ORIGINAL ARTICLE

The Effect of *Piper betle* on Wound Healing in Male Sprague Dawley Rats

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ABSTRAK

Daun sireh atau Piper betle (PB) telah digunakan secara tradisional di banyak negara Asia untuk penyembuhan luka dan penyakit lain. Tujuan kajian ini adalah untuk mengkaji kesan penyembuhan luka PB pada tikus Sprague-Dawley yang diaruhkan kecederaan kulit. Lapan belas tikus jantan Sprague-Dawley diaruhkan luka dan kemudian dibahagikan kepada tiga kumpulan yang terdiri daripada kawalan (penyembuhan luka normal tanpa "dressing"), salina (penyembuhan luka dibalut kain kasa yang direndam dalam air salina) dan PB (kain kasa yang mengandungi pes PB). Luka diaruhkan di belakang tikus dengan jarum biopsi punch steril berukuran 6 mm. Luka diperiksa pada hari 3, 5, 7, 9, 11 dan 13 untuk kekeringan, eksudasi, dan pembentukan parut. Tikus telah dikorbankan pada hari ke-14 dan tisu granulasi yang terbentuk pada luka itu kemudiannya dikeluarkan untuk pemeriksaan histologi menggunakan pewarnaan Hematoxylin dan Eosin (H&E). Pembentukan tisu granulasi dalam tikus yang dirawat dengan PB menunjukkan penutupan luka dengan masa yang lebih pendek (pada hari ke-3) berbanding dengan kumpulan kawalan dan salina (pada hari ke-5). Pembalut luka yang mempunyai PB berupaya meminimumkan pembentukan tisu parut secara signifikan ($p<0.05$) berbanding kumpulan kawalan dan salina. Kesimpulannya, keputusan dari kajian ini menunjukkan bahawa PB mempunyai kesan pemulihan luka yang baik dengan kesan kurang parut dan mungkin ia dapat digunakan secara komplementari untuk merawat luka.

Kata kunci: *Piper betle, penyembuhan luka, tisu parut, tisu granulasi*

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ABSTRACT

Piper betle (PB) leaves have been traditionally used in many Asian countries for the healing of wounds and other ailments. The aim of this study was to examine the wound healing effect of PB on skin injury-inflicted Sprague-Dawley rats. Eighteen male Sprague-Dawley rats were inflicted with wounds and then divided into three groups consisting of a control (normal wound healing without dressing), saline (wound healing aided by gauze soaked in saline) and PB (gauze impregnated with PB paste) dressings. Wounds were created on the back of rats with 6-mm sterilized punch biopsy needle. Wounds were examined on days 3, 5, 7, 9, 11 and 13 for dryness, exudation, and scar formation. Rats were sacrificed on day 14 and the granulation tissue formed on the wound was then excised for histological examination using Hematoxylin and Eosin staining. The formation of granulation tissue in rats treated with PB showed higher progress to wound closure with shorter days (on day 3) compared to the control and saline group (on day 5). PB dressing minimized the formation of scar tissue significantly ($p<0.05$) compared to the control and saline groups. In conclusion, our results suggest that PB has good wound healing effect with less scarring and perhaps it can be used to treat wound complementary to the present method.

Keywords: granulation tissues, *Piper betle,* scar tissues, wound healing

INTRODUCTION

Wound healing mechanism is a complex process involving cellular events that generate resurfacing, reconstitution and restoration of the tensile strength of the damaged tissues (Orsted et al. 2011; Gupta et al. 2004). The healing process of a human adult can be divided into four phases i.e. coagulation or hemostasis, inflammation, proliferation and formation of granulation tissues, and tissue remodeling (re-epithelialization and the formation of new extracellular matrix) (Velnar et al. 2009). How fast a wound heals remains as a serious issue for public health since it will affect their working ability, health, and quality of life. Delay in the healing process may lead to other complications and serious side effects. The duration of each stage in the process of wound healing may vary according to factors such as tissue health, how the wound is managed (Jones et al. 2006), efficiency of the body's immune system (Park & Barbul 2004), and nutrient consumption (Guo & DiPietro 2010). Other factors include growth factors, platelet derived growth factor (PDGF) and transforming growth factor-beta (TGF-β) in stimulating the migration of fibroblasts cells to the wound site (Shankar et al. 2014).

