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Research Article

Pharmacological Evaluation of Topical Gel Containing Plant Proteinases on Wound Healing Using Excision and Incision Wound Model

Gunde M C[1,2*], Gilhotra R M[2], Amnerkar N D[3]

[1]*Kamla Nehru College of Pharmacy, Butibori, Nagpur, Maharashtra, India*
[2]*Suresh GyanVihar University, Mahal, Jagatpura, Jaipur, Rajasthan, India*
[3]*Adv.V.R.Manohar Institute of Diploma in Pharmacy, Wanadongari, Nagpur, Mahrashtra,* India

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ABSTRACT
The aim of present study was to evaluate wound healing activity of topical gel containing plant proteinases using excision and incision wound model. In excision wound model, Circular wounds of about 2.5cm diameter were made on depilated dorsal thoracic region of anaesthetized rat under aseptic condition. All the topical gels were applied once daily starting from the day of wounding. The percentage of wound contraction was calculated from the measured wound area in 3day interval and epithelization time was also noted. Histopathological study performed to check healing markers. In incisio wound model, Paravertebral long incision of about 6 cm length were made on depilated back of the rats. The parte skin was kept together and stitched at about 0.5 cm intervals continuously and tightly using non absorbable surgical sutures. When the wounds were cured thoroughly, the sutures were removed on day 9 and the tensile strength of the healed wounds were measured on day 10. Data obtained were analyzed using oneway ANOVA and post hoc test done using graph pad prism version 5. The results of present study indicated that chitosan gel containing mixture of plant proteinases shows good wound healing activity as compare to other groups by increasing percentage of wound contraction, reducing epithelization time and improving breaking strength. Histopathological studies also support the results. The more wound healing activity may be due to synergistic effects of plant proteinases and chitosan with each other.

Keywords: Plant proteinases, Excision, Incision, Wound healing.

INTRODUCTION

A wound may be defined as loss or breaking of cellular, anatomical or functional continuity of living tissue. Everyday it became a critical clinical problem with initial and late complications result into important cause of morbidity and mortality[1]. Wound healing is a complex process take place in all parts of body. Although it is a continues process, divided into different phases (Coagulation and haemostasis phase, inflammatory phase, proliferative phase, remodeling phase) for better understanding[2,3]. In India, traditionally people use the plants for cure of many diseases and now researchers are taking interest to know the potential of natural products for the development of drugs. Many medicinal plants have been shown good wound healing activity in animal studies[4]. Modern wound medicine should integrate and support the body for regeneration of loss or break tissues[5]. Chitosan used as a gelling agent in present study is a linear polysaccharide composed of β-(1-4) linked D-glucosamine and N-acetyl-D-glucosamine units. It is a second most abundant natural biopolymer after cellulose[6,7]. Cationic primary amino group of chitosan binds with anionic substrate of mucus and shows mucoadhesion property which is important for topical use[8]. Furthermore, it shows wound healing activity by its haemostatic effect, activate macrophage, stimulate cell proliferation and tissue regeneration[9,10]. Plantcysteine proteinases shows different pharmacological activities[11]. Papain, a nonspecific cysteine proteinase obtained from latex of Carica papaya is able to break variety of necrotic tissue in wound healing over a wide pH range 3.0-12.0[12]. It can enhance wound healing abilities of chitosan[13] and also

Table 1: Grouping of animals for experimental design

Group	Treatment
Group- I	Negative control received no treatment
Group- II	Treated with empty chitosan gel
Group- III	Treated with chitosan gel containing papain
Group- IV	Treated with chitosan gel containing bromelain
Group- V	Treated with chitosan gel containing papain-bromelain mixture
Group- VI	Positive control received standard drug (Framycetin Sulphate IP).

**Author for Correspondence*

Table 2: Photomicrography equipment specifications for histopathological study

Slide No.	Particular's	Magnification			Remark Colour	Exposure Speed
		Eye piece	objective	Total		
1.	Treated group	10 x	10x	100 x	P,R,B	1/15 sec.
2.	Treated group	10 x	40 x	400 x	P, B, Bl	1/8 sec.
3.	Control group	10 x	10 x	100 x	P,Bl,	1/15 sec.
4.	Control group	10 x	40 x	400 x	P, B	1/4 sec.

