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Research article

EVALUATION OF WOUND HEALING ACTIVITY OF *ACACIA AURICULIFORMIS* A. CUNN. STEM BARK

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ABSTRACT

Objective: To evaluate *in vivo* wound healing activity of ointment containing ethanol and aqueous bark extracts of *Acacia auriculiformis* A. Cunn. (Family: Mimosaceae).

Methods: The presence of phytochemicals like carbohydrates, phenols, flavonoids, tannins and saponins was determined by preliminary phytochemical screening. Wound healing effect of ointment containing 5% w/w ethanol and aqueous stem bark extracts was determined by using excision and incision wound models in Swiss albino mice. Hydroxyproline content determination and histopathological studies of treated groups were carried out.

Results: The results showed that both formulations possess significant wound healing activity, which was evidenced by decreased period of epithelialization, increased rate of wound contraction, tensile strength, hydroxyproline content, granulation tissue and collagen fibre formation in all treated animals. The activity may be due to presence of phenols, tannins, and flavonoids.

Conclusion: The ointment containing ethanol extract showed better wound healing activity than the ointment containing aqueous extract.

Keywords: *Acacia auriculiformis*, excision wound model, hydroxyproline, incision wound model, tensile strength, wound contraction.

INTRODUCTION

Wound is a physical trauma which occurs when the integrity of any tissue is compromised [1]. Wound healing is a dynamic physiological process that involves a series of phases and mediates through the interaction of a complex cascade of a cellular and biochemical actions leading to the restoration of structural and functional integrity with regain of strength of injured tissues. It involves continuous cell-cell interaction and cell-matrix interactions that allow the process to proceed in phases including inflammation, wound contraction, re-epithelialization, tissue remodeling and formation of granulation tissue with angiogenesis. If the phases of wound healing do not proceed in this way, healing may progress inappropriately to a chronic wound [2]. The main goals of the researches in wound healing are to evaluate the influence of various measures in wound management programs on healing and to screen drugs that encourage healing process efficiently [3]. Several medicinal plants have been used since time immemorial for treatment of wounds and showed promising effects [4]. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body. These constituents include various chemical families like alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins, and phenolic compounds [5]. The herbal extracts and fractions effectively arrests bleeding from fresh wounds, inhibit microbial growth and accelerate wound healing [6]. *Acacia auriculiformis* A. Cunn. (Mimosaceae) is a straight, medium-sized tree, native plant of Australia and was first introduced to India in 1946 in West Bengal [7]. The plant have showed various pharmacological activities like antioxidant [8], antimicrobial [9], antimalarial [10], antifilarial [11], cestocidal [12], antimutagenic and chemopreventive [13], spermicidal [14], hepatoprotective and anti diabetic activity [15]. There is no previous report on wound healing activity of *Acacia auriculiformis* in literature to the best of our knowledge and in this paper, we report for the first time, the efficacy of *Acacia auriculiformis* stem bark extracts in the treatment and management of wounds. The present study was carried out to assess the wound healing properties of hydrophilic ointments prepared with 5% ethanol and aqueous extracts of *Acacia auriculiformis* stem bark extracts.

MATERIAL AND METHODS

Preparation of extracts

The powdered stem bark of *A.auriculiformis* A. Cunn. (500g) was defatted by extracting with petroleum ether and then extracted with ethanol in soxhlet extractor. The total aqueous extract was prepared by cold maceration method. The extracts obtained were concentrated by distilling off the solvents and recovering the same. The percentage yield of ethanol and aqueous extracts were 7.56 % w/w and 3.94% w/w respectively. These extracts were further used for evaluation of wound healing activity.

Drug and Chemicals

Sodium lauryl sulphate, propylene glycol, stearyl alcohol, white petrolatum, hydrochloric acid, copper sulphate, sodium hydroxide, sulphuric acid, hydrogen peroxide (SD Fine Chemicals Limited, Mumbai, India, p-dimethylaminobenzaldehyde (Thermal Fisher Scientific Pvt. Ltd. Mumbai, India) and hydroxyproline (Himedia, Mumbai, India). All chemicals used were of analytical grade.

Preliminary phytochemical screening

Dried ethanol and aqueous extracts were subjected to various chemical tests to detect the presence of various phytoconstituents like carbohydrates, alkaloids, glycosides, phenols, tannins, flavonoids and saponins.