The use of herbal plants to treat injuries is known from archaic times. However, only in the last decade, scientific findings have confirmed the benefit of some herbal plants in treating wounds. Leaves and fruits from herbal plants are known to contain polyphenol antioxidants and this is the main factor in promoting wound healing (Galeano et al. 2001). Karthik & Banerjee noted that patients with skin ulcers given *Datura metel* extract showed a reduction in ulcer area and slough, along with the improvement of granulation tissue (Karthik & Banerjee 2014). It was reported that leaf extract of *Terminalia chebula*, when applied on rat dermal wounds, showed high rates of wound contraction along with decreased period of epithelialization and increased tensile strength (Suguna et al. 2002). Beneficial effects of *Portulaca oleracea* on wound repair in *Mus musculus* JVI-1 was reported to decrease the surface area of the wound and increased the tensile strength (Rashed et al. 2003). *Momordica charantia* [bitter gourd], a traditional herb with the antidiabetic property, exhibited wound closure significantly faster on wound inflicted diabetic rats compared to untreated diabetic rats (Teoh et al. 2009).

Piper betle (PB) or commonly known as “sireh” in Malaysia and Indonesia, belongs to the kingdom *Plantae,* a family of *Piperaceae* (Chaveerach et al. 2006; Fazal et al. 2014). The leaves of PB have been commonly used for healing of wounds in many parts of Asian countries. Leaf of PB extract is proven to have polyphenols such as hydroxychavicol, chavibetol, chavicol, that have the following properties: anti-microbial, anti-inflammatory, antioxidant, antiseptic, wound healing agent as well as pain relieving properties (Fazal et al. 2014; Arambewela et al. 2005). Moreover, PB has the ability to promote wound contraction and reduction of healing time in Wistar rats by enhancing epithelialization process of the wound (Nilugal et al. 2014).

Dressings that create and preserve a moist environment provide the optimal conditions for the process of wound healing. The moisture under occlusive dressings increases the rate of epithelialization while promoting healing through moisture itself and the presence of a low oxygen tension (Jones et al. 2006). Normal saline is a favored wound-cleansing solution. This is due to its isotonicity that does not interfere with the normal repair process, injure tissues, cause sensitisation or allergies, or even alter normal bacterial flora of the skin, which can trigger the growth of more virulent organisms (Fernandez & Griffiths 2012).

The possible wound healing effect of PB has not been explored in great detail and thus the main aim of this study was to investigate the wound healing properties of PB in a dressing form and to compare it with normal saline dressing.

MATERIALS AND METHODS

PREPARATION OF AQUEOUS PB GAUZE

The powdered form of PB leaves was purchased from Ethno Resources Sdn. Bhd. (Sungai Buloh, Selangor, Malaysia). Five grams of powdered PB leaves were mixed with 5 ml of 0.9% sterilized normal saline (1 g/ml).

Non-medicated sterilized gauze was impregnated with the mixture to cover the rats` wounds.

EXPERIMENTAL RATS

A total of 18 male Sprague Dawley rats (250-300 g) were purchased from the Laboratory Animals Resource Unit (LARU), Faculty of Medicine, Universiti Kebangsaan Malaysia. Animal care was adhered strictly as in the protocols approved by UKM Animal Ethics Committee (UKMAEC) for the study (FP/BIOK/2015/YASMIN/9-DEC./717-DEC.-2015-JUNE-2016). The rats were placed individually in one cage each and maintained on the pellet diet and water *ad libitum*, in a standard room condition, relatively humid and 12-hour light dark cycle. The rats were randomly divided into 3 groups with 6 rats in each group; control (normal wound healing without dressing), saline (wound healing aided by gauze soaked in saline) and PB (gauze impregnated with PB paste) after acclimatized for 1 week and the rats were given dressings for 14 days.

PUNCH BIOPSY WOUND

On day one, four 6 mm wounds were created on the dorsal part of rats under anesthesia with 0.1 ml dose of ketamine, xylazine, and zolatile (LARU, Faculty of Medicine, Universiti Kebangsaan Malaysia) using punch biopsy needle. To keep dressings at the place, adhesive tape and cotton crepe bandage were used. Dressings were replaced every alternate day (day 3, 5, 7, 9, 11, and 13). Parametric measurements, comprising evaluation of wound condition macroscopically, measurement of wound contraction, period of wound closure, and epithelialization process were conducted every alternate day (on days 3, 5, 7, 9, 11, and 13).

SKIN TISSUE COLLECTION

The rats were sacrificed under anesthesia by cervical dislocation on day 14 after wound infliction, and the wounded areas were collected using a punch biopsy needle with the same diameter as that used for wound creation. Care was taken to excise only the newly formed regenerated tissues without any contamination from normal skin.

