Journal of Entomology and Zoology Studies

Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2018; 6(1): 1204-1213

Received: 16-11-2017
Accepted: 17-12-2017

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Wound healing and anti-bacterial efficacy of *Rheum emodi* and its different extracts on excisional wounds in rabbits

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Abstract
Encouraging results from the past studies regarding the use of *R. emodi* as a wound healing agent, necessitates the full exploration of this herb for its efficacy in wound healing. The present study was aimed to evaluate the wound healing and anti-bacterial efficacy of R.*emodi* and its ethanolic, aqueous and petroleum ether extracts in excisional wounds in thirty healthy rabbits randomly assigned in five equal groups. Three excisional wounds (1.5×1.5 cm) on the dorsal spine were created in all under general anesthesia. Except for the first group treated with povidone iodine which served as control, all the other groups were treated with *R.emodi* and its extracts. Animals in the treated groups showed prompt and better healing in the following order: Petroleum ether extract > Aqueous extract > Ethanolic extract > Crude dust powder > Control group.

Keywords: *R. emodi*, rabbit, wound, crude dust powder, ethanolic extract, petroleum ether extract, aqueous extract

Introduction
India has a rich tradition of plant-based knowledge on the healthcare. A large number of plants/plant extracts/decoctions or pastes are equally used by tribal and folklore traditions in India for treatment of cuts, wounds, and burns [1]. *Rheum sps*. Commonly known as Rhubarb, has been cultivated for over 5000 years for its medicinal purposes, originating in the mountains of the North-western provinces of China and Tibet [2]. Indian Rhubarb, which is official in the Indian Pharmacopoeia, consists of the died rhizomes of *R. emodi* [3].
Wound healing involves continuous cell–cell and cell–matrix interactions that allow the process to proceed in three overlapping phases namely, inflammation (0-3 days), cellular proliferation (3-12 days) and remodeling (3-6 months) [4-6]. Matrix formation requires the removal of granulation tissue with revascularization. A framework of collagen and elastin fibers replaces the granulation tissue. This framework is then saturated with proteoglycans and glycoproteins. This is followed by tissue remodeling involving the synthesis of new collagen mediated by TGF-β. The final product of this process is scar tissue [7]. The production of wound exudate is a normal part of the inflammatory process [8]. Fluid from acute wounds plays an essential part in the healing process and the exudate from these wounds is rich in growth factors that promote tissue repair [9]. Bacterial infection reduces wound contraction [10] and consequently delays the wound healing process [11, 12]. Chrysophanol, physcion and emodin in Rhubarb so reported to have anti- bacterial activity and anti-fungal activity [13, 14]. On the perusal of the available literature no work comparing the wound healing efficacy of different extracts of *R. emodi* was found. The present study was thus aimed to evaluate the wound healing and anti-bacterial efficacy of R. *emodi* and its different extracts (ethanolic, aqueous and petroleum ether) in excisional wounds in rabbits.

Material and methods
Selection and preparation of animals
Thirty (30) clinically healthy rabbits of either sex, 9-15 months age with their body weight ranging between 2-3 kg were used for the study. The rabbits were randomly divided into 5 equal groups, of 6 animals each. All the animals were reared under identical managemental conditions. The dorsal thoraco-lumbar portion was shaved, cleaned and prepared for aseptic surgical procedures.

Anesthesia

General anesthesia was induced in animals for creation of wounds. Each animal was given xylazine @ 10 mg/kg I/M, left in calm environment for 5 minutes and then administered Ketamine Hydrochloride @ 50 mg/kg I/M.

Experimental Design

Full-thickness cutaneous wounds were created (1.5× 1.5 cm) on dorsal spine in the thoraco-lumbar region, one on the right side (R) and two on the left side (LI & L2) with 2.5 cm distance in between. All wounds were flushed with normal saline solution followed by treatment with medicaments, (Table A). The medicaments were applied for 12 consecutive days and then on alternate days till complete healing of wound.

Table A

Group	No. of animals (No. of wounds)	Treatment
Group 1	6 (18)	Flushing of wound with Normal saline solution + Povidone Iodine Ointment.
Group 2	6 (18)	Flushing of wound with Normal saline solution + Dusting the wound with *Rheum emodi* powder.
Group 3	6 (18)	Flushing of wound with Normal saline solution + 10% ethanolic extract ointment (*Rheum emodi*).
Group 4	6 (18)	Flushing of wound with Normal saline solution + 10% aqueous extract ointment (*Rheum emodi).*
Group 5	6 (18)	Flushing of wound with Normal saline solution + 10% petroleum ether extract ointment (*Rheum emodi).*

Parameters recorded

Gross Examination of wound

The wounds were grossly evaluated subjectively on day 0, 3rd, 7th, 14th and 21st day post wounding. These wounds were individually evaluated for the following parameters grossly; Wound Edges, Skin color of surrounding wound, Degree of Inflammation, Appearance of granulation tissue, Extent of cicatrization and Presence and type of exudates, using a modified Bates-Jensen Wound Assessment Tool (BWAT-m) [15].

Bacterial Colony Count

For total bacterial viable count, tissue from each wound on the day of creation and then at day 3rd, 7th, 14th and 21stwas collected under local anesthesia in sterile test tubes. The tissue were triturated, slurry made and then serially diluted in 10% P.B.S for estimating total bacterial count. As obtained of each dilution, it was spread on nutrient agar plates in duplicate. Agar plates were then incubated for 24hrs at 37 °C. Bacterial colonies from required plates were counted and the total viable count was calculated as:

$$TVC = \frac{\text{Average No. of colonies in the desired dilution x Dilution factor}}{\text{Volume used}}$$

The total viable count is expressed as cfu/g of tissue sample.

