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In vivo assessment of lipid peroxidation and antioxidant profile in aqueous and ethanolic extract ointment of *Salix acmophylla* leaves in rabbits for wound healing improvement on excisional full thickness skin wounds

Qumaila Sakeena, Md. Moin Ansari, Sadaf Sakeena, Shawkeen Muzamil and DM Makhdoomi

Abstract
The present study has been designed to estimate and evaluate the concentration of oxidant i.e. lipid peroxidation (nmol of melonialdehyde/g of tissue) coloured adduct (melonialdehyde) was read at 532 nm on spectrophotometer and antioxidants i.e. 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 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 Lowest concentration of lipid peroxidation (melonialdehyde) and highest concentration of catalase enzyme and trace metals was seen in 5% ethanolic extract ointment treated group with fastest wound closure in just 14.50± 0.42 days followed by 5% aqueous extract ointment treated group in in 17.16±0.30 compared to control group 20.16±0.30. These *in vivo* results strongly suggest the significant and beneficial effects of *Salix acmophylla* leaves ointment for the improvement of wound healing and can be exploited to accelerate excision wound healing.

Keywords: Lipid peroxidation, catalase, copper, zinc, 5% aqueous extract, 5% ethanolic extract, atomic absorption spectroscopy, salix acmophylla, wounds, healing, rabbit

1. Introduction
In developing countries, the treatment of wounds poses a significant problem. Wounds is defined as physical injuries which results in either opening or breaking of the skin. Wound-healing is a complex, natural and dynamic process that comprises three phases, e.g. inflammation, proliferation and remodeling, and proceeds with overlapping successive stages and well organized various tissue and cellular interactions (Jorge *et al*. 2008, Judith *et al*. 2010, Singh *et al*. 2006, Ansari, 2014) [17,18,43, 3]. The basic principle for adequate 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) [33, 4]. As soon as injury occurs in a tissue, reactive oxygen species (ROS) are secreted although these ROS are very important in combating infection but when secreted in large amount have deleterious effect on wound healing process due to its harmful effects on cells and tissues. When reactive oxygen species are overproduced in case oxidative stress it results in cytotoxic effects which in turn cause delayed wound healing. Therefore, elimination of ROS could be an important strategy to improve wound healing. Wound healing process can be accelerated by manipulate the oxidation stress in the wound area. Free-radical scavenging enzymes (FRSE) are a group of cytoprotective enzymes that not only have an essential role in the reduction, deactivation, and removal of ROS but also in regulating the wound healing process. Also, it involves a complex phenomenon which involves self-generating autocoids and hormones working in a systematic synchrony leading to wound healing (Meenakshi *et al*. 2006) [26]. Currently therapeutic strategies which aim on targeting ROS by using antioxidants in the treatment of wounds. Natural compounds from medicinal plants having antioxidant and immunomodulatory activities are used as therapeutic agents in treatment of these wounds. Plants are also a rich source of phytochemicals, which not only have wound healing but also antioxidant properties and if their are depleted levels of various antioxidants it may contribute to delayed healing.

(Pascoe *et al.* 1987) [33]. Various workers have reported wound healing properties of some plants among them the chosen ornamental plant *Salix acmophylla* used in the present study is found in almost all regions of Kashmir and locally known as Wir/Veer Kani (Rather *et al.* 2010) [38]. This *Salix* plant is 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) [20, 11, 6]. These vast medicinal properties could be attributed to asprin, salicylic acid, etc. found in *Salix* leaves and bark (Pojar and Mackinnon, 1994) [36] and the bark and leaves can be pounded and applied directly to the wounds as healing agents (Moerman, 1998) [28]. The active ingredient of the *Salix* bark is called salicin and it hydrolyzes in aqueous media to glucose and salicylic alcohol (saligenin). Besides, salicin, it contains a wide variety of phytochemicals (catechins, flavonoids and proanthocyanidins) which are potent antioxidants and have wound healing properties. Although thousands of phytochemicals have been identified but only a small fraction have been studied thoroughly (Zarger *et al*. 2014) [49]. These polyphenolic bioflavonoids and trace elements both act as antioxidants and in turn facilitate wound healing as these elements act as free radical enzyme scavengers, helps in facilitating and inducing vascular endothelial growth factor (VEGF) expression which is a key element supporting wound angiogenesis. The leaves of *Salix acmophylla* also contains copper and zinc in high levels (Ali and Aboud, 2010) [2]. These trace metals have important role in wound healing as they increase the expression of VEGF and are potent antioxidants (Chandan *et al*. 2002) [10]. On phytochemical screening of *Salix acmophylla*, moderate concentration of alkaloids, coumarines, cardiacglycosides, ratenges, phenols, flavonoids, saponins, tannins, essential oil and terpenes were found. 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) [31]. Thus the present study was aimed to assessed of lipid peroxidation and antioxidant profile in aqueous and ethanolic extract ointment of *Salix acmophylla* leaves for wound healing improvement on excisional full thickness skin wounds in rabbits.

