EFFECT OF ETHANOLIC EXTRACT OF CYANOTIS CRISTATA LEAVES APPLIED TOPICALLY ON WOUND HEALING IN WISTAR RATS

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Received: 15 June 2017, Revised and Accepted: 12 August 2017

ABSTRACT

Objective: The objective of this study was to assess the effect of ethanolic extract of Cyanotis cristata leaves when applied topically on healing of wound in Wistar rats.

Methods: C. cristata leaves were evaluated for its effect on healing of wound in three models, namely, incision, excision, and burn wound. Each model included five groups, each consisting of 6 rats. The five groups were as follows: Group I rats (control and ointment base), Group II rats (standard and silver sulfadiazine), and Group III, Group IV, Group V (treated with C. cristata extract ointment of 0.5%, 1%, and 2%, respectively). Variables evaluated included breaking strength in incision wound while it was contraction rate and epithelialization in excision and burn wound. One-way analysis of variance and Tukey’s post hoc test was used to analyze data.

Result: In incision wound, breaking strength in test group was significantly increased (p<0.001) as compared to control. In excision and burn wound of test group, time to epithelialization and contraction rate was significantly decreased (p<0.001). The granulation tissue from excision and burn wound showed increased collagen and less inflammatory cells in test groups in comparison to the control.

Conclusion: The ethanolic extract of leaves of C. cristata, when applied topically, enhanced wound strength and accelerated healing of incision, excision, and burn wounds in rats.

Keywords: Incision wound, Excision, Burn wound, Breaking strength, Contraction, Epithelialization.

INTRODUCTION

Wound healing involves various processes, cells, and mediators that help to restore structure and function of the damaged tissue [1]. The processes of healing such as inflammation, wound contraction, epithelialization, and remodeling occur simultaneously, resulting in closure of the wound [2]. Any deviation from normal process adversely affects wound healing [3].

Various factors can result in delayed or non-healing of the wound [3]. Wound impair the quality of life [4]. The management of burn wound is a challenge since it has high morbidity and mortality. Delayed and non-healing burn wound is associated with high risk of mortality [5-7]. Therefore, for improving the management of wound including burn wound, acceleration of wound closure and healing is essential.

Various agents have been used to expedite the healing process which is either proceeding normally or delayed [8-14]. Traditional medicine involves the use of medicinal herbs as they are easily accessible and cheap. The plant Cyanotis cristata belongs to the family Commelinaceae. A member of this family, Commelina benghalensis possess wound healing activity [15]. The root paste of C. cristata is used for relief of swelling and snake bite [16]. C. cristata leaves possess antioxidant effect [17]. Physicochemical analysis of the plant has shown the presence of flavonoids and tannins [18]. There is abundant literature showing that flavonoids and tannins have wound healing property [19-21].

A thorough literature search revealed no documentation of wound healing activity of C. cristata. Therefore, we evaluated the effect of topical administration of ethanolic extract of leaves of C. cristata on wound healing in Wistar rats.

MATERIALS AND METHODS

Materials

Ketamine (Neon Laboratories Ltd., Thane), xylazine (Indian Immunological Limited, Hyderabad), paraffin wax (Meta Wares India Private Limited, Delhi), silver sulfadiazine (JK Biochem Health Care Pvt. Limited, Uttar Pradesh), and ethyl alcohol (Hi-tech Chemicals, Mumbai) were used in the study.

Animals

Healthy, adult Wistar rats, either gender, reared in the Central Animal Research Facility, Manipal, with weight ranging from 180 to 200 g were used. Animals were kept in separate cages which had sterile husk bedding under standard environment conditions [22]. Food and water were administered freely. The research work was undertaken following approval from the Institutional Animal Ethics Committee, Manipal (letter number IAEC/KMC/82/2014).

Preparation of extract

The plant, C. cristata, was obtained locally and validated by a Professor of botany, Udupi. The leaves were kept in a shaded area to allow for drying. Dried leaves were powdered. Soxhlet extraction of 200 g powder with 95% ethanol at 60-80°C for 30–40 h was done [23]. This was followed by distillation, and later, heating on a water bath below 50°C. The amount of extract obtained was approximately 10% (w/w).
**Preparation of formulation**
White soft paraffin was triturated with ethanolic extract of *C. cristata* leaves to obtain a different concentration of ointment on a w/w basis, namely, 0.5%, 1%, and 2% ointment [24]. The prepared herbal ointment was put in an airtight, appropriately labeled, plastic container and was maintained at room temperature.

