Vol. 18(3), pp. 17-24, April 2024
DOI: 10.5897/AJPP2024.5380
Article Number: DCC7A4F72001
ISSN 1996-0816

http://www.academicjournals.org/AJPP

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African Journal of Pharmacy and Pharmacology

Full Length Research Paper

Effects of the use of *piper betle* l. leaf extract associated with adipose-derived stem cells on ulcers in *streptozotocin*-induced diabetic mice

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Received 6 January, 2024; Accepted 14 March, 2024

Diabetic ulcers often present challenges in healing or may heal incompletely without the restoration of proper function. *Piper betle L*. is recognized as a valuable traditional medicine for treating open wounds due to its antibacterial and anti-inflammatory properties, as well as its ability to stimulate fibroblast proliferation. Additionally, adipose-derived stem cells (ADSCs) have the capacity to self-renew and differentiate into various cell types, facilitating the repair of wounded organs in the body. Therefore, this study evaluates the therapeutic effect of combining ADSCs with *Piper betle L*. extract on the process of healing chronic wounds. Mice with chronic diabetes and induced ulcers were randomly divided into four groups: Group 1 (control, n=15), Group 2 (*Piper betle L*. extraction, n=15), Group 3 (ADSCs injections, n=15), and Group 4 (ADSCs injections combined with *Piper betle L.* extraction, n=15). The wounds in the control group, the *Piper betle L.* extraction group, and the ADSCs group healed on days 18, 17, and 16, respectively. However, the group treated with ADSCs combined with *Piper betle L.* extraction healed as early as the 14th day. *Piper betle L.* extraction and ADSCs are regarded as paracrine signals that promote the proliferation and migration of fibroblasts and keratinocytes, leading to faster healing. Moreover, *Piper betle L.* possesses antibacterial properties, thus, when combined with ADSCs in treatment, it accelerates the healing process of diabetic wounds.

Key words: *Piper betle* L., diabetes, ulcers, adipose-derived stem cells (ADSCs).

INTRODUCTION

According to the 2021 report of the International Diabetes Federation, approximately 10.5% of the adult population worldwide (aged 20 to 79 years) has diabetes, and this number is projected to rise to approximately 783 million

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people by 2045. It is estimated that a quarter of individuals with diabetes will develop a foot ulcer at some point. Despite receiving the best standard treatment, about 50% of patients are still not completely healed after 12 weeks [https://idf.org/]. In Vietnam, in 2017, more than 3.5 million patients were hospitalized due to diabetes, with this number increasing, leading to hospital overload. Among these cases, diabetic ulcer complications account for approximately 16 to 66%. Currently, the cost of treating each diabetes episode (lasting 2 to 4 days) is around 2 million VND, and this cost increases fourfold if the patient experiences ulcer complications (Thu et al., 2021).

Wound healing poses a significant challenge for diabetic patients (Jhamb et al., 2016), often leading to limb amputation due to the lack of effective therapies. Cell-based therapies, particularly those utilizing stem cells, are emerging as promising options for wound treatment in diabetic skin. Adipose-derived stem cells (ADSCs) offer significant potential in regenerative medicine due to their immunomodulatory, renewal, and differentiation capabilities. They are known to secrete cytokines and growth factors such as VEGF, TGF-β, and PDGF, contributing to angiogenesis and tissue regeneration (Mazini et al., 2019). *Piper betle L.,* a tropical vine in the Piperaceae family found in South and Southeast Asian countries has been traditionally used for various medicinal purposes, including treating ulcers, joint pain, and hemorrhoids. Recent research has highlighted the components of *Piper betle L*. leaves for their antioxidant properties, thrombosis resistance without compromising hemostasis, and promotion of dermal fibroblast formation (Nayaka et al., 2021). This study provides new insights into the mechanism and accelerated wound healing potential of ADSCs in conjunction with *Piper betle L.* extract, aiming to develop an effective and safe treatment for chronic skin ulcers, particularly diabetic ulcers.

