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Research Article

EVALUATION OF *Salix acmophylla* LEAVES OINTMENT FOR FULL THICKNESS SKIN WOUND HEALING IN RABBITS

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Abstract: In present study, four full thickness excisional skin wounds (1.5×1.5 cm2), 2.5 cm apart from each other was created (dorsal spine of thoraco-lumbar region) on each of 18 rabbits (three groups having six animals) of either sex and weighing 1.8 to 2.5 kg under standard anaesthetic protocol to evaluate the wound healing potential of *Salix acmophylla* leaves ointment and their response on wound morphometry and histomorphological parameters. The total number of wounds evaluated in the study was 72 and thus each treatment groups was evaluated on 24 wounds (6 wounds per treatment). The experimental study was conducted in three groups (I, II and III). Group I animals were treated with sterile normal saline solution (negative control). Groups II and III were dressed topically with thin layer of 5% Povidone iodine and 5% *Salix acmophylla* leaves ointment, respectively. Povidone iodine treated wounds were used as a positive control. Rabbits were evaluated for gross examination, wound morphometry and histomorphological examination. No physiological side effects and other complications were observed in any group. It was interesting to note on day 7 after post-wounding that there was appreciable reduction in size of wound treated with *Salix* and Betadin as compared to control groups. Furthermore, complete filling of the wound with granulation tissue without scab and demonstrable wound contraction was noticed in *Salix* on day 14 post-wounding that Betadin and normal group. Complete epithelialization and closure of *Salix* and Betadin treated wounds could be distinguished on day 21 and percentage healing 100.00% and 95.66% respectively were observed, whereas in NS complete healing was noticed in only 61.00% of wounds. Grossly, wounds treated with 5%, *Salix acmophylla* leaves ointment significantly accelerated the rate of wound healing compared to wounds treated with sterile normal saline solution or dressed with Povidone iodine ointment. Overall results indicated that there were wounds dressed with *Salix acmophylla* leaves ointment (group III) showed considerable signs of full thickness skin wound healing and significantly($p<0.05$) healed earlier in 17.83days followed by Povidone iodine treated wounds (group II) in 19.00 days than wounds dressed with sterile normal saline solution (group I) in 20.83 days. These results strongly document the beneficial and significant effects of *Salix acmophylla* leaves ointment for the acceleration of wound healing enclosure in rabbits.

Keywords: Wound healing, Full-thickness wounds, *Salix acmophylla*, Gross examination, Wound morphometry, Histomorphological study, Rabbits

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Introduction

A wound may be defined as disruption of the cellular or anatomical continuity of the normal organ structure [1]. It is a common and frequent reason for seeking veterinary attention. The basic principle of optimal wound healing is to minimize tissue damage and provide adequate tissue perfusion and oxygenation, proper nutrition and moist environment to restore the anatomical continuity and function of the affected part [2,3]. Cutaneous wound repair involves migration, infiltration, proliferation, and differentiation of several cell types like keratinocytes, fibroblasts, endothelial cells, macrophages and platelets which culminates in an inflammatory response, the formation of new tissue and wound closure [4]. Veterinary practitioners often encountered animals with traumatic wounds that are infected, too large to close immediately or both. The way in which wounds are managed can affect the rate of healing, the time to return to normal function, the final cosmetic appearance, and hence the satisfaction of customers [5]. The management of wounds depends on the stage of wound healing and can include irrigation, mechanical and chemical debridement, use of antiseptic and antimicrobials, adherent and non-adherent dressings, and miscellaneous topical applications. In many cases, the best way to treat such wounds is to manage them as an open wound [6]. Consequently, there exists a need for new agents which may be useful in proper wound management. In this direction a number of herbal products are being reported on the use of various indigenous medicinal plants to possess wound healing, anti-inflammatory, antibacterial, antifungal and analgesic properties and have been used for therapeutic purposes [7]. The indigenous herbal drug preparations contribute sufficient increasing interest in wound healing alternative to conventional antiseptic, antibiotic and anti-inflammatory agents [8]. It is therefore, important that the dressing used should not promote the growth of harmful bacteria and at the same time it should not have deleterious effect on the tissue. Therefore, products selected to create a healing environment must be chosen thoughtfully and scientific rationale must support their use. The over usage of synthetic drug resulting in higher incidences of adverse drug reactions have provoked mankind to go back to nature for safer remedies.

Nature provides a wide variety of plants that contain medicinal properties. The powerful ingredients found in the stems, leaves, roots, flowers, and seeds of certain plants have natural healing properties that have been found to cure various diseases. Herbal medicines (phytomedicinals) are the outcome of therapeutic experiences of generations of practicing physicians of indigenous systems of medicine for over hundreds of years. Herbal drugs being much less expensive than their synthetic counterparts also have better cultural acceptability, better compatibility with the human body and minimal side effects [9,10]. The herbal drugs have been used throughout the world and have received greater attention in recent times, because of their diverse nature of curing diseases, safety and tolerance compared to conventional drugs. The utilization of herbal drugs is on the flow [11]. Herbal drugs have been used for wound healing in either crude form or in the form of different extracts. According to the World Health Organization (WHO), the use of herbal drugs throughout the world has increased tremendously [9]. In India, medicines based on herbal origin have been the basis of treatment and cure for various diseases [12]. Plants are exploited in many ways such as food, fodder, fuel wood, timber wood, medicinal, etc [13]. The therapeutic use of medicinal plants has gained a considerable energy in the past few decades. It shows that there is a huge contact of human life with local flora as well as local flora influence human beings. Medicinal plants are also important for the livelihoods of deprived communities all over the world. Continual exposure to stressful conditions generates free radicals, which may over power the inbuilt protective mechanisms and cause tissue damage. There are reports that plants possessing free radical scavenging activity are known to have anti-inflammatory, anti-tumour, wound healing and many other activities. Of these, *Salix* spps. is distributed throughout tropical and sub-tropical parts of India, Sumathra and Java [14]. Species of *Salix* found in almost all regions of Kashmir as *Salix acmophylla*. Willow and *"bains/ Wir/ Veer Kani"* respectively, are common English and vernacular names for a number of sister trees of the genera *Salix* representative of family Salicaceae. These are fast growing and yet medium-sized deciduous trees. It grows primarily in the cool, fertile, irrigated lands as it requires larger quantities of water, though it can withstand cold winter frost [15]. As per the Unani system of medicine the leaves of willows give "*cold dry*" effect while the flowers display "cold wet" effect. *Salix* plants contain a wide variety of compounds called phytochemicals, mainly described as those compounds having medicinal properties. Scientists have identified thousands of phytochemicals, although only a small fraction has been studied closely [16]. The active ingredient of the *Salix* bark is called salicin. Salicin hydrolyzes in aqueous media to glucose and salicylic alcohol (saligenin). *Salix* spps. have abundant watery bark sap, which is heavily charged with salicylic acid. Besides, salicin, it contains flavonoids and proanthocyanidins, which are potent wound healing agents [16]. Experimental surgery offers the freedom of biopsying wounds, doing histopathological and histochemical studies, which facilitates objective evaluation of progress of wound healing. The Indian traditional system of medicine is based on pragmatic facts of the observations and the experience over millennia. More than 1200 diseases are mentioned in different classical texts. Traditional medicine, being a significant element in the cultural patrimony, still remains the main choice for a large majority of people for treating various diseases and ailments. From the review of literature, it is clear that experimental wound healing potential for the *Salix acmophylla* leaves selected seems to be fewer and needs further investigation. This necessitates the exploration of this herb *Salix acmophylla* leaves ointment for its efficacy in wound healing.

