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Wound healing activity of aqueous extract of *Crotalaria verrucosa* in Wistar albino rats

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ABSTRACT

Objective: To evaluate the wound healing effect of aqueous extract of *Crotalaria verrucosa (C. verrucosa)* in rats. **Methods:** Three wound models including incision, excision and dead space wounds were used in this study. The parameters studied were breaking strength in incision models, granulation tissue dry weight, breaking strength and hydroxyproline content in dead space wounds, percentage of wound contraction and period of epithelialization in excision wound model. **Results:** Two doses of the extract with and without dexamethasone showed significant increases in mean hydroxyproline, total protein content and dry weight of granulation tissue but it was higher with dose 800 mg/kg comparing with the control. The dexamethasone treated group showed a significant (P<0.001) reduction in the wound breaking strength when compared to control group in incision type of wound model. Coadministration of *C. verrucosa* with dexamethasone significantly (P<0.001) increased the breaking strength compared to the dexamethasone treated only group. In excision wound model, the percentage of the wound contraction was significantly (P<0.01) increased by two doses of test extract on all the days except the lower dose which exhibited only on 12th, 16th days of drug treatment and it also reversed the dexamethasone suppressed wound contraction. It significantly (P<0.001) reduced the time required for epithelialization and reversed the epithelialization delaying effect of dexamethasone (P<0.001). **Conclusions:** *C. verrucosa* was found to possess significant wound healing property. This was evident by decrease in the period of epithelialization, increase in the rate of wound contraction, skin breaking strength, and granulation tissue dry weight content. Hence *C. verrucosa* could be a good wound healing agent.

1. Introduction

The process of wound healing occurs in three stages, inflammation, proliferation and remodeling. The basic principles of wound healing like minimizing tissue damage, debriding non viable tissue, maximizing tissue perfusion and oxygenation, proper nutrition and moist wound healing environment have been recognized for many years[1]. Great number of drugs are being administered from simple and less expensive analgesics to complex chemotherapeutic agents in the management of wound healing[2]. Appropriate method for healing of wounds is essential for the restoration of damaged anatomical continuity of tissue and disturbed functional status of the skin[3].

Crotalaria verrucosa (C. verrucosa) belongs to family of fabacea, well known as blue rattlesnake. It is an erect shrub which grows to 80–100 cm high with angular branches. It has oviate to triangular leaves. The juice of its leaves is used in scabies and impetigo, and is considered efficacious in diminishing salivation. In Nigeria it is used for various skin infection, colic and flatulence. It is widely spread in tropics and is found in waste places of native India. However to the best of our knowledge a systematic study on wound healing activity of *C. verrucosa* has not been undertaken to evaluate the wound healing property of aqueous extract of *C. verrucosa* on various animal models in Wistar rats[4].

2. Materials and methods

2.1. Animal care and handling

This was done as per the guidelines set by the Indian

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National Science Academy, New Delhi, India. A total of 108 healthy Wistar rats (150–200 g), aged twelve weeks of either sex, bred locally in the animal house of Kasturba Medical College, Manipal were selected for the study. They were housed under controlled conditions of temperature of (23±2) ℃, humidity of 50% and 10–14 h of light and dark cycles respectively. The animals were housed individually in polypropylene cages containing sterile paddy husk (procured locally) as bedding throughout the experiment and had free access to sterile food and water *ad libitum*. Animals were kept under fasting for overnight and weighed before the experiment. The study was undertaken after obtaining the approval of Institutional Animal Ethical Committee (IAEC approval letter No.IAEC/KMC/ 13/2009–2010 dated 14 th February 2009).

2.2. Collection and preparation of aqueous extract of *C. verrucosa*

Dried leaves of *C. verrucosa* were crushed and soaked in water overnight which was later subjected to boiling for 6 hours. The resultant extract is then drained and concentrated on water bath and desiccators sequentially to yield 5% of concentrated extract. *C. verrucosa* plants were procured locally in January and authenticated by the professor of Botany, Mahatma Gandhi Memorial College, Udupi.

