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Evaluation of Wound Healing Activity of *Piper Betle* Leaves and Stem Extract In Experimental *Wistar* Rats

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ABSTRACT

The present work aims at evaluating the wound healing potential of a traditional plant *Piper betle* (PB) of both leaves and stem. The ethanolic extract of both *Piper betle* leaves and stem were investigated to evaluate the rate of wound healing enclosure and the histology of healed wounds in rats. Four groups of adult female *albino wisttar* rats were experimentally wounded at the dorsal region of the rats. Group 1 animals were left with untreated and set as negative control. Group 2 animals were treated with 10% Povidone Iodine, and set as positive control. Group 3 and 4 treated with ointment formulation containing 10% *Piper betle* leaves and 10% *Piper betle* stem leaves extract respectively. All the four groups are treated with for 21 days. Wound healing was assessed by rate of wound closure estimation and histology studies on the 21st day of post wounding. 10% *Piper betle* stem enhanced the wound healing process by increasing rate of wound closure compared to negative control group. Histological observation also showed better organized tissue and more collagen fibers in 10% *Piper betle* stem treated group. These results strongly document the significant effect of 10% *Piper betle* stem extract to accelerates the rate of wound healing and closure in the experimentally induced wound rats.

Keywords: Piper betle, Wound healing, Hematoxylin & Eosin stain and Massion Trichrome stain the rate of wound healing and closure in the experimentally induced wound rats.

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INTRODUCTION

Wound healing is the process of repair that follows injury to the skin and other soft tissues. Healing is the interaction of a complex cascade of cellular events that generate resurfacing, reconstitution and restoration of the tensile strength of injured tissue [1]. Under the most ideal circumstances, healing is a systematic process, traditionally explained in term of three classic phases known as inflammation, proliferation and maturation [1].

The prevalence of wound complication has increased tremendously worldwide and has become a serious issue for public health [2]. Wound can be associated from the minor injury such as hitting a table or cut by knife and it also can be a major problem such as accident and even after some surgical procedure. Wound healing is an important process by which delay to this process, can lead to many other illness.

Herbal medicines are currently in demand and their popularity is increasing day-by-day. Herbal plants are contains or produce large amount of chemical compounds that are needed by human body to treat illness or diseases or to promote health and well being[3]. As the people become aware of the potency and side effect of the synthetic drugs, there is an increase in interest in the natural remedies with a basic approach towards the nature. Today, estimated 80% of people in developing countries still rely on traditional medicines[3].

Plants are the principle source of raw material for plant based medicines since ancient times the traditional medicines are receiving great importance in the health care sector over the world. This research is conducted by using a well known medicinal plant which is *Piper betle*. *Piper betle* is tropical medicinal plant closely related to the common pepper and belongs to kingdom of *Plantae*, division of *Mangnoliphypa*, class of *Mangnolipisida*, order of *Piperals*, family of *Piperaceae*, and genus of *Piper*, species of *betle* [4.]

The whole plant of Piper betle which are leaves, stem, roots, flowers and fruits has its own medicinal usage. The whole plant is known to cure many disorders such as antioxidant activity, anti ulcerogenic activity, anti platelet activity, anti inflammatory activity, anti amoebic activity, anti filarial activity [5]

However, very less publication indicating scientific findings *Piper betle* stem part have been published. They were no reports regarding the wound healing effect of *Piper betle* stem, thus the present study aims to investigate the medicinal use of *Piper betle* leaves and stem as a wound healing promoter that has been cited in folkloric literature.

MATERIALS AND METHODS

Plant material

Piper betle leaves and stem were collected from locally from Klang, Selangor. Both the *Piper betle* leaves and stem were sent to Institute of Bioscience, University Putra Malaysia for species authentication. The voucher specimen number is SK 2189/13.

Plant extraction

Both the *Piper betle* leaves and stem were washed with water and dried under shed for 3-4 weeks. Prior to dried, both the leaves and stem were cut into small sizes. Then, both parts were powdered in an electric blender separately[6]. Methanol extraction of both part obtained by soxhlet extraction. Required solvent which is 200ml of 70% ethanol is filled into boiling flask and the 20g of course powder of *Piper betle* leaves filled in the thimble. Boiling temperature 35^0C was set. The mixture was filtered using a fine muslin cloth. The step was followed by rotor vaporized to remove ethanol. Then filtered was dried at 50^0C. The dried extract was used for the study [7].

Drug formulation and grouping

A type of drug formulation was prepared for topical administration of wound which is ointment base. Emulsifying ointment was prepared by using emulsifying wax BP as base. Two different types ointment were prepared with same dosage which are 10% *Piper betle* leaves and 10% *Piper betle* stem ointments [8].