Materials and Methods

Animals of the study

This study was permitted by Institute Animal Ethics Committee (IAEC), Sher-e-Kashmir University of Agricultural Sciences and Technology, Faculty of Veterinary Sciences and animal Husbandry, Jammu and Kashmir, India. Twenty four clinically healthy rabbits of either sex, adult with their body weight ranging between 1.8 to 2.5 kg were used for the study. The animals already tagged and housed individually in cages. The animals were provided with commercial diet and water ad libitum, maintained under uniform conditions and acclimatized to approaching and handling for a period of 5-10 days prior to the study.

Preparation of *Salix* leaves ointment

The leaves [Fig-2] of the plant [Fig-1] *Salix* collected were dried and crushed into coarse powder. For topical application 5% w/w ointment will be prepared in Vaseline base.

Fig-1 *Salix acmophylla* plant

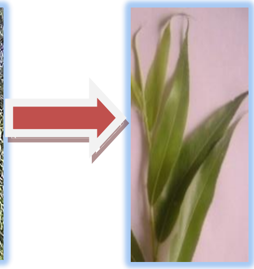

Fig-2 *Salix acmophylla* leaves

Grouping and preparation of animals

The rabbits were randomly divided into 3 groups, of 6 animals each. The animals were weighed and subjected to thorough physical and clinical examination before wounding. The wound was prepared for proper aseptic surgical procedures.

Induction of anaesthesia

Animals were kept off feed for 8 hours and water was withheld before surgery. Each animal was given xylazine dosed at 10 mg/kg I/M and left in calm environment for five minutes and then administered ketamine hydrocholoride dosed at 50mg/kg I/M [19]. Each animal was positioned in sterna recumbency and the dorsal area from the cranial aspect of the thorax to the lumbosacral region was prepared for aseptic surgery.

Surgical procedure

The animals were placed in sternal position on the operative table. The location of wound edges was outlined by a locally fabricated metal marker and millimeter ruler [Fig-3]. Four full thickness excisional wounds (1.5×1.5), 2.5 cm apart from each other was created using a no. 15 BP blade on either side of dorsal spine in the thoraco-lumber region [Fig-4]. The wounds were named as R1 and R2 on right side and L1 and L2 on left side. Haemorrahge, if any, was controlled by digital pressure. Each wound was cleaned with sterile normal saline solution and dressed with as per the scheduled therapy.

Fig-3 Metal Marker

Fig-4 Nomenclature of the wounds induced on right (R1 and R2) and left (L1 and L2) side

Fig-5 Marking the wound edges on cellophane paper

Therapy of wounds

All the wounds were washed with sterile normal saline solution followed by therapy as indicated below:

Table-1 *Therapeutic modalities used in different groups*

Group	No. of animals (No. of wounds)	Therapy
I	6 (24)	No treatment was given (Control)
II	6 (24)	Application of wound with 5% Povidone-iodine (Betadin) ointment till healing
III	6 (24)	Application of wound with 5% Salix leaves ointment till healing

Each treatment group consisted of 6 animals. Thus, each treatment was evaluated on a total of 24 wounds.

Evaluation of wound healing

Parameters to be recorded

The wound healing efficacy of therapy was evaluated on the basis gross examination, wound morphometry and histomorphological studies.

Wound Morphometry [17]:

Wound Size:

The wound boundaries were marked with Indian ink permanent market and tracing was taken on sterile cellophane paper before starting the treatment and subsequently on day 3rd, 7th, 14th and 21st [Fig-5]. These tracings were placed on graph paper and wound area was calculated.

Percentage Healing: The evaluated surface area was used to calculate the percentage healing using the below formula:

$$H = \frac{A - B}{A} \times 100$$

Where,

H = Percentage healing.

A = Area of wound at the beginning of the experiment.

B = Area of wound at the end of particular period.

Percentage healing was calculated on the day of creation and subsequently on day 3rd, 7th, 14th and 21st.

Histomorphological examination: The biopsy samples were collected from each group on day 7th, 14th and 21st of wounding and preserved in 10% formalin solution followed by routine procedure for histomorphological examination. Paraffin embedding technique was used and 5-6μ thick sections were cut and stained using H & E stain [18] and Mason's Trichrome stain [19]. Time required for wound healing: It was recorded on the day on which wound healed completely.

Statistical Analysis

All results were expressed as Mean ± standard error. The data was analyzed using the suitable statistical program for Social analysis 16 for Windows software (SPSS Inc, Chicago, IL) [20]. One-way Analysis of Variance (ANOVA) and multiple Duncan range tests were used to compare the means at different time intervals among different group. A value of P<0.05 was considered significant.

