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PREPARATION AND EVALUATION OF GENTAMICIN LOADED CHITOSAN-GELATIN COMPOSITE FILMS FOR WOUND HEALING ACTIVITY

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ABSTRACT : Natural polymers are used as lead compounds for design of therapeutic drug delivery systems for treatment of different ailments. Chitosan and gelatin have proven wound healing properties individually. As both have wound healing property, the combination of these two polymers and incorporation of drugs into the composite films may show improvement in wound healing property. Thus, the composite films and drug loaded films were evaluated for various *in vitro* evaluation tests and to ascertain the applicability of prepared combination for wound healing activity. The composite films were prepared with increase in gelatin concentration. The drug loaded films were prepared with increasing concentrations of drug. These films were evaluated for thickness, folding endurance, water absorption capacity, antibacterial activity, tensile strength, *in vitro* drug release by diffusion studies and *in vivo* studies by excision wound model. The drug loaded films shown significant difference in folding endurance, water absorption capacity, antibacterial activity when compared to optimized composite film. There was no significant difference in thickness and tensile strength of drug loaded films when compared to blank composite films. Percentage of wound contraction was more for wounds treated with gentamicin loaded chitosan-gelatin composite film than blank composite film. With the above results, gentamicin loaded chitosan-gelatin combination had shown better results when compared to chitosan-gelatin film, chitosan film alone in wound healing activity.
Key words: Gentamicin, Chitosan, gelatin, chitosan-gelatin, wound healing

INTRODUCTION

Natural polymers are useful as biomaterials and these are biodegradable polymers. These are used in regenerative medicine, implantable materials, controlled release carriers or scaffolds for tissue engineering. Cellulose, chitin, chitosan and gelatin are widely used natural polymers. Natural polymers when used as drug delivery carriers, they are degraded into biologically accepted compounds, often through the process of hydrolysis, which leave the incorporated medications behind (Cullen T Vogelson 2001). Advantages of natural polymers are good cytocompatibility (Dang et al. 2006), biodegradable in nature and do not require any surgery for removal of polymers (Shi C et al 2009).

The present research work concentrated on the preparation of films from chitosan, gelatin, chitosan-gelatin composite films and gentamicin loaded chitosan-gelatin films at different concentrations of drug were prepared, tested to ascertain the applicability of prepared combination for wound healing activity.

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-Glucosamine and N-acetyl-D-Glucosamine. It is commercially produced by deacetylation of chitin, which is structural element in exoskeleton of crustaceans (crabs, shrimps etc). The amino group in the chitosan has a pKa value of 6.5. Thus chitosan is positively charged and soluble in acidic to neutral solution, which makes chitosan a bioadhesive and readily binds to negatively charged surfaces such as mucosal membranes.

Chitosan is nontoxic, biocompatible (Shelma R et al 2008, In-Yong K et al 2008, Emir BD, Raphael MO 2006), biodegradable polymer(Shi C et al 2009, Sarawathy G 2001). It has reported uses in drug delivery, cell delivery systems, orthopedics, wound healing (Daniela E and Camelia EO 2008), ophthalmology, bone healing (Senel S and Mc Clure SJ 2004). It enhances function of polymorpho nuclear cells, macrophages(Ueno H et al.1999) and fibroblastic proliferation of migration(Su CH et al. 1997). It exhibits antimicrobial activity against bacteria (Mohy Eldin MS 2008), fungi (Qi L 2004), and yeast. It is hypoallergenic, has rapid blood clotting property, haemostatic and acts as fat attractor by binding dietary lipids (Koide SS 1998).

Gelatin is also a natural polymer derived from collagen of animal skin and bones. It is translucent, colorless, brittle and tasteless. It is biodegradable in nature. It has good film forming property and known for its wound healing properties (Tanaka A et al. 2005) by preventing fluid loss due to exudation (Sinha RN et al. 1972). It is commonly used as gelling agent in food, pharmaceutical, photography and cosmetic manufacturing. It is used in manufacture of shells of pharmaceutical capsules. It is used as stabilizer, thickener, texturizer in foods such as ice creams, jams, yogurt, cream cheese. It is used as carrier, coating or separating agent for other substances. Gelatin is used as biological substrate to culture adherent cells. It is a good source of protein. It promotes general joint health and stiffness in athletes.