MATERIALS AND METHODS

Induction of diabetes using Streptozotocin

Streptozotocin (STZ) was dissolved in 0.1 M citrate buffer (pH 4.5) at a concentration of 4 mg/mL. Twenty male Swiss mice, aged 5 weeks and weighing 25±3 grams, were used for the study. Prior to inducing diabetes, their food consisted of 40 to 60% lipid for two weeks. Environmental conditions were maintained at a temperature of 24 ± 1ºC, humidity at 55 ± 5%, and light-dark cycles of 12 h (from 8 am to 8 pm). The mice underwent a 24-h fasting period before injection.

STZ was injected intraperitoneally at a dosage of 60 mg/kg (Huong et al., 2021). Within 24 h post-injection, the mice were given access to 10% glucose solution and resumed their diet of 40 to 60% lipid as specified above. Blood samples were collected from the tails after 1 week for glucose concentration measurement using a blood glucose meter (Onetouch Ultra 2, Johnson and Johnson, USA). Mice with blood glucose levels exceeding 200 mg/dL were considered to have developed diabetes. The injection procedure was repeated once a week for a total of four times before cessation of treatment.

Dispense (collect) the extraction from *Piper betle* L. leaves

Piper betle L. leaves collected from Nui Thanh District, Quang Nam Province, Vietnam were identified and classified by Botany laboratories, Hue University of Sciences. Prepare 500 g fresh *Piper betle* L. leaves washed twice by distilled water. Comminute the leaves, then compost with ethanol 70% (ratio 1:10) for 5 h, at 50ºC for evaporating oil. Next is the concentration by evaporation using a rotary evaporator at 70ºC until collecting a kind of solid light brown color powder?

Quantitative analysis of hydroxychavicol in the *Piper betle* L. leaves extract by HPLC

The quantitative estimation of hydroxychavicol in *Piper betle L*. leaves extract was analyzed using high performance liquid chromatography (HPLC) with a Waters 2695 System controller coupled with a photodiode array detector. An Agilent Zorbax Eclipse XDB-C18 column (4.6×150 mm, 5 µm) served as the stationary phase. The mobile phase operated in gradient mode consisting of 0.1% orthophosphoric acid (solvent A) and 100% acetonitrile (solvent B). The flow rate of the mobile phase was maintained at 1 mL/min, with detection performed at a wavelength of 280 nm. A calibration curve was generated using concentrations of 100, 200, and 300 µg/mL of hydroxychavicol. A 1 mg/mL aqueous extract was analyzed qualitatively using the HPLC system. The percentage content of hydroxychavicol in the *Piper betle L*. leaves extract was determined using the calibration equation. The quantitative estimation of hydroxychavicol (mg/g) in the Piper betle L. leaves extract was conducted via HPLC, with a calibration equation of $Y = 26100x + 260000$ ($R^2 = 0.9976$).

Test the anti-microbial and anti-fungal potential

The antibiotic-resistant bacterial strains utilized in this study included Escherichia coli (ATCC 25922), Pseudomonas aeruginosa (ATCC 27853), Enterococcus faecalis (ATCC 29212), and Staphylococcus aureus (ATCC 25923), provided by the Department of Microbiology at Hue Central Hospital, Vietnam, as well as Microsporum gypseum and Candida albicans, provided by the Department of Microbiology at the University of Medicine Pham Ngoc Thach. The procedure began by suspending the strain of microorganisms in tubes containing 0.9% NaCl solution, followed by vortexing for homogenization. The optical density (OD) of the bacterial solution was adjusted to 0.55. Next, 200 µL of each bacterial solution was inoculated onto agar petri dishes using sterile swabs, ensuring even distribution across the plates. Perforations with a diameter of 4 mm were then made on the agar surface.

Subsequently, 300, 200, and 100 mg/mL solutions of *Piper betle* L. leaf extract were aspirated and placed into the perforated holes on the petri dishes. Betadine and NaCl were employed as positive and negative controls, respectively. The petri dishes were then incubated at 4ºC for duration of 8 to 15 h, followed by transfer to a 37ºC incubator for an additional 24 h. Finally, the diameter of the antibacterial rings formed around the perforated holes was measured to assess the antimicrobial activity.