2. Materials and Methods

2.1 Plant material

The leaves of *Salix acmophylla* (Figure 1 and 2) were collected from the fields surrounding Faculty of Veterinary Sciences, Kashmir (J&K), India, in the month of May and identified by Division of Environmental Science, SKUAST-Kashmir, Shalimar and Department of Botany, Kashmir University, Hazratbal.

Fig 1: Salix acmophylla tree

Fig 2: Salix acmophylla leaves

2.2 Preparation of extracts and extract ointments

The leaves of the plant *Salix spp.* were collected and then shade dried for a week followed by drying in oven pre-set at 37 ^{o}C for 4 days (Figure 3), the samples were then powdered in electric mill (Figure 4) and stored in airtight container. The fine powder of leaves was then subjected to extraction with boiled distilled water and ethanol respectively in Soxhlet apparatus (figure 5) for 7hrs. The solution was then filtered through Whattman filter paper using Buchner funnel under vacuum evaporator at 40 ^{o}C to obtain the extract. Then the resulting extract was stored, protected from light in refrigerator at 4 ^{o}C in a glass container till further use. Aqueous extract (figure 6) and Ethanolic (figure 7) of *Salix acmophylla* (5g) was mixed with simple ointment (soft paraffin) (95g) to get a 5% extract ointment (w/w) respectively.

Fig 3: Dried leaves

Fig 4: Making of powder by Electric mill

Fig 5: Soxhlet extraction apparatus

Fig 6: Aqueous extract

Fig 7: Ethanolic extract

2.3 Experimental protocol

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 were 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 locally using a fabricated metal marker (figure 8) and wounds (1.5cm×1.5cm and 2.5 cm apart), were 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 (figure 9). Haemorrahge, if any, was controlled by digital pressure.

Fig 8: Metal marker

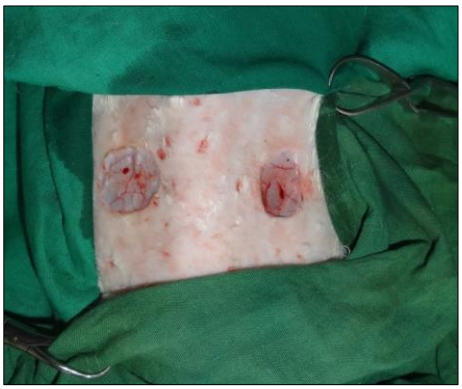

Fig 9: Full thickness excisional wounds

2.4 Anaesthesia and Post-operative care

Procedures were carried out under aseptic conditions and under proper anaesthesia (Ketamine Hydrochloride at the dose rate of 50mg/kg and Xylazine at the dose rate of 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.

2.5 Therapy schedule

Wounded rabbits were divided into three groups (Table 1). 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 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 NSS as accordingly followed by application of wounds with 5% *Salix* leaves ethanolic extract ointment on wound till healing.

Table 1: Therapeutic modalities used in different groups

Group	No. of animals	Therapeutic protocol
I	6 (12)	No treatment will be given to (Control)
II	6 (12)	Application of wound with 5% *Salix* leaves aqueous extract ointment on wound till healing.
III	6 (12)	Application of wound with 5% *Salix* leaves ethanolic ointment on wound till healing.

2.6 Tissue collection and processing for assay of different enzymatic antioxidants
Wound tissues were collected on day 7, 14, 21 under mild anaesthesia.

2.6.1 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) [32]. This coloured adduct was read at 532 nm.