Results

Creation of excisional wounds resulted in variable extent of bleeding and formation of clot. The clot was dried and formed a cover over the wounds, which rendered the evaluation of colour of granulation tissue difficult. The scab, which covered the underlying granulation tissue, was detached in majority of the wounds after 14th post-wounding days. The scar became paler with passage of time, which indicated stage of maturation. Gradual decrease in the size of the wounds and increase in percent wound contraction (healing) was recorded in all the groups up to day 21 post-wounding. It was interesting to note on day 7 after post-wounding that there was appreciable reduction in size of wound treated with *Salix* and Betadin as compared to control group/wounds. Furthermore, complete filling of the wound with granulation tissue without scab and demonstrable wound contraction was noticed in *Salix* on day 14 post-wounding that Betadin and normal group. Complete epithelialization and closure of *Salix* and Povidone iodine treated wounds could be distinguished on day 21 and percentage healing 100.00% and 95.66% respectively was observed, whereas in NS complete healing was noticed in only 61.00% of wounds. The Mean±SE values of wound size (cm^2) in animals of different groups at different observation intervals are depicted in [Table-2] and [Fig-6]. Post wounding a significant (p<0.05) decreasing trend in the area of wound size was noted in all the groups on all observation intervals. The complete closure of cutaneous excisional wounds was noted by day 21 in the animals of group III. Comparison among the groups revealed significant (p<0.05) decrease in the wound size in the animals of treated groups (II and III) from those of control group (I) on all corresponding observation intervals. Furthermore, the wound size value was significantly (p<0.05) lower in the animals of groups II and III from those of group I on all corresponding observation intervals from day 3 to 21 post wounding.

Table-2 *The Mean±SE values of wound size (cm2) in the rabbits of different groups at different observation intervals*

Group	Observation Intervals (Days)				
	0	3	7	14	21
I	2.50±0.04aE	1.76±0.08bD	1.33±0.05cC	1.05±0.08cB	0.62±0.04cA
II	2.47±0.04aE	1.45±0.05aD	0.83±0.01bC	0.69±0.01aB	0.09±0.04bA
III	2.51±0.02aE	1.50±0.06aD	0.73±0.01aC	0.38±0.04aB	0.00±0.00aA

Figures with different superscript (capital letters) differ significantly (P<0.05) between days within the groups; Figures with different superscript (small letters) differ significantly (P<0.05) between groups; n = 6 animals in each group

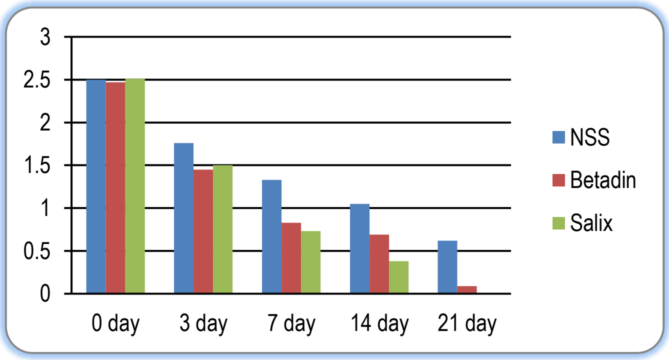

Fig-6 Effect of different therapy on wound size (cm2) in the rabbits of different groups at different observation intervals

The Mean±S.E values of percentage healing (%) in animals of different groups at different observation intervals are depicted in [Table-3] and [Fig-7]. Post wounding the percentage healing increased significantly (p<0.05) in all groups, at all observation interval with the result cent percent healing was achieved in the animals of group III by the end of the observation period. The healing was still incomplete in the animals of group I and II by the end of the observation period. Comparison among the group showed significant (p<0.05) increase in percentage healing of all treated wounds (groups II and III) as compared to control wounds (group I). Among treated groups percent wound healing was significantly (p<0.05) faster in the animals of group III as compared to the animals of group I and II.

Table-3 *The Mean±SE values of percentage healing (%) in the rabbits of different groups at different observation intervals*

Group	Observation Intervals (Days)				
	0	3	7	14	21
I	0.00±0.00aA	12.50±0.54aB	25.50±1.04aC	51.83±0.75aD	61.00±1.67aE
II	0.00±0.00aA	15.00±0.89bB	45.50±1.04bC	71.83±0.75bD	95.66±1.63bE
III	0.00±0.00aA	16.00±2.09bB	55.50±1.04cC	81.83±0.75cD	100.00±0.00cE

Figures with different superscript (capital letters) differ significantly (P<0.05) between days within the groups; Figures with different superscript (small letters) differ significantly (P<0.05) between groups; n = 6 animals in each group

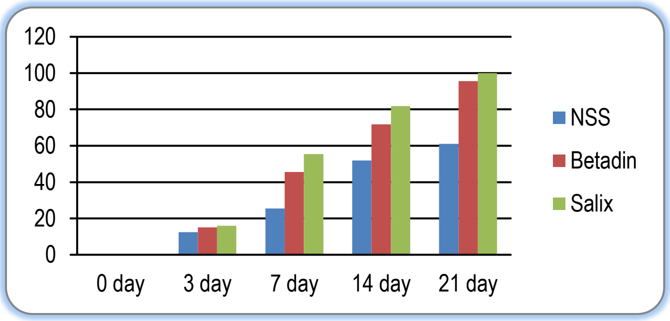

Fig-7 Effect of different therapy on percentage healing (%) in the rabbits of different groups at different observation intervals

Gross appearance of wounds of different rabbits of different groups on different intervals is shown in [Plate-1]. By day 7th post-wounding, granulation tissue associated with admixture of mononuclear and polymorphonuclear cells was seen in *Salix* treated wounds which was thicker than adjacent skin [Plate-4]. The Povidone iodine treated wounds showed mild to moderate inflammatory reaction with predominant heterophilic infiltration (3), underneath the scab, whereas wounds treated by NSS group showed necrosed tissue having pus cells underneath the scab [Plate-2]. By day 14 post-wounding *Salix* treated wounds showed marked granulation tissue associated with vascularisation and heterophilic infiltration [Plate-4].