2.3. Acute toxicity studies

Healthy Wistar rats of either sex were chosen and divided into six groups (n=6). They were starved overnight. They were orally fed with graded doses of aqueous extract of *C. verrucosa*. Following the administration, the animals were closely observed during first 24 hours. The doses up to 3 g/kg were well tolerated without producing any signs of toxicity and mortality thus maximum tolerated dose was found. In this study two doses of 400 mg and 800 mg were taken as lower and higher dose respectively.

2.4. Study design

The animals were randomly allocated into six groups with six animals each for the three experimental animal wound models. Group 1 received 2 mL of pure water by oral through intragastric tube. Group 2 and 3 received 400 mg/kg and 800 mg/kg of *C. verrucosa* extract by oral route respectively. The dose selection was based on the acute toxicity studies. Group 4 received dexamethasone, (0.17 mg/kg, IM) (DEXA group). Group 5 and 6 received DEXA (0.17 mg/kg, IM) + plants extract 400 mg/kg and DEXA (0.17 mg/kg, IM) + plants extract 800 mg/kg respectively.

Dosing schedule – *C. verrucosa* extract and dexamethasone were administered orally and intramuscularly respectively once daily from day 0 to day 9 in the incision and dead space wound models and from day 0 to the day of complete wound healing or the 21st postoperative day, whichever occurred earlier in the excision wound model. In group 5 and 6, *C. verrucosa* extract was given after the injection of dexamethasone.

2.5. Experimental wound models

All wounding procedures were carried out under ketamine anaesthesia (10 mg/kg, im).

Incision wound: Two paravertebral incisions of 6 cm length were made through the skin and muscles at a distance of about 1.5 cm from the midline on either side of the vertebral column. After the incision was made the parted skin is kept together and stitched at 0.5 cm intervals interrupted sutures using surgical thread and a curved needle. All the groups were treated in the same manner as per protocol. The sutures were removed on day 9 and the breaking strength of the wound was measured on day 10 by continuous and constant water flow technique[5].

Dead space wound: Physical changes in the granuloma tissue were studied in this model. The dead space was inflicted on either side in the lumbar region through a small nick in the skin. Sterilized glass cylinder measuring (2.5× 0.5) cm was introduced into the pouch[6]. The wounds were sutured and mopped with alcoholic swabs. Animals were placed in individual cages after recovery from anesthesia. The day of the wound creation was considered as day zero. On 10^{th} post wounding day, the granulation tissue formed on the implanted tube is carefully dissected under anesthesia. Granuloma tissue from one tube was used for the estimation of hydroxyproline[7] and protein[8]. Granuloma from the other tube was cut into pieces measuring 15 mm in length and 5 mm in breadth and used for determination of wound tensile strength. As part of granulation tissue was sent for estimation of SOD catalase and reduled glutathione GSH levels.

Excision wound: The excision wound was created on rats as described by Morton and Malon[9] by cutting away a 4.9 cm^2 full thickness of skin from a predetermined area on the back of selected rat[10,11]. The excised wound was left open. Wound healing potential was determined by wound contraction and wound closure time (Period of epithelization). Wound area was measured by tracing the wound margin using 1 mm^2 graph paper on the day of wounding and subsequently on alternative days until healing was complete. The healed area was calculated by subtracting wound area from the original wound area. The percentage of wound contraction was calculated using the formula:

Percentage of wound contraction = (Healed area / Total wound area) × 100

Number of days required for falling of eschar without any residual raw gives the period of epithelialization[12].

2.6. Statistical analysis

Values are expressed in mean±SEM. Results were analysed by one way analysis of variance (ANOVA) followed by Scheffe's test using SPSS computer package version 16. A P–value <0.05 was considered significant.

3. Results

3.1. Incision wound model

The mean breaking strength in the control group was (278.95 ±2.40) g. The two doses of aqueous extract of *C. verrucosa*

showed significant increase in mean breaking strength when compared to control (P<0.001) (Table 1). In the dexamethasone treated group the mean breaking strength was (151.16±0.87) g which was significantly (P<0.001) decreased compared to control group. Coadministration of dexamethasone with *C. verrucosa* 400 and 800 mg has significantly (P<.001) increased the breaking strength to (359.20±2.59) g and (388.35±2.70) g respectively (Table 1).