Results

Evaluation of wound

The evaluation of wound was recorded based upon the appearance of wound, degree of inflammation, extent of granulation tissue and cicatrization and lastly, the presence and type of exudate.

The wound appearance was recorded based upon the condition of wound edges and the color of the skin surrounding the wound. The percent score of wound edge status score in animals of different groups at different observation intervals are depicted in Table-1. On the day of wounding the edges of the wounds of all the animals were clear, well defined and not attached to the wound base (Fig. 2). On day 3 post-wounding 100% of animals of groups 1, 2 and 5 showed distinct and clearly visible wound edges that were attached to wound base. In the rest of the group, only 50% wounds showed improvement while the rest 50% wounds were similar to those of the day 0 (Fig. 3). On day 7, 100% wounds of the first 4 groups had clear wound edges that were attached to the wound base while in group 5 only 50% of the wound showed improvement (Fig.4). On day 14 and 21 all the wounds of extract treated groups were indistinct and diffuse with edges not visible, whereas in 1st two groups only 16.67% of the wounds had such appearance.(Fig.5 and Fig. 6) No alteration from normal skin color of skin surrounding the wound area was observed in any animal of any group at any observation interval.

The percent score for degree of inflammation in animals of different groups at different observation intervals are depicted in Table-2. On day 7 all the animals of group 1 showed the same picture as on day 3. In treatment groups improvement was seen on day 7. There was only redness while swelling had subsided. The improvement was seen in maximum percentage of animals of group 5. Redness and inflammation in all the wounds of extract treated groups was absent on day 14 postwounding whereas, in dust treated group such improvement was seen on day 21 postwounding. In treated groups 100% wounds and in control group only 16.67% wounds showed such improvement on day 21 only (Fig. 6).

The percent score of the extent of granulation tissues in animals of different groups at different observation intervals depicted in Table-3, represents that on day 3. 66.66% of wounds showed beefy red bright granulation tissue that covered 25-75% of the wound surface in the animals of groups 3-5 leaving the rest 33.33% of wounds of these groups the granulation tissue covered more than 75% of the wound surface. In control group all the wounds had dull red granulation tissue that covered only less than 25% of the wound surface. In dust treated groups 66.66% of the wounds resembled those of control wounds while 33.33% wounds had bright granulation tissue that covered 25-75% of wound surface. On day 7 same pictures as on day 3 was repeated in treatment groups but in control group 33.33% of wounds showed improvement. On day 14 post-wounding 16.67% of wounds of the control group had bright red granulation tissue that covered more than 75% of wound surface while in 83.33% of wounds less than 75% of wound surface was covered. In dust treated groups less than 75% of wound surface was covered at 66.66% of the animals. Similarly in group 4 and 5 only 16.67% of animals had less than 75% wound surface covered by granulation tissue while at rest 83.33% the skin was intact and there was partial thickening of skin. In group 3, all wounds were less than 75% covered. On day 21 all the wounds of extract treated groups had intact skin that had partially thickened. In dust treated group 66.66% of wounds were similar to those of extract treated groups. Similarly 16.67% of wounds of the control group were similar

to those of extract treated wounds (Fig. 6).

The percent score values of extent of the cicatrization in animals of different groups at different observation intervals are depicted in Table-4. On day 3 wounds of group 1 and 2 animals showed scab formation and in extract treated groups 50% of animals showed red, larger and thicker areas and 50% showed scab formation. On day 7, in group 1 animals the wounds showed red, larger and thicker areas, while in group 2 animals 33.33% wounds showed light pink, smaller and softened scar and 66.66% wounds showed red, larger and thicker areas. In group 3 wounds of 66.66% animals showed fade-thin out areas and 33.33% wounds showed light pink, smaller and softened scar. In group 4 and 5 animals showed fade thin out areas. On day 14 all the wounds of group 1 and 2 animals showed small and softened scar. On day 21 in group 1, 16.67% animals showed normal skin and 83.33% fade thin out areas while group 2, 50% animals showed normal skin and 50% animals showed fade, thin out areas. On day 14 and 21 extracts treated groups animals showed normal skin color (Fig. 5 and 6).

The percent score of exudates in animals of different groups at different observation intervals, which are depicted in Table-5, clearly show that initially there was no exudate in any wound of any group on day 0. Eventually, on day 3 it changed to moist wound base and moisture was evenly distributed in all the groups. Proceeding towards day 7 groups 1 and 2 showed the same status as that of day 3, whereas the extract treated groups showed no exudate. Wounds of group 1 and 2 showed moist bases on day 14. On day 21 wounds of group 1 animals didn't show any improvement from day 14 but wounds of dust treated groups were dry without any exudation. In extract treated group 100% dry wounds were seen on day 14 onwards

Bacterialogical analysis

The Mean± SE values of Bacterial colony count (cfu/g of tissue) in animals of different groups at different observation intervals are depicted in Table-6.