Fibroblast and keratinocyte cell culture and treatment with *Piper betle* L. extraction

Fibroblast cells (NIH3T3) and Keratinocyte cells (HaCaT) obtained from the Vietnam Academy of Science and Technology were

seeded into 6-well plates containing DMEM/F12 medium (Sigma-Aldrich Corporation, USA) supplemented with 10% FBS (Sigma-Aldrich Corporation, USA) and 1% penicillin. The plates were then incubated at 37°C with 5% CO2 until the cell density reached approximately 80%.

Upon reaching the desired cell density, wounds were created by scratching the cell monolayers, followed by rinsing twice with phosphate-buffered saline (PBS) from Sigma-Aldrich Corporation, USA. Subsequently, fresh medium was added to each well, and *Piper betle L*. leaf extract at concentrations of 1, 2, and 3 mg/mL was supplemented into the wells. The plates were then placed in a humidified atmosphere at 37ºC with 5% CO2 and monitored continuously for 48 h, with observations recorded every 3 h.

Isolation and culture of ADSCs

Retrieve 1 gram of adipose tissue from under the skin of mice and place it into a sterilized 15 mL Falcon tube. Add 1 mL of DMEM/collagenase (mg/mL) solution obtained from Sigma-Aldrich Corporation, USA, and vortex for 3 min. Incubate the mixture for 15 min at room temperature, then transfer it to a heated cabinet and maintain at 37ºC for 50 min, stirring constantly for 10 min.

After the incubation period, filter the suspension and centrifuge at 2500 rpm for 5 min at 37°C. Discard the supernatant, add 1 mL of PBS, shake, and centrifuge again at 2500 rpm for 3 min at 37°C to collect the cells. Use DMEM/F12 medium supplemented with 10% FBS and 1% penicillin for cell culture.

Assess the viability of the cells and conduct cell seeding at a density of 50,000 cells/cm2 in DMEM/F12 medium supplemented with 10% FBS and 1% penicillin. Maintain the cells at 37°C with 5% CO2. After 3 days, replace the medium once.

Characterization of ADSCs

The cells were harvested by dissociating adipose-derived stem cells (ADSCs) using 0.25% trypsin-EDTA solution obtained from Sigma-Aldrich Corporation, USA. Subsequently, approximately 1×105 cells were washed and labeled with fluorescence-conjugated antibodies targeting specific cell surface markers, including CD34, CD36, CD45, CD73, CD79a, CD90, CD105, CD106, and HLA-DR. The labeled cells were then incubated at room temperature for 30 min. Isotype control IgG was utilized to stain the cells for control purposes.

After incubation, the cells were washed twice with PBS and subjected to analysis using fluorescence-activated cell sorting (FACS) equipment from BD - Becton Dickinson, USA, to quantify and characterize the expression levels of the respective cell surface markers.

Healing potential test combining the *Piper betle* L. extraction and ADSCs

Mice with diabetes were anesthetized via peritoneal injection with a mixture of Xylazil, Ketamil, and NaCl in a ratio of 2:3:3 (Llium, Australia). Subsequently, the mice were shaved, and ulcers were created using a biopsy tube to produce 7 mm diameter wounds. The ulceration process involved exposing the skin to hot water at 100°C for 10 s followed by perforation. *Piper betle L*. balm was diluted to a concentration of 10% with distilled water.

Twenty diabetic mice, post skin excision, were divided into four groups: Group 1 received injections of NaCl only, Group 2 received NaCl injections combined with *Piper betle L*. extract, Group 3 received injections of 106 adipose-derived stem cells (ADSCs), and Group 4 received injections of 106 ADSCs combined with *Piper betle L*. extract. All injections were administered via the caudal veins.

Mice in Groups 2 and 4 were additionally anointed with *Piper betle L*. extract at a concentration of 10% after excision, with applications performed twice a day. The wound morphology was observed over an 18-day period, and histological changes were evaluated.

