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WOUND HEALING ACTIVITY OF METHANOLIC EXTRACT OF *ARNEBIA BENTHAMII*

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ABSTRACT: *Arnebia benthamii* (synonym - *Microtomia benthamii*) commonly known as Ratanjot or Laljari found in western Himalayan region and used as wound healer roots by local vaids and tribes. The roots of *Arnebia benthamii* contain a red dye that is used in various health disorders like fungal infection, inflammation, fever and coloring/flavoring agent in Indian curries. To estimate wound healing potential of root extract of *Arnebia benthamii*, percentage wound contraction, wound area, epithelization time, tensile strength and wound index were measured in 13 days of wound healing study. The results showed highly significant wound healing in excision as well as incision wound healing models ($P<0.01$). The *Arnebia benthamii* extract-treated wounds showed about 41% higher wound contraction rate and 44% increased tensile strength in comparison with negative control animals. The epithelization time decreased by 43% and wound index value decreased by 89% in comparison to negative control animals which indicated that methanolic root extract of *Arnebia benthamii* is very helpful in faster and high-quality wound healing ($P < 0.05$).

INTRODUCTION: Plants are traditionally used to treat wounds, cuts, and burns by folklore traditions and tribes all over the world. Modern research showed that plants could heal wounds by various mechanisms like angiogenesis, fibroblast proliferation, up-regulation of iNOS, activation of NF-kB, favour of proinflammatory cytokines, alpha 1 type 1 collagen synthesis or/and antioxidant activity [1]. The skin injury by cut, tear or puncture in epidermis or dermis of skin, results in wound [2]. Wound healing is a complex process to restore the damaged structures of skin in the injured area.

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The healing cascade for wounds can be divided into inflammatory, proliferative and remodeling phases in which many different and connected healing mechanisms are involved [3]. The rapid hemostasis, inflammation, migration, and differentiation of mesenchymal cells at wounded sites occur then angiogenesis process starts with re-epithelization and collagen synthesis and finally remodeling of healed skin completed [4].

The impaired healing of wounds is mainly due to defects in the healing process, which may be caused by many factors like oxygenation, infection, stress, sex hormones, and age while some diseases also impair wound healing like diabetes, obesity, ischemia, uremia, and fibrosis. Some medications may also retard the healing of wounds includes glucocorticoid steroids, non-steroidal anti-inflammatory drugs, and chemotherapy. The alcoholism, smoking and malnutrition also affect

healing of wounds [3-4]. *Arnebia benthamii* is a perennial herb grows in open moist slopes at altitude of 3000 to 3900 meter in India, Pakistan [5], Afghanistan, and Nepal. In India, the roots of this plant sold under local names Ratanjot, Balchhadi, Laldori, and Laljari, especially in Kinnaur, Chitkul, Chamba, Spiti, and Chanshal regions of Himachal Pradesh, Ladakh, Anantnag, Kargil of Jammu and Kashmir. At Gangotri, Har Ki Doon, Ralam, Daruma, Badrinath, and Kandara of Uttarakhand state, this plant is found in dry sandy rocky places [6].

Arnebia benthamii is 30-90 cm in height, bristly or pubescent with erect or prostrate stem. Leaves are alternate, roots contain purple color dye; chymes are bractate with heterostylous, long-styled flowers with inserted stamens at middle of corolla tube. Calyx is spotted to base while corolla is in funnel form, hairy outside with 4 lobed ovaries [7]. *Arnebia benthamii* roots yield shikonin and alkanin, the red pigments are responsible for plant color and therapeutic activity. The active phytoconstituents of *Arnebia benthamii* are naphthoquinones, flavonoids, triterpenoids, benzoquinones, steroids and alkaloids. The main chemical constituents are artemidiol, hosluridol, shikonin, ganoderiol, alkanin and 2-hexaprenyl-6-hydroxy phenol and an essential oil arnebinus in dry plant [8].

Arnebia benthamii scientifically validated for its antipyretic, antioxidant, antiseptic, antimicrobial, antifungal [9], anthelmintic and antidepressant properties. The plant also used for stimulant, diuretic, expectorant and tonic properties. The jam and sherbet (sugar syrup) of the flowering shoot is used to treat throat, tongue, fever and cardiac disorders but prohibited in liver disorders. The oil extract of dried roots is used as traditional medicine for wound care by tribes [9-10]. In Indian kitchen, roots are used as coloring and flavoring agent in various curries under name Ratanjot. The commercial preparation of *Arnebia benthamii* is Gule Khazaban or Gaozaban, a costly medicine used to treat fungal infection, inflammation, fever and wound healing [11].