Preparation of ointment

The ointment was prepared according to formula: Sodium lauryl sulphate (1%), propylene glycol (12.5%), stearyl alcohol (25%), white petrolatum (25%), extract (5%) and purified water (31.5%). Stearyl alcohol and white petrolatum were melted together at about 75°C to form oleaginous phase. The other agents including extracts were dissolved in purified water and heated at the same temperature. Then, the oleaginous phase was added to the aqueous phase with continuous stirring until the two phases were mixed properly. Sodium lauryl sulphate acts as emulsifying agent with stearyl alcohol and white petrolatum, comprising the oleaginous phase of emulsion and the other ingredients in aqueous phase [16].

Hydrophilic ointment base without extracts was used as control. The prepared ointments were then tested for homogeneity by visual inspection. These were tested for their appearance with no lumps [17].

Experimental Animals

Swiss albino mice (20-25 g) were purchased from Disease free small animal house, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar (Haryana) and were used for biological activity as per the experimental protocol approved by Institutional Animal Ethical committee (Guru Jambheshwar University of Science and Technology, Hisar, Haryana), Registration number 0436, dated 20th December 2012. The animals were housed under standard environmental conditions of temperature and humidity (25 ± 2°C and 50 ± 5% respectively) and were fed with standard pellet diet and water *ad libitum.*

The animals were grouped into four major groups:

Group I - Control.
Group II – Standard (treated with betadine 5% w/w povidone iodine ointment).
Group III - Test group treated with 5% w/w ethanol extract ointment.
Group IV - Test group treated with 5% w/w aqueous extract ointment.

Acute dermal toxicity studies

The study was carried out to determine the therapeutic dose of the ethanol and total aqueous extracts. The acute dermal toxicity testing of the ethanol extract and the total aqueous extract was done by applying the ointments containing ethanol and total aqueous extracts of the highest concentrations of 12% (w/w) on the shaved back of the mice. The OECD guidelines no. 402 was followed for the study [18].

Wound healing studies

Excision Wound Model

The mice were anesthetized by administering ketamine (0.5 ml/kg b. w. i.p.). A full thickness of the excision wound of circular area of 10 mm diameter and 2 mm depth was made on the shaved back of the mice 30 minutes later the administration of ketamine injection. The wounding day was considered as day 0. The wounds were treated with topical application of the ointments till the wounds were completely healed. The wounds were monitored and the area of wound was measured on 4th, 8th, 12th and 16th post-wounding days and the mean percentage of wound contraction was reported. The period of epithelialization was calculated as the number of days required for filling of the dead tissue remnants without any residual raw wound.

Percentage wound contraction = 100× (wound area on 0th day-wound area on nth day)/ Wound area on day 0; where n = number of days 4th, 8th, 12th, and 16th day [19, 20].

Incision Wound Model

In the incision model, the mice were anesthetized by administering ketamine (0.5 ml/kg b.w. i.p.) and one longitudinal peravertebral incision of about 2 cm length was made through the skin and cutaneous muscle at a distance about 1.5 cm from the midline on the each side of the depilated back of the mice 30 minutes later the administration of ketamine injection. After the incision, the parted skin was sutured 1cm apart. The wounds of animals in the different groups were treated with topical application of the ointments once a day. The sutures were removed on the 9th day after wounding and the breaking strength was measured on the 10th day. Tensile strength was calculated using the following formula: Tensile strength = Breaking strength (g)/ cross-sectional area of skin (mm^2) [21].

Estimation of Biochemical Marker

Circular wound was created treated with topical application of ointments for 10 days. On the 11th day, the hydroxyproline content was determined in the wound tissue. The wound tissue was excised and dried in oven at 60 ^{0}C for 12 hours. It was hydrolyzed in 6N hydrochloric acid for 24 hours at 110 ^{0}C in sealed glass tubes. The hydrolysate was neutralized to pH 7.0. The samples were mixed with 1ml of 0.01M copper sulphate followed by the addition of 1ml of 2.5N sodium hydroxide and then 1ml of 6% hydrogen peroxide. The solution was mixed and shaken occasionally for 5 minutes. All the tubes were incubated at 80 ^{0}C for 5 minutes with frequent vigorous shaking. Upon cooling, 4 ml of 3N sulphuric acid was added with agitation. Finally, 2ml of 5% *p*-dimethylaminobenzaldehyde was added. The samples were incubated at 70 ^{0}C for 16 minutes, cooled by placing the tubes in water at 20 ^{0}C for 12 hours and developed red colour [22]. The red colour thus measured on UV Spectrophotometer at 558 nm. The amount of hydroxyproline in the samples was calculated using a standard curve prepared with pure L-hydroxyproline at the same time [23].