Table 3: Effect of topical gels on wound contraction in excision wound model

Area of wound closure (sq. mm.± SEM) (Percentage of wound contraction in sq. mm.± SEM)							
Days → / Groups ↓	0 day	3rd day	6th day	9th day	12th day	15th day	18th day
Group-I	493.41±2.31 (0.00)	461.75±2.37 (6.41±0.39)	422.58±4.12 (14.15±0.78)	371.58±4.23 (24.67±1.10)	278.08±4.76 (43.63±0.85)	157.41±4.44 (68.09±0.96)	64.91±4.24 (86.87±0.82)
Group-II	494.41±1.31 (0.00)	450.50±4.02 (8.89±1.01)	413.08±3.65 (16.44±0.84)	344.41±7.01 (30.33±1.41)	247.16±5.93 (50.00±1.15)	134.08±3.83 (72.87±0.82)	13.66±3.65 (97.23±0.73)
Group-III	493.75±2.38 (0.00)	444.25±6.02 (10.01±1.16)	402.33±3.02 (18.50±0.73)	318.75±5.40 (35.43±0.77)	220.41±5.74 (55.34±0.76)	103.41±3.89 (79.04±0.80)	4.83±2.71 (98.99±0.56)
Group-IV	493.20±2.46 (0.00)	441.58±4.00 (10.45±1.06)	400.83±1.86 (18.72±0.48)	315.16±3.77 (36.09±0.75)	218.41±3.55 (55.71±0.62)	101.33±4.81 (79.44±1.02)	4.66±2.16 (99.05±0.43)
Group-V	493.70±2.08 (0.00)	432.63±5.51 (12.36±1.20)	384.58±4.30 (22.09±1.03)	286.66±5.26 (41.92±1.57)	186.91±2.92 (62.13±0.55)	74.83±5.31 (84.84±1.09)	0.00 (100.00)
Group-VI	494.03±2.11 (0.00)	431.88±5.18 (12.51±1.18)	386.16±3.23 (21.77±0.58)	295.08±3.64 (40.26±1.34)	193.08±5.92 (60.91±0.98)	78.08±4.77 (84.18±1.02)	0.50±0.83 (99.89±0.17)

Initial wound area approx. 495sq mm

≈ n = 6 animals in each groups.

≠ Result expressed as Mean Area ± S.E.M

* P≤ 0.01 indicates significant when compared with control.

Ψ Figure in parenthesis indicate percent wound contraction.

Table 4: Effect of topical gel on epithelization time

Groups	Epithelization time
Group - I	24.83±0.81
Group - II	21.83±0.81
Group - III	20.33±1.21
Group - IV	19.58±0.66
Group - V	16.91±0.66
Group - VI	17.83±0.75

P < 0.01 vs.control. Values are mean ± SEM (n=6)

shows anti-inflammatory, antioxidant and antibacterial property[14]. It may be useful for decreasing bacterial burden, reduction of exudates and to increase granulation tissue formation[15,16]. Bromelain another complex mixture of proteinases obtained from pineapple plant (*Ananus cosmosus*). Due to its proteolytic activity it removes some cell surface molecules and that affect lymphocyte activation and migration at wounding site[17]. It may decreases neutrophil movements towards site of inflammation and shows anti-inflammatory activity[18]. It also shows antioxidant activity which is required for wound healing[19]. Therefore, in present study we evaluate wound healing activity of plant proteinases (papain and bromelain) and chitosan which used as a gelling agent.

MATERIAL AND METHODS

Materials

Chitosan purchased from Sigma Aldrich, Mumbai, India. Papain kindly provided as gift sample by Advanced Enzymes Technology Ltd. Thane (W), India and Bromelain purchased from Prisha Herbals, Indore, India. Other solvents and chemicals used in present study were of analytical grade.

Preparation of topical gels

Topical gels were prepared by adding chitosan into half of water containing glacial acetic acid (1%v/v) stirred it slowly. After the swelling remaining amount of water was added and mixed properly, methyparaben (0.1%w/w) was added as preservative. After the preparation of gel required amount of papain and bromelain were added. The prepared gels were stirred and sonicated to remove air bubbles.

