THE ABILITY TO STIMULATE PROLIFERATION OF *PIPER BETLE* L. LEAF EXTRACTSON FIBROBLAST

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SUMMARY

Piper betle L. has been used as a folk medicine, the leaves of the *P. betle* L. act as a potential source in the treatment of skin wounds. It can be attributed to the phenolic compounds within the ethanolic extract of *P. betle* L. Fibroblasts play a very important role in supporting wound healing. Acceleration of the wound withdrawal process is is closely related to fibroblast migration and proliferation. However, research on medicinal plants' ability to stimulate proliferation and wound healing and their effects on fibroblasts has not been thoroughly studied. Therefore, we investigated the antibacterial ability and possibility of wound healing of *P. betle* L. extracts on fibroblasts isolated from umbilical cord stem. Evaluate the antibacterial properties of *P. betle* L. extract by agar disk diffusion method. Evaluate the ability to stimulate cell proliferation of *P. betle* L. extract by scratch healing assay on fibroblasts in vitro. The results of this study show that the *P. betle* L. extracts has good resistance to pathogenic microorganisms including *Escherichia coli*, *Staphylococcus aureus* and Salmonella. Studies on the ability to stimulate proliferation showed that extracts at a concentration of 0.06 µL/mL inhibited cell proliferation. The concentration of 0.025 µL/mL stimulates fibroblast proliferation better than concentrations 0.03 µL/mL, 0.02 µL/mL; facilitating the scratch healing process 2.8 times faster than the control group *in vitro*. Our results show that *P. betle* L. has great potential in being used as a raw material in the development of drugs of natural origin for the treatment of skin wounds.

Keywords: *Piper betle* L., fibroblasts, heal scratches.

INTRODUCTION

The skin is one of the largest organs and plays important a physical barrier that protects the body against negative external influences. When this barrier is damaged, pathogens have a direct route to infiltrate the body, potentially resulting in infection. Wound healing is a complex process that can be divided into at least 3 continuous and overlapping processes: an inflammatory reaction, a proliferative process leading to tissue restoration, and, eventually, tissue remodeling (Li *et al*., 2007). During proliferation, fibroblasts play a very important role in supporting wound healing. At the end of the inflammatory phase and the onset of proliferation (after 24 to 48 hours of injury), the first fibroblasts appear at the site of injury, they infiltrate and degrade the fibrin clot.The conversion from fibroblasts to myofibroblasts controls a delicate balance between contraction and re-epithelialization that promotes fibroblast proliferation and migration, accelerates wound retraction (Matthew *et al*., 2015). However, accelerating wound withdrawal can be interrupted for a variety of reasons including increased free-radicals-mediated damage, delayed granulation tissue formation, reduced angiogenesis and decreased collagen reorganization leading to chronic wound healing (Matthew *et al*., 2015).

Piper betle L. is cultivated widely in most of the humid tropical climate of Southeast Asia including Vietnam. Their leaves could be used as a folk medicine because of its medicinal properties (Prabhu *et al*., 1995). *P. betle* L.extracts contain bio-compounds that have been shown to have positive effects in the treatment of diabetes, conjunctivitis, mastitis, burns, sores, antibacterial, anti-inflammatory, antioxidant and anti-cancer properties. (Duraniet *et al*., 2017). Other studies have reported that the *P. betle* L.extracts also shows potential in wound healing and anti-aging treatment (Guha Majumdar, Subramanian., 2015). However, scientists are continuing to explore the mechanisms involved, especially their role in stimulating proliferation of cell lines in the treatment of open wounds, which are still poorly understood.

Current medications have shown good efficacy against antisepsis in open wounds but cause side effects. Therefore, research and creation of drugs of medicinal origin to replace and assist in wound treatment and wound pathology is necessary. Therefore, our study was conducted to study the antibacterial effect of betel leaf extracts and the ability to stimulate proliferation of fibroblast cells in human.

MATERIAL AND METHOD

Material

P. betle L. was collected in Hue city, Thua Thien Hue province. The sample was tested and classified by the Plant Department, Department of Biology, University of Science - Hue University.

Preparation of *P. betle* L. extracts

500g fresh betel leaves are cleaned and washed thoroughly with water and rinsed with distilled water. Washed fresh leaves are dried at room temperature. Grind the sample, place in an incubator with 70% ethylic alcohol in a ratio of 1:10 and incubate for 5 hours at a temperature of 50ºC. After incubation, the extracts is filtered and the solvent is removed by a vacuum rotary evaporator at a temperature of 70ºC to 5%, a *P. betle* L. extracts is obtained, stored at 4ºC.