The Povidone iodine treated wounds showed granulation tissue with fibroblasts proliferation varying moderate to predominant [Plate-3], whereas wounds treated by NSS group showed severe heterophilic infiltration [Plate-2]. By day 21 post-wounding, wounds treated with *Salix*, showed no inflammatory reaction had denser and thicker collagen fibres in granulation tissue and are partial to complete epithelialization tendency to form hair follicle similar to normal skin [Plate-4]. The Povidone iodine treated wounds dense collagen deposition with partial epithelialization was observed [Plate-3], whereas wounds treated with NSS showed predominant amount of collagen deposition associated with partial to complete but immature epithelialization [Plate-2]. Wounds dressed with *Salix acmophylla* leaves ointment (group III) showed considerable signs of full thickness skin wound healing and significantly (P<0.05) healed earlier in 17.83 days followed by Povidone iodine treated wounds (group II) in 19.00 days than wounds dressed with sterile normal saline solution (group I) in 20.83 days [Table-4].

Table-4 *Mean±S.E. of time required for wound healing*

Group	Healing time (days)
I	20.83± 0.40a
II	19. 00± 0.89b
III	17.83± 0.75c

Means with different superscripts within a column were statistically significantly different (P<0.05)

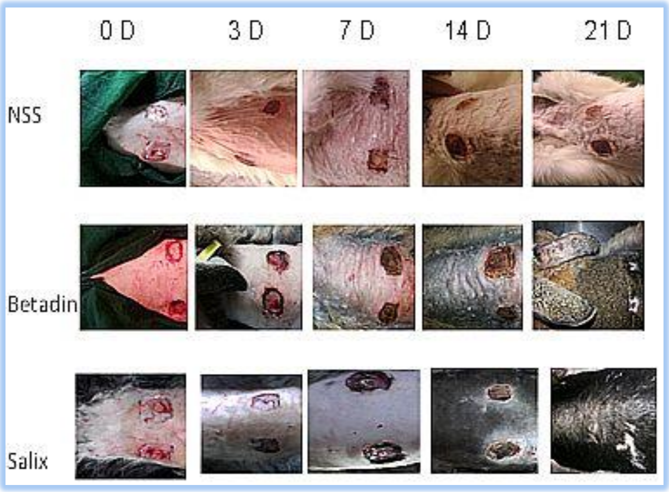

Plate-1 Gross appearance of wounds of different rabbits of different groups on different intervals

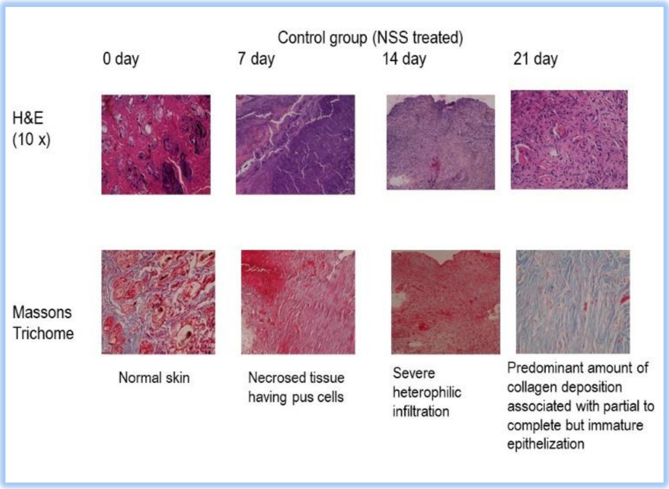

Plate-2 Histomorphology of wounds of different rabbits of Normal Saline Solution (NSS) treated on different intervals

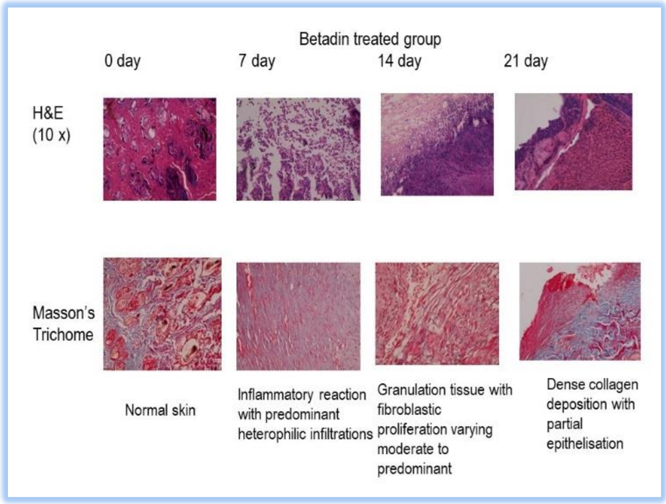

Plate-3 Histomorphology of wounds of different rabbits of Povidone Iodine (betadin) treated on different intervals

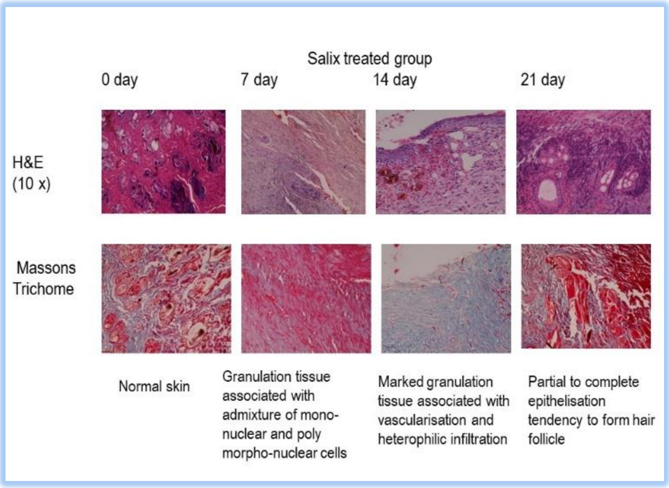

Plate-4 Histomorphology of wounds of different rabbits of *Salix* treated on different intervals

