

Full Length Research Paper

Acute toxicity study and wound healing potential of *Gynura procumbens* leaf extract in rats

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This study was conducted to evaluate the effects of topical application of ethanol extract of *Gynura procumbens* leaf extract on the rate of wound healing closure and histology of wound area. An area of uniform wound 2.00 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 with 0.2 ml of each vehicle (gum acacia), Intrasite gel, 100 and 200 mg/ml of ethanol extract, respectively. Macroscopically, wound dressed with *G. procumbens* leaf extract and Intrasite gel significantly healed earlier than those treated with vehicle. On day 14 post-surgery, 6 animals from each group were sacrificed and histological analysis of wounds area showed that wounds dressed with leaf extract showed comparatively less scar width at wound closure and granulation tissue contained less inflammatory cells and more collagen with angiogenesis compared to wounds dressed with vehicle. Furthermore, acute toxicity study has indicated no mortality with 5 g/kg dose of *G. procumbens* leaf extract in Sprague Dawley and did not produce any major clinical signs of toxicity. In conclusion, wounds dressed with leaf extract significantly enhanced and accelerated the rate of wound healing enclosure in rats.

Key words: *Gynura procumbens*, ethanol leaf extract, acute toxicity, liver function test, renal function test, wound healing, histology.

INTRODUCTION

Normal wound healing response begins as soon as the tissue is injured. Wound healing is the process of repair that follows injury to the skin and other soft tissues. Following injury, an inflammatory response occurs and the cells below the dermis begin to increase collagen production. Later, the epithelial tissue is regenerated (Souba and Wilmore, 1999). It is accepted that wound

repair is an immune-mediated physiologic mechanism (Singer and Clark, 1999). Wound healing, or wound repair, is an intricate process in which the skin repairs itself after injury (Nguyen et al., 2009). Several plants and herbs have been used experimentally to treat skin disorders, including wound injuries, in traditional medicine (Abdulla et al., 2009; Mahmood et al., 2010).

Gynura procumbens (Merr.) (family Compositae) known as "Sambung nyawa", is widely distributed in South East Asian countries such as Indonesia, Malaysia, and Thailand. The plant has traditionally been used for the treatment of eruptive fevers, rash, kidney disease,

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migraine, constipation, hypertension, diabetes mellitus and cancer (Perry, 1980). Pharmacological studies have indicated that *G. procumbens* has antioxidant (Rosidah et al., 2008), anti-herpes simplex virus (Nawawi et al., 1999), anti-hyperglycemic (Hassan et al., 2010), anti-inflammatory (Iskander et al., 2002), antiulcer (Mahmood et al., 2010), anti hyperlipidemic (Zhang and Tan, 2000) and blood pressure reduction capabilities (Kim et al., 2006). Several studies have shown that *G. procumbens* leaf extract contains several pharmaceutically active chemical constituents, such as flavonoids, saponins, tannins, and terpenoids and sterol glycosides (Akowuah et al., 2002). Thus so far, there is no data available on wound healing effect of *G. procumbens* leaf extract. This encourages us to assess the rate of wound healing enclosure of *G. procumbens* leaf extract macroscopically and microscopically in rats.

MATERIALS AND METHODS

Intrasite gel

Intrasite gel was purchased from University Malaya Medical Centre Pharmacy. Intrasite gel is a colorless transparent aqueous gel, which contains 2.3% of a modified carboxymethyl cellulose polymer together with propylene glycol (20%) as a humectants and preservative. When placed in contact with a wound, the dressing absorbs excess exudates and produces a moist environment at the surface of the wound, without causing tissue maceration. 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.

G. procumbens leaf extract preparation

Fresh leaves of *G. procumbens* were obtained from Ethno Resources Sdn Bhd, Selangor Malaysia, and identified by comparison with the Voucher specimen deposited at the Herbarium of Rimba Ilmu, Institute of Science Biology, University of Malaya, Kuala Lumpur. The leaves were tap washed followed by washing with distilled water. The leaves were shade-dried for 7 to 10 days and were then finely powdered using electrical blender. 100 g of fine powder were 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 40°C and the clear semi solid extract was dissolved by using the vehicle, gum acacia in normal saline as described by Mahmood et al. (2010). 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 one gram and two grams of ethanolic 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 *G. procumbens*. Thirty six healthy *Sprague Dawley* rats (18 males and 18 females) were obtained from the Experimental Animal House, Faculty of Medicine, University of Malaya, and were assigned equally into 3 groups labeled as vehicle (gum acacia in normal saline); 2 and 5 g/kg of *G. procumbens* in vehicle, respectively (Perry, 1980). 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 two weeks.