Design of animal experiments

After taking permission from IAEC (Institutional Animal Ethical Committee) proposal no. KNCOP/R&D/AN-PROT/14-15/05, all animal experiments were performed asperguidelines given by CPCSEA (Committee for the Purpose of Control and Supervision of Experiments on Animals). Albino wistar rats weighing 150-200gm were used for present study. Free access to food and water were given to rats and they kept in clean polyethylene cages separately. Cages were kept in well ventilated animal house.The animals were divided as shown in Table 1.

Formation of Excision wounds

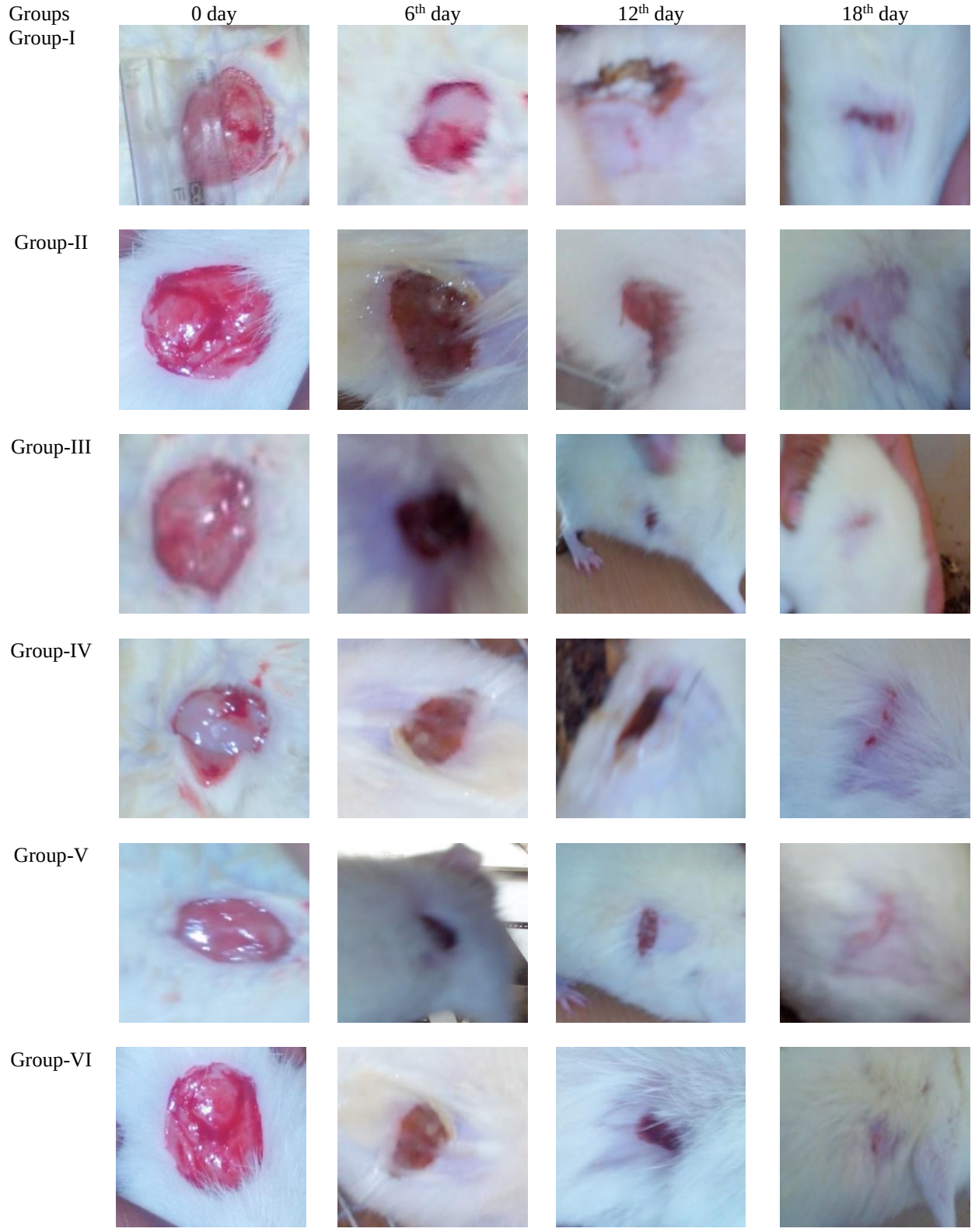


Figure 1: Effects of topical gels on wound contraction in excision wound model

Rats were locally anaesthetized by diethyl-ether and depilated by removing hairs at the dorsal thoracic region by sterile blade prior to excision. Circular wounds of about 2.5cm diameter were made on depilated dorsal thoracic region under aseptic condition and were observed throughout the study. The areas of the wounds were measured (in sq. mm) immediately by placing a transparent polythene graph paper over the wound and then tracing the area of the wound on it (Approx. area 490 sq. mm). This was taken as the initial wound area reading. All the topical gels were