Discussion

Rabbits provide useful models for studies designed to develop new bandage materials [21] or to investigate novel therapies, such as cell therapy [22, 23], Platelet concentrates [7] and laser therapy [24, 25]. In the present study rabbits were used for evaluation of wound healing efficacy of *Salix acmophylla*. The rabbits used were of either sex, aged between 1.8-2.5 kg. Animals were found easy to handle and wounds were created without any problem. These findings match with those of [26] findings. Larger wounds of 1.5 x 1.5 cm dimensions could be created due to availability of larger body surface area of animals owing to their more body size. Different wound models have been used for evaluation of wound efficacy in the previous studies which include incisional [27], excisional [28, 17], full-thickness wounds [29], partial thickness wounds [30]. Full-thickness wounds proved to be better and easier to create. Harvested wounds can be examined histologically for both the epithelial gap and granulation bed characteristics [31]. Different researchers have used different techniques and instruments for creation of wounds e.g., metal marker [17], laser technique (32), tracing paper [28]. Also, different shapes of wounds have been created in previous studies which include triangular, circular, rectangular, square shapes by different workers. Circular wounds, however, contracted at a perceptibly slower rate; because it is argued that they present a greatest resistance to the contractile forces. In order to have a suitable model for evaluation of *Salix acmophylla* on wound healing in present study full-thickness 1.5 x 1.5 cm square excisional of wounds e.g., epidermis and dermis [33]. 2.5 cm apart and two wounds on each on each side e.g. total four wounds per animal. The animals were kept in sterna recumbency easily reproducible position, the outline of the intended wound was first marked accurately on the prepared skin using an instrument made according to the required measurements by placing it on position and marking the outline using BP blade. This was then incised down through the epidermis and dermis. The full thickness of the skin within the incision was then carefully stripped away by sharp dissection from its underlying muscle and discarded [34]. In the rabbit, the healing process involves maximum wound contraction, prior to the initiation of the cell migration and matrix remodelling [35]. Few safe and effective anaesthesia regimens have been described for use in rabbits, partially because of the susceptibility of this species to sometimes fatal respiratory depression. Although inhalant anaesthetics are generally safer than injectable anaesthetics, their use may be limited by lack of equipment or facilities. Different anaesthetic regimes have been evaluated in rabbits under laboratory conditions. Many drugs are used intramuscularly in rabbits in combinations such as Ketamine-Xylazine, Xylazine-ethyl-(1-methyl-propyl) malonyl-thio-urea salt, ketamine-EMTU, Xylazine-acepromazine-ketamine and ketamine-chloral hydrate are used in combination with one another [36]. In present study Ketamine- Xylazine combination was used. Ketamine at the dose rate of 50mg/kg body weight intramuscularly and Xylazine at the dose rate of 10 mg/kg body weight intramuscularly. This combination used gave the wanted results. The dose used created the suitable depth and duration of anaesthesia as required for the surgical manipulation. The animal remainded anaesthetized up to the completion of the surgical procedure (30 minutes) and no maintenance dose was required. The combination produces adequate analgesia and muscle relaxation. There was no complication of anaesthesia and surgery observed, as the standard protocol was followed in rabbit excisional skin wounds, which is in accordance with previous studies conducted by [17].

The wound edges and the skin surrounding the wound area give important clue about the gross appearancce of the wound. In the current study, on day 0 the wound edges were well-defined and not-attached to wound base in any group. Skin surrounding the wound area showed normal colouration in the entire groups throughout the study period. Post-wounding the edges changed from well defined to diffuse and barely visible. These changes were faster in treated groups than in control group. In case of positive control wound treated groupPovidone iodine solution was used. Povidone-iodine also allowed the normal healing of wound edges when applied to the surgical wound and is reported to be safe from the standpoint of wound healing, and also decreases the number of wound infection [37]. *Salix* treated wound showed faster improvement in wound edge status as compared to control group. There is a priority of nature inherent in healing. Factors critical to survival such as phagocytosis, blood flow and surface covering occur early in wound healing. The wounds in which the edges are not together because of extensive tissue loss or large surface areas those wounds heal by secondary intention. These wounds heal slowly through the process of granulation and epithelialisation [38].

Inflammation is an immune response to cellular/tissue injury or infection by pathogens. It is clinically characterized by features such as redness, warmth, swelling, and pain. The process itself is not considered a disease, but failure to contain it and successfully resolute it in a timely fashion results in exacerbation of tissue damage and modulation of cell signalling pathways [39]. Inflammation involves a portfolio of cellular and molecular components collectively referred to as inflammation mediators. If there is no inflammation, healing does not begin. If too little inflammation occurs, healing is slow. If too much inflammation occurs, excessive scar is produced (40). In current study all the groups showed signs of inflammation on day 3 post-wounding; eventually it subsided in all the groups by the end of the study period. This was faster in *Salix* treated wounds than the Betadin treated wounds followed by control group. Extent of granulation tissue means of repair for all vertebrates. Special cells in our body respond to injury by forming a collagenous “glue”. This body glue is called granulation tissue [40]. In full-thickness wounds the epidermis can resurface from the margins only after adequate granulation tissue has formed [41]. In current study the extent of granulation tissue increased in all the treated groups form day 0 post-wounding till 7 except control group (on day 14). On day 21 post-wounding only, *Salix* treated wounds showed areas with intact skin or partial to complete thickness. As healing proceeds, the newly formed granulation tissue which is red due to blood vessels fades with healing because the vessels disintegrate due to apoptosis [42]. Therefore, healing is better in *Salix* treated wounds as the granulation tissue was complete as early as day 14 and then started fading and normal skin started appearing. This could be due to early completion of inflammatory phase in *Salix* treated wounds and change to proliferative phase which constitute formation of new blood vessels and increased fibroblastic proliferation which together constitute granulation tissue [41]. Cicatrisation is the process of formation of scar. Matrix formation requires the removal of granulation tissue with revascularization. A framework of collagen and elastin fibres replaces the granulation tissue. This framework is then saturated with proteoglycans and glycoproteins. This is followed by tissue remodelling involving the synthesis of new collagen mediated by TGF-β. The final product of this process is scar tissue [42]. In the current study, the *Salix* treated wounds showed light pink, smaller and softened scar on day 7 post-wounding whereas the control group showed thicker and red wound areas. Towards the end of study period on 21, all wounds treated with *Salix* showed fade thin to white line appearance of wound site, whereas in Povidone iodine treated wounds showed softened scar formation and there is no clear cut scar formation in control group. The production of wound exudates is a normal part of the inflammatory process [43]. In present study no, measurable exudates were found in wounds treated with *Salix* and Betadin except control group.

