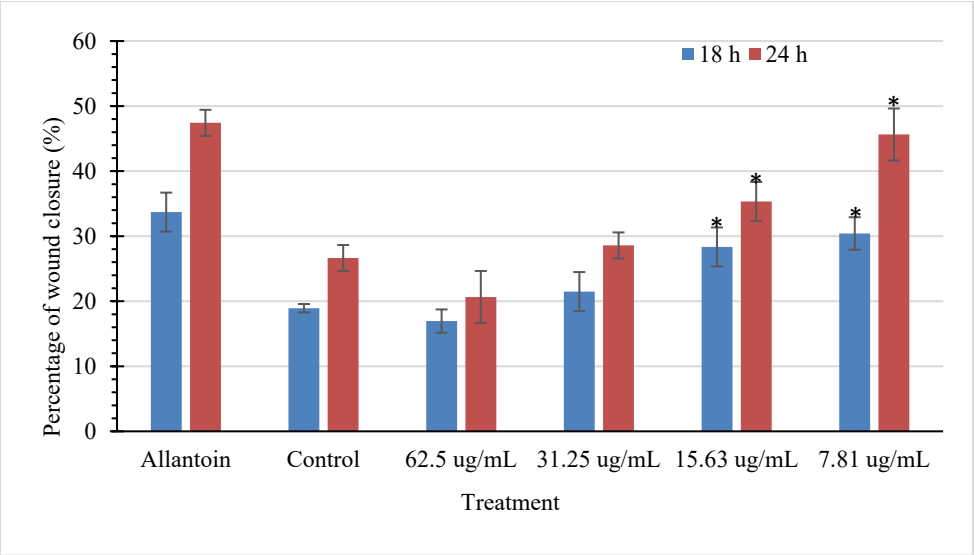

Figure 3 Effect of the *P. minus* aqueous extract on the migration rate of HaCaT cells following 24 hours after treatment as determined by the scratch assay. Cell migration was quantified as the remaining scratch wound area relative to the initial wound area using ImageJ software. Data are expressed as mean ± SD of three independent experiments. Statistically significant differences are indicated as $*P < 0.05$ as compared to control.

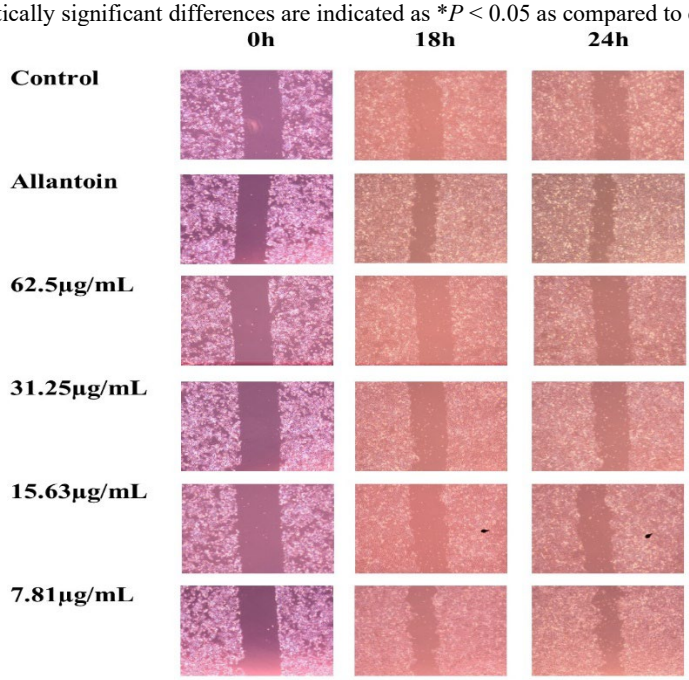

Figure 4 Representative images for migration of HaCaT cells treated with different concentrations of the *P. minus* aqueous extract at 18 and 24 hours after incubation (x4 magnification).

5. Conclusions

In summary, this study underscores the potential of *P. minus* aqueous extract for wound healing by stimulating the cell proliferation and migration of keratinocytes. The observed wound healing properties of the extract could be attributed to its antioxidant properties and its high phenolic contents. These preliminary data point towards the potential of *P. minus* aqueous extract as a subject for further exploration in the context of wound healing materials. Subsequent studies are necessary to validate its efficacy as a wound healing agent.

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