The animals were sacrificed on the 15th day. Serum biochemical and histological (liver and kidney) parameters were determined following standard methods (Perry, 1980; Rosidah et al., 2008). 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 12 rats each. Each rat that weighted between 230 to 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).

Experimentally induced excision wounds

The animals were anesthetized with 0.09 ml of Ketamine i.m injection (30 mg/kg, 100 mg/ml) and 0.01 ml of Xylazil i.m. injection (3 mg/kg, 100 mg/ml) prior to creation of the wounds. The skin shaved by electrical clipper, disinfected with 70% alcohol and injected with 1 ml of Lignocaine HCl s.c injection (2%, 100 mg/5 ml). An area of uniform wound 2.00 cm in diameter (circular area = 3.14 cm²) was excised from the nape of the dorsal neck of all rats with the aid of round seal under local and general anesthesia as described previously (Abdulla et al., 2009). Avoid incision of the muscle layer and tension of skin was kept constant during the procedure, the entire wound left open, the wound area was measured immediately under 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 was recorded, as described previously (Abdulla et al.,

2009).

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 (Mahmood et al., 2010). 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 and 200 mg/ml of ethanol extract of *G. procumbens* in vehicle each were applied topically twice daily to the wound of Groups 3 and 4, respectively. The wound was observed daily and 6 animals from each group were sacrificed when complete wound-healing enclosure occurs. The remaining animals were observed for complete wound healing.

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, 5, 10, and 15 post-wounding surgery and the wound closure rate was expressed as the percentage of wound area compared with that on post-operative day by using a transparent paper and a permanent marker under general anesthesia (Ketamine and Xylazil) as described previously (Mahmood et al., 2010). The wound areas recorded were measured using a graph paper. The percent of wounds healing on these days were determined (Mahmood et al., 2010). Number of days required for falling of scar without any residual raw wound gave the period of epithelization.

Histological evaluation of healed wounds

The skin specimen from wounds healed areas were fixed in 10% buffered formalin and processed by paraffin tissue processing machine. The healed skin was assessed by taking a 5 μ section, stained with hematoxylin and eosin.

Statistical analysis

All values were reported as mean \pm S.E.M. and the statistical significance of differences among groups were assessed using one-way ANOVA. 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 *G. procumbens* extract at a dose of 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, serum biochemistry, and histopathology data did not show any significant differences between control and treated groups (Figure 1, and Table 1). We concluded that *G. procumbens* leaf extract 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 *G. procumbens*-treated groups or with reference standard control showed considerable signs of dermal healing and significantly healed faster compared to group received the placebo control treatment (gum acacia in normal saline) (Table 2 and Figure 2). Table 3 showed the effects of *G. procumbens* extract on the percentage of wound healed on days post surgery. Throughout the experiment, the percentage of healing in placebo control group wounds was significantly lower than those of *G. procumbens* extract-treated groups and reference standard control wounds. Histology of wound area on day 14 post-surgery showed that wound dressed with *G. procumbens* extract showed comparatively less scar width at wound closure compared to the placebo-treated group (Figure 3), and the granulation tissue of wound area contained comparatively few inflammatory cells, and more collagen and proliferating blood capillaries (angiogenesis) compared with placebo-treated group (Figure 4).

DISCUSSION

It is important to note that throughout the period of wound treatment, the *G. procumbens* 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. 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. The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction resulting in a smaller amount of apparent scar tissue (Wasman et al., 2010). The results of the current study showed that topical application of *G. procumbens* extract significantly accelerated the rate of wound healing, and in histology, granulation tissue contain comparatively less inflammatory, more collagen and angiogenesis. This is in line with the results of Abdulla et al. (2009) and Mahmood et al. (2010) who showed that wound treated with plant extract of granulation tissue contain less inflammatory, more collagen and angiogenesis. Wound healing effects may be due to regulation of collagen expression and increase in tensile strength of the wounds (Abdulla et al., 2010; Midwood et al., 2004). Similarly, enhanced healing activity has been attributed to collagen formation and angiogenesis (Abdulla et al., 2009; Mahmood et al., 2010). 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 (Bonte et al., 1993). Angiogenesis in granulation tissues