Wound healing is the body's natural process of regenerating dermal and epidermal tissue. It is the process where by the body restores the injured part to as near its normal condition as possible.Though wound healing takes place naturally on its own, some of complications like sepsis, disruption of tissue and skin layer, maggot's formation, extension of infection to adjacent and interior organs occur in major cases. To prevent extensive loss and damage to the tissue, skin grafting (Anjaiah A et al. 2001) and biological dressings (Varshney AC et al 1998) were developed.

The ability of the skin to repair itself after a minor wound is remarkable, but when the damage is severe or occurs in large amounts of skin area, proper and immediate coverage of wound surface with an adequate dressing is needed to protect the wound and accelerate wound healing. Ultimately the immediate wound coverage, temporary or permanent, is one of the principal goals of wound management.

MATERIALS AND METHODS

Materials

Gentamicin is obtained as gift sample from Integrated Marketing Co., Hyderabad. Chitosan is extracted from crab shells. Gelatin is procured from Qualigens fine chemicals, Mumbai, India. Ethylene glycol is procured from Central drug House, Mumbai. Two gram positive bacteria: *bacillus subtilis, staphylococcus aureus*, and two gram negative bacteria: *escherichia coli, pseudomonas aeruginosa* were selected and obtained from NCL, Pune.

Methods

Preparation of films

Preparation of chitosan films: The chitosan films were prepared by casting chitosan (which is collected from crab shell (Felicity B et al 2007)) (1%) solution in the plastic tray and air dried at room temperature.

Preparation of gelatin films: The gelatin films were prepared by casting gelatin (10%) solution in the plastic tray and air dried at room temperature.

Preparation of chitosan-gelatin composite films: All the chitosan-gelatin composite films (of 6 inch length and 4.5 inch width) were prepared by solvent casting technique. Chitosan (1%) and gelatin (10%) solutions were prepared individually. They were mixed in the ratios of 1:10, 1:20, 1:30, and 1:40. The polymer solution was mixed thoroughly and 0.2ml. of ethylene glycol was added as plasticizer. The solution was filtered under vacuum for the removal of any entrapped air bubbles. Then the solution was cast into the film on the plastic tray and air dried at room temperature (Tanwar YS 2005). The composition of different films was shown in Table-1.

Table-1: Composition of blank films

S.No	Code of the film	No. of parts	
		Chitosan	Gelatin
Films			
1	F_1	1	-
2	F_2	-	10
Composite films			
3	F_3	1	10
4	F_4	1	20
5	F_5	1	30
6	F_6	1	40

Based on the tensile strength of the films, the ratio of 1:40 was selected for further process. The drug loaded films were prepared by using this ratio.

Preparation of drug loaded chitosan-gelatin composite films: Stock solutions of gentamicin hydrochloride were prepared in the concentrations of 1, 2, 3, 4 %w/v. From each concentration 0.86ml. of drug solution was added to the polymer solution of 1:40 ratio of chitosan and gelatin such that 10, 20, 30, 40 µg. of drug was present in 0.19 sq.cm. area of the film respectively (this area is equal to the area of standard antibiotic disc). Different drug loaded composite films were prepared with composition as shown in Table .2.

Evaluation of films

Thickness: The thickness of the film influence the amount of drug available and also the time required to absorb the polymer into the body. The thickness of each film was measured using screw gauge at three different positions of the film and the mean was calculated.

Table-2: Composition of drug loaded films

S.No	Code of film	µg of drug/0.19 sq.cm
Gentamicin hydrochloride		
1	GF_{11}	10
2	GF_{12}	20
3	GF_{13}	30
4	GF_{14}	40
5	GF_{15}	50

Folding endurance: The folding endurance was determined to determine flexibility of film. The flexibility of the film is needed to handle the film easily and for comfortable, secured application of film on the wound. It was determined by repeatedly folding one film at same place till it breaks or folded up to 300 times manually. The number of times of film could be folded at the same place without breaking give the value of folding endurance.

Water absorption capacity: It is of utmost importance, if they are used for biological applications and wound healing. It is used to measure the capacity of film to absorb wound exudates. The initial weight of 1inch of dry film was noted. Then this film was placed in 15ml. of distilled water taken in petri plate. The weight of the film was noted periodically at first hour, second hour, third hour and 24th hour. Every time after noting the weight, the film was placed in fresh water. Water absorption capacity of the film was calculated using a formula

$$\%\ \text{Water absorption capacity} = \frac{\text{Final weight - Initial weight}}{\text{Initial weight}} \times 100$$

Antibacterial activity: The prepared films were evaluated for antibacterial activity against four different strains with agar plate diffusion method by measuring the zone of growth inhibition of micro organisms. The antimicrobial activity of a compound is expressed in terms of ability to inhibit growth of bacteria in nutrient agar.