At day 0 i.e. immediately after creation of wounds, the viable bacterial colony count did not differ significantly among the groups. Subsequently the bacterial colony count decreased in all the groups from day 3 onwards till day 14 post-wounding. By the end of the study period the viable bacterial colony count was negligible. Comparison within the groups showed significant (p<0.05) on day 7 in all the groups from their respective base values except group 2 which continued till the end of the study period. Comparison among the groups revealed significantly higher values of bacterial count on day 3 and 7 post-wounding in groups 2 and 4 than in other groups and significantly higher values in the animals of group 3 than those of other groups on day 14 and 21 post-wounding.

Discussion

Wound healing has 3 phases, inflammatory, proliferative and maturational and is dependent upon the type and extent of damage, the general state of the host's health and the ability of the tissue to repair [16]. The present study showed that the topical application of R.*emodi* and its different extracts could significantly enhance the rate of wound healing. Post wounding the edges changed from well-defined to diffuse and barely visible, these changes being fastest in extract treated groups followed by dust *R.emodi* treated group and then the control group. Emodin obtained from Rheum species has been found to accelerate the wound healing activity in an excisional wound model in rats. Emodin derived from Rhubarb topically and recorded increased content of hydroxyproline on day 7 post-wounding and more tensile strength at day 14 post-wounding in treated wounds[17]. Inflammation was resolved more quickly in petroleum ether extract and aqueous extract treated group. This could be because of their greater concentrations of anti-inflammatory agent in these extracts [18] and better anti-bacterial activity [19].

In present study granulation in treated groups completed as soon as 14th day post wounding after which normal skin started resurfacing. In full-thickness wounds the epidermis can resurface from the margins only after adequate granulation tissue has formed [20]. Matrix formation requires the removal of granulation tissue with revascularization. A framework of collagen and elastin fibers replaces the granulation tissue. This framework is then saturated with proteoglycans and glycoproteins. The final product of this process is scar tissue [7]. This process of scar formation was fastest in petroleum ether extract and aqueous extract treated group, as these extracts are reported to contain proteins and amino acids [21]. The present study results are in accordance with Aakhoon (2001) who reported fastest rate of epithelialization and wound contraction in rhubarb treated groups [22].

The production of wound exudate is a normal part of the inflammatory process [8]. In present study wounds were found to be moist, allowing proper nutrition and healing process although healing was better in treated groups due to its wound healing potential. Povidone iodine used in control group as well as rhubarb and its extracts are reported to possess antibacterial activity [23]. So, no infection was produced either in control group or treated groups.

Microbial analysis of *R.emodi* and its extracts proved their anti-microbial activity. The bacterial colony count decreased in all the groups including positive control in which povidone-iodine was applied. Povidone-iodine has a good anti-bacterial action. These observations are in consonance with Lacey (1979) who reported use of povidone-iodine in stimulated ulcer experiments and concluded povidone-iodine as a potent and persistent bactericidal activity and recommended its use in treatments of wound infection [23]. Among treated groups dust *R.emodi* showed least anti-microbial activity. Highest was shown by Petroleum ether followed by ethanolic and then aqueous extract treated groups. These findings match with those of Lu *et al.* (2011) who reported high anti-bacterial activity of anthraquinones extracted in ethanolic extract [24]. Similar findings were reported by Mastuda *et al.* (2001) who showed scavenging activity of alcoholic extract of five kinds of Rhubarb [25]. The anti-microbial activity of *R. emodi* is attributed to the presence of anthraquinones especially, oxantrone esters, revandchinone-1, revandchinone-2, revandchinone-3 and revandchinone-4 which are reported to be present in higher concentrations in petroleum ether extract [19].

Conclusion

R. emodi dust treated group and different extract treated groups showed better healing than control group. Complete healing was noted in extract treated groups at the end of the study period. Among the extract treated groups petroleum ether extract treated group showed better healing than the aqueous extract treated group followed by ethanolic extract treated group.

Highest anti-bacterial activity was recorded in Petroleum ether extract treated group followed by ethanolic extract and aqueous extract treated groups respectively.

Acknowledgment
We are highly thankful to Division of Veterinary Surgery & Radiology and Mountain Research Centre for Sheep and Goat for providing all the necessary help.

Table 1: Percent Score of wound edges in animals of different groups at various observation intervals

Group No.	Score No.	Observation Intervals (Days)				
		0	3	7	14	21
1	0				16.67	16.67
	1		100	100	83.33	83.33
	2	100				
	3					
2	0				16.67	16.67
	1		100	100	83.33	83.33
	2	100				
	3					
3	0				100	100
	1		50	100		
	2	100	50			
	3					
4	0				100	100
	1		50	100		
	2	100	50			
	3					
5	0			50	100	100
	1		100	50		
	2	100				
	3					

Wound Edges
0 = Indistinct, diffuse, outline not visible.
1 = Distinct, outline clearly visible, attached with wound base.
2 = Well-defined, not-attached to wound base.
3 = Well-defined, not-attached to wound base/rolled under, thickened.
4 = Well-defined, fibrotic, scarred. (Barbara, 2001)

Table 2: Percent score of Degree of Inflammation in animals of different groups at various observation intervals

Group No.	Score No.	Observation Intervals (Days)				
		0	3	7	14	21
1	0	100				16.67
	1				100	83.33
	2		100	100		
	3					
2	0	100			33.33	100
	1			33.33	66.66	
	2		100	66.66		
	3					
3	0	100		16.67	100	100
	1			83.33		
	2		100			
	3					
4	0	100		33.33	100	100
	1			66.66		
	2		100			
	3					
5	0	100			100	100
	1			66.66		
	2		100	33.33		
	3					

Degree of Inflammation
0 = Clean wound
1 = Redness, no swelling, no discharge
2 = Redness, swelling, no discharge.
3 = Redness, swelling, discharge present.

