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Evaluating the effect of wound healing and antioxidant property of different extract ointments and trace elements present in *Salix acmophylla* leaves on full thickness excisional skin wounds present in rabbits.

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ABSTRACT

The search for "natural remedies" has drawn attention to herbal drugs and plants in todays world. *Salix acmophylla* leaves have been reported to possess high levels of Proanthocyanidins or condensed tannins which are a group of biologically active polyphenolic bioflavonoids that are synthesized by not only *Salix* spp. but by many plants and also it has been reported that *Salix* spp. contain high level of trace elements mostly zinc and copper. These Proanthocyanidins, tannins, zinc and copper altogether act as antioxidants and facilitate wound healing as these elements are used by the body to make free radical enzyme scavengers, which neutralize the free radicals thus helps in facilitating and inducing VEGF expression, a key element supporting wound angiogenesis. Strategies to manipulate the redox environment in the wound are likely to be of outstanding significance in wound healing process. The four most important enzymes that neutralize the free radicals are the superoxide dismutase (SOD) enzyme, methionine reductase, catalase, and glutathione peroxidase. Thus the present study aimed to estimate the concentration of Catalase (IU/mg of skin tissue) in the skin tissue using spectrophotometer at 240nm and levels of zinc (µg/ml of serum) and copper (µg/ml of serum) using atomic absorption spectroscopy (AAS) method at 213.9nm and 324.8nm respectively in rabbits after creation of excisional full thickness skin wound and post treatment with the 5% ethanolic and 5% aqueous extract ointment group (6 animals each) and comparing the results with the control group (6 animals). The highest concentration of Catalase (CAT) enzyme and trace elements i.e Zinc and Copper was seen in 5% ethanolic extract ointment treated group with the fastest wound closure in 14.50± 0.42 days followed by 5% aqueous extract ointment treated group in 17.16± 0.30 compared to control group 20.16 ± 0.30.

Keywords**:** *5% aqueous extract, 5% ethanolic extract, Atomic Absorption Spectroscopy (AAS), Catalase (CAT), Copper (Cu), Polyphenolic bioflavonoids, Proanthocyanidins, Salix acmophylla, Tannins, Vascular endothelial growth factor (VEGF), Wounds, Zinc.*

1. INTRODUCTION

A wound may be defined as disruption of the cellular or anatomical continuity of the normal organ structure (Bennet, 1988).The basic principle of optimal wound healing is to minimize tissue damage and provide adequate tissue perfusion, oxygenation, proper nutrition, moist environment to restore the anatomical continuity and function of the affected part (Pierce and Mustoe, 1995; Begum and Nath, 2000). Cutaneous wound repair involves migration, infiltration, proliferation and differentiation of several cell types like keratinocytes, fibroblasts, endothelial cells, macrophages and platelets which culminate in an inflammatory response, the formation of new tissue and wound closure (Phillips *et al.*, 1991; Barrentios *et al.*, 2008). Wound healing is a complex multifactorial process that results in the contraction and closure of the wound and restoration of a functional barrier. Repair of injured tissues occurs as a sequence of events, which includes inflammation, proliferation, and migration of different cell types. The reactive oxygen species (ROS) are deleterious to wound healing process due to the harmful effects on cells and tissues. Free-radical scavenging enzymes (FRSE) are a cytoprotective enzyme group that has an essential role in the reduction, deactivation, and removal of ROS as well as regulating the wound healing process. Also, this complex phenomenon involves self-generated autocoids and hormones working in a systematic synchrony leading to wound healing (Meenakshi *et al*., 2006). Plants are a rich source of phytochemicals, which can have wound healing and antioxidant properties. Depleted levels of various antioxidants may contribute to delayed healing. (Pascoe *et al.*, 1987) Several indigenous drugs have been described in folkloric Indian medicine for the management of cuts, bruises, burns, and wounds. Various workers have reported wound healing properties of some plants among them the chosen ornamental plant *Salix acmophylla* that is found in almost all regions of Kashmir and locally known as Wir/ Veer Kani (Rather *et al.*, 2010). This *Salix* grows primarily in the cool, fertile, irrigated lands (as it requires larger quantities of water), and can withstand cold winter frost, reported to possess strong anti-inflammatory property and is used as astringent, antiseptic, antipyretic, analgesic and cardiotonic in Indian System of Medicine (Kallman, 1994; Chopra *et al.*, 1996; Bhattcharhjee, 1998). Willow (*Salix spps*.) are the source of the natural precursor to asprin, salicylic acid, found in its leaves and bark (Pojar and Mackinnon, 1994). The bark and leaves can be pounded and applied to wounds as healing agents (Moerman, 1998). 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 antioxidants and have wound healing properties. *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 have been studied thoroughly (Zarger *et al*., 2014). *Salix acmophylla* contains copper and zinc at high levels (Ali and Aboud, 2010).These elements have important role in wound healing as they increase the expression of vascular endothelial growth factor and are potent antioxidants(Chandan *et al*., 2002). The result of the phytochemical screening for *Salvia officinalis* and *Salix acmophylla* reveals a moderate concentration of alkaloids, coumarines, cardiacglycosides, ratenges, phenols, flavonoids, saponins, tannins, essential oil and terpenes. Some of these chemical compounds have been associated to have antibacterial activities, some have antioxidant activity and some to have curative properties against pathogens (Nweze *et al*., 2004). So, the present study is focused on wound healing activity of *Salix acmophylla* leaves to justify its traditional use and antioxidant activity, and also studied the mechanism behind the wound healing activity.