MATERIALS AND METHODS:

Materials:

Collection of Plant Material: The *Arnebia benthamii* plant was collected from Uttarkashi of Uttarakhand, India, in September 2017 and dried. Another sample was purchased from local market of Dehradun under name Ratanjot/Lalchhadi. The plant specimens were prepared and identified from the Botanical Survey of India, Dehradun, Uttarakhand (Voucher No. 118376).

Preparation of Methanolic Extract: 200 gm of dried root coarse powder was extracted with methanol by hot Soxhlet method. The extract after extraction was concentrated by rotary drum evaporator (Buchi type) at 50 °C till total volume decreased to one-third of original volume. The concentrated extract then dried at 45 °C in vacuum oven till dryness. The dried extract was stored in airtight container [12].

Preparation of Gel for Topical Application: Appropriate quantity of carbopol 934 was soaked in water for a period of 2 h. Carbopol was then neutralized with triethanolamine with stirring. Then specified amount of drug was dissolved in appropriate and pre weighted amounts of propylene glycol and ethanol. The solvent blend was transferred to carbopol container and agitated for additional 20 min. The dispersion was then allowed to hydrate and swell for 60 min, finally adjusted the pH with 98% TEA until the desired pH value was approximately reached (6.8-7). During pH adjustment, the mixture was stirred gently with a spatula until homogeneous gel was formed. All the samples were allowed to equilibrate for at least 24 h at room temperature prior to use in experimentation.

Animals: The healthy adult Sprague Dowley rats with average weight of 200-250 g and age of 3-4 months were procured from the animal house of Devsthali Vidyapeeth College of Pharmacy, Lalpur Kichha Road, Rudrapur, Uttarakhand, India and housed at 24-26 °C with humidity, not more than 75%. The animals thoroughly checked for any disease, physical wound with or without infection and allowed to feed food pallets with water ad libitum. Before study, animals were acclimatized for at least one week in group of 3 animals in each polypropylene cage. The complete study of wound healing, the ethical clearance for animal use, care and study guidelines were approved from Institutional Animal Ethical Committee of Devsthali Vidyapeeth College of Pharmacy,

Rudrapur, Uttarakhand, India (DVCP/IAEC/ 2018/02) under the supervision of Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment, Forest & Climate Change, Government of India, New Delhi (1452/PO/Re/S/ 11/CPCSEA).

Methods: Acute dermal irritation/corrosion: According to OECD TG 404 acute dermal irritation/corrosion, a single dose of test substance was tested on skin of animals in two groups (3 animals in each group) one for *Arnebia benthamii* and second for untreated control. Sprague Dowley rats were used for study. The fur was removed by electronic clipper from dorsal area of trunk approximately 24 h before the test. The herbal extract gel (500 mg) was applied on approximately 6 cm^2 area and covered with gauze patch. At 3 min, 1 h, 4, 8, 12 h of herbal extract gel application, animals were observed for any possible toxicity. The first patch was removed after 3 min of application then second patch after 1 h and third patch after 4 h was removed. At every patch removal and completion of study, animals were observed for toxic effects and for 14 days for late toxic effects. All animals were examined for erythema, edema, irritation and other local and systemic toxic effects [13].

Excision Wound Healing Model: The animals were anesthetized and fur was removed by electronic clipper in dorsal thoracic region 1 cm away from vertebral column and 5 cm away from ear then a circular area of 500 mm^3 were marked by permanent marker in all animals then divide into four groups (3 animals in each group). The marked area was excised in full-thickness and blotting of wound with cotton soaked in normal saline to achieve hemostasis [14].

Treatment Group I: animals were treated with methanolic root extract gel of *Arnebia benthamii* by topical application. Positive control group I: animals were treated with 10% w/w standard *Aloe vera* gel. Negative vehicle control I: animals treated with pure gel without any medication. Negative control I: animals were untreated but wounds were cleaned to prevent external infection. The treatment of the wounded area of all animals except negative control group was applied twice daily from day of surgery till the epithelization was completed [15]. At 0, 3, 5, 7, 9, 11, and 13th day after wounding, wounds area were marked on transparent sheet by permanent marker. The marked wound area again photocopied on millimeter-scale graph paper then wound area was calculated for each wound.