Table 3: Percent Score of Extent of Granulation Tissue in animals of different groups at various observation intervals

Group No.	Score No.	Observation Intervals (Days)				
		0	3	7	14	21
1	0					16.67
	1				16.67	83.33
	2			33.33	83.33	
	3		100	66.66		
	4	100				
2	0					66.66
	1				66.66	33.33
	2		66.66	33.33	33.33	
	3		33.33	66.66		
	4	100				
3	0					100
	1		33.33	33.33	100	
	2		66.66	66.66		
	3					
	4	100				
4	0				83.33	100
	1		33.33	33.33	16.67	
	2		66.66	66.66		
	3					
	4	100				
5	0				83.33	100
	1		33.33	33.33	16.67	
	2		66.66	66.66		
	3					
	4	100				

Appearance of granulation tissue
0 = Skin intact, partial skin thickness
1 = Bright, beefy red, 75% -100% of wound filled
2 = Bright, beefy red, < 75% &> 25% wound filled.
3 = Pink or dull red less or equal to 25% of wound healed.
4 = No granulation tissue. (BWAT-m, 2001)

Table 4: Percent Score of Extent of Cicatrization in animals of different groups at various observation intervals

Group No.	Score No.	Observation Intervals (Days)				
		0	3	7	14	21
1	0					16.67
	1					83.33
	2				100	
	3			100		
	4		100			
	5	100				
2	0					50
	1					50
	2			33.33	100	
	3			66.66		
	4		100			
	5	100				
3	0				100	100
	1			66.66		
	2			33.33		
	3		50			
	4		50			
	5	100				
4	0				100	100
	1			100		
	2					
	3		50			
	4		50			
	5	100				
5	0				100	100
	1			100		
	2					
	3		50			
	4		50			
	5	100				

Extent of cicatriation

0 = Normal skin color.
1 = Fade, thin –out or small white line.
2 = Light pink, smaller and softened scar
3 = Red, larger and thicker areas.
4 = Scab formation

Table 5: Percent score of Presence of Exudate in animals of different groups at various observation intervals

Group No.	Score No.	Observation Intervals (Days)				
		0	3	7	14	21
1	0					
	1				100	100
	2		100	100		
	3					
2	0					100
	1	100			100	
	2		100	100		
	3					
3	0				100	100
	1	100		100		
	2		100			
	3					
4	0				100	100
	1	100		100		
	2		100			
	3					
5	0				100	100
	1	100		100		
	2		100			
	3					

Presence of exudates

1 = None
2 = Scant-wound base moist, no measurable exudate
3 = Small-wound tissue moist, moisture evenly distributed.
4 = Moderate-wound base saturated
5 = Large-wound tissue bathed in fluid (Barbara, 2001)

Table 6: Mean±SE of Bacterial colony count (cfu/g of tissue) in the animals of different groups at different observation intervals

Group No.	Observation Intervals (Days)				
	0	3	7	14	21
1	1.88 ± 0.16^{aA}	0.90 ± 0.20^{aB}	0.11 ± 0.06^{aC}	0.043 ± 0.00^{aC}	0.067 ± 0.02^{aC}
2	1.78 ± 0.13^{aA}	1.65 ± 0.23^{bA}	1.19 ± 0.07^{bB}	1.14 ± 0.15^{bB}	1.06 ± 0.15^{bB}
3	1.76 ± 0.14^{aA}	0.42 ± 0.16^{cB}	0.03 ± 0.00^{aC}	0.46 ± 0.01^{aC}	0.04 ± 0.11^{aC}
4	1.81 ± 0.12^{aA}	1.53 ± 0.14^{bA}	1.14 ± 0.09^{bB}	0.049 ± 0.11^{cC}	0.08 ± 0.03^{aD}
5	1.93 ± 0.13^{aA}	0.11 ± 0.03^{cB}	0.06 ± 0.02^{aB}	0.04 ± 0.01^{aB}	0.04 ± 0.01^{aB}