Isolation of fibroblasts from umbilical cord

The umbilical cord, tissues normally discarded at birth, were collected under sterile conditions at Hue Central Hospital and transported to the laboratory in an icebox. Donors were negative for hepatitis virus markers, syphilis and HIV.

Complete placenta tissue samples were washed three times with sterile PBS. The coarse surface of the umbilical cord lining membrane (UCLM) close to the maternal side were scraped using surgical instruments and soaked in PBS. A total of 2g UCLM tissue was transferred to a 50 mL centrifuge tube and mechanically fragmented into 1cm^2 sections with ophthalmic scissors. Subsequently, tissue fragments were seeded in 6-well plates at a density of 5×10^4 cells/mL , and cultured in DMEM/F12 (Sigma-Aldrich, Inc.) supplemented with 10% FBS (Sigma-Aldrich, Inc.). Cells were cultured in an incubator at 37°C in a humidified atmosphere containing 5% CO_2 and passaged upon reaching 80% confluence (P3). The medium was replaced every 3 days and the morphology was observed under an inverted optical microscope (Olympus Corporation).

Anti-microbial potential test

The vaccine-auditing strains used include: *Staphylococcus aureus, Escherichia coli* and *Salmonella*. These microorganisms were provided and evaluated at Department of Biology, Hue University of Scienc.

The minimum inhibitory concentration of *P. betle* L. extracts for bacterial strains was determined by diffusion method on agar plates with diluted concentrations of 100%, 25%, 6.25%. Agar plate containing microorganisms is perforated with a diameter of 15 mm, drawing 1 mL of diluted betel leaf into the perforated hole. The cold dish is cooled at 4°C for 12 hours, transferred to a 37°C incubator for 24 hours. Measure the diameter of the antibacterial ring. The experiment was repeated 3 times.

Evalation the composition of the extracts

P. betle L. extracts (3 mL) after collection will be taken to the Drug, cosmetic and food quality control center of Thua Thien Hue province (HueQC). The extracts is checked for composition by Gas Chromatography-Mass Spectrometry system (GC-MS).

Qualification the effect of *P. betel* L. extracts on fibroblasts

Fibroblast were cultured in primary medium in DMEM / F12 + 1% penicillin, 37°C, 5% CO_2. When the cell density reaches 70% - 80%, the subculture to a 6-well plate was carried out. The *P. betel* L. extracts were added at different concentrations to the culture medium. The sample was evaluated every 3 hours for 48 hours.

Crystal violet assay is used to evaluate cytotoxic effects of *P. betel* L. extracts on fibroblasts. The Fibroblasts were fixed with 10% formalin and stained with 0.1% crystal violet. The absorbance at 595 nm in the stained cells solubilized with 0.1% SDS was measured using a microplate reader (Taguchi *et al*., 2011).

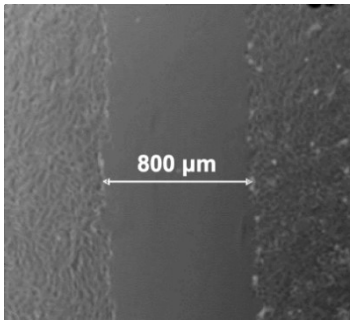


Figure 1. Illustration of scratch method according to Hussain *et al*. (2016)

Assessement the ability of the *P. betel* L. extracts to heal scratches on fibroblasts

During the secondary culture, when the cell population is relatively homogeneous and the density reaches 80 - 90%. Scratches with a width of 800 µm are created on the cell surface using a sterile pipette tip.

The cells are then washed with PBS to remove suspended cells. Supplement DMEM/F12 + 10% FBS culture medium and *P. betle* L. extracts at different dilution concentrations. The cells were cultured at 37°C, CO_2 5% and monitorred every 3 hours for 48 hours. The experiment was repeated 3 times.