**Study design**
Three models of the wound were used - incision, excision, and burn wound. A total of 90 animals were used. Each model had 30 rats which were allocated randomly into 5 groups. Each group had 6 rats. Drugs were applied topically in each model as follows:

- **Control group** - paraffin wax, standard - silver sulfadiazine, and test Groups III (test 1), IV (test 2), and V (test 3) received - 0.5%, 1%, and 2% of the extract, respectively.

**Incision wound model**
Under ketamine anesthesia (80 mg/kg, intraperitoneally [i.p.]), the back of each rat was shaved. Two full thickness, straight incision 6 cm long, on either side of midline were made. They were sutured intermittently, 1 cm apart [9]. Removal of sutures was on 7th day. Drugs were applied daily on the wound up to nine days post-wounding. On the 10th day, breaking strength was assessed using water flow technique of Lee [11].

**Excision wound model**
A 500 mm² round area of full thickness skin was excised from an area marked out by a seal having a diameter of 2.5 cm on the dorsum between the scapulas following administration of ketamine i.p. [25]. Drugs were applied daily up to the 21st post-operative day or until total healing whichever was earlier. The wound was traced on alternate day from day 0 to 21st post-operative day, or till, the wound was healed and fixed on a graph paper. The rate of contraction was expressed as a percentage of the initial area of wound [26]. The time (days) taken for eschar to fall was epithelialization period [26].

**Burn wound model**
Under i.p. ketamine xylazine anesthesia (0.1 ml/100 g; 91 mg/kg ketamine and 9.1 mg/kg xylazine), molten wax (80°C) was poured through a metal cylinder having a circular opening of 500 mm². After 8 minutes, the cylinder and wax were removed to produce a burn wound of partial thickness [21]. Drugs were administered and parameters measured as described above for excision wound [27]. In both excision and burn wound, granulation tissue was obtained under ketamine anesthesia (80 mg/kg, i.p.) using a punch biopsy needle following completion of healing and subjected to histopathological analysis [28].

**Statistics**
Data were expressed as mean ± standard error of mean. One-way analysis of variance followed by Tukey's post hoc test was used to analyze data. *P < 0.05* was considered as statistically significant.

**RESULTS**

**Incision wound model**
The wound of rats in control group had a breaking strength of 322.59±4.44 g. In all test groups, mean breaking strength was significantly (*p<0.001*) more than control and standard groups. It was significantly (*p<0.001*) better in rats who received 2% preparation as compared to rats treated with 0.5% and 1% preparation (Table 1).

**Excision wound model**
**Period of epithelialization**
The mean period of epithelialization in control group was 18.83±0.60 days. It was significantly lower (*p<0.001*) in all test groups relative to control and standard (Table 2).

**Table 1: Effect of ethanolic extract of leaves of *C. cristata* on the breaking strength of a ten day old incision wound in rats**

| Group (n=6); drug | Mean±SEM (Breaking strength (g)) |
|------------------|---------------------------------|
| Control; paraffin wax | 322.59±4.44 |
| Standard; silver sulfadiazine | 323.06±5.45 |
| Test 1; *C. cristata* leaves (0.5% ointment) | 388.18±3.84*** |
| Test 2; *C. cristata* leaves (1% ointment) | 421.58±4.39** ** |
| Test 3; *C. cristata* leaves (2% ointment) | 457.53±4.19*** |

Values are mean±SEM. Statistics - one-way ANOVA followed by Tukey's post hoc test. *p<0.001 versus control, **p<0.001 versus standard, ***p<0.001 versus test 1, ¶p<0.001 versus test 2. n=Number of rats in each group. *C. cristata*: Cyanotis cristata. SEM: Standard error of the mean

**Table 2: Effect of ethanolic extract of leaves of *C. cristata* on the period of epithelialization in an excision wound in rats**

| Group (n=6); drug | Mean±SEM (Period of epithelialization (days)) |
|------------------|-----------------------------------------------|
| Control; paraffin wax | 18.83±0.60 |
| Standard; silver sulfadiazine | 18.50±0.76 |
| Test 1; *C. cristata* leaves (0.5% ointment) | 12.33±0.42*** |
| Test 2; *C. cristata* leaves (1% ointment) | 12.00±0.44*** |
| Test 3; *C. cristata* leaves (2% ointment) | 11.00±0.36*** |