Tensile strength: Tensile strength measures the ability of film to withstand rupture, mechanical pressures or the force required to break the film. Tensile strength of the film was determined by using the *Instron* tensile testing machine at SDDC section in CLRI. It was expressed in MPa units. Tensile strength was done for all composite films.

Diffusion studies: Diffusion studies were conducted with selected drug loaded films CF_{10} to find the time taken to release the total drug. The diffusion studies were carried out using diffusion membrane. For every 10 minutes, 5ml. of sample was withdrawn from the beaker. The concentration of drug in the samples was estimated using UV/Visible spectrophotometer at respective wavelength. An equal volume of fresh distilled water was replaced after with drawl of each sample.

***In vivo* studies**

Pathogen free adult female albino rats weighing 150-200 gm. were selected. The rats were housed in polypropylene cages under standard laboratory conditions with 12-hour light dark cycle. The rats were fed with standard laboratory chow (Hindustan Lever limited, Mumbai) and water *ad libitum.* The wound healing activity was conducted with the protocol as shown in Table .3.

Table-3: Protocol for *in vivo* wound healing studies

S.No.	Group No.	Purpose	Code of film used for application
1	I	Normal control	-
2	II	Wound control	-
3	III	Treatment group with blank chitosan film	F_1
4	IV	Treatment group with blank Composite film	F_6
5	V	Treatment group with gentamicin loaded composite film	GF_{14}

The anaesthetized animal was placed on the operation table in normal position. The dorsal fur of the animals was shaved with an electric clipper and the anticipated area (2 cms. length and 1cm. width) of the wound to be created was outlined on the back of the animals on interscapular region i.e, 5mm. away from ears. Full thickness skin from the demarked area was excised to get a wound area of 2sq.cm. After achieving haemostasis, the wound was blotted with sterile gauze in control group, the respective film on the wound of animals in treatment groups (Group-II, III).
Then the following parameters were determined at specific time intervals.

Percentage of wound contraction: This is measured to determine the reduction in wound area at different period of treatment. It was determined by graphical method. Wound area was calculated on 7^{th}, 14^{th}, 21^{st} and 28^{th} post wounding day by counting number of squares of retraced wound area on graph paper. The degree of wound healing was calculated as % closure of the wound area from the original wound using a formula

$$\% \text{ closure} = 1-(A_d/A_0) \text{ X } 100$$

A_0 – Wound area on day zero.
A_d – Wound area on corresponding days.

Histopathological studies: Biopsy specimens for histopathological examination were collected at 7, 14, and 28^{th} day post-treatment from all the groups and preserved in 10% buffered formalin. They were processed by routine paraffin embedding technique i.e, 5-6 microns thick sections were cut and stained with haematoxylin and eosin (Carleton and Drury, 1965). The specimens were collected by trephining that involved skin tissue of both healing and normal skin using corneal trephiner.

Photography: The photographs of wound from different groups were taken at specific intervals for visual comparison.

Statistical Analysis

The results are expressed as mean ± S.D. Statistical analysis was performed by paired t-test, one-way and two-way analysis of variance (ANOVA) test for multiple comparisons. Statistical significance was set accordingly at p (0.05) level.

RESULTS AND DISCUSSION

All the films were prepared by solvent casting technique and were evaluated for thickness, folding endurance, water absorption capacity, antibacterial activity, tensile strength and wound healing activity.

In composite films, with increase in gelatin concentration, thickness also increased significantly. There is no significant difference in thickness of drug loaded films when compared to blank composite films which indicated that the loading of drug did not influence the thickness of films as shown in Table. 4. All the films have uniform thickness.

Table. 4 shows the folding endurance values of all prepared films. With increase in concentration of gelatin, there was significant increase in folding endurance of composite films. There was significant difference ($p<0.05$) in folding endurance between optimized blank composite film F_6 and the drug loaded films may be due to alteration of flexibility of films by drugs. GF_{14} shown maximum folding endurance among drug loaded films, which indicated that it may have maximum flexibility as shown in Table. 4.