II MATERIALS AND METHODS

2.1 Plant material

The leaves of *Salix acmophylla* were collected from the fields surrounding Faculty of Veterinary Sciences, Kashmir (J&K), India, in the month of May and identified by Department of Botany, Kashmir University ,Hazratbal .

2.2 Preparation of extracts and extract ointments

The leaves of the plant *Salix spp* collected were shade dried for a week followed by drying in oven pre-set at 37° C for 4 days, the samples were powdered in electric mill and stored in airtight container. The fine powder of leaves was extracted with boiled distilled water and Ethanol respectively by Soxhlet apparatus for 7hrs. The solution was filtered through Whattman filter paper using Buchner funnel under vacuum evaporator at 40° C to obtain the extract . Then the resulting extract was stored , protected from light in refrigerator at 4° C in a glass container till further use. Ethanolic and Aqueous extract of *Salix acmophylla*(5g) was mixed with simple ointment (soft paraffin) (95g) to get a 5% extract ointment(w/w) respectively.

2.3 Creation of Wound

The experimental study was conducted in eighteen (18) adult rabbits of either sex (1.5-2.5kg) purchased from the rabbit section of Mountain Research Centre for Sheep and Goat, FVSc& AH, Shuhama/Wussan rabbitary farm, Pattan. The animals already tagged and housed individually in cages. The animals were acclimatized to approaching and handling for a period of 5-10 days prior to the study. The location of wound edges was outlined by a locally fabricated metal marker was created using a no. 15 BP blade on either side of dorsal spine in the thoraco-lumber region. The wounds were named as R on right side and L on left side. Haemorrahge, if any, was controlled by digital pressure. Procedures

were carried out under aseptic conditions and in proper anaesthesia (Ketamine Hydrochloride@ 50mg/kg and Xylazine @10 mg/kg). Post-operative analgesic (Butorphanol tartrate at the rate of 0.1-0.5mg/kg s/c qid)was given for 3 days. All the ethical issues were considered in the surgical procedures and during the treatment. Each treatment group consisted of 6 animals. Thus each treatment was evaluated on a total of 12 wounds.The animals were allowed to recover and were housed individually in metallic cages containing autoclaved drapes and received food and water *ad libitum*. Each wound was cleaned with sterile normal saline solution and dressed with as per the scheduled therapy.

2.4 Treatment schedule

Wounded rabbits were divided into three groups. The wounds of group I to serve as a control were topically washed with Normal Saline Solution (NSS) and no treatment was given, wounds of group II were washed with Normal Saline Solution (NSS) followed by application of wounds with 5% *Salix* leaves aqueous extract ointment on wound till healing and wounds of group III were washed with Normal Saline Solution (NSS) as accordingly followed by application of wounds with 5% *Salix* leaves ethanolic extract ointment on wound till healing.

2.5 Wound Morphometry (Handoo *et al*., 2014):

2.5.1 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 3^{rd}, 7^{th}, 14^{th} and 21^{st}. These tracings were placed on graph paper and wound area was calculated.

2.6 Tissue collection and processing for assay of different enzymatic antioxidants

Wound tissues were collected on day 7, 14, 21 under anaesthesia. Sample preparation for studying oxidants and antioxidants was done by the method described earlier (Shukla *et al.,* 1997).