Values are mean±SEM. Statistics - one-way ANOVA followed by Tukey's post hoc test. *p<0.001 versus control, **p<0.001 versus standard, ***p<0.001 versus test 1, ¶,§p<0.001 versus test 2. n=Number of rats in each group. *C. cristata*: Cyanotis cristata. SEM: Standard error of the mean

**Rate of wound contraction**
Wound contraction rate (%) on the 4th and 8th day was significantly (p<0.001) greater in rats who received 2% preparation of extract than other groups (Table 3). It was complete on day 12 in rats treated with 2% extract and day 16 in rats treated with 0.5% and 1% extract (Table 3).

**Burn wound model**
**Period of epithelialization**
Epithelialization occurred in a significantly (p<0.001) shorter period of time in all test groups as compared to control and standard groups (Table 4).

**Rate of wound contraction**
On the day 4, 8, 12, and 16, contraction of wound was significantly more (p<0.05) in rats treated with test drug as compared to the rats in control group. Furthermore, on these days, rats treated with 2% preparation showed faster healing than the rats treated with 0.5% and 1% preparation (Table 5).

**Histopathological evaluation**
Granulation tissue obtained from excision and burn wound was examined under light microscope.

**Excision wound (Fig. 1)**
Control group showed dermal scar tissue effacing the adnexal structures (Fig. 1a). In standard group treated with silver sulfadiazine, there was a focally eroded epidermis overlying the dermal stroma showing a fibrotic scar tissue (Fig. 1b). Test group treated with 0.5% extract of *C. cristata* showed focally atrophied epidermis overlying dermis with early scar tissue formation and minimal inflammatory cells (Fig. 1c). Wounds of rats treated with 1% extract showed regular epidermis overlying granulation tissue with prominent capillary proliferation (Fig. 1d). In the section of wound in rats treated with 2% extract, epidermis ranged from regular to focally eroded with dermal granulation tissue (Fig. 1e).
Table 3: Effect of ethanolic extract of C. cristata leaves on contraction rate of an excision wound in rats

| Group (n=6); drug                        | Wound contraction rate (%) | Mean±SEM |
|------------------------------------------|----------------------------|----------|
| Control; paraffin wax                    |                            | 23.00±0.25 | 48.66±0.33 | 86.16±0.30 | 92.16±0.40 |
| Standard; silver sulfadiazine            |                            | 23.66±0.42 | 50.00±0.57 | 87.33±0.42 | 94.16±0.40* |
| Test 1; C. cristata leaves (0.5% ointment) |                            | 44.16±0.47** | 68.16±0.30*** | 87.33±0.49 | 100*** |
| Test 2; C. cristata leaves (1% ointment)  |                            | 48.50±0.22*** | 73.16±0.30*** | 92.33±0.42*** | 100*** |
| Test 3; C. cristata leaves (2% ointment)  |                            | 54.33±0.42** | 74.66±0.49** | 100** | - |

Values are mean±SEM. Statistics - one-way ANOVA followed by Tukey’s post hoc test. p<0.001 versus control, P<0.05 versus standard, ¶p<0.001 versus test 1, ¶p<0.001 versus test 2, n=Number of rats in each group, C. cristata: Cyanotis cristata, SEM: Standard error of the mean

Table 4: Effect of ethanolic extract of C. cristata leaves on epithelialization of burn wound in rats

| Group (n=6); drug                        | Period of epithelialization (days) | Mean±SEM |
|------------------------------------------|-----------------------------------|----------|
| Control; paraffin wax                    | 17.66±0.95                        |          |
| Standard; silver sulfadiazine            | 17.16±0.60                        |          |
| Test 1; C. cristata leaves (0.5% ointment) | 14.83±0.79**                      |          |
| Test 2; C. cristata leaves (1% ointment)  | 13.16±0.70**                      |          |
| Test 3; C. cristata leaves (2% ointment)  | 12.66±0.55**                      |          |