Histopathological Studies

For histological study, granulation tissue were fixed in 10% neutral formalin solution for 24 hours and dehydrated with a sequence of ethanol - xylene series of solution. The materials were infiltered and embedded with paraffin (40-60 ^{0}C). Microtome sections were taken at 10µ thickness and stained with hematoxylin - eosin dye. The histological changes were observed under the Carl Zeiss Micro imaging GmbH Hingen, Germany microscope [24, 25].

Statistical Analysis

The results obtained from the wound models have been expressed as mean ± standard error mean (SEM) and were compared with the corresponding control group (hydrophilic ointment base) by applying ANOVA test.

RESULTS

Preliminary phytochemical screening

Preliminary phytochemical screening revealed the presence of carbohydrates, phenols, tannins, saponins and flavonoids.

Acute Dermal Toxicity Study

There was no change in general behavior or appearance, loss in body weight etc. Toxicity study showed no mortality up to the maximum selected dose of 12% (w/w) till the end of experiment.

Excision Wound Study

Both the test samples exhibited wound healing properties when compared with control group. The formulated ointment with ethanol extract was more effective and significant ($p < 0.01$) than aqueous extract throughout the test days with 18 days as period of epithelialization i.e. A better healing pattern with complete wound closure as compared to aqueous extract treated group and control group with 21 and 25 days respectively as period of epithelialization (Table 1).

Table 1: Effect of ointments containing extracts of *Acacia auriculiformis* A. Cunn. stem bark on wound contraction

Groups	Percentage wound contraction				Epithelialization period (days)
	4th day	8th day	12th day	16th day	
Group I	10.72±0.41	28.13±1.45	45.26±0.65	64.66±1.52	25
Group II	45.37±1.11**	64.2±0.49**	81.23±0.57**	99.69±0.87***	17
Group III	37.95±1.56*	58.39±1.94**	76.16±1.09**	91.21±1.24**	18
Group IV	25.69±1.52*	46.85±0.79*	65.57±1.43*	81.93±1.49**	21

Values are expressed as Mean±S.E.M. n=6 in each group, analyzed by ANOVA followed by Dunnett's t-test, *p<0.05, **p<0.01, ***p<0.0001.

Incision wound study

There was significant increase in tensile strength of wounds when treated with ointments containing ethanol and aqueous extracts. The animals treated with ointment containing 5% (w/w) ethanol extract indicated significantly high ($P < 0.01$) and 5% (w/w) total aqueous extract indicated significant tensile strength ($P < 0.05$) as compared to the control group. The increase in tensile strength of wounded skin indicates the promotion of collagen fibres (Table 2).

Table 2: Effect of ointments containing ethanol extracts of *Acacia auriculiformis* A. Cunn. stem bark on breaking strength and tensile strength

Groups	Breaking strength (g)	Tensile strength (g)
Group I	426 ± 7.33	14.2 ± 1.24
Group II	619 ± 5.52**	20.63 ± 0.69**
Group III	553 ± 5.61**	18.43 ± 0.52**
Group IV	468 ± 8.37*	15.6 ± 0.47*

Values are expressed as Mean±S.E.M. n=6 in each group, analyzed by ANOVA followed by Dunnett's t-test, *p<0.05, **p<0.01.

Biochemical Marker Estimation

Biochemical analysis showed increased hydroxyproline content, which reflects increased cellular proliferation and collagen synthesis. The results indicate that the animals treated with ointment containing 5% (w/w) ethanol extract have shown better wound healing activity than control and 5% (w/w) aqueous extract (Table 3).