2.6.1 Catalase activity (nmoles of H_2O_2consumed/min/mg of protein)

was assayed by the method of Claiborne 1985. The rate of decomposition of hydrogen peroxide was measured spectrophotometrically at 240 nm.

2.6.2 Lipid peroxidation (LPO)

Malondialdehyde (MDA) an end product of lipid peroxidation was measured by a reaction with thiobarbituric acid (TBA) yielding a coloured substance (Ohkawa *et al.,* 1979). This coloured adduct was read at 532 nm. The protein content of tissue was determined by the method of Nichans and Samuelson, 1968 using bovine serum albumin (BSA) as standard.

2.7 Estimation of serum micrometals (antioxidants)

Serum Zinc (µg/ml) and Copper (µg/ml) were estimated by Atomic Absorption Spectroscopy at 213.9nm and 324.8nm.

3. Statistical analysis

The results are expressed as Mean ± standard error. The data was analyzed using the suitable statistical program for Social analysis 20 for Windows software (SPSS Inc, Chicago, IL) (Snecador and Cochran, 1989). One way Analysis of Variance (ANOVA) test was used to compare the means at different time intervals among different groups . A value of $P<0.05$ was considered significant.

4. Results and Discussion

Wound size

The Wound size denoted the amount of wound contraction. Unlike epithelialization, 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 (Hardy, 1989). Wound 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 extract of *Salix* contain glycosides (1.5-11%) (Bissett, 1994; Mc Guffin *et al.,* 1997); salicylates (salicin, salicortin, populin, fragilin, tremulacin) (Meier *et al.,* 1985); tannins (8-20%) (Thieme, 1968); aromatic aldehydes and acids: salidroside, vanillin, syringin, salicylic acid, caffeic and ferulic acids; salicyl alcohol (saligenin); flavonoids (Bissett, 1994; Mc Guffin *et al.,* 1997). Tannins are capable of precipitating proteins, resulting in shrinkage of cells. This precipitating protein forms a coagulum. Underneath the coagulum quicker regeneration of tissue takes place. The wound closure is fastest in wounds treated with 5% ethanolic extract ointment of *salix* followed by 5% aqueous extract ointment of *Salix* and then the sterile normal saline solution.(Table 1 and Figure 1)

Table 1: Mean ± S.E. of time required for wound healing

Group	Healing time (days)
I	20.16 ± 0.30^{C}
II	17.83 ± 0.47^{B}
III	14.50 ± 0.50^{A}

Effect of Aqueous and Ethanolic extract ointments of *Salix acmophylla* on enzymatic antioxidants for healing wound

Catalase (nmoles of H_2O_2 metabolised/mg protein/min)

The normal values of catalase in normal tissue is 64.4 nmoles of H_2O_2 metabolised/mg protein/min. In the current study on day 7th post-wounding the Catalase level of wounds treated with 5% ethanolic and aqueous extract ointment of *Salix* were increased, which could be attributed to the anti oxidative property of *Salix* plant and was recorded highest in 5% ethanolic extract ointment treated wounds while the wounds treated with sterile NSS Catalase levels were lower than the both *Salix* extract treated groups.(Table 2)

Alam *et al*., (2006) reported that S. caprea flower extract contain 207 ± 6.1 mg/g total polyphenols expressed as gallic acid equivalents (GAE, mg/g of GAE). Since polyphenols are responsible for the antioxidant activity, the amount of total polyphenols obtained in the extract indicated high antioxidant activity. The scavenging of free radicals by the S. caprea flower extract indicates that it contains compounds that convert the free radicals to more stable products. Pohjamo *et al*., (2003) reported the presence of various phenolic compounds including catechin, gallocatechin, dihydrokaempferol and its glycoside, taxifolin, vanillic acid, 3-p-coumaryl alcohol, coniferyl alcohol, sinapylaldehyde, naringenin-enol and dihydromyrcetin in S. caprea. The compounds mentioned are important constituents of plants and are known for their potent antioxidant activity. Catechin (Lin *et al*., 2003), gallocatechin (Nakagawa and Yokozawa, 2002), dihydrokaempferol (Jung *et al*., 2003) and taxifolin (Kostyuk *et al*., 2003) have been reported to scavenge various ROS and RNS, while coniferyl alcohol (Nenadis *et al*., 2003) and vanillic acid (Sang *et al*., 2002) have been reported to scavenge DPPH radicals.