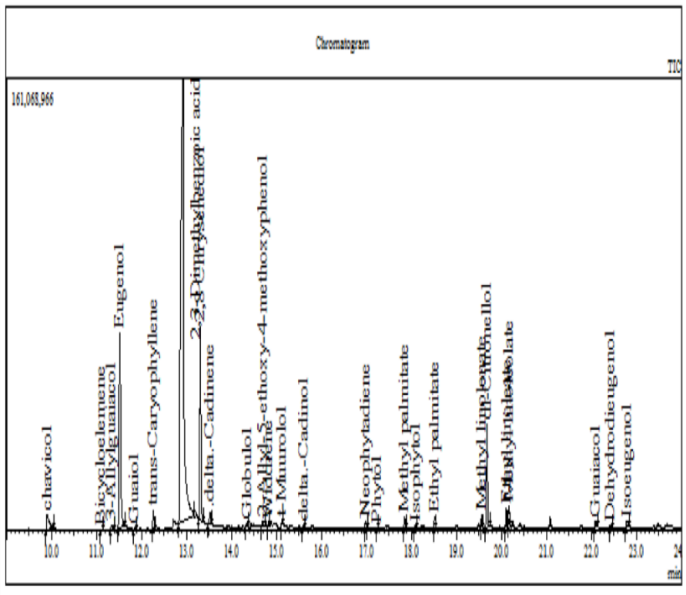
Statistical analysis

In this study, all experiments were repeated three times and the collected data were analyzed using ANOVA statistical software, with significance level P <0.05.

RESULTS

Component test results

GC-MS method has identified 16 compounds in *P. betle* L. extracts. The chemical composition of the extracts includes carbohydrates, tri-terpinoids, steroids, alkaloids, amino acids, tannins and essential oils,... similar to the results of Alam *et al*. (2015). The phenolic compounds are mainly found in fresh betel leaf, it is resistant to nitrification, antioxidant, antiplatelet and anti-thrombotic effects and inhibits cancer (Alam *et al*., 2015).



Peak Report TIC

Peak#	Name	R.Time	Area	Area%
1	chavicol	9.910	22366842	1.80
2	Bicycloelemene	11.113	653187	0.05
3	3-Allylguaiacol	11.341	4652822	0.37
4	Eugenol	11.523	152309643	12.27
5	Guaiol	11.853	781002	0.06
6	trans-Caryophyllene	12.271	10199116	0.82
7	2,3-Dimethylbenzoic acid	12.933	786163512	63.34
8	2,8 Chrysenediol	13.315	121081860	9.76
9	.delta.-Cadinene	13.531	9423260	0.76
10	Globulol	14.350	2489087	0.20
11	2-Allyl-5-ethoxy-4-methoxyphenol	14.701	3589035	0.29
12	Widdrene	14.831	1474164	0.12
13	t-Muurolol	15.128	2398781	0.19
14	delta.-Cadinol	15.607	1269245	0.10
15	Neophytadiene	16.984	3588296	0.29
16	Phytol	17.240	736414	0.06
17	Methyl palmitate	17.856	6223685	0.50
18	Isophytol	18.096	1542406	0.12
19	Ethyl palmitate	18.524	6399763	0.52
20	Methyl linolenate	19.567	7632423	0.61
21	dl-Citronellol	19.684	61970498	4.99
22	Ethyl linoleate	20.116	8838638	0.71
23	Methyl linoleolate	20.176	13028621	1.05
24	Guaiacol	22.098	4904397	0.40
25	Dehydrodieugenol	22.435	1679946	0.14
26	Isoeugenol	22.800	5686360	0.46
			1241083003	100.00

Figure 2. GCMS results

Anti-microbial potential of *P. betle* L.Extractsion

The results of the survey on the antibacterial ability of *P. betle* L. extracts with different concentrations on the isolated bacterial strains showed that the extracts has good resistance against the pathogenic microorganisms on the skin. At the stock concentration, the extracts showed the most lethal resistance to *E. coli* (35.5±0.4 mm) and *Salmonella* (28±0.3 mm).

Particularly bacterium *S. aureus* is one of the most common causes of bacteremia, infective endocarditis and various skin, soft-tissue infections, particularly when skin or mucosal barriers have been breached, the extracts of *P. betle* L. is most resistant to *S. aureus*, at the stock concentration lethal resistance to (35.2±0.6 mm), at a 25% dilution, the extracts is most resistant (20.0±0.5 mm). In contrast, the 6.25% diluted extracts did not have antibacterial properties.