The percentage of wound contraction and epithelization period was calculated [9]. The falling of scare from the wound was considered as completion of epithelization. Wound index was estimated on 13th day by observing the healed wound using a scoring system, healthy healing scored as 0, delayed healing as 1, pus containing wound 2, unhealed wound 3, and necrotic wound scored as 4 [16].

Incision Wound Healing Model: The shaved animals divide into four groups to study the tensile strength of incised wound sutured with standard sutures. A 3 cm long paravertebral incision was made in full-thickness on either side of vertebral column then closed with sutures (Mersilk, Ethicon, Aurangabad, India) of 1 cm apart. Treatment group II: animals were treated with methanolic root extract gel of *Arnebia benthamii* by topical application. Positive control group II: animals were treated with 10% w/w standard *Aloe vera* gel. Negative vehicle control II: animals treated with pure gel without any medication. Negative control II: animals were untreated but wounds were cleaned to prevent external infection. The animals with sutured wounds were treated twice daily and on 8th post wounding, day sutures were removed if left in skin [17]. The treatments of unsutured wound continue till 13th post wounding day. At the end of study, wound stripes of full-thickness skin and equal size were made with the help of sharp scissors and two surgical blades (No 11, MediEdge, Pioneer surgical products, Delhi-92, India) fixed at 2 cm distance apart.

Animals were then transferred for rehabilitation to animal house. Both ends of strip were fixed with two clips than one end hang withstand and another end with an empty polyethylene bag. The polyethylene bag then gradually filled with water till breakdown of wound strip from healed incised wound. The weight of water with polyethylene and clip in grams was considered as tensile strength of that wound [18].

RESULTS AND DISCUSSION:

Acute Dermal Irritation/Corrosion: At 500 mg of methanolic root extract of *Arnebia benthamii* in gel preparation was applied on about 6 cm^3 area of shaved skin for different periods of time in separate groups of animals in a single dose and any sign of erythema or redness or any reversible or irreversible damage to skin, ulcers, bleeding, bloody scabs were not observed during study period (up to 12 h) and without any discolouration or blanching of skin, alopecia in gel applied area and scars in post-study duration (14 days).

Wound Healing Potential: The methanolic root extract of *Arnebia benthamii* (10% w/w gel) was evaluated for its wound healing potential using excision and incision wound healing models. The results of the study showed that root extract of *Arnebia benthamii* possesses highly significant wound healing activity in both excision and incision wounds. The data of epithelization and wound index also indicated that healing is faster and healthier as compared to control.

Excision Wound Healing: The methanolic root extract of *Arnebia benthamii* showed significant ($p<0.01$) faster-wound closure as sharp contraction of wound area and increase in % wound contraction (41%) as compared to positive (10% w/w *Aloe vera* gel) and negative control (plain gel without medicament) **Fig. 1** and **2**. The comparison of all group data of excision wound study showed in **Table 1**.

