Full Length Research Paper

Histological study of wound healing potential by ethanol leaf extract of *Strobilanthes crispus* in rats

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This study was conducted to evaluate the effects of topical application of ethanol extract of *Strobilanthes crispus* leaf on the rate of wound healing closure and histology of healed wound. Four groups of male Sprague Dawley rats, all were experimentally wound in the posterior neck area. An area of uniform wound 2 cm in diameter using circular stamp, was excised from the nape of the dorsal neck of all rats with the aid of round seal. The animal groups were topically treated respectively with 0.2 ml of each vehicle (gum acacia), intraSite gel, 100 and 200 mg/ml of ethanol extract. Macroscopically, wound dressed with leaf extract and intraSite gel-treated group significantly healed earlier than those treated with vehicle. Histological analysis of healed wounds dresses with leaf extract showed comparatively less scar width at wound closure and healed wound contained less inflammatory cells and more collagen with angiogenesis compared to wounds dressed with vehicle. In conclusion, wounds dressed with leaf extract significantly enhanced the acceleration of wound healing enclosure in rats, and this was ascertain by histological study.

Key words: *Strobilanthes crispus* leaf, ethanol extract, wound healing, histology.

INTRODUCTION

Healing of wounds starts from the moment of injury and can continue for varying periods of time depending on the extent of wounding and the process can be broadly categorized into three stages; inflammatory phase, proliferate phase, and finally the remodeling phase which ultimately determines the strength and appearance of the healed tissue (Sumitra, 2005; Ayyanar, 2009). Wound healing is a complex series of interrelated events that are mediated through the phases by a wide range of chemically co-ordinate cellular processes as well as hormonal influences (Chan et al., 2008). Medicinal plants have been shown to possess wound healing activity in animal studies (Nayak et al., 2009; Mahmood et al., 2010). One of the herbs that have great potential is *Strobilanthes crispus*. Acanthaceae is native plant to tropical countries and the leaves have been used traditional medicine in Malaysia. Traditionally, *S. crispus* leaves were boiled with water and has been used as anti-diabetic, diuretic, antilytic, and laxative (Sunarto, 1977).

In addition, the leaves of *S. crispus* has been shown to possess anti-AIDS and anti-leukemia (Kusumoto et al., 1992), high antioxidant activity, anti-hyperglycemic and hypolipidemic effects (Norfarizan-Hanoon et al., 2009), and anti-carcinogenic (Asmah et al., 2006; Fazdelly et al., 2006). Rahmat et al. (2006) reported that the methanolic extract of this plant displayed strong cytotoxic effect on colon cancer (Caco-2), human breast cancer hormone non-dependent (MDA-MB-231) and liver cancer (HepG-2). This plant has many cystoliths of calcium carbonate and an infusion is mildly alkaline (Perry and Metzger, 1980). The leaves of this plant contained high amount of minerals (potassium, calcium, sodium, iron and phosphorous). These leaves also contained high content of water-soluble vitamins (C, B1 and B2) and it also contains other composition such as catechins, alkaloids, caffeine and tannins. Catechins of *S. crispus* leaves showed highest antioxidant activity compared to vitamin E (Ismail et al., 2000). There have been no reports on histology of wound healing potential of *S. crispus* leaves which encourages us to evaluate the histology of wound healing of this plant extract in rats.

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MATERIALS AND METHODS

Intrasite gel

Intrasite gel was purchased from University Malaya Medical Centre Pharmacy. Intrasite gel is an amorphous hydrogel which gently re-hydrates necrotic tissue and facilitates autolytic debridement while loosening and absorbing slough and exudates, clearing the way for effective wound healing. It is also designed for wounds that are granulating and epithelialising. It can also be used to provide the optimum moist wound management environment during the later stages of wound closure. It is non-adherent and does not harm viable tissue or the skin surrounding the wound. This makes the use of Intrasite gel ideal for every stage in the wound management process. (Intrasite gel is a trademark for Smith and Nephew Healthcare Limited) (Williams, 1994).

Lignocaine HCl (2%, 100 mg/5 ml)

Lignocaine is a local anesthesia and was purchased from the Experimental Animal House, Faculty of Medicine, University of Malaya (Delta Veterinary Laboratory PTY LTD, NSW 20011). 1 ml of Lignocaine was injected subcutaneous.