Table-4: Thickness, folding endurance, water absorption capacity of prepared films

S.No.	Code of the film	Thickness (µm.) (Mean± S.D.)	Folding endurance (Mean±S.D)	Water absorption capacity (%) (Mean±S.D)
Blank films				
1	F_1	60±0.017	>300	1101.87±9.08
2	F_2	126±0.02	177.6±6.55	It was dissolved.
3	F_3	30±0.01	202±5.50	879.60±14.92
4	F_4	43±0.005	215±3.60	951.18±7.92
5	F_5	56±0.011	225±3.51	1266.69±8.86
6	F_6	61±0.01	256±5.50	1411.03±11.99
Gentamicin loaded films				
7	GF_{11}	63±0.02	231.6±6.42	1104.01±15.43
8	GF_{12}	56±0.005	228.3±5.50	1043.78±19.29
9	GF_{13}	83±0.015	225±4	1142.89±11.74
10	GF_{14}	56±0.015	260.3±3.78	1193.62±17.93
11	GF_{15}	60±0.01	259.6±4.72	1109.55±17.08

With increase in concentration of gelatin, water absorption capacity of composite films was significantly increased. The water absorption capacity of drug loaded films was slightly increased with increase in concentration of drug as per table no.4. It was found that, there was significant difference in water absorption capacity between blank composite films and drug loaded films. It indicated that the drug is decreasing the water absorption capacity of film may be due to interference of drugs in water absorption by polymers. Among gentamicin drug loaded films, GF_{14} i.e., 40µg./0.19 sq.cms. loaded film, shown maximum water absorption capacity.

Table. 5 shows the diameter of zone of inhibitions of drug loaded films and optimized blank composite film. The zone of inhibition of drug loaded films against four different bacterial species was different in agar disc diffusion technique. As the concentration of drug in the film was increasing the mean diameter of zone of inhibition was increased in drug loaded films. Among gentamicin drug loaded films GF_{14} shown maximum inhibition against all tested bacteria. The antibacterial activity of drug loaded films was more than the blank chitosan and composite films. It confirmed that the more antibacterial activity of the drug loaded films than blank films as shown in Table. 5, so drug loaded films can show fast wound healing property than blank films.

Table. 6 shows the tensile strength parameters of composite films and selected drug loaded film. As gelatin proportion is increased, parameters like maximum extension, elongation at break (%) and tensile strength were increased. As per paired t-test of statistical analysis there was no significant difference ($p<0.05$) in tensile strength of blank and drug loaded films. It indicated that the tensile strength of film was not changed significantly after loading the drug into film.

Table. 7 shows the cumulative percentage drug release of selected drug loaded film. The percentage of drug release from these film was determined by *in vitro* diffusion studies to find the time taken by film to release the complete loaded drug for eliciting its antibacterial action on wound. 99% of gentamicin was released within 1 ½ hour (90 minutes) indicated that the film is not interfering in drug release on wound

Table-5: Antibacterial activity of optimized composite and drug loaded films against different organisms

Code of film	Diameter of zone of inhibition (in cms) (Mean±S.D.)			
	Bacillus subtilis	***Staphylococcus aureus***	***Escherichia coli***	***Pseudomonas aeruginosa***
Blank films				
F_1	1.70±0.045	1.66±0.09	1.5±0.072	1.52±0.040
F_2	0	0	0	0
F_6	1.38±0.061	1.37±0.060	1.20±0.050	1.26±0.040
Gentamicin loaded films				
GF_{11}	2.22±0.060	2.29±0.050	2.12±0.08	2.02±0.055
GF_{12}	2.46±0.081	2.61±0.055	2.33±0.06	2.43±0.045
GF_{13}	2.80±0.045	2.79±0.035	2.68±0.065	2.72±0.045
GF_{14}	3.13±0.040	2.98±0.03	3.05±0.045	2.91±0.055
GF_{15}	3.34±0.045	3.10±0.035	3.24±0.05	3.07±0.050

Table-6: Tensile strength parameters of blank composite and selected drug loaded films

Tensile strength parameters	F_3	F_4	F_5	F_6	GF_{14}
Maximum load (N)	17.80	43.64	33.29	22.71	20.53
Maximum extension (mm)	0.67	1.17	1.12	2.67	1.56
Elongation at break (%)	2.22	2.33	2.25	5.33	3.13
Tensile strength (mpa)	11.13	39.68	41.62	45.42	41.13

***In vivo* studies:** Table. 8 shows percentage wound contraction of different groups at different time intervals. There was significant ($p<0.05$) difference in percentage of wound contraction between untreated group and treated group (Group-III, IV, V). 100% of wound contraction was observed in groups treated with selected drug loaded film within 28 days. There was significant difference in percentage of wound contraction between wounds treated with chitosan (Group-III), wounds treated with optimized blank composite (Group-IV) and drug loaded films (Group-V). It indicated that chitosan-gelatin composite films and drug loaded chitosan-gelatin films have improved wound healing activity than chitosan alone film. Though there was increased wound contraction with drug loaded films, but no significant difference was observed between drug loaded composite films and blank composite film.