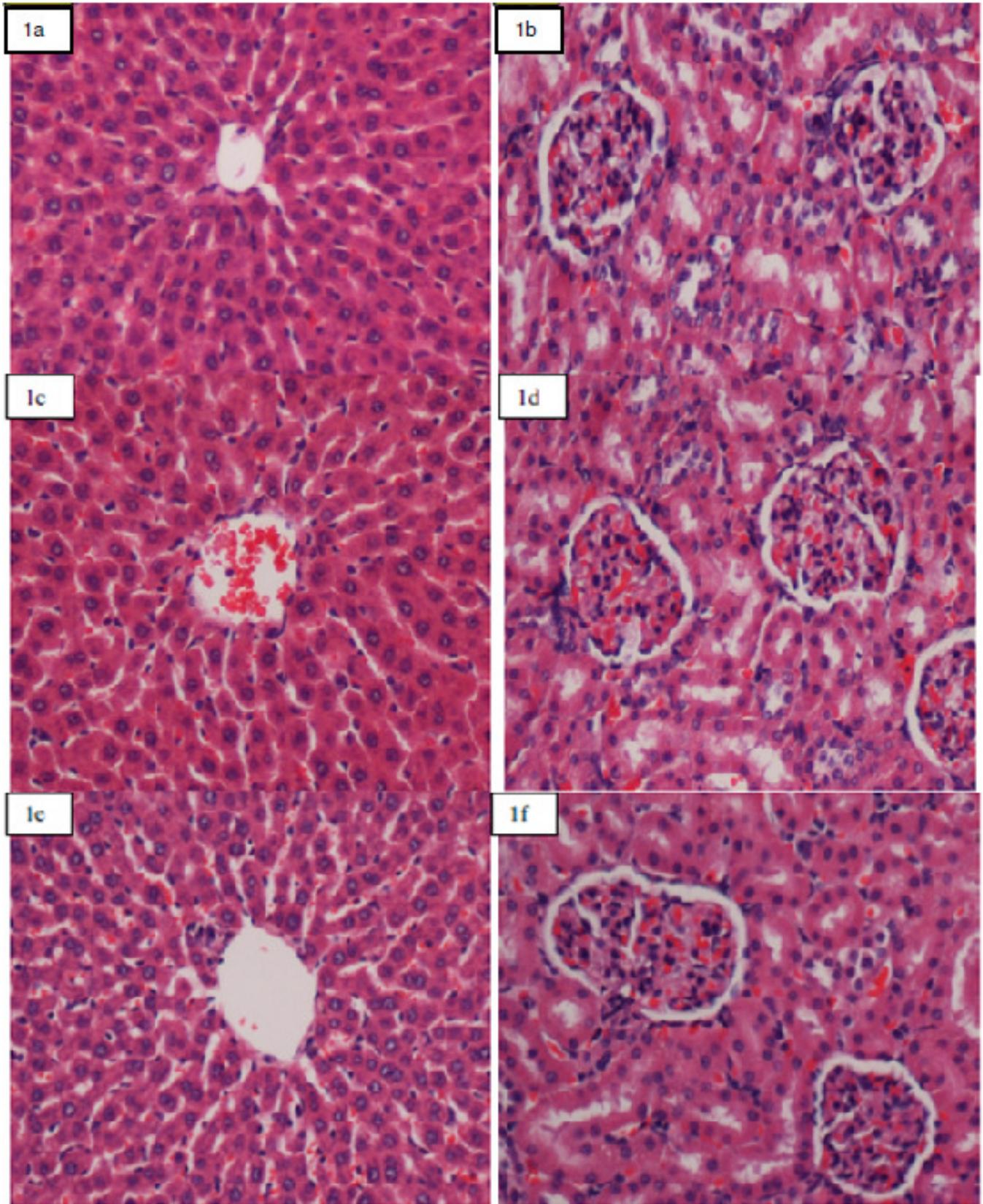


Figure 1. Histological sections of liver and kidney in acute toxicity test. (1a and 1b) Rats treated with 5 ml/kg vehicle (gum acacia in normal saline). (1c and 1d) Rats treated with 2 g/kg (5 ml/kg) *G. procumbens* extract. (1e and 1f) Rats treated with 5 g/kg (5 ml/kg) *G. procumbens* extract. There is no significant differences in structures of liver and kidney between treated and control groups.

Table 1. Acute toxicity test.