3.2. Dead space model

The mean breaking strength of granulation tissue in the control group was (218.94±1.02) g. A marked increase in breaking strength of the granulation tissue was observed in the two *C. verrucosa* groups compared to control (P<0.05) but more with higher dose 800 mg/kg (Table 2). The breaking strength in dexamethasone treated group was decreased significantly to (199.87±0.91) g compared to the control group (Table 2).

The mean hydroxyproline, total protein content and dry weight of granulation tissue in control group were (13.47±0.17) mg/g, (79.56±0.35) mg/L and (40.88±0.52) mg respectively. The two doses of the extract showed significant increase in mean hydroxyproline, total protein content and dry weight of granulation tissue but higher with dose 800 mg/kg compared to control (Table 2). In dexamethasone treated group mean hydroxyproline, total protein content and dry weight of granulation tissue were (10.54±0.30) mg, (48.87±0.29) mg/L and (37.07±0.95) mg which were significantly (P<0.001) less compared to control group. However coadministration of dexamethasone with *C. verrucosa* 400 and 800 mg has significantly (P<0.001) increased the mean hydroxyproline content to (24.43±0.21) g and (30.44±0.39) g respectively compared to only dexamethasone treated group (Table 2). Similar results noted when mean dry weight of granulation tissue and total protein estimations were done (Table 2). The catalase activity and glutathione synthase levels were significantly increased in the test group of 400 mg/kg and 800 mg/kg compared to control (Table 3).

3.3. Excision wound

The percentage of wound contraction was 27.78±0.56, 48.56±0.56, 59.16±0.24 and 72.64±2.08 as measured on 4th, 8th, 12th and 16th day respectively in the control group. The wound contraction was not altered in the 400 mg/kg dose group of *C. verrucosa* on 4th, 8th days of drug treatment but significantly increased on 12th and 16th day as compared to the control group (P<0.001). However a significant increase in wound contraction was seen in the higher drug treated group of 800 mg/kg compared to control on all 4th, 8th, 12th and 16th days (Table 4). Apart from this, we have also noted a positive trend of increasing wound contraction rate in *C. verrucosa* and negative trend of decreasing wound contraction rate in dexamethasone treated group and they were statistically significant on all the days compared to the control. Similar observation was also made in the dexamethasone & *C. verrucosa* treated group when compared to the dexamethasone treated group. The two doses of plant extract 400 mg/kg and 800 mg/kg exhibited statistically significant wound contraction rate where it increased from 24.72±0.29 to 15.76±0.11 and 31.54±0.28 to 12.29±0.17 as compared to dexamethasone treated group from 4th to 16th day respectively (P<0.001) (Table 2).

The mean period of epithelialization in the control group was (16.61±0.13) and it was significantly reduced to (13.43 ±0.11) and (10.98±0.03) in the lower and higher dose group respectively(P<0.001). Similarly in dexamethasone treated only group it was (18.18±0.10) days which was significantly (P<0.001) reduced to (15.76±0.11) and (12.29± 0.17) days with both (dexamethasone + 400 mg/kg plant extract and dexamethasone+800 mg/kg plant extract) treated group respectively (Table 4).

Table 1

Effect of the aqueous extract of *C. verrucosa* on the incision wound model (n=6, Mean±SEM).

Group	Drugs	Dose and route	Breaking strength (g)
Group 1	Control	2 mL of distilled water	278.95±2.38
Group 2	*C. verrucosa*	400 mg/kg, oral	322.16±1.85[a]
Group 3	*C. verrucosa*	800 mg/kg, oral	361.32±1.02[a]
Group 4	Dexamethasone	0.17 mg/kg, im	151.16±0.87[a]
Group 5	Dexa. +*C. verrucosa*	0.17 mg/kg, im + 400 mg/kg, oral	359.20±2.60[a,b]
Group 6	Dexa.+*C.verrucosa*	0.17 mg/kg, im + 800 mg/kg, oral	388.35±2.71[a,b]

Dexa- Dexamethasone; [a]P<0.001 vs control, [b]P<0.001 vs Dexa, One-way ANOVA.