Table 1. Anti-microbial activity of *P. betel* L. extracts

Concentration (%)	Anti-bacterial ring diameter (mm)		
	E. coli	***S. aureus***	***Salmonella***
100%	35.5 ± 0.4	35.2 ± 0.6	28.0 ± 0.3
25%	11.8 ± 0.5	20.0 ± 0.5	8.4 ± 0.6
6,25%	0	0	0

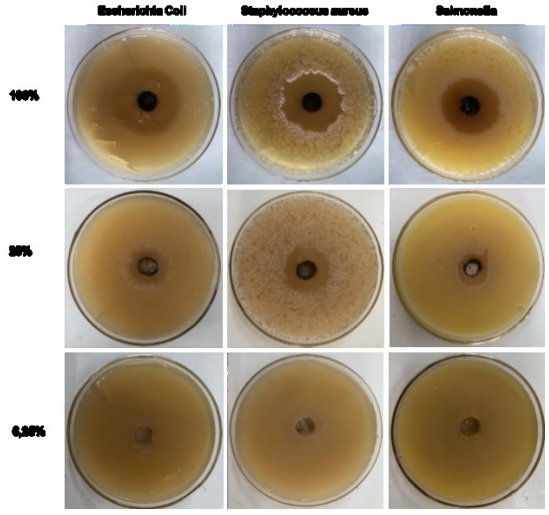


Figure 3. Anti-bacterial ability of *P. betel* L. extracts

Cytotoxic effects of *P. betel* L. extracts on fibroblasts

A fibroblast is a type of biological cell that synthesizes the extracellular matrix and collagen, produces the structural framework (stroma) for animal tissues, and plays a critical role in wound healing. Fibroblasts are the most common cells of connective tissue in animals.In our study, fibroblasts were treated *in vitro* with *P. betle* L. extracts at different concentrations (0.58 µL/mL; 0.24 µL/mL; 0.12 µL/mL; 0.06 µL/mL; 0.03 µL/mL). The results are shown in the image (Figure 4.A.B).

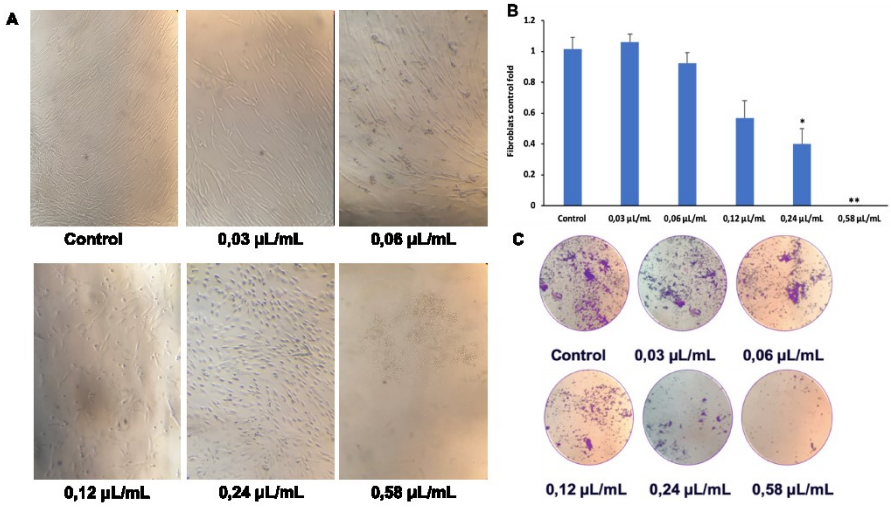


Figure 4. Toxicity ability of *P. betle* L. extracts at different concentrations on fibroblast.
*(A) Morphology of fibroblasts were treated with different concentration of P. betle L. extracts; (B) Effect of P. betle L. extracts at various concentrations on viability of fibroblast * and ** Significant difference (p < 0.05 and 0.01, respectively) between the 5 concentrations 0.03 µL/mL; 0.06 µL/mL; 0.12 µL/mL; 0.24 µL/mL; 0.58 µL/mL compared with control at the same time; (C) Dye with crystal violet after 48h treat with extracts for check cell death*

Concentration of 0.58 µL/mL, after 6 hours of observation, the *P. betle* L. extracts was toxic to fibroblasts with more than 90% of dead cells, the cells were separated into plaque from the stick surface, some of the contractile cells no longer have the characteristic shape of fibroblasts (Figure 4. A).

The concentration of 0.24 µL/mL; 0.12 µL/mL; 0.06 µL/mL, the cells did not shed at the surface of the 6-well plate, but the growth rate was slower than the control group, some cells showed shrinkage and after 48 hours the cells stopped dividing.