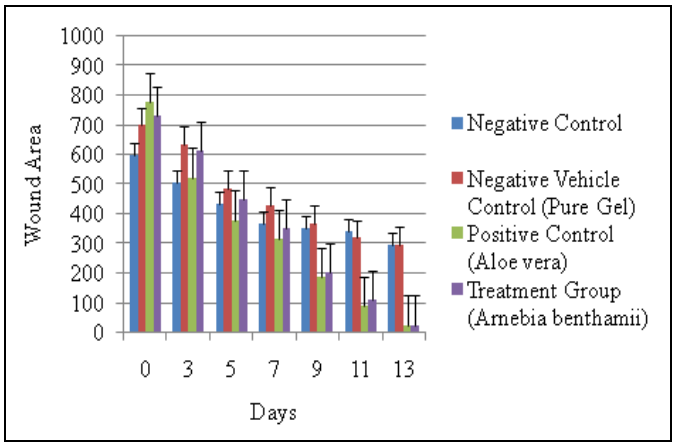

FIG. 1: EFFECT OF METHANOLIC EXTRACT OF *ARNEBIA BENTHAMII* ON WOUND SURFACE AREA

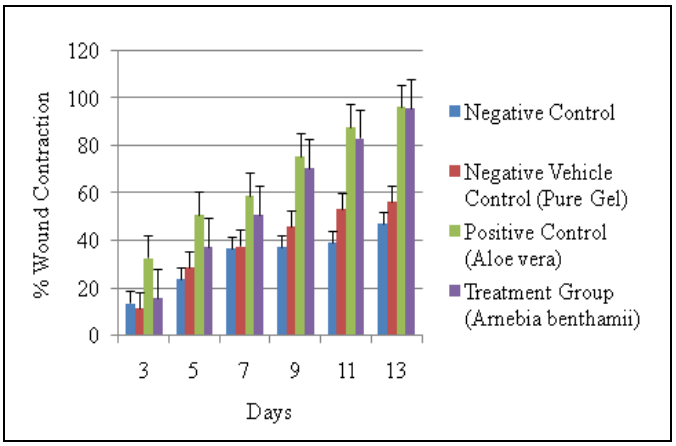

FIG. 2: EFFECT OF METHANOLIC EXTRACT OF *ARNEBIA BENTHAMII* ON % WOUND CONTRACTION

TABLE 1: WOUND HEALING ACTIVITY OF METHANOLIC ROOT EXTRACT OF *ARNEBIA BENTHAMII*

Parameters /Days	Negative Control#		Negative Vehicle Control (Pure gel)#		Positive control (*Aloe vera* 10 % w/w gel)#		Treatment Group *Arnebia benthamii* (10% w/w gel)#	
	Wound Surface Area [mm^2]	% Wound Contraction	Wound Surface Area [mm^2]	% Wound Contraction	Wound Surface Area [mm^2]	% Wound Contraction	Wound Surface Area [mm^2]	% Wound Contraction
Day 0	601 ± 106	-	702 ± 82.0	-	777 ± 54.4	-	733 ± 156	-
Day 3	506 ± 54.6	13.8 ± 16.9	636 ± 77.0	11.8 ± 18.7	524 ± 129	32.884 ± 14.7	613 ± 116	15.88 ± 4.53
Day 5	437 ± 56.3	24.1 ± 22.8	488 ± 67.6	29.0 ± 16.1	381 ± 122	50.87 ± 15.4	449 ± 80.8	37.57 ± 10.7
Day 7	370 ± 33.5	36.7 ± 12.4	431 ± 79.4	37.8 ± 14.0	318 ± 117	58.96 ± 15	354 ± 62.5	51.01 ± 6.38
Day 9	355 ± 85.7	37.6 ± 24.4	370 ± 43.3	46.3 ± 10.9	189 ± 42.7**	75.48 ± 6.0*	204 ± 64.7**	70.6 ± 12.15
Day 11	343 ± 102	39.4 ± 25.5	321 ± 59.4	53.3 ± 12.1	91.6 ± 31.4*	87.96 ± 4.88*	111 ± 57.1*	83.33 ± 10.26*
Day 13	296 ± 87.4	47.4 ± 23.5	299 ± 81.3	56.5 ± 14.0	27.4 ± 5.3**	96.31 ± 2.93**	27.2 ± 21.6**	95.91 ± 3.52**
Tensile Strength (gm)	184 ± 68.0		268 ± 27.7		490 ±2 9.3**		479 ± 42.9**	
Epithelialization time (Days)	20.6 ± 0.89		16.8 ± 1.92		11.2 ± 1.64**		11.6 ± 1.51**	
Wound index	3.8 ± 0.44		3.4 ± 0.54		0.6 ± 0.54**		0.4 ± 0.54**	

#Mean ± SD, n = 3, *P<0.05, **P<0.01

Epithelization Period Study: The epithelization data showed that the application of methanolic root extract gel of *Arnebia benthamii* in excision wounds decreases significantly ($p<0.05$) in epithelization periods (43%) which indicated that healing was faster in treated animals as compared to negative control group animal wounds **Fig. 3**.

