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In vivo wound healing effect of water extract of *Stereospermum kunthianum Cham.* stem bark

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ABSTRACT

The objective of the present study was to evaluate the *in vivo* wound healing effect of water extract of *Stereospermum kunthianum cham.* (SK)-bark. The plant is mainly used by the local people of Mayo–Danay, Cameron as wound healing agent. The healing activity was evaluated in rats using excision and incision wound on normal dexamethasone (DX)-delayed wound healing. Control animals received normal saline topically while other groups received DX (intramuscular [i.m.]), water extract orally or DX with the plant extract. The extract treated wounds were found to epithelize faster than those of controls DX treated rats. A significant increase in skin tensile strength was observed in earlier than in later groups. A histological examination of incision wounds of the extract-treated group showed dense collagen cross-linking and numerous blood vessels. The present study concludes that the stem bark of SK possesses excellent wound healing potential that may substantiate its use in the traditional medicine.

KEY WORDS: Epithelialization, *Stereospermum kunthianum*, surgical wounds, tensile strength

INTRODUCTION

The epidermis, the dermis of the skin normally exists in steady state equilibrium to form a protective barrier of internal organs against entry of infectious or other noxious agents from the environment. Any damage to the skin layers initiates a complex biochemical response that leads to tissue repair or wound healing. This process is characterized by dynamic, interactive events described in three phases: Inflammation, proliferation remodeling (Singer and Clark, 1999). As a natural response, wound healing is a result in any loss of continuity in the skin or any body tissue, following trauma, infection, or pathological process (Martin, 2013). Due to simplicity in the measurement of wound healing responses, the excision and incision skin-wound healing models in rodents are by far the most convenient reliable methods of study for potential new therapeutic agents. Hence, a number of plant extracts from folk medicine have been used to demonstrate some promise using these experimental models. Medicinal plants have also generated much interest in recent studies for treating skin ailments, as they are affordable, purportedly safe due to less hypersensitivity reactions (Raina *et al*., 2008). *Stereospermum kunthianum* (Cham, Srine Petit), belonging to family Bignoniaceae (Juss. nom. Cons.), also referred to as pink jacara in English, is a deciduous tree found in dry tropical areas. In the Far North Region of Cameroon, fresh stem bark, mixed with latex is used locally as a cataplasm to treat wounds by the first intention. Among its traditional medical uses, it was reported that a bark-preparation is applied in some African regions on wounds, ulcers in the treatment of various inflammatory conditions (Burkill, 2009). Ching and Omogbai (2010) did not report overt acute or subacute marked adverse effects following oral administration of the extract to rats. The efficacy of the water extract of SK in human complement system fixation *in vitro* has previously been reported (Drissa *et al*., 2002). The antiplasmodial activities of naphthoquinones and one anthraquinone from the lipophilic root bark extract were also reported (Onegi *et al*., 2002). Anti-diarrheal analgesic activities of stem bark (Burkill, 2009) were also investigated. No research has ever done however to validate the traditional use of SK for treating wound.

The present study was therefore designed to evaluate the wound healing effect of the water extract of SK stem bark in normal dexamethasone (DX) treated rats.

MATERIALS AND METHODS

Plant Material Extraction

The stem bark of SK was collected from Yagoua, Far North Region, 120 km by road from Maroua city, Cameroon, altitude 328 m, latitude 10°00′, longitude 15°08′ authenticated at the Herbarium of Wildlife School of Garoua (No 6298/HEFG) where a voucher was deposited for future reference. The dried powder of the plant material (250 g) was infused in 3 L distilled water for 24 h. The aqueous extract was then filtered and concentrated at 70°C. Qualitative tests were performed for various functional groups in the extract like Alkaloids (Dragendorff's reagent test); amino acids (xanthoproteic test); flavonoids (alkaline reagent test); glycosides (Baljet test); proteins (biuret test); reduced sugars (Fehling test); Saponins (foam test) tannins ($FeCl_3$ test) (Ahuja *et al*., 2011; Tiwari *et al*., 2011; Zohra *et al*., 2012). Flavonoids were also quantified using $AlCl_3$ method rutin as standard, as described by (Makkar *et al*., 1997).

Animal Husbandry and Ethical Considerations

All animal procedures were conducted with strict adherence to the NIH Guide for the care and use of Laboratory Animals (NIH Publication #85-23 Rev. 1985). Male albino-Wistar rats weighing 150-180 g fed on standard chow pellet diet allowed water *ad-libitum* were used. Animals were caged under laboratory environment with 12 h darklight cycles.