Concentration of 0.03 µL/mL, after 48 hours of monitoring did not detect the phenomenon of cell shrinkage and peeling off the adhesion surface. The cells have the typical shape of fibroblasts, spread evenly on the surface of the well, the cells have the same rate of proliferation and development similar to the control group.

After 48 hours of adding extracts to the culture medium, statistical analysis results (* $p < 0.05$, ** $p < 0.01$) showed that there was a significant difference between the concentration group of 0.24 µL/mL, 0.58 µL/mL compared with the control group (0.42, 0.016 - fold compared to control) (Figure 4.B). We carried out dyeing with crystal violet at all concentrations to check for cell death.The results showed that, at the concentration of 0.03 µL/mL extracts cells still existed and developed normally and the *P. betle* L. extract started showing negative effects on cell growth in concentration of 0.06 µL/mL.At the concentration of 0.58 µL/mL extracts no cells were found to stick to the surface of 6-well plate. (Figure 4.C).

The ability of the *P. betel* L. extracts to heal scratches on fibroblasts

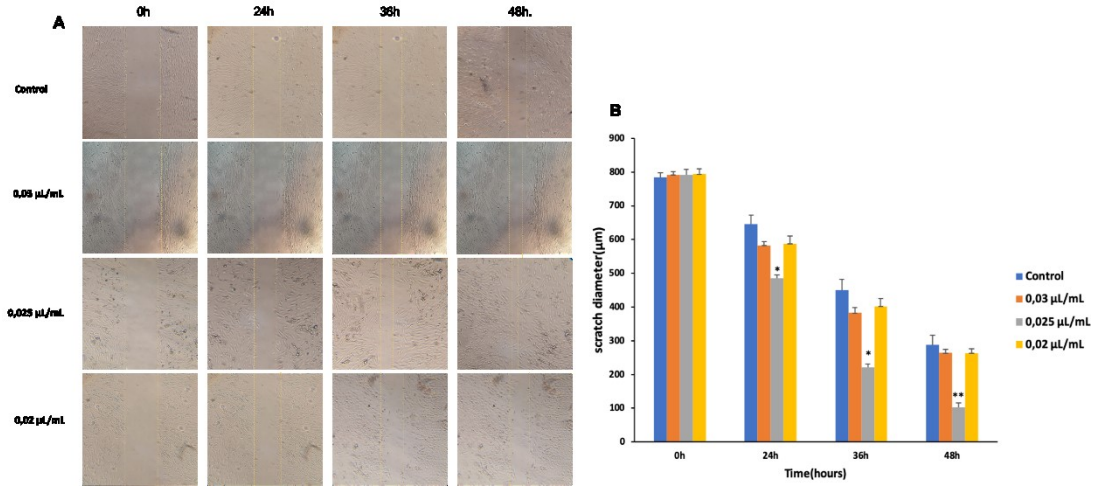


Figure 5.The ability of *P. betle* L. extracts at various concentrations to heal scratch wound of fibroblast were presented in photos (A) and a graph (B). * and ** Significant difference ($p < 0.05$ and 0.01, respectively) between the 3 concentrations 0.03 µL/mL; 0.025 µL/mL; 0.02 µL/mL compared with control at the same time

Among various methods for measuring cell proliferation, we choose scratch assay was used by many authors for both aims: proliferation and migration measuring assay, especially when serum was added for proliferation test (Zubair *et al*., 2012; Jonkman *et al* 2014). Thus,we continue to study the optimal concentration of *P. betle* L. extracts that works on fibroblasts. Fibroblasts were cultured secondary in DMEM/ 12 + 10% FBS medium until density reached 90%, scratched and supplemented with *P. betle* L.extracts at 0.03 µL/mL; 0.025 µL/mL; 0.02 µL/mL observed for 48h. The wound healing activities in treated with extracts groups were compared with the ones in control group at the same times. Statistically significant differences only had in 0.025 µL/mL- treated group in all experimental checkpoint times (24h, 36h, 48h) (Figure 5.B). 24 hours after the addition of the extracts, this time there was a difference in the morphology of the scratch at the concentration of 0.025 µL/mL compared to the other concentrations and the control group, 660 µm diameter of control versus 500 µm at 0.025 µL/mL-treated group. At the remaining concentrations and the control group, scratch morphology has not changed significantly. The proliferation stimulation ability represented by scratch healing speed. (Figure 5.A).