Table 2

Effect of the aqueous extract of *C. verrucosa* on breaking strength, dry weight of granulation tissue, total protein and hydroxyproline in dead space model(n=6, Mean±SEM).

Group	Drugs	Dose and route	Breaking strength of granulationtissue (g)	Dry weight of granulation tissue (mg)	Total proteins (mg/g)	Hydroxyproline (mg/g)
Group 1	Control	2 mL of distilled water	218.94±1.02[c]	40.88±0.52[c]	79.56±0.35	13.47±0.17
Group 2	*C. verrucosa*	400 mg/kg, oral	254.79±3.52[c]	50.15±0.15[c]	82.24±0.68[b]	31.93±0.43[c]
Group 3	*C. verrucosa*	800 mg/kg, oral	284.19±1.73[c]	68.26±0.55[a]	101.56±0.96[d]	38.93±0.29c
Group 4	Dexamethasone	0.17 mg/kg, im	199.87± 0.91[c]	37.07±0.95[a]	48.87±0.29[a]	10.54±0.30[c]
Group 5	Dexa +*C. verrucosa*	0.17 mg/kg, im+400 mg/kg, oral	351.06±1.51[c,d]	46.07±0.82[a,d]	50.71±0.24[c]	24.43±0.21[d]
Group 6	Dexa+*C.verrucosa*	0.17 mg/kg, im+800 mg/kg, oral	388.35± 2.70[c,d]	53.75±1.02[c,d]	73.65±0.54[d]	30.44±0.39[d]

[a]P<0.05 vs control, [b]P<0.05 vs Dexa, [c]P<0.001 vs control , [d]P<0.001 vs Dexa.

Table 3
Antioxidant activity of the aqueous extract of *C. verrucosa* in dead space wound model(n=6, Mean±SEM, units/mg/min).

Groups	Catalase activity	GSH
Control	1.49±0.11	7.62±0.37
C. verrucosa 400 mg/ kg	2.14±0.15	6.23±0.29[a]
C. verrucosa 800 mg/ kg	3.35±0.23[a]	4.22±0.21[a]

[a]P<0.05 compared to control, One-way ANOVA, SEM =Standard error of mean, GSH=glutathione.

Table 4
Effect of the aqueous extract of *C. verrucosa* in excision wound model(n=6, Mean±SEM).

Group		Dose and route	% of wound contraction				
			4th day	8th day	12th day	16th day	POE
Group1	Control	2 mL of distilled water	27.78±0.56	48.56±0.56	59.16±0.24	72.64±2.08	16.61±0.13
Group2	*C. verrucosa*	400 mg/kg, oral	28.13±0.72	48.56±0.56	69.04±0.27c	81.67±0.35[c]	13.43±0.11[c]
Group3	*C. verrucosa*	800 mg/kg, oral	33.08±1.03[c]	57.78±0.72[c]	81.38±0.49[c]	94.13±0.48[c]	10.98±0.03[c]
Group4	Dexa	0.17 mg/kg, im	22.16±0.60[c]	40.69±0.95[c]	50.31±0.29[c]	64.22±0.39[c]	18.18±0.10[c]
Group5	Dexa+ *C. verrucosa*	0.17 mg/kg, im+400 mg/kg, oral	24.72±0.29	44.03±1.21[a]	63.62±0.37[cd]	78.25±0.46[ad]	15.76±0.11[ad]
Group6	Dexa+*C.verrucosa*	0.17 mg/kg, im+800 mg/kg, oral	31.54±0.28[ad]	51.87±4.96[d]	72.51±0.76[cd]	83.12±0.65[cd]	12.29±0.17[cd]

Dexa- Dexamethasone, [a]P<0.05 vs control, [c]P<0.001 vs control , [d]P<0.001 vs Dexa; POE- Period of epithelialization.