Drugs

Drugs, as well as surgical procedures, were conducted as previously described (Tsala *et al*., 2014). Accordingly, ketamine (Rotexmedica-Trittau-Germany), diazepam (Renaudin-France), dexamethasome (Guangdong Medecine and Health Products I/E corp.) nylon surgical treat size 1 (Agary Pharmaceutical Ltd.) were purchased from a local pharmacy store). All other chemicals were of laboratory grade freshly prepared.

Determination of *In Vivo* Cicatrizant Activity

Grouping of animals

Animals were divided into 4 groups consisting 5 rats each as follows: Group 1: Water (2 mL); Group 2: DX (0.34 mg/kg i.m. on the 1st day, thereafter 0.17 mg/kg on alternate days); Group 3: SK (50 mg topically); Group 4: DX (0.34 mg/kg i.m. on the 1st day, thereafter 0.17 mg/kg on alternate days) + SK (50 mg topically).

Excisional wound model

Animals were anaesthetized by i.m. injection of ketamine/diazepam (ketamine 25 mg/kg diazepam 10 mg/kg). An area (4 cm^2) was marked using a frame marker pen. The required area of the dorsal fur of the animals was shaved with an electric clipper area sterilization achieved by spraying with 70% alcohol in water. A full thickness skin (4 cm^2) was excised from the predetermined area by removing the epidermis and dermis layers until the subcutaneous fat (avoiding *Panniculus carnosus*). All treatments in the four groups were given every 2 days until the wound was completely healed. Special care was taken to avoid variation in the dose given.

Animals were monitored on daily basis. Wound diameter was recorded in vertical, horizontal planes as well as epithelialization time that indicate the formation of new epithelial tissue to cover the wound. The lesions on each rat were also rated using the following parameters (1) the presence of exudates, (2) erythema, (3) swelling, (4) ulceration, (5) crust formation (Masoko *et al*., 2010). The degree of wound healing was calculated using the formula:

$$\text{Degree of wound healing (\%)} = \frac{\text{WA on zero day (mm}^2\text{)} - \text{WA on corresponding day (mm}^2\text{)}}{\text{WA on zero day (mm}^2\text{)}} \times 100$$

Where WA is wound area in mm^2

A number of days for complete epithelialization were noted. Wounds were considered closed (completely healed) if moist granulation tissue was no longer apparent and the wound was covered with new epithelium.

Incisional wound model

A 5 cm incision was made perpendicular to the axis of symmetry of the animal, and the two borders of the wound were stitched together at its center with interrupted sutures at a distance of 1 cm. Treatments started immediately. The experimental agent being tested was applied to the wound every 48 h. On the 10th day of post-wounding, animals were sacrificed by chloroform overdose and wound areas from each animal were dissected carefully. Stripes of equal size (width) from one side were cut, and a line was drawn on either side, 3 mm away from the wound, for breaking strength determination. One piece of tissue was fixed in 10% formalin for histopathological examination; the other was used to quantify the wound breaking strength (WBS).

Determination of Wound Tensile Strength

Both ends of each skin stripe were fixed with a pair of steel clip; one clip was allowed hanging on another clip with a freely suspended polyethylene bag through a string, run over the pulley. It was then gradually filled with water from a polyethylene reservoir until the wound stripe was broken at the site of wound. The amount of water required to break the wound was noted and expressed as the tensile strength of wound in grams (Kumar *et al.*, 2010). The tensile strength was calculated according to the following equation:

$$\text{Tensile strength} = \frac{\text{Total breaking load (g)}}{\text{Cross sectional area (mm}^2\text{)}}$$

For preliminary screening, an activity greater than 25% was considered a positive wound healing activity. The percentage of activity was calculated according to the following formula:

$$\text{Activity (\%)} = \frac{WBS_c - WBS_t}{WBS_c} \times 100$$

Where,
WBS_t = Average of the force necessary to open the wound of a treated rat
WBS_c = Average of the force necessary to open the wound of an untreated rat (control).

Histopathological Studies

Skin specimens were immediately fixed in 10% (v/v) neutral formalin until the tissues hardened. Each specimen was embedded in a paraffin block thin sections (5 μm) were prepared and stained with hematoxylin and eosin (for general morphological observations). Slides were examined qualitatively under a light microscope, for collagen formation, fibroblast proliferation, angiogenesis, and epithelialization.

Statistical Analysis

All data were expressed as mean ± standard deviation. Statistical analysis was performed with analysis of variance followed by Dunett *post-hoc* test, using SPSS 16.0 software. $P < 0.05$ was considered significant.