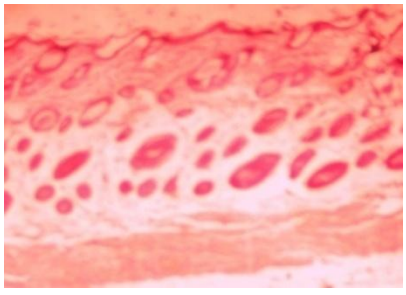
Table 3: Effect of ointments containing extracts of *Acacia auriculiformis* A. Cunn. stem bark on hydroxyproline content in the excision wound

Groups	**Hydroxyproline content (µg/100mg)**
Group I	11. 48 ± 0.21
Group II	22.86 ± 0.54**
Group III	19.86 ± 0.67**
Group IV	15.34 ± 0.66*

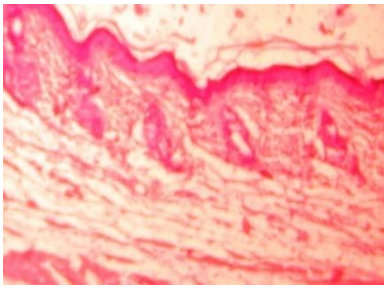
Values are expressed as Mean±S.E.M. n=6 in each group, analyzed by ANOVA followed by Dunnett's t-test, *p<0.05, **p<0.01, ***p<0.0001

Histopathological Study

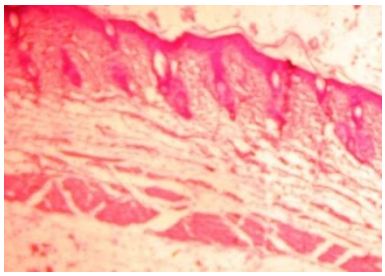
Histopathological studies showed that there was a marked infiltration of inflammatory cells, increased blood vessel formation, and enhanced proliferation of cells as a result of treatment with prepared medicated ointment of *Acacia auriculiformis* A. Cunn. stem bark with ethanol and aqueous extracts. The study showed the presence of fibroblasts and keratinocytes. A well-advanced organization of granulation tissue, collagen synthesis and on-going epithelialization was observed in treated groups with 5% w/w ethanol and aqueous extracts ointments. More collagen synthesis was recorded in ointment with ethanol extract treated group than the ointment with aqueous extract treated group (Figure 1).



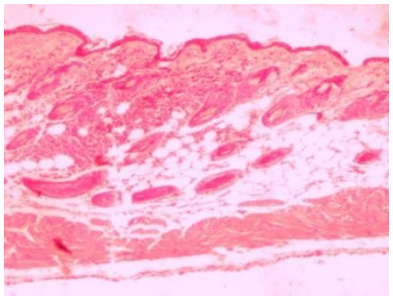
A



B



C



D

Fig. 1: Histopathological study of skin tissue treated with Control (A), Standard (B), Ointment containing Ethanol (C) and Aqueous extract (D) on 16th day of treatment

DISCUSSION

The ointments prepared with ethanol and aqueous extracts of stem bark of *Acacia auriculiformis* A. Cunn. were evaluated for wound healing studies. Both the extracts showed wound healing activity by enhancing wound contraction, shortened epithelialization period and increased tensile strength. In excision wound healing model, the study demonstrated that there was a significant increase in rate of wound contraction. The rate was higher in the group of animals treated with ointment containing ethanol extract than the ointment containing aqueous extract. This increased rate may be due to collagen synthesis which is evidenced by increased protein content which was determined by hydroxyproline content in the granulation tissues. The enhanced rate of wound healing may be due to formation of fibroblasts because fibroblast is a main cell content involved in the synthesis and deposition of extracellular matrix [26]. In incision wound healing model, both the extracts showed increase in tensile strength as compared to the control group. The activity of ointment containing ethanol extract was significantly higher than the ointment containing aqueous extract. The tensile strength may be increased due to stabilization of collagen fibres. The presence of collagen fibres, fibroblasts and formation of capillary vessels was confirmed by histopathological studies. The wound healing activity of the extracts may be due to presence of various phytoconstituents like phenolic constituents, flavonoids, tannins etc. They promote the wound healing through several cellular mechanisms, chelating of the free radicals and reactive species of oxygen, promoting contraction of the wound and increasing the formation of capillary vessels and fibroblasts [27]. Since no detailed scientific data was available

regarding the wound healing activity of *Acacia auriculiformis*, the present study was designed to explore the same.

CONCLUSION

The ointment containing ethanol extract showed better wound healing activity than the ointment containing aqueous extract. The future scope of study involves the need for isolation of phytoconstituents responsible for the activity.

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