Grouping of animals for topical administration includes:-

Group 1 – Consists of 5 rats untreated for 21 days and set as negative control

Group 2 – Consists of 5 rats treated with 10% Povidone Iodine for 21 days and set as positive control

Group 3 – Consists of 5 rats treated with 10% *Piper betle* leaves for 21 days

Group 4 – Consists of 5 rats treated with 10% *Piper betle* stem for 21 days

Experimental rats

Wistar albino adult female rats were obtained from the experimental animal house, faculty of Biomedicine, Asia Metropolitan University. The rats were divided randomly into 4 groups of 5 rats each. Each rat with body weight between 150g-250g was selected and housed separately (one rat, one cage). The animals were maintained on standard pellet diet and tap water. They were maintained in standard room condition, relatively humidity and 12 hour light dark cycle. The rat cages were cleaned changed bedding every day. Animals were weighed on the particular days of experiment. All animals are closely observed for any infection [9].

Animal ethics

University animal ethics committee approved the experimental protocol & animals were maintained well in an animal house approved by AMU animal ethics committee (AMU/AEC/HSFBH/2013/3).

Excision wound model

The animals were firstly anesthetized with diethyl ether. The hair of the dorsal neck region was shaved with an electrical shaver and disinfected with 70% alcohol. The area of wound to be created was outlined on the back of the animals with permanent marker. A uniform wound area of 2.00cm in diameter was excised roundly with scissor from nape of the dorsal neck of all rats aseptically. The day of wound creation was considered as day zero. The animals were allowed to recover and were housed individually in different cages. Rate of wound closure were measured and histology studies done on the 21 day of post wounding [10].

Topical application of vehicles

Wound of group 1 rats were untreated for whole 21 days and set as negative control. Group 2 wounds were dressed once daily with 10% Povidone Iodine. Group 3 and 4 were dressed with 10% *Piper betle* leaves and 10% *Piper betle* stem extract once daily respectively. The wounds were observed daily until complete wound healing enclosure occurs.

Rate of wound closure determination

The rate of wound closure was measured on day 0, 5^{th}, 10^{th}, 15^{th} and 20^{th} of post wounding. The rate of wound contraction area was recorded by transparent paper and marker pen. Recordings of the wound areas were measured on graph paper[10]. The percentage of wound reduction from the original wound was calculated using the following formula:-

$$\text{Rate of wound closure} = \frac{\text{Wound area day 0} - \text{Wound area day X}}{\text{Wound area day 0}} \times 100$$

Histology studies

Specimens of skin from healed wounds from each rat was taken and fixed in 10% buffered formalin solution for histopathology studies. Sections of the healed skin were made at a thickness of 3 micrometer and stained with H&E stain and MT stain and assessed for the histopathology changes. The slides were examined and photomicrographs was obtained using motic camera 250 that attached with to light microscope [10].

Statistical analysis

All values are reported as mean ±S.E.M. the means of wound area measurements between groups at different time intervals were compared using one way ANOVA, followed by Tukey one way ANOVA was used to examine the mean differences in wound healing between the groups. Data analysis was performed using statistical package programme SPSS version 12.0. A p value <0.05 was considered as statistically significant [10].

RESULTS AND DISCUSSION

Rate of wound closure

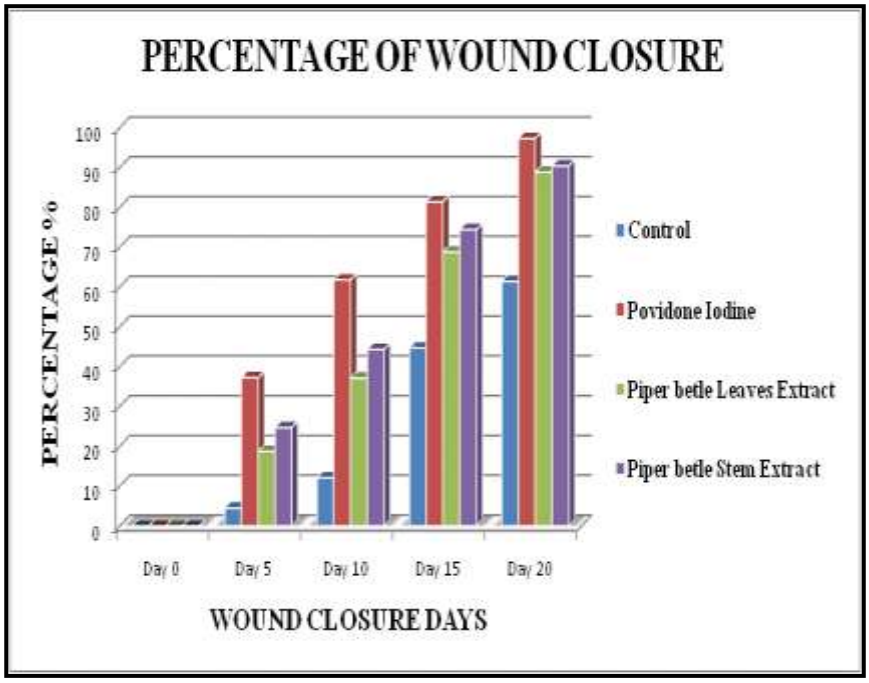
A significant increase in the wound healing activity was observed in the animals treated with both 10% *Piper betle* leaves and 10% *Piper betle* stem. However, higher percentage of wound closure was observed in 10% *Piper betle* stem compared control group.