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

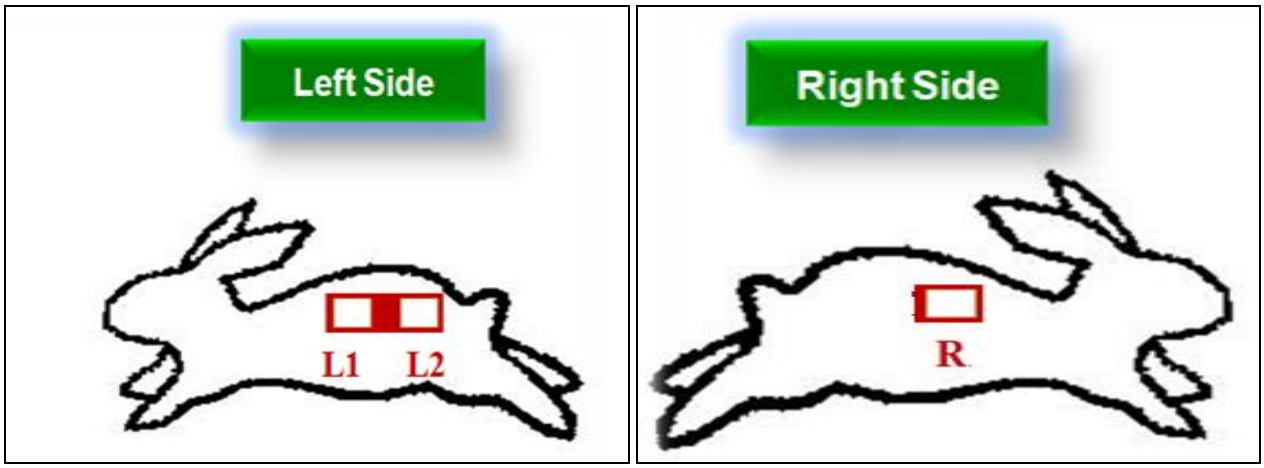
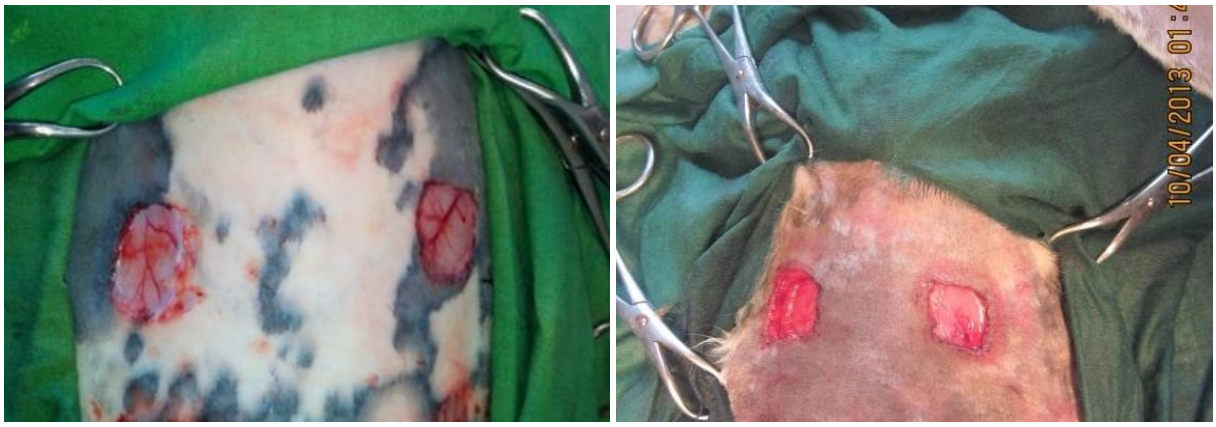
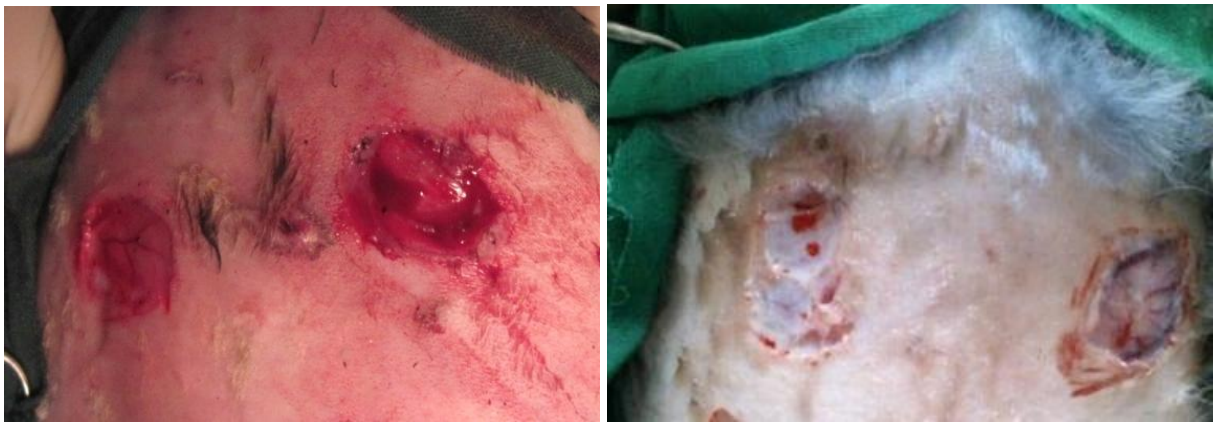


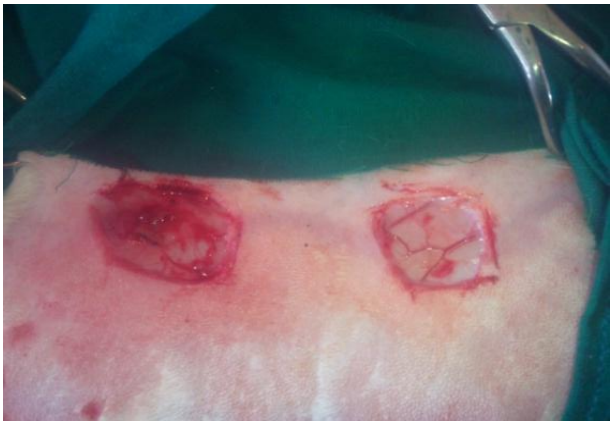
Fig 1: Showing location of the wounds on animal model