36 hours after addition of the extracts, the 0.025 µL/mL concentration group had a faster healing rate than the remaining extracts groups and the control group. Cell proliferation can made some scratch sites almost completely, at this time the width of the scratch narrowed more than 70%. In contrast, in the groups of concentrations of 0.03 µL/mL, 0.02 µL/mL and the control groups, the width of the scratches narrowed by nearly 50%. 48 hours after addition of extracts, the concentration group of 0.025 µL/mL had the fastest healing rate, about the level of scratch was nearly completely narrowed and 2.8 times higher than the control group. At the concentration of 0.03 µL/mL and 0.02 µL/mL, the rate of healing of scratches is equivalent to the control group and is equal to 60%.

DISCUSSION

The role of medicinal plants as a source of medicinal materials has been recognized since ancient times (Prabhu *et al*., 1995). Although major scientific and technological advances have been applied in the field of combination chemistry, medicinal plants are the basis for many drugs currently being used commercially or in the development stage (Balandrin *et al*., 1993) . These drugs are helping us find entirely new chemistry classes of therapeutic agents and new mechanisms of action. Several compounds isolated from plants are currently used to treat cancer and other diseases (Guha Majumdar, Subramanian, 2019; Balandrin *et al*., 1993)

P. betle L. is a famous medicinal herb classified among Vietnam's precious medicinal herbs. They have been using for the treatment of a range of diseases such as halitosis, boils and abscesses, conjunctivitis, constipation,

headache, mastoiditis, leucorrhea, abrasion, cuts (Prabhu *et al*., 1995; Guha Majumdar, Subramanian., 2019). In the present study, our results are similar to those of Syahidah *et al*. (2017), *P. betel* L. extracts showed strong resistance to skin pathogenic microorganisms including *E. coli, S. aureus, Salmonella* (Syahidah *et al*., 2017). *S. aureus* infections can spread through contact with pus from an infected wound, skin-to-skin contact with an infected person. *S. aureus* usually acts as a commensal bacterium, asymptomatically colonizing about 30% of the human population (Wollina *et al*., 2017). Localized infection is a not uncommon complication of *Salmonella* septicaemia, particularly occurring in immunocompromised patients. There are clinical manifestation of infection with *S. typhi* and highlight the possible *Salmonella* aetiology for unusual cutaneous lesions in individuals coming from endemic areas (Marzano *et al*., 2003).

The results of our scratch healing model show that the *P. betle* L. extracts at a concentration of 0.025 µL/mL has the effect of promoting the healing process in an *in vitro* model. This has been demonstrated by a significant increase in the rate of scratch reduction, ANOVA analysis results and images showing scratch healing depends on the concentration of the extracts. After 24 hours of scratching, the concentration of 0.025 µL/mL showed the strongest ability to stimulate proliferation, the cells moved outside and the width of the scratch was narrowed by more than 35%. Meanwhile, the other two concentrations, the cell began to tend to proliferate, a small number of cells had moved to the edge of the scratch.After 48 hours of the experiment, there was a clear difference between the concentration group and the control group, the concentration of 0.025 µL/mL showed the ability to promote the movement of cells better than the concentration group. other and control groups. At this time, the scratch size was narrowed by more than 80%, 2.5 times higher than the other two concentrations and 2.8 times higher than the control group.

The increased shrinkage may be due to proliferation stimulation activity in fibroblasts of extracts in the scratch model. Myofibroblasts are believed to play a key role in wound contraction by exerting tension on the surrounding extracellular matrix (ECM) and secreting ECM proteins such as collagen to stabilize the contraction (Matthew *et al*., 2015). Durani *et al*. (2017) showed that *P. betle* L. extracts modulates senescence-associated genes expression in replicative senescent Human Diploid Fibroblasts (HDFs). Their results showed that *P. betle* L. extracts can improve cell proliferation of young (143%), presenescent (127.3%), and senescent (157.3%) HDFs (Durani *et al*., 2017).

P. betle L. extracts contain phenolic components that have been shown to reduce reactive oxygen species (ROS) including peroxide, superoxide and hydroxyl radicals by inhibiting lipid peroxidation (Ali *et al*., 2018). Injury processes are often associated with oxidative stress, which triggers negative responses to structure and cell repair. By removing excess free radicals in the cell, *P. betle* L. extracts alleviate oxidative stress, thus protecting the structure and promoting the proliferation of fibroblasts (Durani *et al*., 2017). Savsani *et al*. (2020) demonstrated that *P. betle* L. extracts in vitro can promote cardiac proliferation and counteract the cytotoxic effect of H_2O_2 thereby significantly reducing intracellular ROS and apoptosis process (Savsani *et al*., 2020).