4. Discussion

Wound healing is an orderly progression of events that establish the integrity of the tissues. Many studies have shown that plant products are preferred in wound healing since they are devoid of side effects and are more effective[13]. Wound healing process begins with the restoration of a damaged tissue as closely as possible to its natural state and wound contraction is the course of shrinkage in wounded area. The healing primarily depends on the repairing ability of the tissue in addition to type and degree of damage and general health status of the tissue.

Granulation, collagen maturation and scar formation are some of the many phases of wound healing which run concurrently, but independent of each other. Up to date there is no reference standard exists and the use of single model is inadequate to conclude which can represent distinctively the various phases of wound healing. Hence three different models have been chosen in our study to assess the effect of *C. verrucosa* on wound healing.

The granulation tissue of the wound is primarily composed of edema, fibroblast, collagen and new blood vessels. The mesenchymal cells of the wound area adjust themselves into fibroblast then begin migrating into the wound gap together with the fibrin strands. The collagen is the main constituent of extra cellular tissue, which is responsible for support and strength. Free hydroxyproline and its peptides are released with collapse of collagen. Thus, measurement of the hydroxyproline could be used as an indicator for collagen turnover. Furthermore, increase in dry tissue also indicates the presence of elevated protein content.

The wound breaking strength is determined by the rate of collagen synthesis and maturation process, wherein there is covalent binding of collagen fibrils through inter and intra molecular cross linking. In our study dead space model, there is not only marked increase in breaking strength of the granulation tissue but also significant increase in hydroxyproline content, total proteins and dry weight of granulation tissue was observed in the two doses of *C. verrucosa* compared to control. By this we can presume that the *C. verrucosa* might have increased the collagen content and probably altered the maturation process, by affecting the cross linking of collagen or improving the quality of collagen fibrils[14].

Wound contraction is the process of mobilizing healthy skin surrounding the wound to cover the denuded area and involves complex and superbly orchesterated interactions of cells, extracellular matrix and cytokines. This centripetal movement of wound margin is believed to be due to the activity of myofibroblast[15]. Since *C. verrucosa* enhanced wound contraction, it would have either enhanced contractile property of myofibroblasts or increased the number of myofibroblasts recruited into the wound area. Granulation, collagen maturation and scar formation are some of the many phases of wound healing which run concurrently, but independent of each other[16]. A significant increase in wound contraction was seen in both doses of *C. verrucosa* compared to control. Hence it appears that *C. verrucosa* has prohealing effect as evidenced by the above findings.

In excision wound model *C. verrucosa* and *C. verrucosa* with dexamethasone hastened the period of epithelialization significantly during initial and later stages of wound contraction. It appears that *C. verrucosa* has prohealing effect as evidenced by the above findings and was able to promote epithelialization either by facilitating the proliferation of epithelial cells or by increasing the viability

of epithelial cells. It is difficult to draw any conclusion from the study regarding the dexamethasone & *C. verrucosa* effect in dexamethasone suppressed wound model.

Oxidative stress is associated with many acute and chronic inflammatory conditions such as wound healing[17]. *C. verrucosa* has shown to possess anti-oxidant property. Flavonoids responsible for free radical scavenging activity were believed to be one of the important components in wound healing, however confirmation needs to be done by further phytochemical analysis. This could be the probable reason for pro-healing effect of *C. verrucosa*. Due to the various properties as discussed above *C. verrucosa* could be used to treat open wounds. However confirmation of this conclusion requires clinical evaluation.

In conclusion, the present study demonstrated that the aqueous parts of *C. verrucosa* promote wound healing activity in animal as a preclinical study. The aqueous extract showed remarkable wound healing activity and it may be suggested for treating various types of wounds in animal and human beings. Further studies with purified constituents compared to the crude extracts might be needed to comprehend the complete mechanism of wound healing activity of *C. verrucosa*.

Conflict of interest statement

We declare that we have no conflict of interest.

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