RESULTS

Phytochemistry

The removal of water from the extract gave 23.76 g of a dark brown paste with a yield of 9.50%. Amino acids, alkaloids, flavonoids, glycosides, proteins, reduced sugars, catechic tannins were identified while saponins gallate tannins were not detected in the extract. The extract was tested to contain 515 mg rutin equivalent/mg extract.

Epithalializationrate and Time

As shown in Figure 1, wound contraction was the first recorded in the control group, 6 days post-wounding and the epithelialization lasted up to about 22 days in this group. DX markedly delayed contraction in rats when compared to the other groups of rats. DX also significantly prolonged the epithelialization time to 31 days as compared with the control group. When given alone, the extract stimulated wound contraction in rats from day 2 post-wounding. The same extract also significantly reduced the epithelialization period to 12-day, to about 18 days in DX treated rats. The epithelialization time of all groups is also reported in Table 1 where the wound healing effect of the extract is clearly demonstrated.

Effect of the Extract on Some Parameters of Inflammation

The effect of the water extract of the stem bark of SK on the exudate and erythema of rats wounds is shown in Table 2. The exudate was observed in the control group up to day-12 of post-wounding. DX caused a prolonged

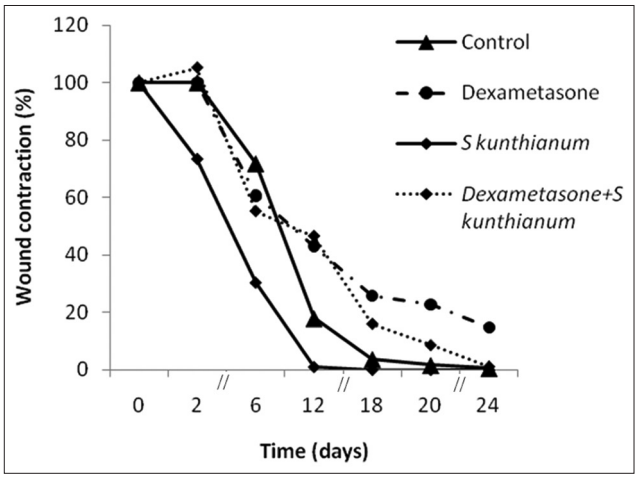

Figure 1: Effect of the water extract of the stem bark of *Stereosperm**u**m kunthianum* on wound contraction in rats

Table 1: Effect of the water extract of the stem bark of SK on the epithelialization time of rats

Groups	Control	DX	SK	DX+SK
Epithelialization time (days)	21.60±1.26	31.20±1.26*	12±0.63*	17.6±0.40*

Each value represents mean±SEM, n=5. *P<0.05: Difference significant when compared to control. *SK: Stereospermum kunthianum,* DX: Dexamethasone

Table 2: Effect of the water extract of the stem bark of *Stereospermum kunthianum* on the exudate and erythema of rats wounds

Group	J0	J2	J4	J6	J8	J10	J12	J14	J16	J18
Exsudate										
Control	0.00±0.00	0.5±0.25	2±0.36	1.75±0.22	1.5±0.44	0.5±0.25	0.25±0.2	00±0.00	00±0.00	00±0.00
DX	00±0.00	00±0.00	1.25±0.22	0.00±0.00*	0.75±0.42	00±0.00	1±0.36	0.5±0.25	1.25±0.22	0.5±0.25
SK	00±0.00	1±0.00	1.75±0.25	0.75±0.25	0.25±0.25	00±0.00	00±0.00	00±0.00	00±0.00*	00±0.00
SK+DX	00±0.00	00±0.00	0.75±0.25	2.5±0.28	1±0.00	0.5±0.28	1.25±0.47	0.5±0.28	0.25±0.25	00±0.00
Erythema										
Control	00±0.00	1±0.00	1.5±0.25	1.25±0.22	2±0.63	0.25±0.22	00±0.00	00±0.00	00±0.00	00±0.00
DX	00±0.00	1±0.00	1.25±0.22	1±0.36	00±0.00	00±0.00	0.25±0.22	00±0.00	0.5±0.25	0.5±0.25
SK	00±0.00	1.25±0.25	1±0.00	0.25±0.25	0.25±0.25	00±0.00	00±0.00	00±0.00	00±0.00	00±0.00
DX+SK	00±0.00	1.25±0.25	2.5±0.28	0.75±0.25	0.00±0.00	0.75±0.25	1±0.40	0.00±0.00	0.00±0.00	00±0.00

Each value represents mean SEM, n=5. *P<0.05: Difference significant when compared to control. DX: Dexamethasone; *SK: Stereospermum kunthianum,* SEM: Standard error of the mean

exudation that lasted for 18 days. The plant extract, when administered alone reduced it to 8 days, and to 16 days when given in with DX. The erythema was present in control rats' wound until the 10th day post-wounding. Extract treated rats recovered from erythema at day-10 post-wounding. All the rats treated with DX recovered from erythema after the 6th day.