Renal function test of rats in acute toxicity study of <i>G. procumbens</i> extract							
Dose	Sodium (mmol/L)	Pottasium (mmol/L)	Chloride (mmol/L)	CO ₂ (mmol/L)	Anion gap (mmol/L)	Urea (mmol/L)	Creatinine (µmol/L)
Vehicle (Gum acacia 20 mg/ml)	138.25 ± 0.45	5.03 ± 0.19	104.03 ± 0.15	23.03 ± 0.82	18.16 ± 0.72	5.63 ± 0.41	50.18 ± 1.34
LD (2 g/kg)	137.65 ± 0.43	5.21 ± 0.16	102.61 ± 1.22	21.74 ± 0.17	18.07 ± 1.35	4.96 ± 0.43	48.97 ± 0.81
HD(5 g/kg)	137.21 ± 0.51	4.89 ± 0.15	102.67 ± 0.76	22.8 ± 0.86	17.73 ± 0.51	5.93 ± 0.39	48.60 ± 1.80

Liver function test of rats in acute toxicity study of <i>G. procumbens</i> extract									
Dose	Total protein (g/L)	Albumin (g/L)	Globulin (g/L)	TB (µmol/L)	CB (µmol/L)	AP (IU/L)	ALT (IU/L)	AST (IU/L)	GGT (IU/L)
Vehicle (Gum acacia 20 mg/ml)	71.37 ± 1.44	11.36 ± 0.53	59.91 ± 1.33	1.91 ± 0.17	0.89 ± 0.15	134.78 ± 9.57	53.05 ± 3.27	153.65 ± 9.35	4.91 ± 0.93
LD(2 g/kg)	71.47 ± 0.52	11.61 ± 0.34	59.61 ± 0.35	2.18 ± 0.16	1.00 ± 0.00	133.37 ± 8.63	51.90 ± 1.33	156.07 ± 3.56	5.00 ± 1.23
HD (5 g/kg)	71.81 ± 1.03	11.72 ± 0.16	60.01 ± 0.67	1.88 ± 0.21	1.00 ± 0.00	135.13 ± 6.52	52.27 ± 3.25	155.00 ± 5.35	5.32 ± 1.07

Values expressed as mean ± S.E.M. There are no significant differences between groups. Significant value at $p < 0.05$. TB: Total bilirubin; CB: conjugated bilirubin; AP: alkaline phosphatase; ALT: alanine aminotransferase; AST: aspartate aminotransferase; GGT: G-glutamyl Transferase.

Table 2. Time required for wound healing by *G. procumbens* leaf extract in rats.

Animal groups	No of animals	Type of dressings (twice daily) (0.2 ml/animal)	Healing time (days) (Mean ± S.E.M)
Group 1	8	Gum acacia in normal saline(20 mg/ml)	19.73 ± 0.92 ^a
Group 2	8	Intrasit gel (standard control)	13.75 ± 0.32 ^b
Group 3	8	<i>G. procumbens</i> extract 100 mg/ml	14.25 ± 0.57 ^b
Group 4	8	<i>G. procumbens</i> extract 200 mg/ml	13.92 ± 0.25 ^b

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

improves circulation to the wound site thus providing oxygen and nutrients essential for the healing process that include-re-epithelization (Suguna et al., 1996). Stimulate epithelial cell proliferation and angiogenesis are important for wound healing process (Cohen et al., 1992). Habibipour et al. (2003) showed that histological analysis of the treated wound contained a large amount of fibroblast proliferation, collagen

synthesis, and neovascularization, which resulted in an increased wound tensile strength and accelerated healing wound.

Phytochemical constituents present in *G. procumbens* extract may be responsible for wound-healing activity and studies with plant extracts have shown that constituent like 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 (Akowuah et al., 2002). 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,