Group 1 **Group 2**

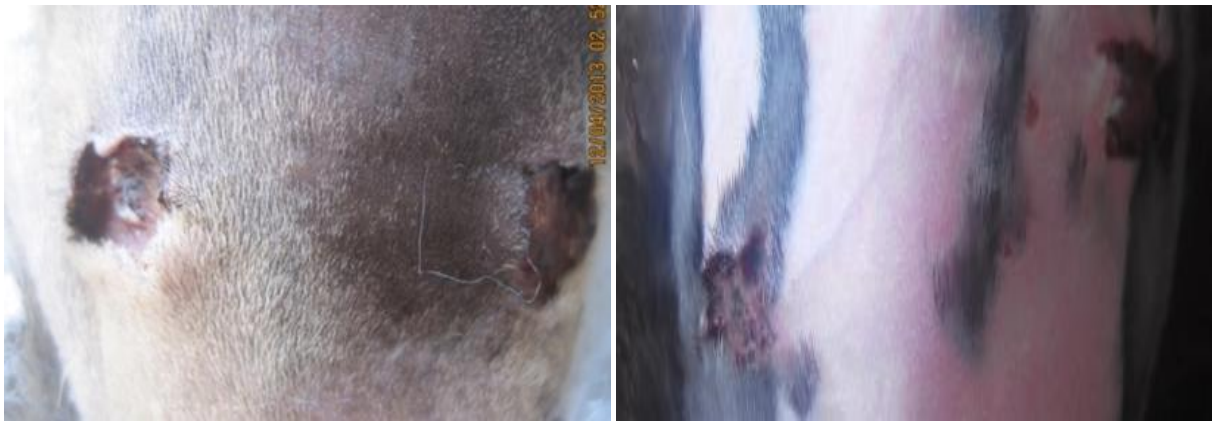


Group 3 **Group 4**

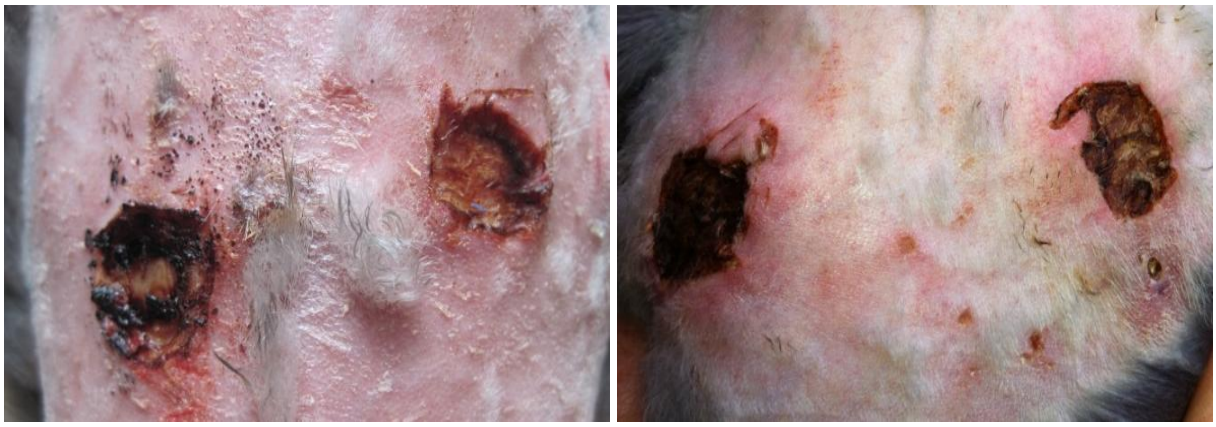


Group 5

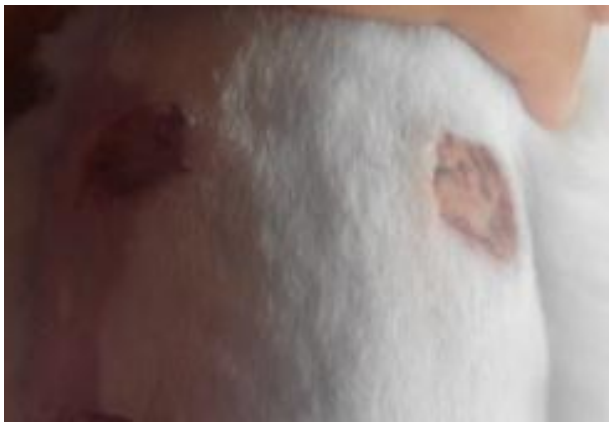
Fig 2: Gross appearance of wounds of different animals of different groups on day 0