Wounds have been shown to re-epithelialize more rapidly under moist conditions rather than dry conditions [44]. Increased production of exudates may be associated with high bacterial growth in the wound. Wounds treated with Povidone iodine as well as *Salix* are reported to possess antibacterial activity [45]. So, no infection was produced either in Betadin treated wounds or *Salix* treated wounds. However, the wound bed remained moist allowing proper nutrition and healing process although healing was better in *Salix* treated wounds due to its wound healing potential. In the present study though the original wound were created by measuring 1.5 x 1.5 cm area, almost all the wounds expanded to some extent possibly due to lose nature of skin on the dorsal thoracolumbar region of rabbits (*i.e*., flaccidity/pliability). Thus, immediately after the surgery, all the wounds had an area greater that 2.25 cm2. Therefore, the percentage of wound size (contraction) was calculated by measuring the actual size of wound on day '0' (not the predetermined area). Further, on day '0', difference in the mean wound area of all the treatment groups was statistically significant. As described by [46] and [17], the tracing of the wounds on transparency sheet/cellophane paper and graph paper gave accurate data regarding the size of the wounds and it was found cheap, reliable and easy to calculate the wound size (length and width measurements) and percentage healing from the tracings. It involved no hi-tech methods and also it was simple to calculate wound area by adopting this technique. These observations substantiate with the findings of [47]. Gradual decrease in the size of the wounds and increase in percent as wound contraction was recorded in both the treated groups up to day 21 post-wounding. Reduction in the wound size was minimal in group NSS. The wounds of *Salix* treated groups evinced complete healing (100% contraction) which was followed closely by Povidone iodine and distantly by NSS on day 21 post-wounding, where mean wound contraction/size of 100%. 95.66% and 61.00% respectively was recorded as observed by [30]. During this study wounds treated with *Salix* showed better wound healing followed by Betadin and then the sterile normal saline solution. This could be attributed to anti-inflammatory, anti-rheumatic, antipyretic, antidote, antigenic, antiseptic properties and wound healing agent of *Salix*. Enhanced healing activity was attributed to increased collagen formation and angiogenesis [48].Angiogenesis in granulation tissues improves circulation to the wound site thus providing oxygen and essential nutrients for the healing process with enhanced epithelial cell proliferation [49]. Contraction occurs simultaneously with granulation and epithelialization, but independent of epithelialization [50]. The percentage healing denoted the amount of wound contraction. Unlike epitheliazation, which closes the wound surface, contraction is a process that actually pulls the entire wound together, in effect shrinking the defect. Successful contraction results in a smaller wound to be repaired by scar formation [40]. Percentage healing varies as according to different observation intervals in each group significantly, depicting an increase in healing from start to the end of observation period. The percentage healing is more in wounds treated with *Salix* followed by Povidone iodine and then the sterile normal saline solution (NSS). Histomorphological analysis of healed wounds confirmed the gross observations. Healing of full thickness skin/excisional wound occurs by epithelialization and wound contraction. Epithelialization begins almost immediately (24 to 48 hours) in sutured wounds with good edge to edge apposition because there is no defect for granulation tissue to fill whereas in open wounds, epithelialization begins when an adequate granulation bed has formed (usually in 4 to 5 days) [50]. Wound contraction is mediated by myofibroblasts [51] which have the features of both fibroblasts and of smooth muscle cells and contain alpha-actin, which play major role in wound contraction [52]. In present study the macrophages showed a decreasing trend in all the groups towards the end of observation period. However, in control group the macrophage number remained higher on day 14 and 21 although comparatively lower in *Salix* treated wounds group. Similar findings were observed by [17], who reported that Rhuberb treated groups showed decreasing trend of macrophage score as compared to that of control group. Present study showed that the *Salix* treated wounds showed better healing than control. Granulation tissue is important for the healing of open wounds because it is extremely resistant to infection and serves as a barrier against systemic infection, provides a surface over which epithelium is able to migrate, plays a role in wound contraction and contains the fibroblasts that produce the collagen for wound healing [53]. [54], suggested that histopathological assessment of mode and rate of healing in open wounds allows more precision that clinical and high-resolution ultrasound, though it does not allow serial examination of wound sites. In the present study, however serial examination was possible as the biopsies were collected at different stage of wound healing. The biopsies were taken for each treatment on 7th, 14th and 21st post-wounding days which helped in providing evaluation of wound healing as reported by [55]. After routine histological processing biopsy samples were stained by H&E [18] to detect morphological changes and Mason's Trichrome stain (MTS) [19], to assess the collagen content of the healing wounds as collagen is a good marker for wound healing. In the initial stage of repair (immature scar) than collagen fibers are seen. There was decrease in formation of exudates in all the treatment groups as the days passed. However, *Salix* treated group resulted in faster reduction in wound exudates (serous type) and drying of the wound other than treatment groups by day 7, which was suggestive of accelerated wound healing [56, 57]. This might be due to the presence of active agents in the respective treatment which has helped in the early enhancement of wound healing. The differences between the groups were significant and from day 14 onwards treatment groups showed similar results with apparent drying of the wounds. Irrespective of the given, there was nil to moderate inflammation at the periphery of the wound of some of the animals on day 1 and day 2 while only in a few wounds by day 3 post-wounding, which subsided to nil on 7th day post-wounding. The slight swelling recorded in few cases may be considered as normal body response to trauma. The granulation tissue as seen grossly appeared in significantly lesser duration in wound treated with *Salix* than other treatment and control group. The findings were also supported by the histopathological findings of early maturation of granulation tissue in *Salix* treated group. Regardless of the treatments used, granulation tissue was below the level of skin between 7 and 14 and proliferated to the level of skin edges and completely filled the blood bed by day 21 post-wounding in Povidone iodine treated group whereas *Salix* treated group granulation tissue was proliferated to the level of skin edges by 14th day post- wounding. In the treatment groups, *Salix* and Povidone iodine treated the scab fell off the wound by 21 post-wounding days except NSS treated group. At this stage, the classical shiny, rose pink/pale red granulation tissue was seen indicating healthy healing progress as described by [54]. The scar colour changed from rosy pink to paler in the later stage, which reflected the progression of blood vessels during maturation and remodelling as suggested by [50]. By day 7th post-wounding, granulation tissue associated with admixture of mononuclear and polymorphonuclear cells was seen in *Salix* treated wounds which was thicker than adjacent skin.