EXAMINATION OF WOUND HEALING

WOUND CLOSURE

The number of days taken for excised wound area to be replaced with granulation tissue during healing process was determined through observation.

CONDITION OF WOUND AREA

Condition of the wound were examined and photographed on day 3, 5, 7, 9, 11, and 13. Wounds were washed with normal saline solution and then dry-wiped before any evaluation. Scores of 1 to 3 were awarded for wound dryness, exudation and scar formation (Table 1). The maximum score of 3 was given to preferable condition in wound healing (dry skin and no

Table 1: Scoring of wound healing. Scores of 1 to 3 were awarded for wound dryness, exudation and scar formation (Table 1). Maximum value of 3 was given for an optimum condition for wound healing on dryness and exudation while for scarring the value of 1 signifies no scarring effect.

Condition	Score 1	Score 2	Score 3
Dryness	Wet	Mild	Dry
Exudation	Exudates	Mild exudates	No Exudation
Scar	No Scar	Transitional Repair	Scarring

formation of exudates) while for scar formations score 1 signified good minimum scarring effect.

HISTOLOGICAL OBSERVATION

Skin tissues collected on day 14 were fixed in 10% buffered formalin solution (R&M, Malaysia), then dehydrated through a series of graded alcohol (50-100%) (HmbG Chemicals, Germany) immersed in xylene (Systerm, Malaysia)and embedded in paraffin wax(Leica, Germany). The 5 μm thick sections cut via microtome were stained with Hematoxylin and Eosin (H&E). The stained sections were viewed microscopically under magnification of 2.5X (DMRXA2, Leica Microsystems, Germany). The images were captured using the camera attached to the microscope and the acquired images are transferred to the AxioVision SE64 Rel. 4.9.1 software for automatic scaling and measurement of the distance of fibroblast tissues. Briefly, The distance of fibroblast or scar tissue was measured from the normal histological structure of the skin on both sides of the scar tissue. The fibroblast tissue can be differentiated by observing and comparing the intensity of purple-blue colour using the H&E staining of the two tissues, normal and fibroblast. The cytoplasm of the fibroblast tissue is basophilic, showing intense purple-blue staining than the normal surrounding tissue. The normal tissue has less active fibroblasts, called fibrocytes with pale staining of the cytoplasm.

STATISTICAL ANALYSIS

All data were presented as mean±SD. Statistical analysis was conducted using SPSS software (version 20, SPSS Inc, USA). The results were analysed statistically using one-way ANOVA to identify the differences between treated groups and control. The two-way ANOVA was tested for any interaction between treatments and day of treatment. The data were considered significant at $p<0.05$ for all ANOVA comparisons.

RESULTS

COMPLETE WOUND CLOSURE

On day 3, the formation of granulation tissue was seen in PB group while it was only seen at day 5 in the control and saline groups (Figure 1). However,

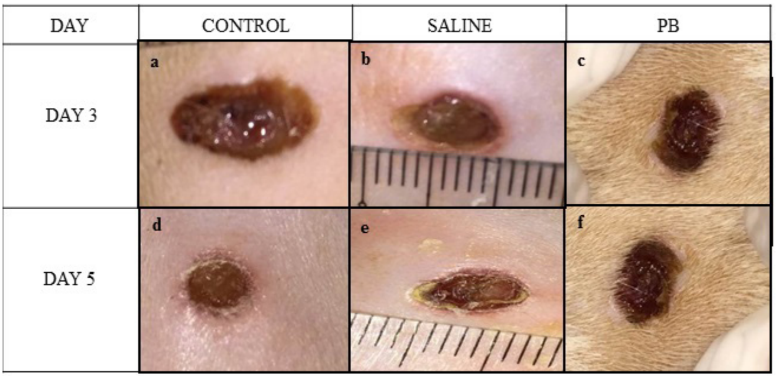


Figure 1: Effects of PB on wound healing of the skin. The figure shows the wounded area on days 3 (a-c) and 5 (d-f) for all three groups (control, saline; PB) with the formation of granulation tissues. Four 6 mm wounds were created on the dorsal part of each rat under anesthesia using punch biopsy needle. Treated groups were washed with saline and wiped dry before evaluation.

there was not much difference in the duration of wound closure between the groups (day 5).