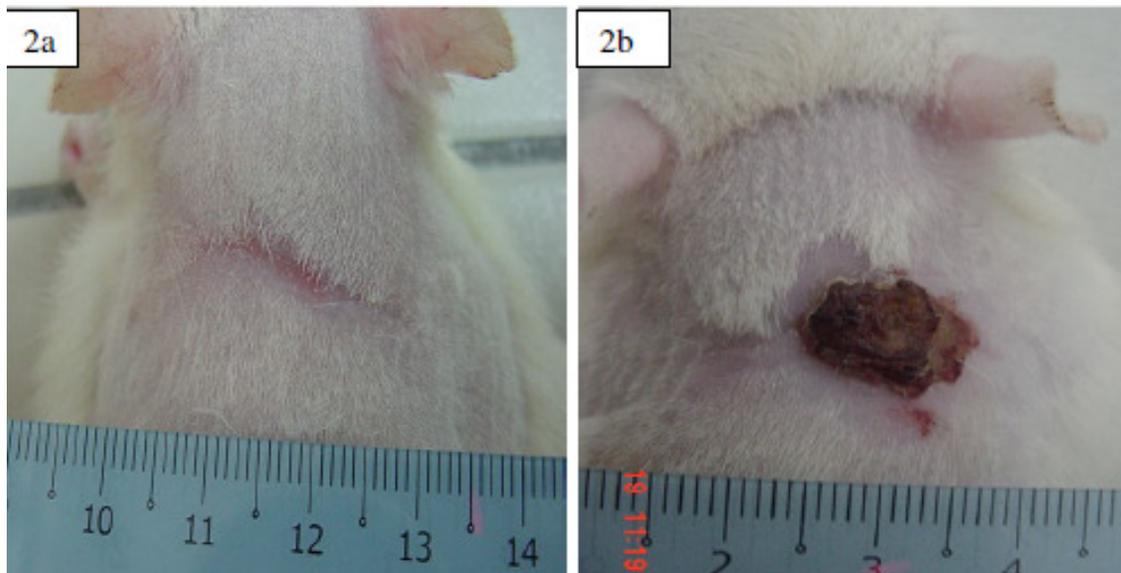


Figure 2. Macroscopically appearance of wound healing on day 14 post-surgery. (a) 200 mg/ml of *G. procumbens*-treated group showing complete wound healing. (b). Gum acacia-treated group showing incomplete wound healing.

Table 3. Effect of *G. procumbens* leaf extract on percentage (%) wound healing in experimental rats.

Animal groups	Vehicles (twice daily) (0.2 ml)	Percentage of wound healing (Mean \pm S.E.M) on day post surgery		
		5	10	15
Group 1 (N= 8)	Gum acacia in normal saline	34.33 \pm 0.62 ^a	72.17 \pm 0.72 ^a	87.00 \pm 0.41 ^a
Group 2 (N = 8)	Intrasite gel	68.67 \pm 1.44 ^b	94.54 \pm 1.17 ^b	100.00 \pm 0.00 ^b
Group 3 (N = 8)	<i>G. procumbens</i> 100 mg/ml	62.00 \pm 0.85 ^b	86.17 \pm 0.99 ^b	100.00 \pm 0.00 ^b
Group 4 (N = 8)	<i>G. procumbens</i> 200 mg/ml	65.17 \pm 0.67 ^b	88.33 \pm 0.83 ^b	100.00 \pm 0.00 ^b

All values were expressed as mean and \pm standard error mean. Mean in columns with different letters were significantly different ($P < 0.05$)