Statistical analysis

All experiments were repeated three times. Differences between groups were determined using a one-way statistical software SPSS version 18.0 with significance level $P < 0.05$. The results were expressed as mean ± SD.

RESULTS

Quantification of hydroxychavicol in the *Piper betle* L. extracted by HPLC

Piper betle L. is recognized as one of the valuable medicinal herbs abundant in northern and central Vietnam. Its fresh leaves contain numerous bioactive compounds, with hydroxychavicol being a major phenolic compound renowned for its reported anti-nitrosation, anti-mutagenic, and anti-carcinogenic properties. Additionally, it exhibits significant anti-inflammatory and antioxidant effects. The content of hydroxychavicol in *Piper betle L*. extract was determined using the HPL method, with a retention time of 3.891 minutes for the controlled hydroxychavicol sample (Figure 1a). Under identical conditions, the analysis of the extract yielded the same retention time as the controlled sample (Figure 1b). The hydroxychavicol content in the *Piper betle* L. leaf extract was found to be 11.47%.

Anti-microbial and anti-fungal potential of *Piper betle* L. extract

The results presented in Table 1 indicate that the antibacterial activity of *Piper betle L*. leaf extract was effective against the four tested microorganisms at a concentration of 300 mg/mL, exhibiting inhibition zones ranging from 13.2 to 25.3 mm. Furthermore, at this concentration, the extract demonstrated inhibitory activity against *Microsporum gypseum* strains as well. However, at concentrations of 100 mg/mL, the extract was unlikely to inhibit the growth of all four tested microorganisms. Previous studies have consistently affirmed the antibacterial potential of *Piper betle L*. extract. For instance, research by Chakraborty et al. (n.d) highlighted the extract's strong inhibitory effects against strains such as *Staphylococcus aureus and Escherichia coli* (Nayaka et al., 2021). Similarly, other studies have reported the extract's ability to inhibit the proliferation of various strains including *E. coli, Pseudomonas aeruginosa, and S. aureus* (Nair and Chanda, 2008).

These findings are consistent with numerous previous

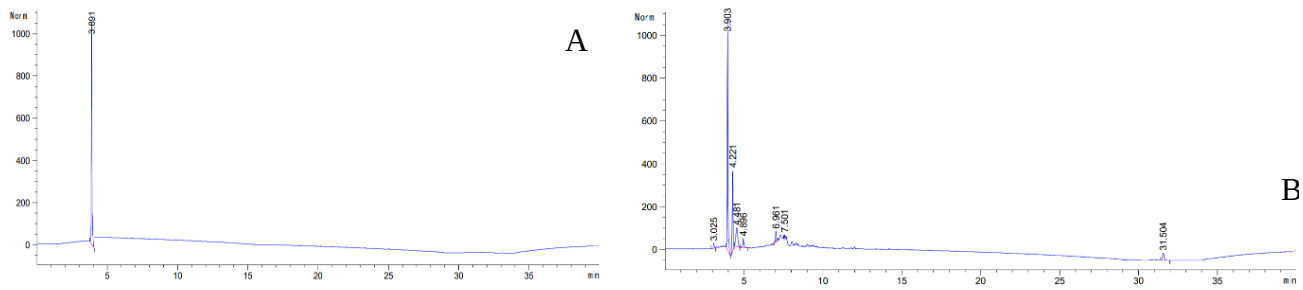

Figure 1. HPLC analysis.
A. HPLC Chromatogram of hydroxychavicol B. HPLC Chromatogram of *Piper betle* L. leaf extract.

Table 1. Antibacterial and anti-fungal activity of crude ethanol extract of *Piper betle* L. by agar well diffusion method.