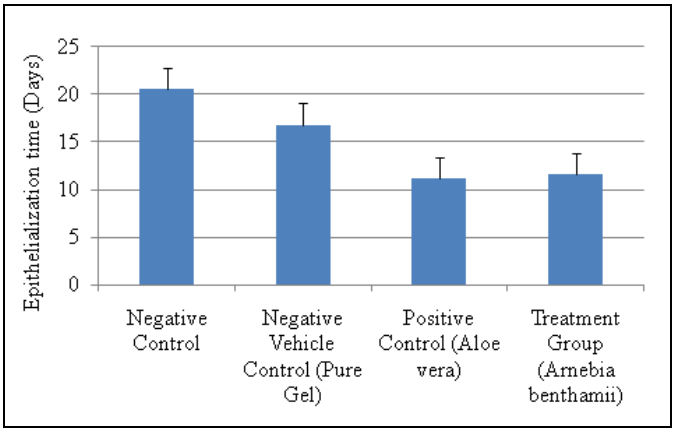

FIG. 3: EFFECT OF METHANOLIC EXTRACT OF *ARNEBIA BENTHAMII* ON EPITHELIALIZATION TIME

Wound Index Estimation: The wound index data showed that the environment around wounded area was clean, without pus formation and complete absence of necrosis in methanolic root extract gel of *Arnebia benthamii* treated animals both in excision and incision wounds. The quality of healing was increased significantly as wound index decreased (89%) sharply ($p<0.05$) in comparison to control group animals **Fig. 4**.

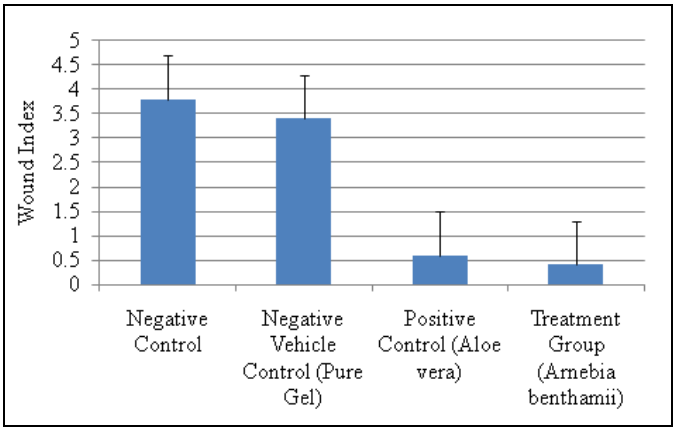

FIG. 4: EFFECT OF METHANOLIC ROOT EXTRACT OF *ARNEBIA BENTHAMII* ON WOUND INDEX

Incision Wound Healing: The methanolic root extract gel of *Arnebia benthamii* (10% w/w) treatment of incision wounds showed increased tensile strength (44%) in comparison to negative control group animals. The incision wounds were clean and free from pus and dead material around the wounded area without any necrotic symptom in experimental and positive control group animals. The wound strip preparations were showed highly significant increase ($p<0.01$) in tensile strength, which was obtained from incision wounds of experimental and positive control group animals when compared with negative control group animal wound tensile strength. The increase in % wound contraction, reduced epithelization time, and lower wound index in excision wounds with increased tensile strength in incision wound, which were treated by methanolic root extract of *Arnebia benthamii* and positive control *Aloe vera* gel showed the significant promotion in wound healing of excision and incision wounds **Fig. 5**.

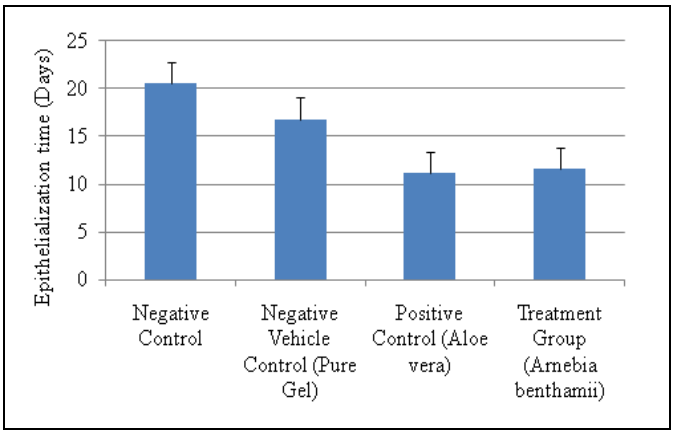

FIG. 5: EFFECT OF METHANOLIC EXTRACT OF *ARNEBIA BENTHAMII* ON TENSILE STRENGTH

CONCLUSION: This study concluded that the methanolic root extract of *Arnebia benthamii* or Ratanjot possesses significant wound healing potential which confers the claim of wound healer plant in Himalayan region. The research data also suggest that *Arnebia benthamii* can be a better choice for polyherbal wound healer preparation.

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CONFLICTS OF INTEREST: We declare no conflict of interest for this research.

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