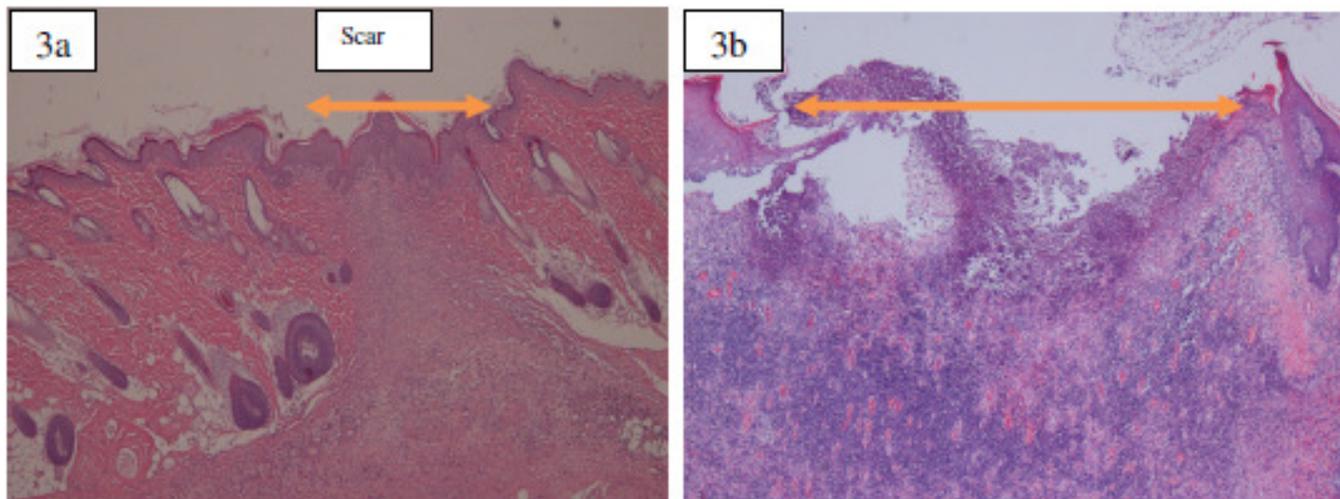


Figure 3. Histological section of healed wound on day 14 post-surgery. (a) 200 mg/kg of *G. procumbens*-treated group showing narrow scar region of wound closure (arrow). (b) Gum acacia-treated group showing incomplete wound healing enclosure (arrow) (H & E stain 4x).

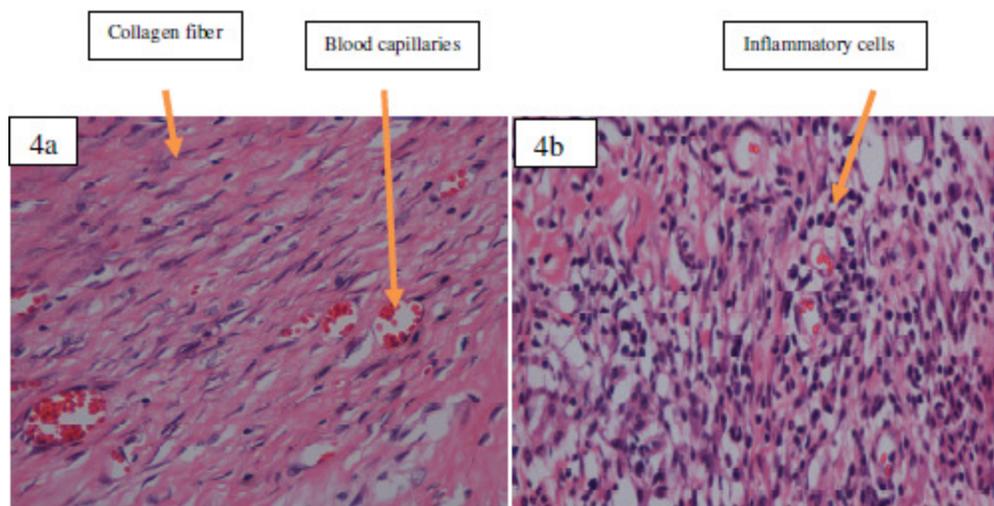


Figure 4. Histological section of healed wound on day 14 post-surgery (a) 200 mg/kg of *G. procumbens* extract-treated group. Granulation tissue contains comparatively more collagen, fibroblast and blood capillaries, and few inflammatory cells. (b) Gum acacia-treated group. Granulation tissue contains comparatively less collagen, fibroblast and blood capillaries, and more inflammatory cells (H & E stain 40x).

preventing the cell damage and by promoting the DNA synthesis (Buntrock et al., 1982).

Mechanisms of wound healing may be contributed to stimulate the production of antioxidants in wound site and provides a favorable environment for tissue healing (Habibipour et al., 2003). *G. procumbens* extract has shown antioxidant activity (Rosidah et al., 2008). It has 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 (Habibipour et al., 2003). 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 (Shukla et al., 1999; Getie et al., 2002). *G. procumbens* contain a wide array of free radical scavenging molecules and flavonoids were the major naturally occurring antioxidant components in this plant (Martin, 1996; Shukla et al., 1999). The higher the 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; Akowuah et al., 2009). *G. procumbens* have been shown to contain anti-inflammatory activity and it is speculated that the acceleration of wound healing potential exerted by this plant extract could be attributed to its anti-inflammatory activity (Iskander 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.

Liver and kidney of the treated rats showed no significant change as compared to the control group. Clinical biochemistry values were within the range of the control animals tested and similar to some of the control reference values published elsewhere (Witthawaskul et al., 2003). The highest dose of *G. procumbens* 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).

Conclusion

G. procumbens extract showed remarkable wound healing activity grossly, and histology of wound area on day 14 post-surgery showed less scar on the wound enclosure and granulation tissue contain markedly less inflammatory cells and more fibroblast and collagen fiber, and blood capillaries compared to gum acacia-treated rats. The acute toxicity profile of this plant could be considered favorable judging from the absence of adverse clinical manifestations. Further studies with purified constituents are needed to understand the complete mechanism of wound healing activity of *G. procumbens* extract.

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