EVALUATION OF WOUND CONDITION: DRYNESS, EXUDATION AND SCAR

A two-way ANOVA was run to examine the effect of treatment and treatment days on scarring, dryness, and exudation. There was a significant interaction between the effect of treatment and treatment days on dryness and exudation but not on scarring of the wound, $F(10,24) = 2.942$, $P=0.015$. Simple main effect analysis showed that control is better than PB and saline treatment in reducing dryness and exudation as early as day 3 (Figure 2). However, from day 7 onwards, PB group showed significant dryness compared to the saline group ($P<0.05$).

There was no significant interaction in scarring evaluation for all groups except for day 7, where PB showed significantly low scar formation (the least score value) compared to the control group (Figure 3). On day 13, the measurement of fibroblast tissue showed less scarring effect with more granulation tissues seen in all groups.

HISTOLOGICAL EVALUATION OF WOUND

On day 13, the tissue sample of each group was taken and stained with H&E. All groups showed the presence of scar tissue, however, PB group showed minimal scarring effect and better epithelial integrity (Figure 4). Statistical analysis showed a significant difference of average distance of scar tissue among the groups (Figure 5). PB group showed significantly the lowest distance of scar tissue compared to the control and saline groups ($p<0.05$). The distance of scar tissue was significantly lower in the saline group compared to the control group ($p<0.05$).

DISCUSSION

Wound healing is a complicated

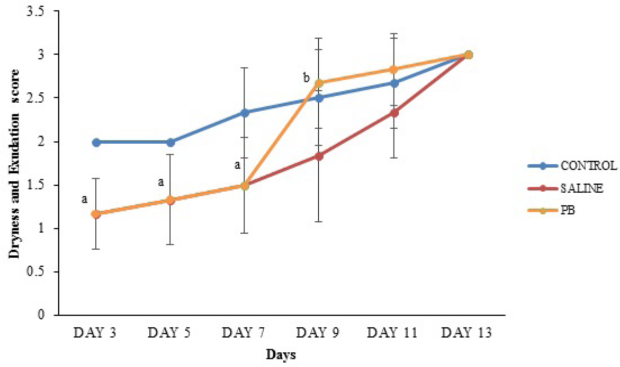


Figure 2: Effects of PB on dryness and exudation of wound. Scores of 1 to 3 were awarded for wound dryness, exudation and scar formation. Maximum value of 3 was given for an optimum condition for wound healing on dryness and exudation. Data are presented as mean±SD with $p<0.05$ (a - significant compared to control for PB and saline; b – PB significant compared to saline)

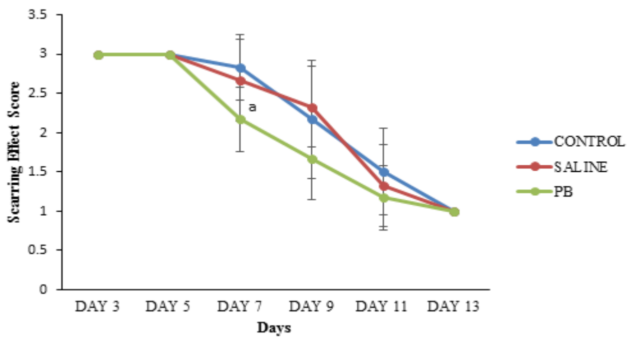
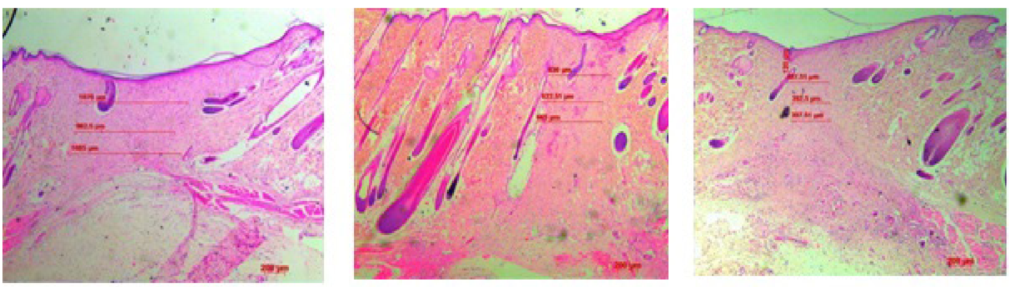


Figure 3: Effects of PB on scar formation. Scores of 1 to 3 were awarded for scar formation. Score value of 1 indicates good and minimum scarring effect. Data are presented as mean±SD with $p<0.05$ (a - significant compared to control)



(a) Control group (b) *Saline group* (c) *PB group*

Figure 4: Effect of PB on histological changes of scar tissues. H&E staining of scar tissues on day 13 for (a) Control group (b) Saline group (c) PB group.