2.6.2 Catalase activity (nmoles of H_2O_2consumed/min/mg of protein)
Catalase was assayed by the method of Claiborne 1985 [9]. The rate of decomposition of hydrogen peroxide was measured spectrophotometrically at 240 nm.

2.7 Estimation of serum micrometals/Trace metals (antioxidants)
Serum Zinc (µg/ml) and Copper (µg/ml) were estimated by Atomic Absorption Spectroscopy at 213.9nm and 324.8nm.

3. Time required for wound healing
Continuous measurement of wound reduction and size contraction helps to predict healing time and aids monitoring of treatment efficacy and evaluation.

4. Statistical analysis
The results were 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). 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.

5. Results and Discussion
5.1 Effect of Aqueous and Ethanolic extract ointments of *Salix acmophylla* on enzymatic antioxidants for healing wound

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* were decreased, which could be attributed to the anti-oxidative property of *Salix* plant while the wounds treated with sterile NSS, LPO levels were higher (Table 2 and figure 10). Lipid peroxidation is common step in several types of injuries like burn, inflicted wound and skin ulcers. The drugs that inhibits lipid peroxidation are believed to increase the viability of collagen fibrils, thus increasing the strength of collagen fibers which in turn is caused by increase in circulation, thereby preventing the cellular damage and promoting the DNA synthesis as according to (Rao and Ghosh, 1997) [37]. Wound healing can be stimulated by the production of antioxidants at wound site and provides a favorable environment for tissue healing (Shukla *et al.* 1999) [42] and wound healing effects can be due to up- regulation of human collagen I expression (Bonte *et al.* 1993) [8] and also due to increase in tensile strength of the wounds (Suguna *et al.* 1996) [44]. Angiogenesis in granulation tissues have said to improves circulation at the wound site thereby providing oxygen and essential nutrients for the healing process which in turn enhances epithelial cell proliferation (Szabo *et al.* 1995) [45]. Salix has been reported to posses antioxidant and anticancer potential (Enayat, 2013) [15], which have further helped in accelerating wound healing process (Elias, 1998) [14].

Table 2: 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

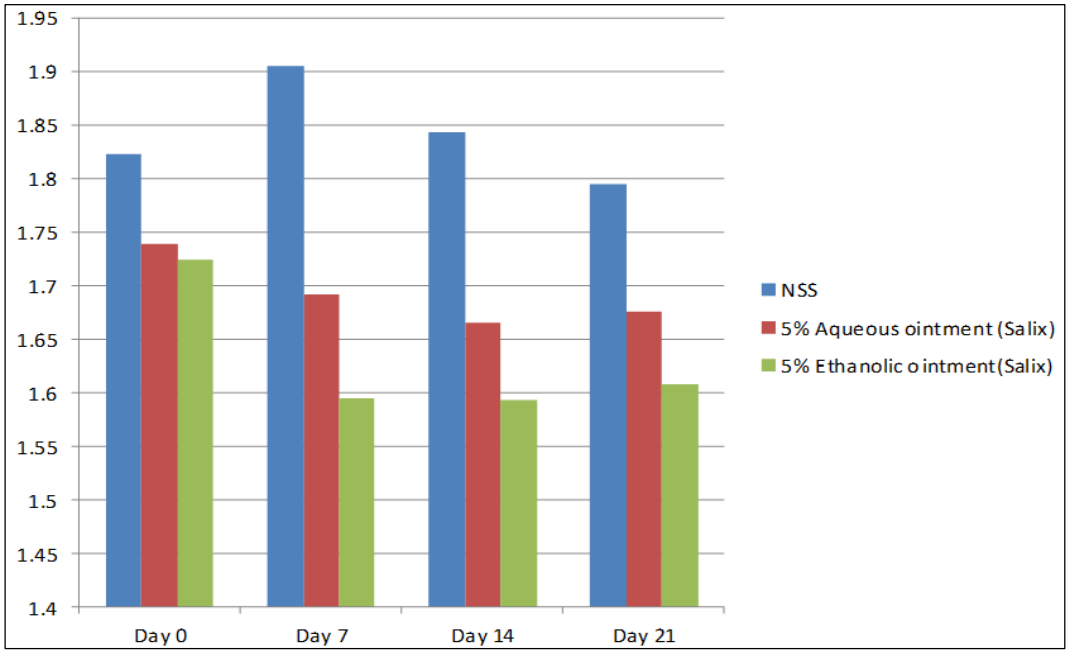

Fig 10: Effect of different therapies on LPO (nmoles of MDA formed/g tissue) in the rabbits of different groups at different observation intervals