Concentration (mg/mL)	Anti-bacterial ring diameter gram (-) (mm)		Anti-bacterial ring diameter gram (+) (mm)		Anti-fungal ring diameter (mm)	
	E. coli	***P. aeruginosa***	***S. aureus***	***E. faecalis***	***C. Albicans***	***M. Gypseum***
300	17.3 ± 0.4	15.0 ± 0.7	25.3 ± 0.9	13.2 ± 0.2	18.1 ± 0.4	+
200	8.3 ± 0.2	10.0 ± 0.3	18.0 ± 0.7	+	+	-
100	-	-	-	-	-	-

(+): presence of activity; (-): absence of activity

studies investigating the antifungal properties of *Piper betel* L. leaf extracts and its bioactive constituents. Pawar et al. (2017) and Syahidah et al. (2017) demonstrated the effective antifungal properties of ethanol-extracted *Piper betel* leaf extract. Additionally, Ali et al. (2016) demonstrated the extract's efficacy against *Candida albicans* by isolating hydroxychavicol, suggesting that this active ingredient disrupts cell membrane integrity.

Scratch assay to determine mobilization of NIH3T3 and HaCaT

To assess the potential rejuvenating and wound-treating effects of *Piper betle L*. leaf extract, wounds were induced in NIH3T3 and HaCaT monolayer cultures, and then treated with the extract. Figure 2a depicts the effects of the extract on NIH3T3 cells, where at 9 post-treatment, some cells began to migrate towards the edges of the scratch. After 48 h, the fastest closure of the scratch area was observed in the group treated with a concentration of 2 mg/mL, with most cells covering the scratch area, which was 4 times faster than the control group. At a concentration of 1 mg/mL, the extract accelerated scratch closure by 2.7 times compared to the group without *Piper betel L*. extract. Similar results were observed for HaCaT cells (Figure 2b), with additional cells migrating towards the edges of the scratch at 9 h post-treatment. After 48 h of extract supplementation, treatment with 2 mg/mL of extract exhibited a faster growth and migration rate compared to the other two concentrations, which was 3.6 times faster than the control. These findings suggest that *Piper betle L*. leaf extract is capable of stimulating the proliferation of fibroblasts and keratinocytes, albeit with differing effects depending on the concentration of the extract.

Flow cytometry

After labeling with fluorescent-compatible monoclonal antibody markers and assessing the degree of consistency using FACS Canto II, the results showed that ADSCs were positive for CD36, CD73, CD90, and CD105, while negative for CD34, CD45, CD79a, CD106, and HLA-DR

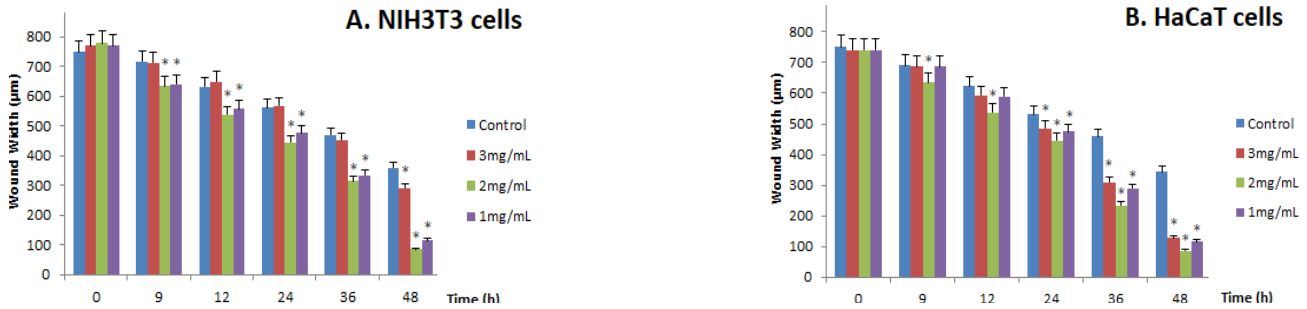

Figure 2. Graph showing average wound size of *Piper betle* L. extraction treated cells over a period of 48 h.

Table 2. Expression of cell surface markers of ADSCs.