Effect on Tensile Strength

In the linear incision wound animals, there was a significant increase in the tensile strength of the scares when treated topically with the aqueous extract of SK. The tensile strength required to disrupt the scare was found to be 866.63 ± 18.02 g/cm^2, versus 699.30 ± 61.82 for vehicle control. DX treated animals needed 544.18 ± 24.69 to tear out the wound (Figure 2).

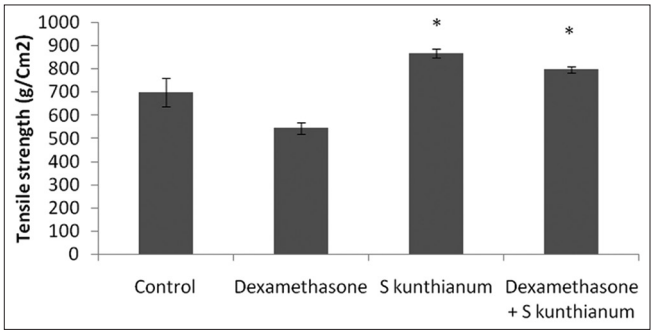

Figure 2: Effect of the water extract of the stem bark of *Stereospermum kunthianum* on the tensile strength of incision wound in rats. Each value represents mean ± standard error of the mean, n = 5. *P < 0.05: Difference significant when compared to control

Histomorphological Analysis of Excisional Incisional Wounds

Dorsal wound area photographed from days 6 to 14 after application of the various treatments on excisional wound model (Figure 3) revealed elevated contraction rate in *S. kunthianum* extract treated rats. Details of the pictures on day 6 showed no crust covered the control wound, whereas DX treated wounds alone or in combination with the plant extract were slightly covered by a crust. At the same time point, the crust over SK extract treated wounds was more developed than with the other treatments. Nevertheless, contraction was already important in this last group. On day 14, only SK extract-treated wounds were completely cicatrized.

Histological slides of incisional wounds displayed in Figure 4 showed that the granular cell layer of negative control rats was thick (sign of hydration). In addition, granulation tissue of DX treated group contains less fibroblast, dilated blood capillary, than in control group rats. In addition, the plant extracts treated group exhibited improvement in the skin structure with a thin well-formed epidermis, a well-organized dermis, with more collagen fibroblasts, no inflammatory feature and numerous blood vessels. In DX + SK treated group, these positive effects were just less prominent when compared to extract treated group alone.

DISCUSSION

The stem bark of SK has been reported to possess antiulcer (Burkill, 2009), analgesic (Ching *et al*., 2009) and complement fixative properties (Onegi *et al*., 2002). As the stem bark of the plant is also used by the people of the northern regions of Cameroon for treating wounds, the present study was designed to evaluate its wound healing potential using the excisional and incisional wound models. Wound healing process constitutes three interactive phases of inflammation, proliferation and remodeling (Singer and Clark, 1999;Tsala *et al*., 2013). Re-epithelialization during the 2nd phase of healing is generally achieved by migration, proliferation, differentiation of epidermal keratinocytes. Simultaneous to the tissue remodeling phase, contraction begins a few days after injury primarily via myofibroblasts (Shai and Maibach, 2005). The water extract of SK stem bark interfered with the primary phases of wound healing by improving swelling, rate of contraction and epithelialization time. Healing features observed in this

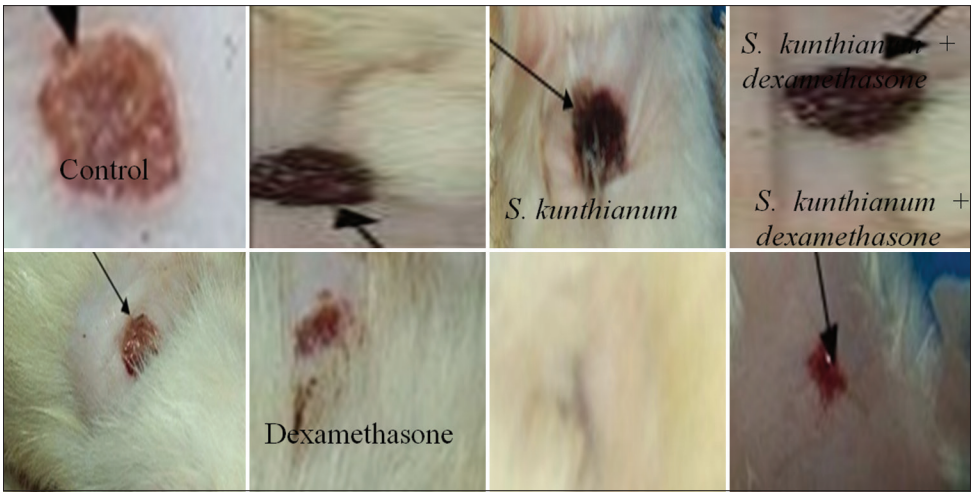

Figure 3: Representation of contraction rate of albino rats dorsal wound area photographed on days 6 (up) 14 (down) after application of the various treatments on excisional wound modelgroup