The Betadin treated wounds showed mild to moderate inflammatory reaction with predominant heterophilic infiltration, underneath the scab, whereas wounds treated by NSS group showed necrosed tissue having pus cells underneath the scab. By day 14 post-wounding *Salix* treated wounds showed marked granulation tissue associated with vascularisation and heterophilic infiltration. The Betadin treated wounds showed granulation tissue with fibroblasts proliferation varying moderate to predominant, whereas wounds treated by NSS group showed severe heterophilic infiltration. By day 21 post-wounding, wounds treated with *Salix*, showed no inflammatory reaction had denser and thicker collagen fibres in granulation tissue and are partial to complete epithelialization tendency to form hair follicle similar to normal skin.

The Betadin treated wounds dense collagen deposition with partial epithelialization was observed, whereas wounds treated with NSS showed predominant amount of collagen deposition associated with partial to complete but immature epithelialization. Angiogenesis, fibroplasias and epithelialization mark the proliferative and maturation phase of wound healing [58]. The change from inflammatory to proliferative phase is marked by increased fibroblasts, increased accumulation of collagen in wound and increased angiogenesis. This change occurs at 3 to 5 days post wounding. In the present study the number of fibroplasias and angiogenesis remained higher in wounds treated with *Salix* group followed by Betadin and then control group on day 7 and 14 post-wounding. A massive angiogenesis allowing the supply of oxygen and nutrients is necessary for the healing process within the tissue [59]. The last stage of wound healing involves the gradual involution and regeneration of dermis.

Collagen content is one of the necessary parameter for determining the pharmacological effects of potential wound healing agents [60]. In present study the collagen content of wounds treated with *Salix* group remained highest throughout the observation period followed by wounds treated with betadine than by control group. Epithelialization showed an increasing trend towards the end of observation period, this trend being highest in *Salix* treated wounds than in Betadin treated wounds with control group showing least amount of epithelialization. Histopatholigcally, epitheliazation, angiogenesis and fibroplasias level were higher in *Salix* treated wounds on day 7, which indicates that *Salix* accelerate the inflammatory reaction and initiate early phase of wound healing. Since the time of collect biopsy sample was fixed to maximum of 21 days in the experimental design, it was not possible to follow the wound healing beyond 21 days to record the day of compare healing in sterile normal saline (control group) treated wounds. However, proportions of wounds with complete healing were calculated at each interval for each treatment. There was significant difference in neovascularization of wounds treated by *Salix* group followed by Povidone iodine treated wounds as compared to control group.

Conclusion

In conclusion, in the sterile normal saline treated wounds (control group), the epithelialization of the wound did not compare well with the normal skin, while in *Salix* treated wounds it compared well with the normal skin followed by Betadin treated wounds. The current study indicates that the dressing with *Salix acmophylla* leaves ointment, as topical application of wounds, enhanced wound healing process significantly in experimental rabbits. Furthermore, *Salix acmophylla* ointment can be used for the wound healing agents in field condition.

Application of research: It has a potential for newer wound healing agent applications in the future.