Restoration and recovery of cells was observed in wounds treated with chitosan film, chitosan-gelatin composite film when compared to untreated wounds in 28th day photomicrographs. Decrease in wound size was observed in wounds treated with gentamicin loaded films on 28th day when compared to all other groups. This suggested that gentamicin drug loaded films may have more capacity for fast recovery and rapid epithelialization of skin than in the untreated and wounds treated with other films (Figure. 1 & 2).

It may be due to its antibacterial action which prevents further infections on wound supporting for fast epithelialization by stimulation of wound healing environment.

On 7th, 14th and 28th day more decrease in wound size was observed in group treated with chitosan-gelatin composite film when compared to in groups treated with chitosan alone. It indicated that the combination of chitosan-gelatin showed better wound healing property than chitosan alone. On 7th, 14th and 28th day the size of wound was more decreased in group treated with gentamicin loaded film when compared to other groups on respective days which indicated that loading of drug into composite films augmented the healing of wound than blank composite films (Figure .3). This may be due to broad antibacterial activity of gentamicin which reduces infections and thus fastens the healing of wound.

Table-7: Cumulative percentage drug release from selected gentamicin loaded film

Time (min)	Drug release (%) (Mean±S.D.)
0	0
10	15.51±0.96
20	29.75±1.10
30	41.80±1.44
40	51.31±0.88
50	62.32±0.66
60	71.63±0.76
70	80.91±0.78
80	87.95±0.87
90	97.98±0.13

Table-8: Percentage of wound contraction at different time intervals in different groups

Group	Wound contraction (%) (Mean±S.D.)			
	7th day	14th day	21st day	28th day
II (Untreated)	21.31±4.20	59.80±2.95	75.91±2.10	92.46±1.11
III (F_1 treated)	40.64±4.12	68.29±1.49	84.75±1.06	95.49±0.29
IV (F_6 treated)	54.33±3.66	76.72±1.33	90.74±0.69	98.78±0.10
V (GF_{14} treated)	60.36±2.42	83.66±2.44	93.20±0.19	99.64±0.08

Figure-1: Photomicrographs of wounds treated with chitosan-gelatin composite film