Table 2: The mean±SE values of CAT in the rabbits of different groups at different observation intervals

Group	Observation intervals in days			
	0	7	14	21
I	90.83 ± 0.47^{aA}	94.66 ± 0.66^{bA}	96.66 ± 0.66^{cA}	99.00 ± 0.57^{dA}
II	91.66 ± 0.49^{aA}	99.50 ± 0.42^{bB}	103.83 ± 0.60^{cB}	107.66 ± 0.42^{dB}
III	92.33 ± 0.82^{aA}	106.16 ± 2.30^{bC}	113.83 ± 1.52^{cC}	108.33 ± 0.49^{bB}

Figures with different superscript (small letters) differ significantly ($P < 0.05$) between days within the groups

Figures with different superscript (capital letters) differ significantly ($P < 0.05$) between groups

n = 6 animals in each group.

Lipid peroxidation (LPO)

The normal levels of lipid peroxidation in normal tissue is 1.75 nmol of melonialdehyde/g of tissue. In the current study on day 7th post-wounding the LPO level of wounds treated with 5% ethanolic and aqueous extract ointment of *Salix* decreased, which could be attributed to the anti oxidative property of *Salix* plant while the wounds treated with sterile NSS, LPO levels were higher than the both *Salix* extract treated groups. (Table 3).

Lipid peroxidation is common step in several types of injuries like burn, inflicted wound and skin ulcers. Any drug that inhibits lipid peroxidation is believed to increase the viability of collagen fibrils, increasing the strength of collagen fibers by an increase in circulation, thereby preventing the cell damage and promoting the DNA synthesis as according to (Rao and Ghosh, 1997).Wound healing mechanisms may be contributed to stimulate the production of antioxidants in wound site and provides a favorable environment for tissue healing (Shukla *et al.,* 1999) and wound healing effects may be due to up- regulation of human collagen I expression (Bonte *et al.,* 1993) and an increase in tensile strength of the wounds (Suguna *et al.,* 1996). Enhanced healing activity was attributed to increased collagen formation and angiogenesis (Shukla *et al.,* 1999; Trabucchi *et al.,* 1986). 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 (Szabo *et al.,* 1995). Salix has been reported to posses antioxidant and anticancer potential (Enayat, 2013), which could have further helped in healing process (Elias , 1998).

Table 3: The mean±SE values of LPO in the rabbits of different groups at different observation intervals

Group	Observation intervals in days			
	0	7	14	21
I	1.82±0.02^{aB}	1.90±0.02^{bC}	1.84±0.00^{aC}	1.79±0.00^{aC}
II	1.74±0.01^{cA}	1.69±0.00^{bB}	1.66±0.00^{aB}	1.67±0.00^{abB}
III	1.72±0.00^{cA}	1.59±0.01^{aA}	1.59±0.00^{aA}	1.60±0.00^{aA}

Figures with different superscript (small letters) differ significantly ($P < 0.05$) between days within the groups

Figures with different superscript (capital letters) differ significantly ($P < 0.05$) between groups

n = 6 animals in each group

Effect of Aqueous and Ethanolic extract ointments of *Salix acmophylla* on serum micrometals (antioxidants) for healing wound

The normal range of Zinc and Copper of rabbits is1.61 µg/ml and 1.03µg/ml of serum respectively. In the current study on day 7th post-wounding the zinc level of wounds treated with 5% ethanolic and 5% aqueous extract ointment of *Salix* increased with respect to wounds treated with sterile NSS, which could be attributed to high zinc levels in the leaves of *Salix* plant while as copper increased on day 7 post wounding in ethanolic extract ointment treated group followed by aqueous extract ointment treated group compared to Sterile NSS group. (Table 4and 5)

Clinical studies have revealed that increased urinary excretions and negative balances for copper (Cu), selenium (Se), and zinc (Zn) in severely injured or burn patients **(**Berger *et al*., 1996), (Selmanpakoglu *et al*.,1994) .Severe injury cause acute decreases in serum TEs levels. Blood Se and serum Fe, Zn, and Cu concentrations were dropped within 2–3weeks after trauma. The relative deficiency may be due to the excessive metabolic demand, increased losses, or reduced intakes **(**Berger *et al*., 1996), (Selmanpakoglu *et al*.,1994). Another cause might be that TEs are redistributed to meet the needs of major organs in acute responses to trauma (Ding *et al*., 2002), (Walsh .2005).