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 3 and figure11). In year 2006 (Alam *et al.*) [24] Reported that *S. caprea* flower extract contain 207±6.1 mg/g total polyphenols expressed as gallic acid equivalents (GAE, mg/g of GAE). These polyphenols have antioxidant activity thus the extract have 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. In 2003 (Pohjamo *et al.*) [35] Reported the presence of Phenolic compounds i.e. catechin, gallocatechin, dihydrokaempferol and its glycoside, taxifolin, vanillic acid, 3-p-coumaryl alcohol, coniferyl alcohol, sinapylaldehyde, naringeninenol and dihydromyrcetin in S. caprea plant, these compounds are important constituents of plants and are known for their potent antioxidant activity. Catechin (Lin *et al*. 2003) [23], gallocatechin (Nakagawa and Yokozawa, 2002) [29], dihydrokaempferol (Jung *et al*. 2003) [19] and taxifolin (Kostyuk *et al*. 2003) [22] scavenge various ROS and RNS, while coniferyl alcohol (Nenadis *et al*. 2003) [30] and vanillic acid (Sang *et al*. 2002) [40] have been reported to scavenge DPPH radicals.

Table 3: 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.

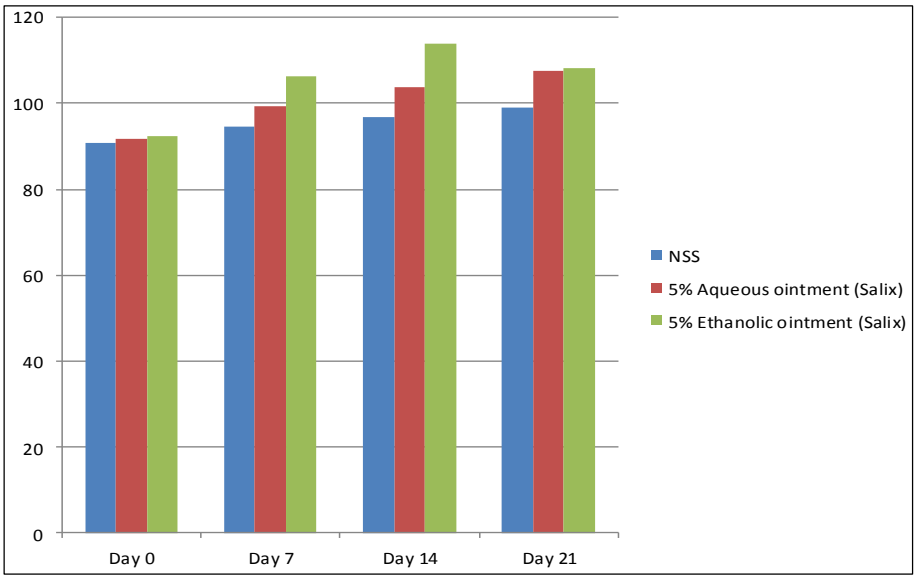

Fig 11: Effect of different therapies on Catalase (nmoles of H_2O_2 metabolised/mg protein/min) in the rabbits of different groups at different observation intervals

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 4, figure 12 and Table 5, figure 13). 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) [5], (Selmanpakoglu *et al*. 1994) [43]. Severe injury cause acute decreases in serum TEs levels. Blood Se and serum Fe, Zn, and Cu concentrations were dropped within 2-3 weeks after trauma. The relative deficiency may be due to the excessive metabolic demand, increased losses, or reduced intakes **(**Berger *et al*. 1996) [5], (Selmanpakoglu *et al*. 1994) [41]. Another cause might be that TEs are redistributed to meet the needs of major organs in acute responses to trauma (Ding *et al*. 2002) [13], (Walsh. 2005) [46].

It has also been studied that 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) [12, 39]. Some temperate Asian species are able to accumulate significant amounts of Fe, Zn, and Pb (Ali *et al*. 1999) [1]. Zinc is the metal moiety in a number of essential: enzyme systems (Underwood, 1962) [47] like Cu/Zn superoxide dismutase (Cu/Zn-SOD) that 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) [21].