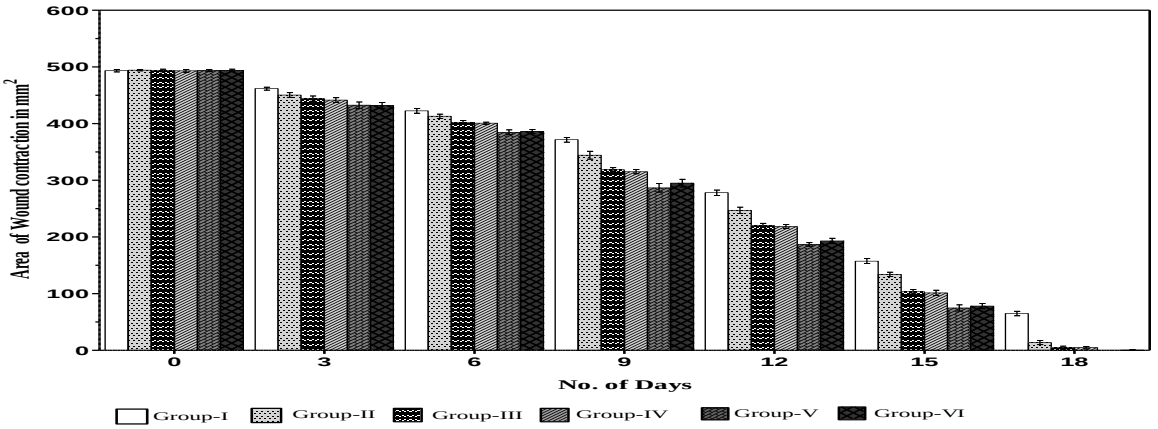


Figure 2: A Effect of topical application of formulated gels on excision wound expressed as area of wound contraction. Values are in mean ± SEM, $p<0.01$ vs control group.

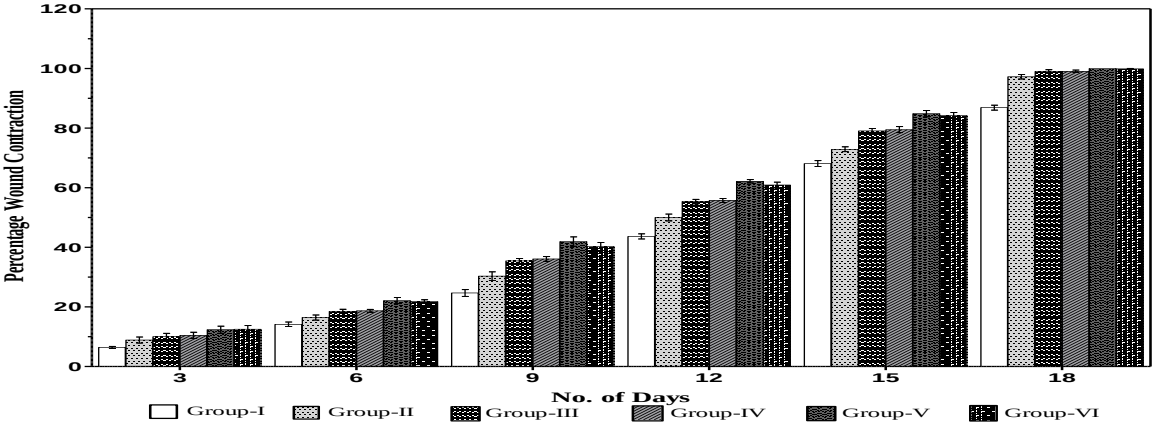
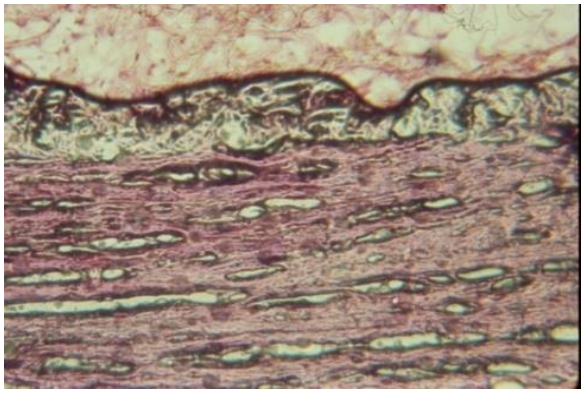
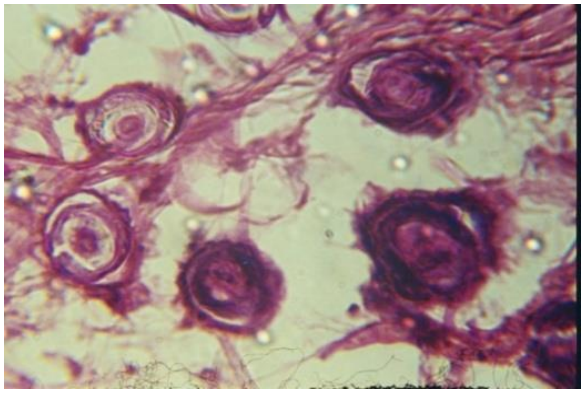