Group 1 **Group 2**

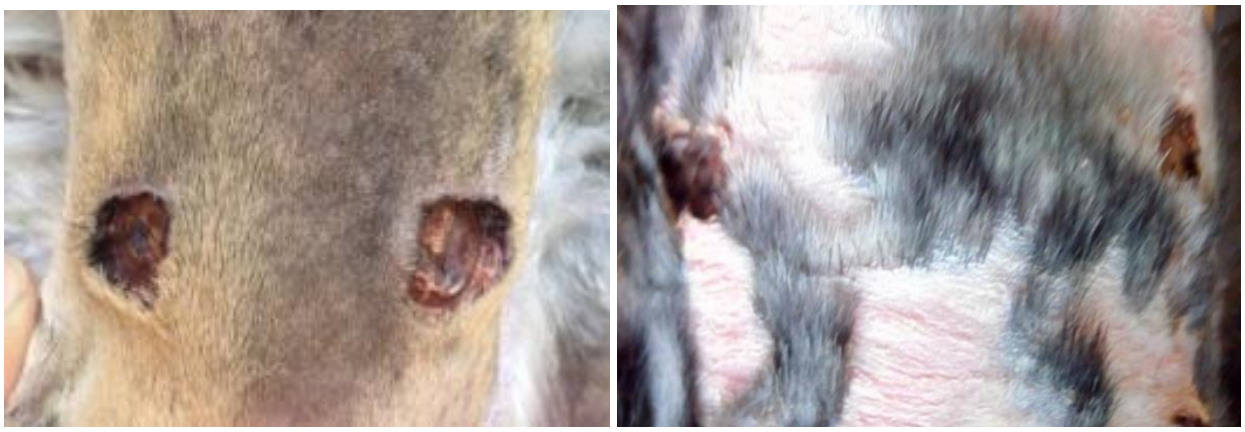


Group 3 **Group 4**

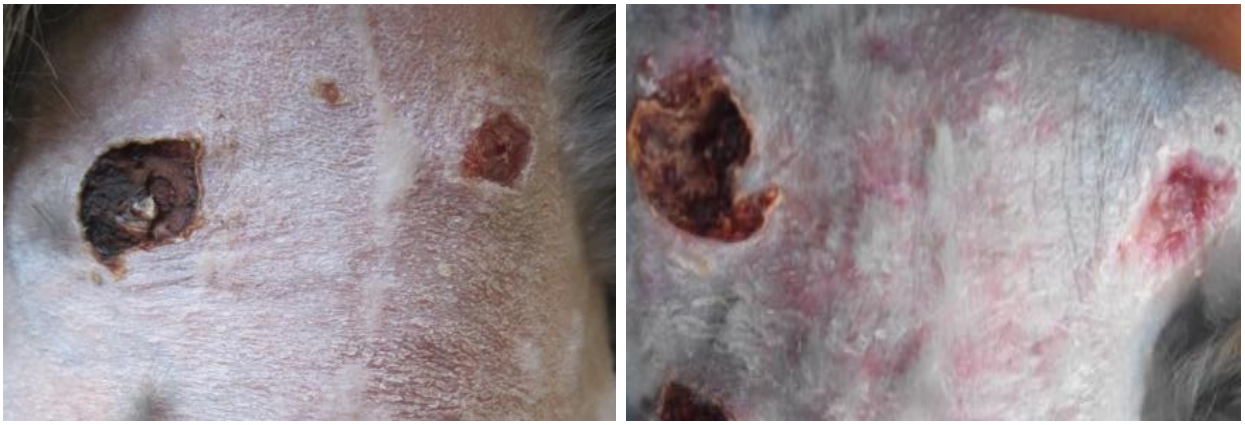


Group 5

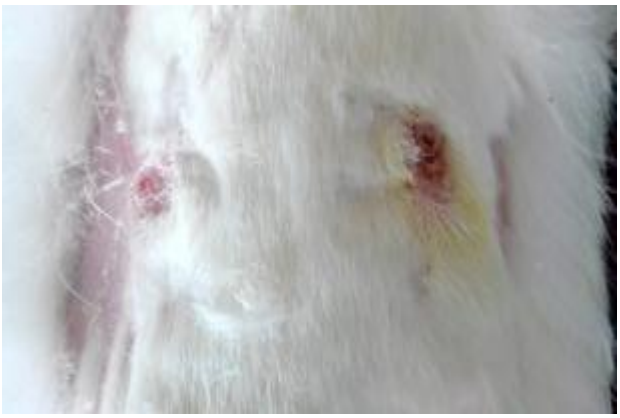
Fig 3: Gross appearance of wounds of different animals of different groups on day 3



Group 1 **Group 2**

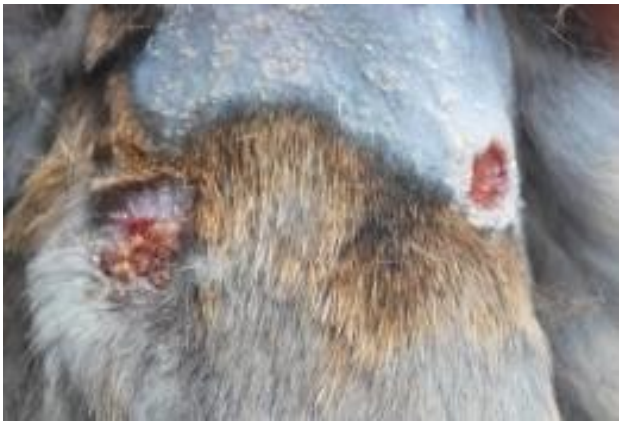


Group 3 **Group 4**

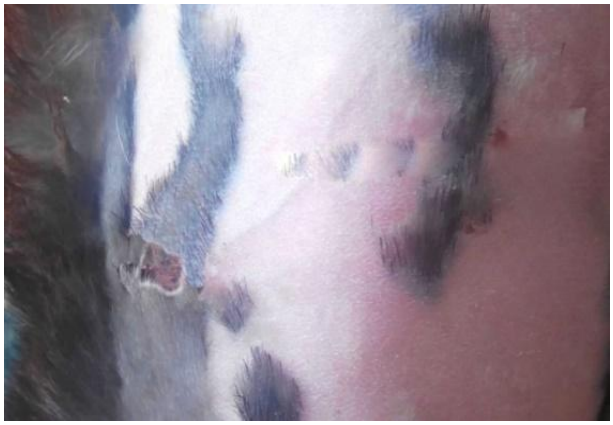


Group 5

Fig 4: Gross appearance of wounds of different animals of different groups on day 7