Marker	(%) expressed
CD36	95.3
CD73	97.8
CD90	98
CD105	96.5
CD34	1
CD45	2
CD79a	0.7
CD106	1.1
HLA-DR	0.4

(Table 2). In 2006, the International Society for Cellular Therapy (ISCT) established standards for MSC identification, including ADSCs. They proposed that MSCs should exhibit plastic adherence properties under standard culture conditions, lack expression (≤2% positive) of CD34, CD45, CD79a, and HLA-DR, and express (≥95% positive) CD73, CD90, and CD105 markers in flow cytometry analysis. Additionally, MSCs should demonstrate trilineage differentiation capacity into chondroblasts, osteoblasts, and adipocytes (Megaloikonomos et al., 2018). In 2013, ISCT issued a revised statement in collaboration with the International Federation for Adipose Therapeutics and Science (IFATS), recommending additional markers for ADSCs, namely negative for CD106 and positive for CD36 (Camilleri et al., 2016).

The combination of *Piper betle* L. extraction and adipose-derived stem cells

Twenty mice were divided into four groups after excision: Group 1 received injections of NaCl (Control), Group 2 received NaCl injections along with *Piper betle L*. extract (EX), Group 3 received injections of 106 ADSCs, and Group 4 received injections of 106 ADSCs along with *Piper betle L.* extract (ADSCs+EX). Morphological and histological results were presented in Figure 3. Initially, the wounds in the ADSCs+EX group appeared dry on the first day. By the 4th day, wounds in the ADSCs+EX group healed the fastest, 1.3 times faster than the control group. Wounds in the ADSCs group healed at a similar rate to the EX group but faster than the control group. Wounds in the groups treated with stem cells and extract became completely dry. In the ADSCs+EX group, wounds were fully healed by the 14th day, while in the EX group and ADSCs group, wounds were fully healed by the 17th and 16th day respectively. In this study, wounds in all treatment groups healed faster than in the control group.

Histological analysis was conducted on day 18 post-ulceration, evaluating the stratum granulosum structure in the mesoderm, epidermis floor structure, and sub-structures such as hair follicles and blood vessels. After 18 days of treatment, mice in the treatment groups exhibited normal and even epidermis, increased keratinocytes, fibroblasts, blood vessels, and hair follicles compared to the control group. Histological analysis of the control group revealed atrophic epidermis, edema in the stromal dermis, and the presence of multiple inflammatory and congestive cells.