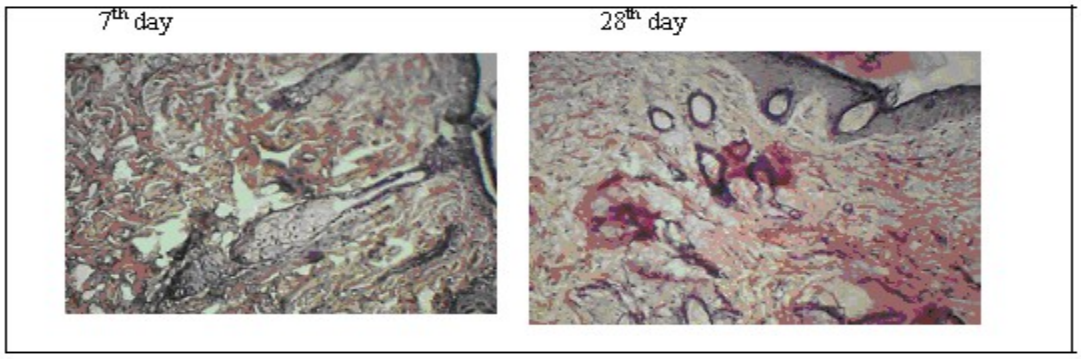


Fig No.2: Photomicrographs of wounds treated with gentamicin loaded films

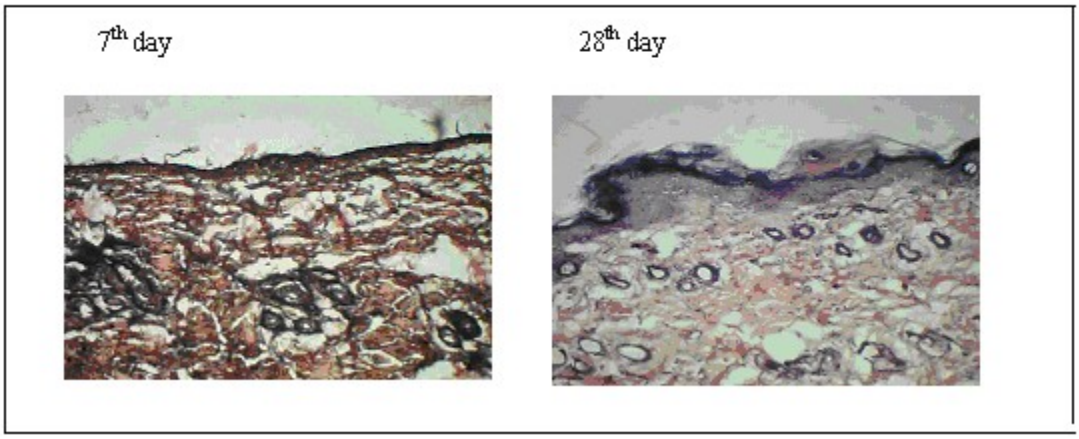
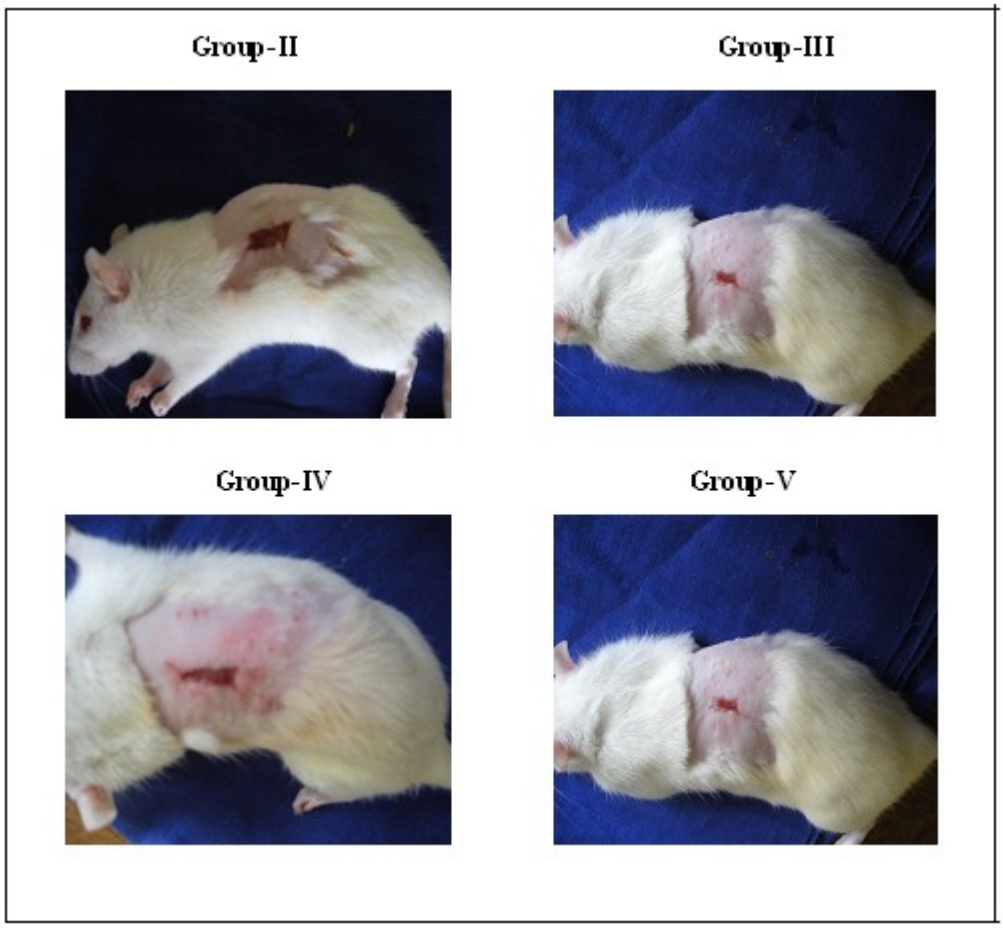


Figure-3: Comparison of photographs of wounds on 14th day in different groups



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