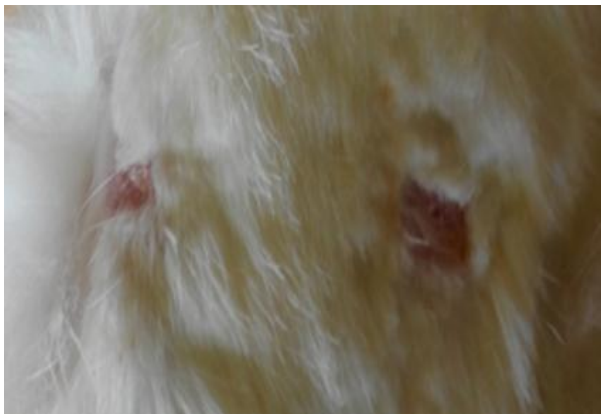
Group 1



Group 2

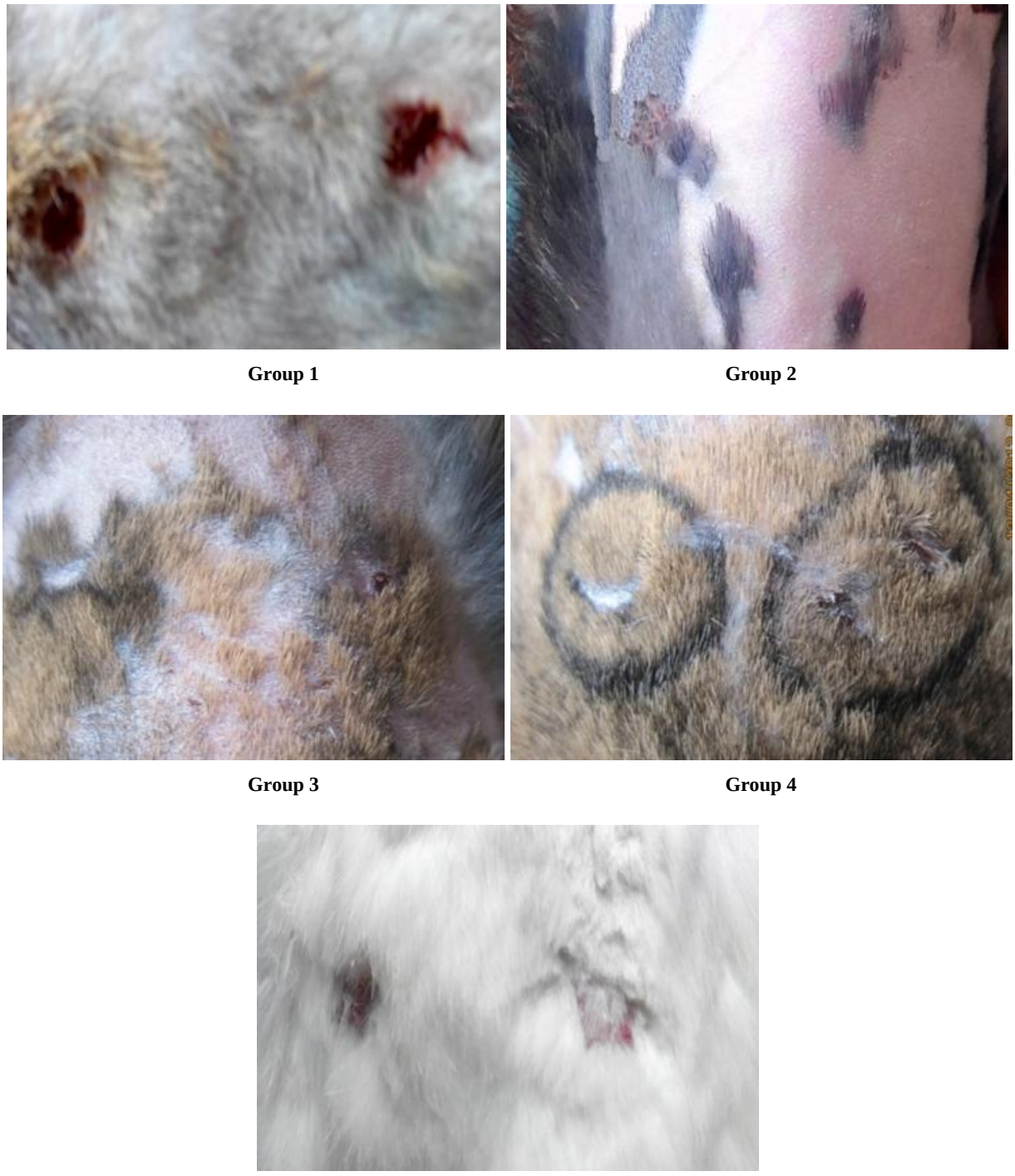
Group 3

Group 4



Group 5

Fig 5: Gross appearance of wounds of different animals of different groups on day 14



Group 1 **Group 2**

Group 3 **Group 4**

Group 5

Fig 6: Gross appearance of wounds of different animals of different groups on day 21

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