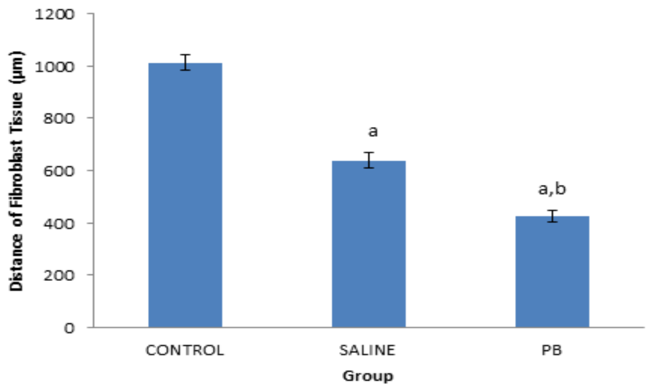


Figure 5: Distance of fibroblast cells/ scar tissue. The images were captured and measured for the distance of fibroblast or scar tissue on day 9 using AxioVision SE64 Rel. 4.9.1 software. Data are presented as mean±SD with $p<0.05$ (a-significant compared to control; b-significant compared to saline)

process involving numerous biological events such as proliferation and migration of keratinocytes, fibroblast and inflammatory cells, angiogenesis, extracellular matrix deposition, granulation tissue formation and wound contraction (Zailan et al. 2010; Orsted et al. 2011). The use of traditional medicine in wound healing has increased during the past decade mainly due to its widespread availability, absence of unwanted side effects and its effectiveness (Jagetia & Rajanikant 2004). Approximately one-third of traditional medicines used for wound healing, compared to only 1-3% of modern medicine, has been found to be effective in treating wounds (Tang et al. 2007). PB is cultivated in most parts of South and Southeast Asia including Malaysia, and it has been used since ancient times for curative purposes such as in the treatment of boils, abscesses, as well as lung diseases (Guha 2006). The leaf is proven to have polyphenols such as hydroxychavicol, chavibetol, chavicol with anti-microbial, antioxidant, anti-inflammatory, and antiseptic properties (Nagori et al. 2011).

We have evaluated in this study the efficacy of PB in wound healing and although there was no significant difference in the duration of wound closure between all groups, however we have shown that wounds treated with PB dressing had promoted early formation of granulation tissue. Treatment with PB has resulted in enhanced wound healing as early as 3 days post wound creation. PB is rich in calcium, phosphorus, iron, iodine, potassium, and vitamins (A, B, C, E) (Rekha et al. 2014) which may help in the formation of a stable collagen fiber and the intracellular matrix (Galeano et al. 2001). The antioxidant polyphenols in PB may help in the elimination of overproduction of reactive oxygen species (ROS) known to delay wound healing and promotes the formation of granulation tissue thus enhancing the healing process (Thangapazham et al. 2016). In addition, PB was also shown to induce proliferation of fibroblast and thus promotes wound healing in rats (Tho et al. 2015)

It was observed that the PB and saline groups showed higher mean score compared to the control for wound dryness and absence of exudation as early as 3 days post wound creation. This could be explained by the fiber found within plant extracts which had the ability to absorb more liquid at the wound area (Boateng et al. 2008) while the saline gives an optimal environment for the healing process to occur as it neither adds nor takes away fluid from the wound bed (Blunt 2001).

Measurement of scar tissue on day 14 showed that rats treated with PB dressing had the least scarring effect compared to the other two groups (control and saline). This may be due to the antioxidant property of PB that may help in reducing oxidative stress and inflammation, thus accelerating the wound healing process. Shorter inflammation phase can accelerate the healing process with less scarring (Liechty et al. 2000). PB was also shown to increase skin contraction and facilitates reepithelialization (Tho et al. 2015) and accelerates wound healing and closure (Nilugal et al. 2014).

Free radical and reactive oxygen species can cause the disruption in the wound healing process. Antioxidant plays an important role in stabilizing and promoting the healing process as observed in this study which was exhibited by PB.

CONCLUSION

In conclusion, this study revealed that PB dressing promoted wound healing by enhancing wound closure with less scarring effect and fibroblast tissue due to faster formation of granulation tissue, as early as three days after the wound creation. Thus, PB can perhaps be used to treat wound complementary to the present method. Further research is needed to determine the mechanisms involved.

The study has a limitation in pursuing further biochemical and histological parameters in relation with wound healing because the students who conducted the experiment were only given three months to perform the study.

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