DISCUSSION

The observation of wound morphology and healing time

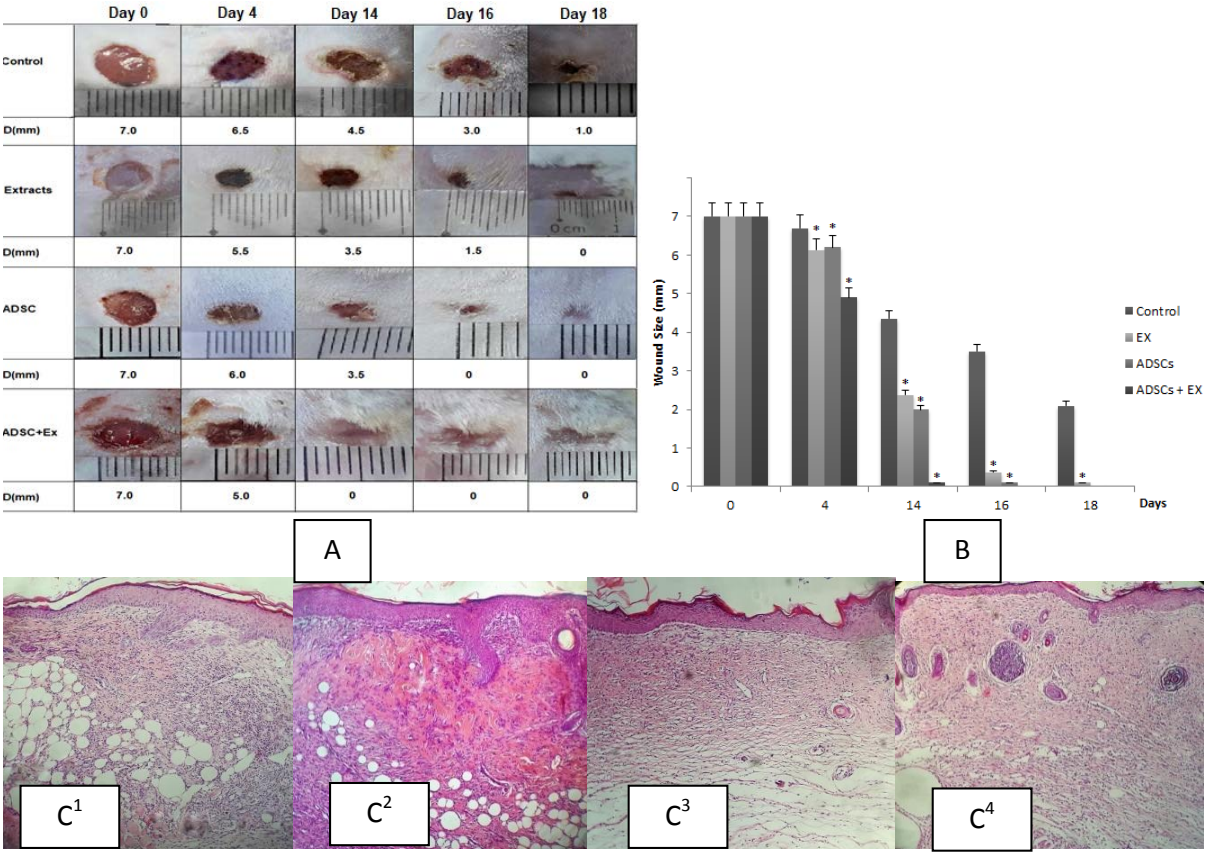

Figure 3. Wound healing in mice at 18 days.
A. Wound morphology within 18 treatment days with *Piper betle* L. extract and ADSC administration; B. Average wound size in different groups on days of treatment; C. Histological structure of different groups after 18 days: C^1. Control group, C^2. Extract group, C^3. ADSCs group, C^4. ADSCs+EX group.

in both the ADSCs + EX group was superior to that of the ADSCs, EX, and control groups. This can be attributed to the simultaneous supply of anti-inflammatory compounds from *Piper betle L.* extract and the enhanced differentiation of administrated ADSCs into skin cells. The interaction and collaboration of these two factors likely facilitated the wound healing process in terms of both structure and function.

Observations of wound morphology indicated that the wound healing process in the ADSCs + EX and ADSCs groups exhibited more positive changes compared to the control group. This could be explained by two main mechanisms: trans-differentiation and paracrine regulation (Rachidi and Damour, 2014). The ability of ADSCs to differentiate into fibroblasts, particularly young fibroblasts, and their potential to secrete cytokines and growth factors contributed to the promotion of fibroblast synthesis and proliferation. Additionally, the rapid re-epithelialization and wound healing ability of ADSCs may be correlated with the up-regulation of CD90, a representative marker of keratinocytes. This acceleration of the wound healing process in the ADSCs group aligns with findings from previous studies (Rodriguez et al., 2015).

Furthermore, the ability to activate skin niche cells and stimulate their proliferation in the administered cells was elucidated through the expression of two proliferation markers: the nuclear marker Ki67 and the membrane marker CD71.

The presence of CD71 in epithelial keratinocytes has been reported by Olszewski and Redvers (2006), and Martin et al. (1997) highlighted that the re-epithelialization stage primarily depends on keratinocyte proliferation. Numerous studies have demonstrated that transplanted adipose stem cells (ADSCs) can promote keratinocyte proliferation, as evidenced by the upregulation of the marker CD71 (Gomathysankar et al., 2014). Moreover, ADSCs express and secrete various cytokines essential for angiogenesis, including SDF-1, Ang-1, IGF-1, PDGF-BB, VEGF, basic FGF, matrix metalloproteinases, IL-6, and IL-8 (Aboulhoda and Abd el Fattah, 2018).