Strobilanthes crispus leaf extracts preparation

Fresh leaves of S. crispus were obtained from Ethno Resources Sdn Bhd, Selangor Malaysia, and identified by comparison with the Voucher specimens deposited at the Herbarium of Rimbab Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur. The leaves were tap washed followed by washing with distilled and shade-dried for 7 to 10 days and was then finely powdered using an electrical blender. 100 g of fine powder was soaked in 1000 ml of 95% ethanol in conical flask for 3 days in room temperature. After that the mixture was filtered using a fine muslin cloth followed by filter paper (Whatman No. 1) and distilled under reduced pressure in an Eyela rotary evaporator (Sigma-Aldrich, USA). The extract was placed in incubator to dry at 45°C and the clear semisolid extract was dissolved by using the vehicle, gum acacia in normal saline as described by Shetty et al. (2007) with slight modification. Two grams of gum acacia was dissolved in 100 ml of normal saline. From this, 10 ml of solution, which contains 200 mg of gum acacia, was used for dissolving 1 g and two grams of ethanol extract each. So 1 ml of each solution contains 100 and 200 mg of extract respectively (100 mg/ml = 20 mg/0.2 ml and 200 mg/ml = 40 mg/0.2 ml).

Acute toxicity studies

The acute toxic study was used to determine a safe dose for S. crispus. Forty eight healthy Sprague Dawley rats (24 males and 24 females) were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya, and were assigned equally into 4 groups labeled as vehicle (gum acacia in normal saline); 1, 2 and 5 g/kg of S. crispus in vehicle, respectively. The animals were fasted overnight (food but not water) prior dosing. Food was withheld for a further 3 to 4 h after dosing. The animals were observed for 30 min and 2, 4, 24 and 48 h after the administration for the onset of clinical or toxicological symptoms. Mortality, if any was observed over a period of 2 weeks. The animals were sacrificed on the 15th day. Hematological, serum biochemical and histological (liver and kidney) parameters were determined following standard methods (Bergmeyer, 1980; Tietz et al., 1983).

The study was approved by the ethics Committee for animal experimentation, Faculty of Medicine, University of Malaya, Malaysia and the Ethic No. PM/07/05/2009/MMA (a) (R). Throughout the experiments, all animals received human care according to the criteria outlined in the “Guide for the Care and Use of laboratory Animals” prepared by the National Academy of Sciences and published by the national Institute of health.

Experimental animals

Sprague Dawley healthy adult male rats were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya, and the rats were divided randomly into 4 groups of 10 rats each. Each rat that weighted between 220 - 250 g (8 weeks old) was housed separately (one rat per cage). The animals were maintained on standard pellet diet and tap water. The study was approved by the ethics Committee for animal experimentation, Faculty of Medicine, University of Malaya, Ethic No. PM/27/07/2009/MAA (R). Throughout the experiments, all animals received human care according to the criteria outlined in the “Guide for the Care and Use of laboratory Animals” prepared by the National Academy of Sciences and published by the national Institute of health.

Experimentally induced excision wounds

The animals were anesthetized with Ketamine and Xylazil prior to creation of the wounds. The skin shaved by electrical clipper, disinfected with 70% alcohol and injected with 1 ml of Lignocaine HCl (2%, 100 mg/5 ml). An area of uniform wound 2 cm in diameter (circular area = 3.14 cm²), using circular stamp, was excised from the nape of the dorsal neck of all rats with the aid of round seal as described by Morton and Melone (1972). Avoid incision of the muscle layer and tension of skin was kept constant during the procedure. The entire wound left open (Nayak et al., 2005). The wound area was measured immediately by placing a transparent tracing paper over the wound and tracing it out. The tracing paper was placed on 1 mm² graph sheet, and traced out. The squares were counted and the area recorded, as described by Chah et al. (2006).

Topical wound application

Wounds of Group 1 animals were topically treated with 0.2 ml of vehicle, gum acacia in normal saline (20 mg/ml), twice daily as placebo control group (Shetty et al., 2007). Wounds of Group 2 rats were topically treated with 0.2 ml of Intrasite gel twice daily as a reference standard control. Moreover, 0.2 ml of 100 mg/ml and 200 mg/ml of ethanol extract of S. crispus each were applied topically twice daily to the wound of Group 3 and 4 respectively. The wound was observed daily and when complete wound-healing all closure occurs, four animals from each group were sacrificed and the wound area was fixed and processed by tissue processing machine.

The remaining 6 animals from each group were observed until complete wound healing wound areas.

Estimation of wound healing (wound closure)

Wound areas were traced manually and calculated in square millimeters. The wound closure area of each animal was assessed by tracing the wound on days 1, 3, 10 and 15 post-wounding surgery and the wound closure rate was expresses as the percentage of wound area compared with that on post-operative day by using transparency paper and a permanent marker under
general anesthesia (a mixture of Ketamine and Xylazil) as described by Nayak and Pinto-Pereira (2006). The wound areas recorded were measured using a graph paper. The percent wounds healing on these days are determined (Chah et al., 2006). Number of days required for falling of scar without any residual raw wound gave the period of epithelization.

**Histological evaluation of healed wounds**

Four animals from each group were sacrificed by overdose of Ketamine and Xylazil when wounds are completely healing. The skin specimen from wound areas were fixed in 10% buffered formalin and processed by paraffin tissue processing machine. The wound area was assessed by taking a 5 µ section, stained with hematoxylin and eosin.