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

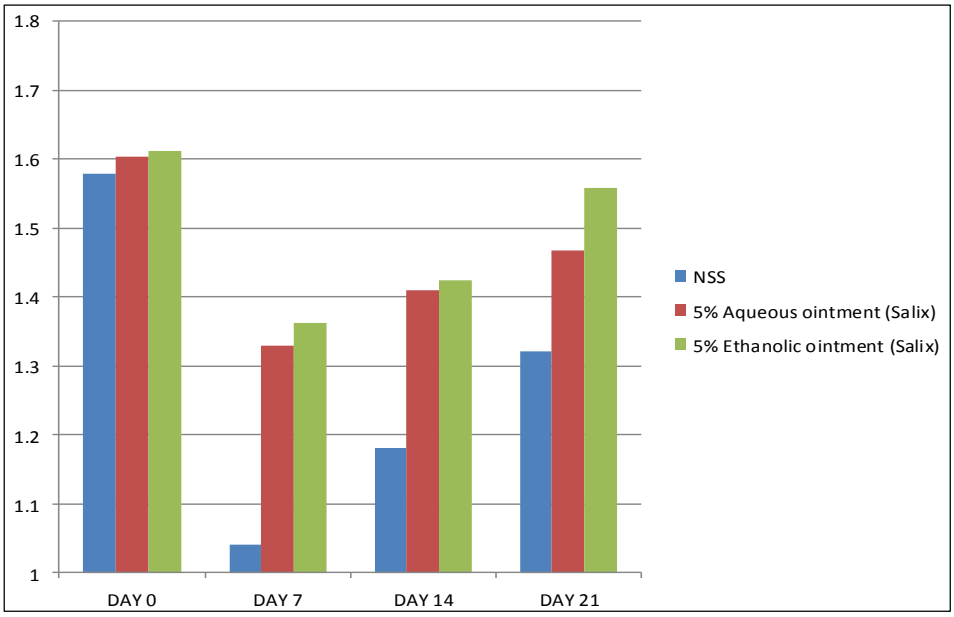

Fig 12: Effect of different therapies on Zinc (µg/ml) in the rabbits of different groups at different observation intervals

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

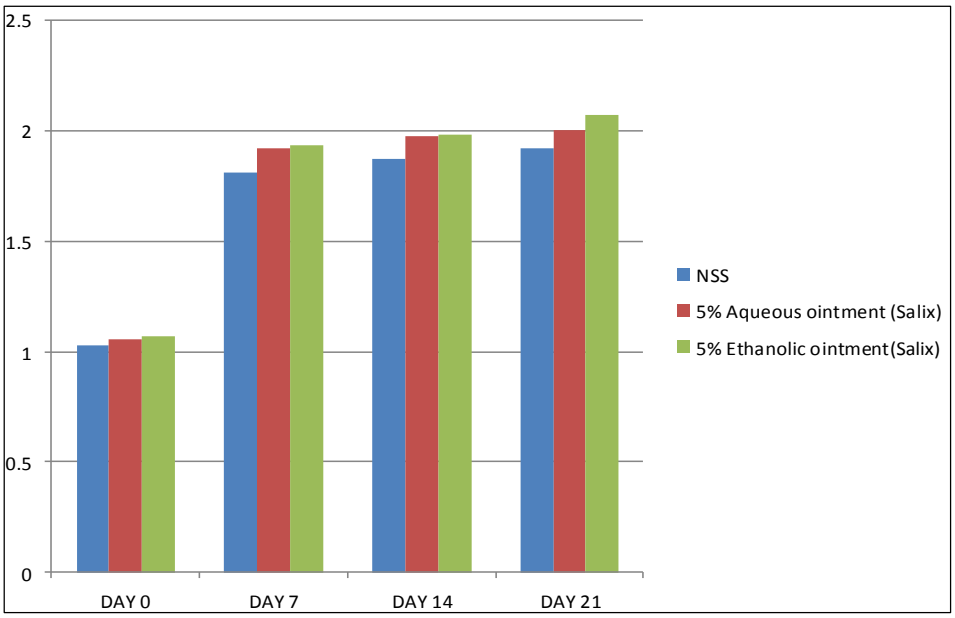

Fig 13: Effect of different therapies on Copper (µg/ml) in the rabbits of different groups at different observation intervals