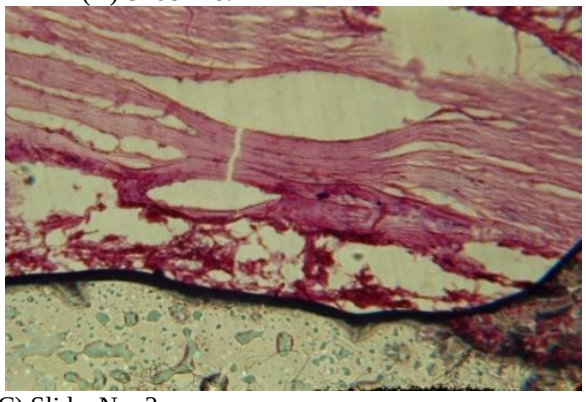
Figure 3: B Effect of topical application of formulated gels on excision wound expressed as percentage wound contraction. Values are in mean ± SEM, $p<0.01$ vs control group.



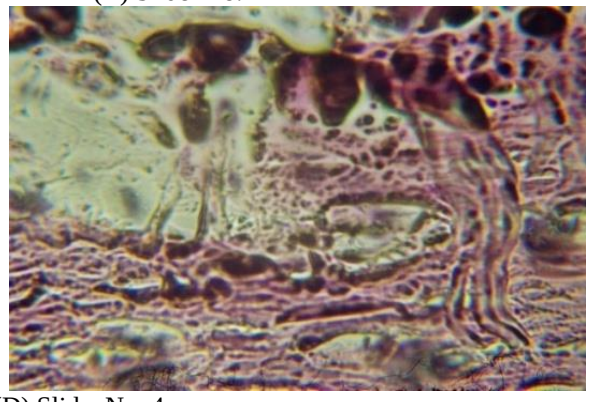
(A) Slide No.1



(B) Slide No. 2



(C) Slide No. 3



(D) Slide No. 4

applied once daily starting from the day of wounding. The wound area were measured on 3rd, 6th, 9th, 12th, 15th and 18th post wounding day (Figure-1). The percentage of wound contraction was calculated from the measured wound area[19]. The epithelization time was noted as the number of days required for scar to fall off and leaving no raw wound behind[20].