**Statistical analysis**

All values are reported as mean ± S.E.M. and the statistical significance of differences among groups were assessed using one-way ANOVA. A value of p < 0.05 was considered significant.

**RESULTS**

**Acute toxicity**

Acute toxicity study is a study in which the animals were treated with the *S. crispus* leaf extract at a dose of 1, 2 and 5 g/kg were kept under observation for 14 days. All the animals remained alive and did not manifest any significant visible of toxicity at these doses. Thus, clinical observations, blood biochemistry, hematology, and histopathology data did not show any significant differences between control and treated groups. We conclude that *S. crispus* orally administered to rats was safe and that no drug-related toxicity was detected even at the highest dose investigated.

**Wound healing activity**

Grossly, wounds dressed with of *S. crispus*-treated groups or reference standard control wounds showed considerable signs of dermal healing and significantly healed faster compared with those who received the placebo control treatment (gum acacia in normal saline) (Table 1, Figures 1a and b). Table 2 shows the effects of *S. crispus* on the percentage of wound healed on days post surgery. Throughout the experiment, the percentage healing in placebo control group wounds was significantly lower than those of *S. crispus*-treated groups and reference standard control wounds.

Histology of wounds on day 13 post surgery. *S. crispus*-treated groups showed complete wound healing closure compared to placebo control group which showed incomplete wound healing closure (no epidermis, arrow) (Figures 2a and b). Granulation tissue of healed wound in *S. crispus*-treated group contained comparatively few inflammatory cells, and more collagen fiber, fibroblasts and proliferating blood capillaries (angiogenesis) compared with placebo-treated group which contain less collagen fibers, fibroblasts and blood capillaries, and more inflammatory cells (Figures 3a and b).

**DISCUSSION**

It is important to note that throughout period of wound treatment, the extract did not cause irritation or pain to the animals as the rats neither show any signs of restlessness nor scratching/biting of wound site when the extract were applied. In the authors laboratory all the surgical interventions were carried out under sterile conditions and animals were closely observed for any infection; those which showed signs of infection were separated and excluded from the study. This is very important and researchers proved that the control microbial infection is necessary for better healing and its management (Muhammad and Muhammad, 2005).

Wound healing is a complex and dynamic process of restoring cellular structures and tissue layers in damaged tissue as closely as possible to its normal state. Wound contracture is a process that occurs throughout the healing process, commencing in the fibroblastic stage whereby the area of the wound undergoes shrinkage. In the maturational phase, the final phase of wound healing the wound undergoes contraction resulting in a smaller amount of apparent scar tissue (Midwood et al., 2004). In the current study, topical application of *S. crispus* significantly accelerated the rate of wound healing, and

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>No of animals</th>
<th>Type of dressings (twice daily) (0.2 ml/animal)</th>
<th>Healing time (days) (Mean ± S.E.M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Group 1</td>
<td>6</td>
<td>Gum acacia in normal saline (20 mg/ml)</td>
<td>20.6 ± 0.37&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 2</td>
<td>6</td>
<td>Intrasil gel (Reference control)</td>
<td>13.17 ± 0.31&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 3</td>
<td>6</td>
<td><em>S. crispus</em> 100 mg/ml</td>
<td>14.80 ± 0.37&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>Group 4</td>
<td>6</td>
<td><em>S. crispus</em> 200 mg/ml</td>
<td>13.00 ± 0.37&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values were expressed as mean and ± standard error mean. Mean with different superscripts were significantly different (P<0.05).
Figure 1. Macroscopically appearance of wound healing on day 13 post surgery in (a) Gum acacia-treated group. (b) 200 mg/ml of *S. crispus*-treated group.

Table 2. Effect of *S. crispus* on percentage (%) of wound healing in experimental rats.

<table>
<thead>
<tr>
<th>Animal groups</th>
<th>Treatment (twice daily) (0.2 ml)</th>
<th>Percentage wound healing (Mean ± S.E.M) on day post surgery</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Day 5</td>
</tr>
<tr>
<td>Group 1(N = 6)</td>
<td>Gum acacia in normal saline</td>
<td>35.17 ± 1.51^a</td>
</tr>
<tr>
<td>Group 2(N = 6)</td>
<td>Intrasite gel</td>
<td>65.17 ± 1.51^b</td>
</tr>
<tr>
<td>Group 3(N = 6)</td>
<td><em>B. rotunda</em> 100 mg/ml</td>
<td>55.17 ± 1.40^c</td>
</tr>
<tr>
<td>Group 4(N = 6)</td>
<td><em>B. rotunda</em> 200 mg/ml</td>
<td>68.00 ± 1.63^b</td>
</tr>
</tbody>
</table>

All values were expressed as mean and ± standard error mean. Mean with different superscripts were significantly different (P<0.05).