From the results of this study, we conclude that, *in vitro* culture, at a concentration of 0.025 µL/mL *P. betle* L.extracts has the ability to stimulate the proliferation of fibroblasts, while at 0.06 µL/mL extracts has the ability to inhibit cell proliferation. This result shows the potential to increase the recovery rate of open wounds of *P. betle* L.extracts, we can use the extracts with the appropriate concentration to serve the wound healing activities and identify. The main active ingredient in the wound healing process of extracts is to develop an optimal clinical regimen by changing the concentration or combining it with other medications.

CONCLUSIONS

P. betle L. has many bioactive compounds. Compounds with high biological properties can be agglomerated by extractsing method and determined by GC-MS analysis.Evaluating *P. betle* L. stimulateactivity on cell proliferation of *P. betle* L. extracts by in vitro scratch assay. *P. betle* L. has strong resistance to pathogenic microorganisms and has the ability to stimulate proliferation of fibroblast cell lines isolated from umbilical cord. Besides, 0.06 µL/mL is the concentration of extract can toxicate the cells. *P. betle* L. extracts at concentration of 0.025 µL/mL is the best stimulating fibroblast proliferation.

Acknowledgments: *This work was supported by the Domestic Master/PhD Scholarship Programme of Vingroup Innovation Foundation.*

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KHẢ NĂNG KÍCH THÍCH TĂNG SINH CỦA CAO CHIẾT LÁ TRẦU *PIPER BETLE* L. LÊN NGUYÊN BÀO SỢI PHÂN LẬP TỪ DÂY RỐN

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Trường Đại học Khoa học, Đại học Huế

TÓM TẮT

Piper betle L. đã được sử dụng như một loại thuốc dân gian, lá của *P. betle* L. hoạt động như một nguồn tiềm năng trong việc điều trị vết thương ngoài da. Điều này được cho là do các hợp chất phenolic trong chiết xuất ethanolic của *P. betle* L. Nguyên bào sợi đóng vai trò rất quan trọng trong việc làm liền vết thương hở. Việc tăng tốc quá trình hỗ trợ liền vết thương có liên quan mật thiết đến sự di chuyển và tăng sinh nguyên bào sợi. Tuy nhiên, nghiên cứu về khả năng kích thích tăng sinh và làm lành vết thương của cây thuốc, tác động của chúng đến nguyên bào sợi chưa được nghiên cứu kỹ lưỡng. Do đó, chúng tôi đã nghiên cứu khả năng kháng khuẩn và khả năng chữa lành vết thương của cao chiết *Piper betle* L. trên nguyên bào sợi phân lập từ dây rốn. Đánh giá tính chất kháng khuẩn của dịch chiết *P. betle* L. bằng phương pháp khuếch tán đĩa thạch. Đánh giá khả năng kích thích tăng sinh tế bào của cao chiết *P. betle* L. bằng các nghiệm thức làm lành vết xước trên nguyên bào sợi trong *in vitro*. Kết quả nghiên cứu cho thấy, cao chiết từ *P. betle* L. có khả năng kháng tốt đối với các vi sinh vật gây bệnh bao gồm *Escherichia coli*, *Staphylococcus aureus* và *Salmonella*. Các nghiên cứu về khả năng kích thích tăng sinh cho thấy cao chiết pha loãng ở nồng độ 0,06 µL/mL đã ức chế sự tăng sinh nguyên bào sợi. Ở nồng độ 0,025 µL/mLcao chiết có khả năng kích thích tăng sinh nguyên bào sợi tốt hơn so với nồng độ 0,03 µL/mL, 0,02 µL/mL; tạo điều kiện cho quá trình làm liền vết xước nhanh hơn 2,8 lần so với nhóm đối chứng trong *in vitro.* Kết quả của chúng tôi cho thấy rằng *P. betle* L. có tiềm năng lớn trong việc được sử dụng như một nguồn nguyên liệu trong việc phát triển các loại thuốc có nguồn gốc tự nhiên để điều trị các vết thương hở.

Từ khóa: Piper betle L., nguyên bào sợi, làm lành vết xước.

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