Time required for wound healing

The time required for complete wound closure can be studied by estimating the wound size which denoted the amount of wound contraction. Unlike epithelialization, which closes the wound surface, contraction is a second most important process that actually pulls the entire wound together thus shrinking the defect. Successful contraction results in a smaller wound which can be repaired by scar formation (Hardy, 1989) [16]. 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 (Bissett, 1994; Mc Guffin *et al.*, 1997) [7, 25], salicylates (Meier *et al.*, 1985) [27], tannins (8-20%) (Thieme, 1968) [46], aromatic aldehydes and flavonoids (Bissett, 1994; Mc Guffin *et al.*, 1997) [7, 25]. Tannins are capable of precipitating proteins which forms a coagulum resulting in shrinkage of cells. 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 slower in sterile normal saline solution treated wounds.(Table 6).

Table 6: 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}

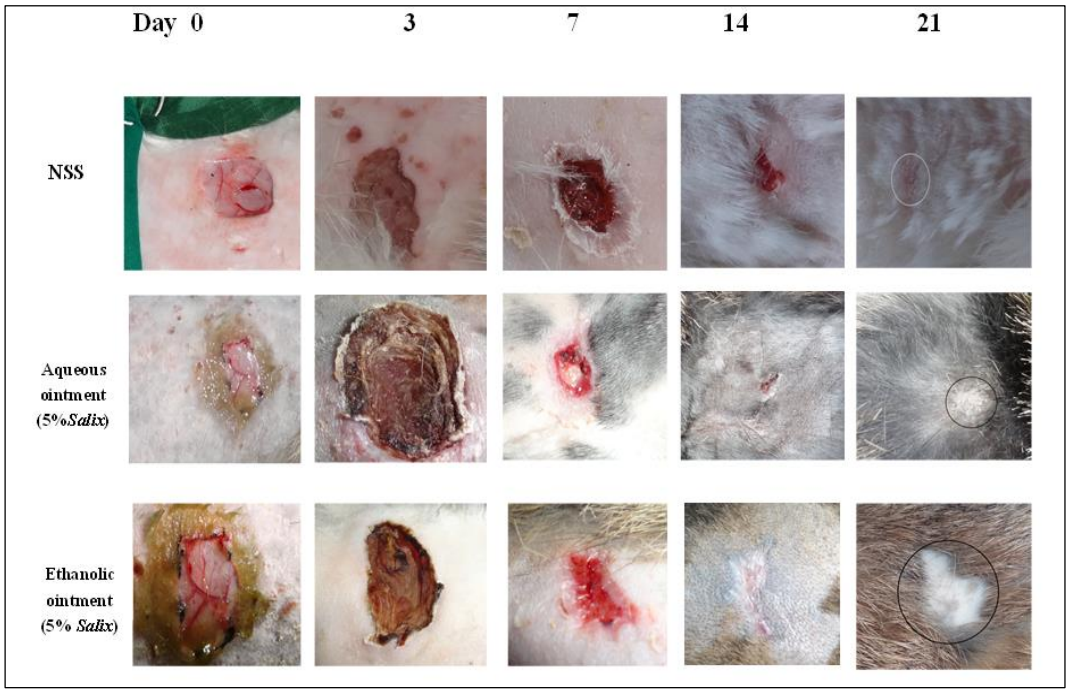

Fig 14: Photographic Evaluation

6. Conclusion

On critical analysis, both 5% aqueous and ethanolic extract ointments of *Salix acmophylla* showed potent wound healing and antioxidant activities suggesting that and ethnopharmacological approach in selecting the plant for study may be useful. Efficacy of this plant as wound healing may also be attributed to its trace metal (Zinc and Copper) concentration which in turn can also be correlated to the effect as antioxidant. *In vivo* results strongly suggest the significant and beneficial effects of *Salix acmophylla* leaves ointment for the improvement of wound healing and can be exploited to accelerate excision wound healing. Furthermore, both 5% aqueous and ethanolic extract ointments of *Salix acmophylla* ointment can be used for the wound healing agents in field condition.

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