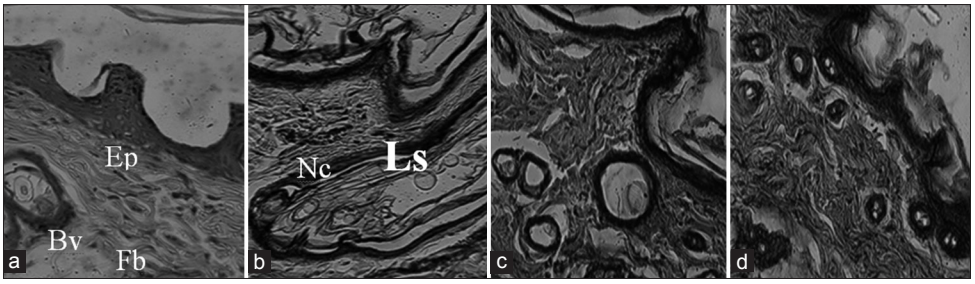

Figure 4: Microscopic view of the section of skin tissue after 10 days of treatment. (a) Negative control group, (b) dexamethasone (DX) treated group, (c) *S. kunthianum* (SK) extract treated group (d) SK extract + DX treated group (H and E stain, ×400 magnification). Bv: Blood vessel; Ep: Epithelial layer; Fb: Fibroblasts; Ls: Lesion; Necrosis

study have been already exhibited in various studies. For example, therapeutic agents that modulate wound repair can be evaluated based on their influence on the development of wound strength (Garmelli and He, 2003). Furthermore, the increasing amount of stable collagen and the alignment of its fibers gradually increase the strength of the healing wound (Shai and Maibach, 2005). The rats treated with the water extract of SK developed tensile strength that suggested a good amount of mature collagen deposition. Histopathological examination further provided additional evidence on the wound healing potential of SK stem bark. Fixation of tissues with formalin followed by hematoxillyn eosin staining is a common method that displays a broad range of cytoplasmic, nuclear, extracellular matrix features (Garmelli and He, 2003). Such data provide additional insights into the status of the healing process, particularly in studies of impaired wound healing (Garmelli and He, 2003). In agreement with previous studies on DX at a higher dose, poor wound healing associated with less epithelialization; fibroblast density were observed in the same rats (Al-Refu, 2011, Murti and Kumar, 2011). In addition, vascularity and the inflammatory pattern recorded for the DX group were higher than that of the control group. Tissue specimen presented here unequivocally showed that the water extract of SK stem bark inhibited vascularity as observed in the DX treated group. The granulation tissue of animal skins treated with the extract also exhibited numerous blood vessels. Many fibroblasts arranged longitudinally to the incision and significant cutaneous tensile strength were also observed when compared with the DX treated rats, suggesting an important collagen cross-linkage (Garmelli and He, 2003; Vidinsk *et al.*, 2006). The extract was effective both on the cutaneous wound healing action of normal DX treated rats; the epithelialization time was not too different between the two groups of rats. These observations were confirmed by the tensile strength measurement. Although the possible mechanism remains to be elucidated, similar wound healing effect were reported on many other medicinal plants (Durmus *et al.*, 2003). Pharmacological activity reported in this study probably comes from the synergistic effect of compounds present in the extract. The observed would healing effects may be attributable to bioactive constituents such as alkaloids, flavonoids, glycosides, proteins, reduced sugar, and catechic tannins. Wound healing potential of these

functional groups has been previously reviewed (Tsala *et al*., 2013). In another hand, saponins gallic tannins were not detected in the stem bark under our experimental conditions.

CONCLUSION

In conclusion, our study shows that an aqueous extract of SK stem bark accelerates cutaneous wound healing in normal DX treated rats. Our findings also indicated that the extract's effect is based on the development of new capillaries on collagen cross-linkage. Although the therapeutic potential of SK was effectively demonstrated in the present study, the precise underlying molecular mechanisms need to be proven through further research.

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