Histology, healed wound contain comparatively less inflammatory, more collagen and angiogenesis.

Wound healing effects may be due to regulation of collagen expression (Bonte et al., 1993) and increase in tensile strength of the wounds (Suguna et al., 1996). Similarly, enhanced healing activity has been attributed to increased collagen formation and angiogenesis (Trabucchi et al., 1986; Shukla et al., 1999). Collagen played a central role in the healing of wounds and it is a principal component of connective tissue and provides a structural framework for the regenerating tissue (Cohen et al., 1992). Angiogenesis in granulation tissues improves circulation to the wound site thus providing oxygen and nutrients essential for the healing process (Szabo et al., 1995) that include re-epithelization. Stimulate epithelial cell proliferation and angiogenesis are important for wound healing process (Buntrock et al., 1982). Habibipour et al. (2003) showed that histological analysis of the treated healed wound group contained a large amount of fibroblast proliferation, collagen synthesis, and neovascularization, which resulted in an increased wound tensile strength and accelerated healing wound.

An ester glycoside compound of caffeic acid, a verbascoside, was isolated from the leaves of *S. crispus* which are known to have analgesic effects internally, and antifungal and antibacterial effects when used externally (Soediro et al., 1983), and may be responsible for...
Figure 2. Histological section of healed wound in (a) Gum acacia-treated group showing incomplete wound closure (no epidermis, arrow), (b) 200 mg/kg of *S. crispus*-treated group showing complete wound healing closure, (H and E stain 4x).

Figure 3. Histological section of wound healed on day 13 post surgery in (a) Gum acacia-treated group. Granulation tissue contains comparatively less collagen, fibroblast and blood capillaries, and more inflammatory cells (b) 200 mg/kg of *S. crispus*-treated group. Granulation tissue contains comparatively more collagen, fibroblast and blood capillaries, and less inflammatory cells. (H and E stain 40x).

wound-healing activity and studies with plant extracts have shown that constituent like catechins, alkaloids, caffeine and tannins have analgesic effects. Catechins of *S. crispus* leaves showed highest antioxidant activity compared to vitamin E (Ismail et al., 2000). Mechanisms of wound healing may be contributed to stimulate the production of antioxidants in wound site and provides a favorable environment for tissue healing (Shukla et al., 1999). *S. crispus* has shown antioxidant activity (Norfarizan-Hanoon et al., 2009). It have been reported that antioxidants may play a significant role in the wound healing process and may be important contributory factor in the wound healing property (Shukla et al., 1999). Antioxidants have been reported to play a significant role in the wound healing process and significantly improve wound healing and protect tissues from oxidative damage (Martin, 1996). *S. crispus* leaf extract contain a wide array of free radical scavenging molecules and flavonoids were the major naturally occurring antioxidant components in this plant (Liza et al., 2010). The higher flavonoids content, the stronger the antioxidant activity. Flavonoids can scavenge for the reactive oxygen species (super-oxide anions) and free radicals produced by ethanol. These reactive intermediates are potentially
implicated in delayed wound healing (Lewis and Hanson, 1991). Flavonoids are known to promote the wound-healing process mainly due to their antimicrobial properties, which appear to be responsible for wound contraction and increased rate of epithelialization (Tsuchiya et al., 1996). Flavonoids are known to reduce lipid peroxidation not only by preventing or slowing the onset of cell necrosis but also by improving vascularity. Hence, any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibers by increasing the strength of collagen fibers, increasing the circulation, preventing the cell damage and by promoting the DNA synthesis (Getie et al., 2002).

To determine the safety of drugs and plant products for human use, toxicological evaluation is carried out in various experimental animals to predict toxicity and to provide guidelines for selecting a ‘safe’ dose in humans. The highest overall concordance of toxicity in animals with humans is with hematological, gastrointestinal, and cardiovascular adverse effects (Olson et al., 2000). Liver and kidney of the treated rats showed no significant change as compared to the control group. Hematology and clinical biochemistry values were within the range of the control animals tested and similar to some of the control reference values published by other researchers (Ringler and Dabich, 1979; Witthawaskul et al., 2003). The highest dose of S. crispus extract which did not cause any toxicity was 5 g/kg body weight, suggesting that this plant is relatively non-toxic since in acute toxicity studies, the product is considered non-toxic if no deaths are registered after 14 days of observation and no clinical signs of toxicity are observed at doses at or below 5 g/kg (Brock et al., 1995).

In conclusion, the extract of this plant showed remarkable wound healing activity and it may be suggested for treating various types of wounds in human beings. The acute toxicity profile of S. crispus extract could be considered favorable judging from the absence of adverse clinical manifestations in experimental animals after. Further studies with purified constituents are needed to understand the complete mechanism of wound healing activity of S. crispus.

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REFERENCES