Figure 1 shows the effect of all groups wound sizes that photographed on 5 consecutive days which are on 0, 5th, 10th, 15th, and 20th of post wounding. In this model, the extract treated animal's shows a more rapid decrease in wound size compared to control rats which is untreated for 21 days.

Percentage of wound reduction from the original wound was calculated on 5 consecutive days by the formulae below:-

$$\text{Rate of wound closure} = \frac{\text{Wound area day 0 - Wound area day X}}{\text{Wound area day 0}} \times 100$$

Bar chart 1 shows the percentage of wound reduction size. The rate of wound closure was significantly increased in all treated group in which P< 0.05. Table 1 shows statistical analysis of one way ANOVA of wound healing activity.



Bar chart 1 shows percentage of wound reduction size.

Table 1 Effect of control, providone iodine ointment and ointment formulation containing *piper betel* leave and stem extract on excision wound model in albino Wistar rats.

Rats	0 Day	5th Day	10th Day	15th Day	20th Day
Control	400.80±0.83	383.02±2.05	352.52±2.08	221.62±1.83	154.60±1.14
Povidone	401.40±1.14	252.20±2.34	153.30±2.17	74.50±1.54	10.78±0.70
Leaf	401.20±0.83	326.06±0.82	252.46±1.93	125.30±1.61	44.70±1.55
Stem	401.20±0.83	302.04±1.63	223.34±1.51	102.62±2.16	38.44±0.53

Histopathology evaluation

Histological evaluation based on hematoxylin and eosin stain was carried out for the treated and untreated groups. There was marked infiltration of the inflammatory cells, increased blood vessels formation and enhanced proliferation of cells as a result of treatment with ointment formulation containing *Piper betle* stem and leaves extract. All groups of experimental rats showed epithelization of wound area. Epithelization in group 1 as negative control shows thin, whereas in other 3 group which are Positive control (10% Povidone Iodine), 10% *Piper betle* leaves extract and 10% *Piper betle* stem extract shows more formation of melanin, melanocytes and fibrous tissue.

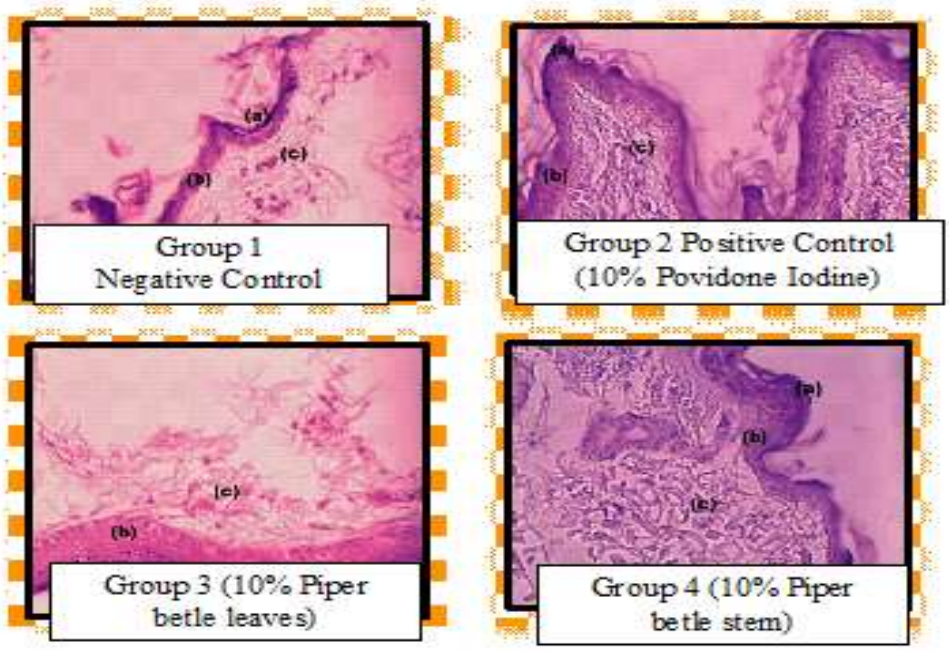


Figure1 shows the histopathology evaluation (Hemotoxylin & Eosin stain) of wound healing tissue from group 1 to group 4. (a) Shows formation of melanin, (b) shows formation of melanocytes and (c) shows formation of fibrous tissue.