Among these cytokines, VEGF stands out as a specific and potent growth factor in angiogenesis. It stimulates the mobilization, recruitment, and migration of progenitor endothelial cells, thereby accelerating the formation of blood vessels (Hoeben et al., 2004). Nie et al. (2011)

demonstrated that ADSCs secrete angiogenic cytokines *in vitro* and *in vivo*, including VEGF, HGF, and FGF-2. SDF-1 activity is crucial for endothelial cell survival, vascular branching, and recruitment of pericytes. Additionally, SDF-1 plays a role in recruiting pericytes and smooth muscle cells, which stabilize and mature newly formed blood vessels (Oudega, 2012).

ADSCs exert their immunoregulatory effects primarily through the secretion of paracrine factors (Kokai et al., 2014). Moreover, *Piper betle L*. extract has demonstrated resistance against bacterial strains causing dermatitis and opportunistic skin diseases, as well as fungal strains causing skin diseases. Particularly, hydroxychavicol, a significant phenolic component in *Piper betle L*. leaves, exhibits strong anti-inflammatory abilities by inhibiting the expression of TNF-α, a pro-inflammatory cytokine (Zamakshshari et al., 2021; Sharma et al., 2020). Nakamura et al. (1998) reported that hydroxychavicol can inhibit TPA-induced H2O2 production and inflammatory responses in mouse skin. Studies have indicated that hydroxychavicol exerts its anti-inflammatory effects by down-regulating pro-inflammatory cytokines (IL-2, IFN-γ, and TNF-α) while stimulating the production of anti-inflammatory cytokines (IL-4 and IL-5) (Pandey et al., 2010). Additionally, *Piper betle* L. extracts containing hydroxychavicol components have been shown to reduce reactive oxygen species, including inhibiting lipid peroxidation (Ali et al., 2018). These findings collectively underscore the significant role of hydroxychavicol in the wound healing process.

Additionally, this study utilized the total leaf extract in wound treatment. *Piper betel* L. leaf extract contains the main ingredient hydroxychavicol, along with alkaloids, steroids, tannins, phenolics, flavonoids, and glycosides (Dwivedi and Tripathi, 2014). Blois et al. (1958) investigated that steroid, terpenoid, and phenolic derivatives, including coumarin, tannin, and flavonoids, could affect proliferation and revascularization.

Bandyopadhyay et al. (2006) demonstrated that the aqueous extract of betel leaf could enhance the Th1-type response, thereby promoting cellular immunity. They also found that the synthesis of interferon-γ (IFN-γ) in human peripheral mononuclear cells was increased by the extract without affecting IL-4 levels. Kanjwani et al. (2008) showed significant suppression of phytohemagglutinin, leading to peripheral blood lymphocyte proliferation *in vitro*, with an extract of *Piper betel* leaf. Another study demonstrated the ability of *Piper betel* leaf extract to decrease antibody titers while simultaneously increasing the suppression of inflammation (Kanjwani et al., 2008).

The extract was also shown to decrease the expression of IL-33, CD248, and VCAM, thus promoting proliferation in fibroblasts and facilitating wound healing (Che et al., n.d). Moreover, Piper betel extraction not only effectively worked as a superficial antibacterial agent but also stimulated *in vitro* fibroblasts and mesenchymal stem cells, as well as accumulated protein at the *in vivo* wound site (Che et al., n.d; Keat et al., 2010).

From this, the extract also has the ability to stimulate stem cells at niches near the wound to expedite wound healing in mice. *Piper betel L.* extract acts as an extracellular signal, supporting cell interaction and stimulating cellular reactions to regulate proliferation, migration, and differentiation. This theoretical explanation elucidates the faster wound healing process in the ADSCs+EX group compared with other experimental groups.

Conclusions

Piper betle L. extract possesses antimicrobial, antifungal, and vasculature regeneration properties, along with the ability to promote cell line proliferation in the skin. When combined with ADSCs, *Piper betle L*. extract treats chronic wounds more effectively and accelerates the healing process compared to the control group.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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