Different species of willow, as well as some clones, vary considerably in their metal translocation patterns and their ultimate resistance of heavy metals (Dickinson *et al*., 1994; Riddell-Black, 1994). Resistance to some metals, such as Cd, Cu, and Zn, has been documented for a few European Salix species (Punshon and Dickinson, 1997; Watson *et al*., 1999). Some temperate Asian species are able to accumulate significant amounts of Fe, Zn, and Pb (Ali *et al*., 1999). . Zinc is the metal moiety in a number of essential : enzyme systems (Underwood, 1962). Cu/Zn superoxide dismutase (Cu/Zn-SOD) catalyzes the dismutation of superoxide, which is constantly formed during aerobic metabolism, to oxygen and hydrogen peroxide. So Cu, Zn, and Se are joined in cellular defense against oxidants (Klotz *et al*., 2003).

Table 4: The mean±SE values of Zinc in the rabbits of different groups at different observation intervals

Group	Observation intervals in days			
	0	7	14	21
I	1.57±0.02^{dA}	1.04±0.01^{aA}	1.18±0.00^{bA}	1.32±0.01^{cA}
II	1.60±0.00^{dA}	1.33±0.01^{aB}	1.41±0.00^{bB}	1.46±0.00^{cB}
III	1.61±0.00^{dA}	1.36±0.01^{aB}	1.42±0.00^{bB}	1.55±0.02^{cC}

Figures with different superscript (small letters) differ significantly ($P < 0.05$) between days within the groups

Figures with different superscript (capital letters) differ significantly ($P < 0.05$) between groups

n = 6 animals in each group

Table 5: The mean±SE values of Copper in the rabbits of different groups at different observation intervals

Group	Observation intervals in days			
	0	7	14	21
I	1.03 ± 0.01^{aA}	1.81 ± 0.00^{bA}	1.87 ± 0.00^{cA}	1.92 ± 0.00^{dA}
II	1.05 ± 0.00^{aAB}	1.92 ± 0.01^{bB}	1.97 ± 0.00^{cB}	2.00 ± 0.00^{cB}
III	1.06 ± 0.01^{aB}	1.93 ± 0.01^{bB}	1.98 ± 0.00^{cB}	2.07 ± 0.01^{dC}

Figures with different superscript (small letters) differ significantly ($P < 0.05$) between days within the groups

Figures with different superscript (capital letters) differ significantly ($P < 0.05$) between groups

n = 6 animals in each group

5. Conclusion

Salix acmophylla extract ointments (Aqueous and Ethanolic) showed potent wound healing and antioxidant activities suggesting that and ethnopharmacological approach in selecting the plant for study may be useful. Furthermore, the chances of success will be more, if the chosen species is used medicinally in the traditional system for the treatment of skin disease. There is not much information available on the phytochemical and pharmacological studies on *Salix acmophylla* . The report of the efficacy of this plant as wound healing may be due to its trace metal (Zinc and Copper) concentration which inturn can also be correlated to the effect on antioxidant enzymes.

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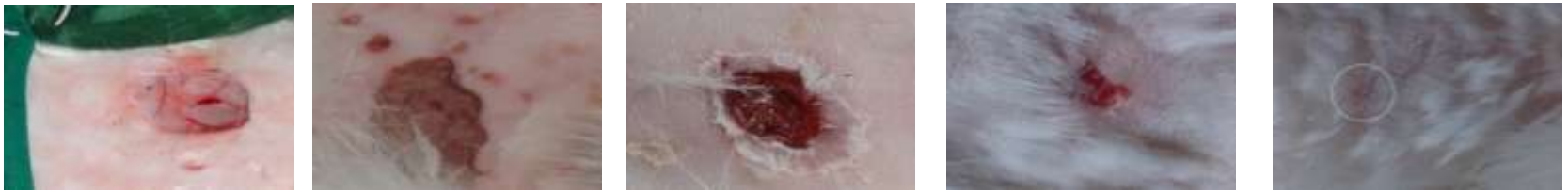

Day 0 **Day 3** **Day 7** **Day 14** **Day 21**

Group I (Control)

Day 0 **Day 3** **Day 7** **Day 14** **Day 21**

Group II (5% Aqueous extract ointment of *Salix acmophylla* leaves)

Day 0 **Day 3** **Day 7** **Day 14** **Day 21**

Group III (5% Ethanolic extract ointment of *Salix acmophylla* leaves)

Figure 1