Massion Trichrome stain was done to evaluate the formation of collagenous tissue. Group 1 as negative control shows thin layer of collagen compared to other 3 groups which are Positive control (10% Povidone Iodine), 10% *Piper betle* leaves extract and 10% *Piper betle* stem extract.

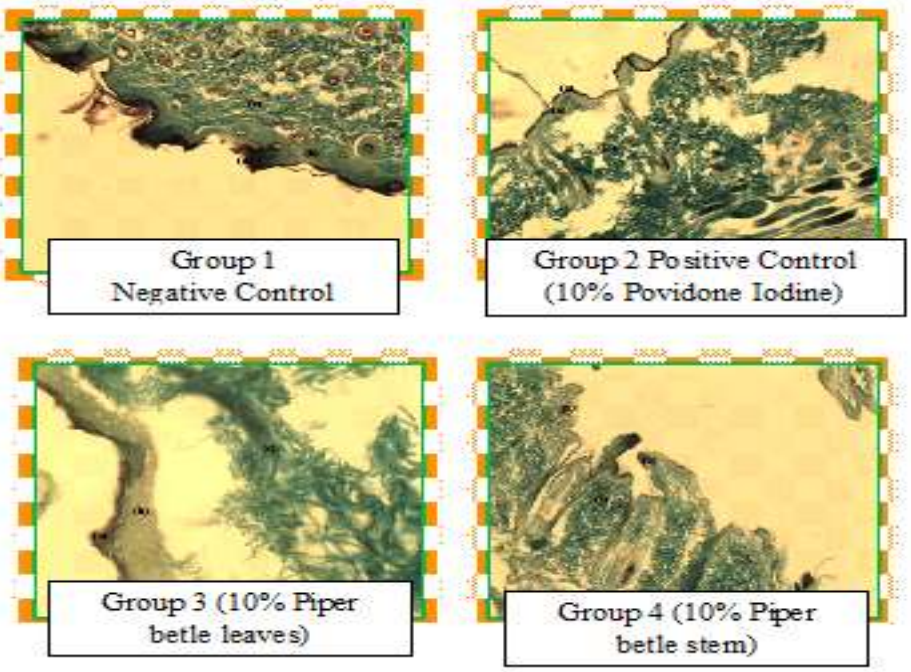


Figure 2 shows the histopathology evaluation (Massion Trichrome stain) of wound healing tissue from group 1 to group 4. (a) Shows formation of melanin, (b) shows formation of melanocytes and (c) shows formation of fibrous tissue.

Wounds are referred to as disruption of normal anatomic structure and function. Skin wounds could happen through several causes like physical injuries resulting in opening and breaking of the skin [11]. The most common symptoms of wounds are bleeding, loss of feeling or function below the wound site, heat and redness around the wound, painful or throbbing sensation, swelling of tissue in the area and pus like drainage [12]. Wound healing is a very complex, multifactor sequence of events involving several cellular and biochemical processes. The aim in these processes is to regenerate and reconstruct the disrupted anatomical continuity and functional status of the skin. Healing process, a natural body reaction to injury, initiates immediately after wounding and occurs in four stages. The first phase is coagulation which controls excessive blood loss from the damaged vessels. The next stage of the healing process is inflammation and debridement of wound followed by re-epitheliasation which includes proliferation, migration and differentiation of squamous epithelial cells of the epidermis. In the final stage of the healing process collagen deposition and remodeling occurs within the dermis [13]. The results showed wound healing and repair, accelerated by applying ointment formulation containing *Piper betle* leaves and stem extract, which was highlighted by the full thickness coverage of the wound area by an organized epidermis. The enhanced capacity of wound healing with the plant could be explained on the basis of anti-inflammatory effects of the plant that are well documented in the literature.

The present study in albino Wistar rats showed enhanced rate of wound contraction and drastic reduction in healing time than control, which might be due to enhanced epitheliasation. The animals treated with ointment formulation containing 10% *Piper betle* leaves and stem showed significant results when compared with providone iodine and control group.

CONCLUSION

The results obtained in the present study indicate that *Piper betle* leaves and stem are more effective in wound healing activity in rat's compared negative control. Hence, the present study accepted the alternate hypothesis which is each part of the *Piper betle* (leaves and stem) are effective in wound healing compared to negative control. Hence, *Piper betle* leaves and stem having a significant wound healing activity in rats compared to control. The wound healing effect of 10% *Piper betle* leaves and stem extract may be due to the presence of more active

molecules. Further pharmacological and biochemical investigation has to be undertaken to clearly elucidate the mechanism of action and will be helpful in projecting this plant as a therapeutic target in wound healing and other diseases. The current study accepted the alternate hypothesis which is each part of *Piper betle* (leaves and stem) are effective in wound healing compared to 10% Povidone Iodine.

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