Research Category: Veterinary Surgery & Radiology

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References

[1] Bennet R.G. (1988) In: Fundamentals of cutaneous surgery. St. Louis, C.V.Mosby, p.778.
[2] Pierce G.F. and Mustoe T.A. (1995) Annual Rev Med, 46, 467–481.
[3] Begum D. and Nath S.C. (2000) Spices Med Plants, 7, 55–93.
[4] Barrentios S., Stojadinovic O., Golinko M.S., Brem H. and Tonic-Canic M. (2008) Wound Repair Regen, 16, 585-601.
[5] Liptak J.M. (1997) Aust Vet J, 78, 408-413.
[6] Lee A.H., Swaim S.F., Yang S.T. and Wilken L.O. (1984) Amer J Vet Res, 45, 1487-1492.
[7] Myers K.A., Marshal R.D., Freidin. (1980) In: Principals of Pathology and Surgery, 1stEdn. Blackwell.Scientific publication, Oxford, Pp. 58-82.
[8] Ahmed I.H., Awad M.A., El-Mehdy M., Gohar H.M. and Ghanem A.M. (1995) Assiut Vet Med J, 32, 236-244 (cited from Vet Bull, 66, 388).
[9] Pal S.K. and Shukla Y. (2003) Asian Pacific J Cancer Prev, 4, 281-284.
[10] Venkatesh S., Reddy G.O., Reddy B.M., Ramesh M. and Appa Rao A.V.N. (2003) Fitoterapia, 74, 274-279.
[11] Kamboj V.P. (2000) Current Sci, 78 (1), 35-39.
[12] Biswas T.K.andMukherjee B. (2003).IntlJ LowerExtreWounds.2,25.
[13] Hussain F. and Khaliq A. (1996) Proceedings of first Training Workshop on Ethnobotany and its application to conservation. NARC, Islamabad, pp. 207-215.
[14] Kirtikar K.R. and Basu B.D. (1975) In: Indian medicinal plants. 2ndedn, international book distributor, Dehradun, India, Pp.377-378,894- 895.
[15] Rather T.A., Qaiser K.N. and Khan M.A. (2010) Ind J TradiKnowl, 9, 403-406.
[16] Zarger M.S.S., Khatoon F. and Akhter N. (2014) World J Pharm Pharmacol, 3, 1320-1330.
[17] Handoo N., Parrah J.D., Moulvi B.A., Ansari M.M., Athar H., Imtiyaz T., Nabi N., Siraj G. and Malik Z.R. (2014) Intl J Vet Sci, 4(2), 50-54.
[18] Luna L. (1968). In: Manual of histologic staining methods of the FIP, 3rd eds. Tata McGrow Hill, New York, p.76.
[19] Masson P.J. (1929) JTechn Methods, 12, 75-90.
[20] Snedecor G.W. and Cochran W.G. (1989) In: Statical methods,6th Ed. The lowa state univer.press Amer. Lowa, U.S., pp; 118-56.
[21] Cangul I.T., Gul N.Y., Topal A. and Yilmaz R. (2006) Vet Dermatol, 17, 417-423.
[22] Sumiyoshi K., Nakao A., Seoguchi Y., Etoguchi K., Okumura K. and Ogawa H. (2004) J Invest Dermatol, 123, 229-236.
[23] Sandeep S., Maiti S.K., Ansari M.M., Kumar N. and Balwada A.K. (2015) Interl J Vet Sci, 3(3), 129-134.
[24] Brosh T., Simhon D., Halpern M., Ravid A., Vasilyev T., Kariv N., Nevo Z. and Katzir A. (2004) Laser Surg Med, 35, 12-17.
[25] Simhon D., Brosh T., Halpern M., Ravid A., Vasilyev T., Kariv N., Katzir A. and Nevo. Z. (2004) Laser Surg Med,. 35, 1-11.
[26] Ramos M.M., Gragnani A. and Ferreira L.M. (2008) J Burn Care Res, 29, 363-368.
[27] Asif A., Kakub G., Mehmood S., Khumun R. and Gulfrajm M. (2007) JPhytother. pp.53-82.
[28] Madhu D.N. (2012) MVSc Thesis IVRI, Uttar Pradesh, India. pp.101.
[29] Nisbet H.O., Nisbet C., Yarim M., Guler A. and Ahmet O. (2010) Wounds. 22, 275-283.
[30] Stephen C., Davis B.S., William H., Eaglstein M.D., Alex L., Cazaniga B.S., Patricia M. and Mertz B.A. (2001) Dermatol Surg, 27, 783-788.
[31] Wang Y., Huang X., Liang Q., Fan R., Qin F., Luo J., Li Y., Liu W., Mao X., Ji H., Liu Z., Zhou H., Fan L. and Gao L. (2011) J Med Plants Res,5(13), 2879-2885.
[32] Manolis E.N., Kaklamanos I.G., Spnaakis N., Filippou D.K., Panagiotaropoulos T., Tsakris A. and Siomos K. (2007) Wound Repair Regen, 15, 252-257.
[33] Lemo N., Marignac G., Rayes-Gomez E., Lilin T., Crosaz O. and Ehrenfest D.M.D. (2010) VeterinarskiArhiv, 80, 737-652.
[34] Billinghama R.E. and Russell P.S. (1956) Skin Annals Surg, 76, 45-67.
[35] Abramov Y., Golden B., Sullivan M., Botros S.M., Miller J.J., Alshahrous A., Goldberg R.P.S. and Sand P.K. (2007) Wound Repair Regen, 15, 80-86.
[36] Hobbs B.A., Rulhal T.G., Sprenkel T.L. and Anthony K.L. (1991) Amer J Vet Res. 52, 669-674.
[37] Viljanto J. (1980) Archive Surg, 115, 253-256.
[38] Shipperley T. and Martin C. (2002) *Nursing Times Net*, 98, 54.
[39] Serhan C.N. and Savil J. (2005) Nature Immunol, 6, 1191-1197.
[40] Hardy M.A. (1989) Phys Therapy, 69, 1014-1024.

[41] Slatter D. (2003) In: Wound repair. In: Textbook of small animal surgery. 3rd Eds. Reprint, pp.66-72.
[42] Konodo T. and Ishida Y. (2010) Foren Sci Interl, 03, 93-98.
[43] Thorman M. (1997) TopicGeriatic Rehab, 13, 33-43.
[44] Dyson M., Young S.R., Hart J., Lynch J.A. and Lang S. (1992) J Invest Dermatol, 99, 729-733.
[45] Ali M.R. and Aboud A.S. (2010) Ibn al- HaithamJ Pure AppliedSci, 23, 3.
[46] Bigbia R.B., Schumacer J., Swaim S.F., Purohit G.C. and Wright J.C. (1991) Ame J Vet Res, 53, 137.
[47] Bohling M.W., Henderson R.A., Swaim S.F., Kincaid S.V. and Wright J.C. (2004) Vet Surg, 33, 579-587.
[48] Bonte F., Dumas M.,Chadgne C.andMeybeck A. (1993) Planta Medica,60,133-135.
[49] SugunaL.,Sivakumar P.andChandrakasan G. (1996) Ind J Exp Biol,34,1208-1211.
[50] Fossum T.W., Hedlund C.S., Johnson A.L., Schulz K.S., Seim H.B., Willard M.D., Baer A. and Carroll G.L.(2007) In: Manual of Small Animal Surgery, Mosby, pp. 150-175.
[51] Yamaguchi Y. and Yoshikano K. (2001) J Dermatol, 28, 521-534.
[52] Swaim S.F. and Henderson K.A.(1990) In: Small animal wound management. Lea and Febiger, Philadelphia. Pp. 1-33.
[53] Abramo F., Mantis P., Lyoyd, D.H., Auxilia S.T., Noli C., Leotta R. and Pfeiffer D. (2004) J Dermatol, 15, 13.
[54] Gupta S.K., Singh, H., Varshney, A.C., Prakash P. and Singh S.P. (1993) Ind Vet J, 70, 45-47.
[55] Tyagi R.P.S. and Singh J. (1993) In: Ruminants surgery, CBS publishers and Distributors. New Delhi, p 39.
[56] Ansari M.M. (2014) In: Fundamentals of General Veterinary Surgery, First edition, published by Satish Serial Publishing House, Delhi-110033 (India), PP.148-178.
[57] Clark R.A. (1993) Dermatol Clin, 11, 647-666.
[58] Singer A.J. and Clarke R.A.(1999) New Engl J Med, 341, 738-746.
[59] Jimenez P.A. and Rampy M.A. (1990) J Surg Res, 81, 238-242.