Values are mean±SEM. Statistics - one-way ANOVA followed by Tukey’s post hoc test. *p<0.001 versus control, **p<0.001 versus standard, ¶p<0.001 versus test 1, n=Number of rats in each group, C. cristata: Cyanotis cristata, SEM: Standard error of the mean

DISCUSSION

The effect of C. cristata on healing of incision, excision, and burn wounds in rats was studied. The mean breaking strength of incision wound in rats of extract treated groups was significantly higher than other groups. The final wound strength depends on quantity and maturation of collagen. Thus, it is a result of fibroplasia and remodeling of collagen [29]. Reactive oxygen species (ROS) generated following wounding can adversely affect healing by various mechanisms including upregulation of matrix degrading enzymes [30-32]. Antioxidant effect of plant extract has been shown to promote healing of wounds [33]. The extracts of leaves of C. cristata have rich antioxidant property [17]. In a previous study, phytochemistry of C. cristata revealed the presence of flavonoids and tannins [18]. Flavonoids increase collagen content of wound through multiple mechanisms. Injury results in the generation of ROS that impairs the strength of collagen making it susceptible to lysis. Flavonoids, being antioxidants, prevent this and increase the viability of collagen [34]. Further, Vitamin C, that is required for collagen formation and maturation, is up-regulated in flavonoid-treated wound [35]. Flavonoids have also been shown to increase the viability and proliferation of fibroblasts [36]. Tannins have antioxidant effect and may act in similar fashion to promote collagen deposition [37]. They also increase angiogenesis by increasing vascular endothelial growth factor formation, thus maintaining adequate supply of oxygen necessary for collagen stability [38].

Epithelialization is the process, wherein the stratified epithelium is restored following injury [39]. C. cristata leaves decreased the period of epithelialization of excision wound in rats. This process involves migration and proliferation of epithelial cells [39]. Flavonoids induce various signalling pathways involved in cell migration - extracellular signal-regulated kinase, protein kinase B, and NADPH oxidase. This results in increased level of E-cadherin, a junctional protein, which...
plays an important role in collective migration of keratinocytes. Further, it also induces several metalloproteinas, which degrades extracellular matrix, thereby facilitating migration [40]. Furthermore, both tannins and flavonoids increase collagen deposition [34-37] that provides scaffolding for the process of epithelialization [41].

Wound contraction, manifesting as shrinkage of wound area, starts around 5th day post wounding [42]. C. cristata leaves significantly accelerated wound healing in excision wound as shown by greater percentage of wound contraction in our study. This enhanced wound contraction may be either due to enhanced activity of fibroblasts which provide the force responsible for wound contraction, or it may be due to increased deposition of collagen. The force generated by fibroblasts, thus compacting the formed collagen [43].

Flavonoids and tannins are considered to be groups rather than individual entities [44,45]. Thus, the individual member(s) responsible for the enhanced wound healing effect needs to be determined. Infection may prolong the healing by prolonging inflammation. Plants with antibacterial action have been shown to promote wound healing [46]. Flavonoids and tannins can prevent bacterial infection of wound. This is another mechanism by which they can enhance healing [47]. However, no group in our study showed any sign of infection.

In burn wound model, C. cristata extract-treated group enhanced epithelialization as well as rate of wound contraction. Burn wound is usually associated with ischemia followed by restoration of blood flow. This results in the generation of ROS that can delay healing [48]. C. cristata leaves have been demonstrated to have antioxidant effect [17]. Furthermore, phytoconstituents such as flavonoids and tannins can accelerate healing [18].

Effect of silver sulfadiazine on healing is a controversial topic with evidence for both its healing and anti-healing effect. Studies have shown that silver sulfadiazine enhances healing by stimulating epidermal migration and granulation tissue formation while another reported that it inhibits fibroblast proliferation and collagen maturation, while some studies demonstrated the effect that was comparable to control [49-51]. In our study, wound healing parameters in silver sulfadiazine-treated group and control group were comparable.

The limitation of the study was that estimation was not done of hydroxyproline (a marker of collagen content) and antioxidants in granulation tissue of wound.

CONCLUSION

Treatment with ethanolic extract of C. cristata leaves topically enhanced wound strength and accelerated healing of incision, excision, and burn wounds in rats. Further studies are required to isolate and evaluate individual phytoconstituents in C. cristata for their wound healing potential.

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