Histopathological studies

The present experimental study was supported with the histopathological study that involve examination of markers responsible for healing like epithelization, collagen formation, fibrosis and neovascularization. On 10th post wounding day regenerated tissue was removed and fixed with 10% formalin solution then it was dehydrated with 90% ethanol, embedded in paraffin, cut into thin sliced section (7 mm thick) stained with haemotoxyline and eosin dye and saw under Olympus P.M.6 Photomicrography equipmentfor above healing markers[21]. Equipment Model- Olympus P.M.6, Film used- Kodak 400 ASA

Formation of incision wound

The animals in each group were anaesthetized by diethyl-ether and depilated by removing hairs by sterile blade prior to incision. Paravertebral long incision of about 6 cm length were made on depilated back of the rats. Afterthe incision, the separated skin was kept together and stitched at about 0.5 cm intervals continuously and tightly using non absorbable surgical sutures USP (monofilament polyamide black)and 26 mm 3/8 circle reverse cutting needle (Figure 2). All the groups were treated in same manner as in the case of excision wound. When the wounds were cured thoroughly, the sutures were removed on day 9 and the tensile strength of the healed wounds were measured on 10th day[22].

Statistical analysis

All experimental data was expressed as mean ± SEM. Statistical analysis of data was done by using one way – ANOVA with *post hoc*test. Probability (p) value is estimated by using Instat graphpad prism 5, window (USA).

RESULTS

Rate of wound contraction

The rate of wound contraction is important parameter for evaluation of wound healing activity. In present study, the significant wound contraction was found in groups treated with chitosan gel containing plant proteinases as compared to untreated group. However group treated with gel containing mixture of plant proteinases shows highest wound contraction. (Table 3/ Figure 3).

Epithelization time

It is another parameter for evaluation of wound healing activity. In present study, it was found significantly reduced in groups treated with chitosan gel containing plant proteinases as compared to untreated group. However group treated with gel containing mixture of plant proteinases shows highest reduction.

Histopathological study

In present study, histopathological observations supports the above results by comparing untreated group with group treated with gel containing plant proteinases mixture.

Slide No.1- Group-V showing thin wellformed epidermis, complete reepitheliazation, increase in collagen fibre, fibroblast cells and new blood vessels formation on 18th post wounding day (100 X)

Slide No.2- Group-V showing thin well formed epidermis, complete reepitheliazation, increase in collagen fibre, fibroblast cells and new blood vessels formation on 18th post wounding day (400 X)

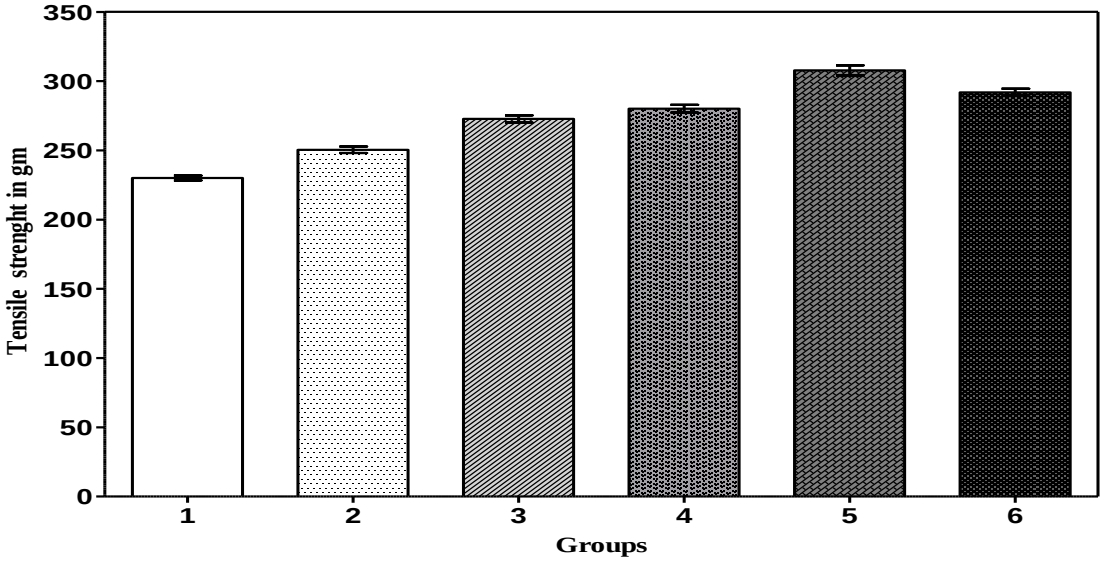


Figure 4: Effect of topical application of formulated gels on tensile strength of the skin in incision wound on 10th post wounding day.

Slide No.3- Group-I showing absence of reepitheliazation with immature granulation tissue formation, less blood vessels formation and poor collagenfibres. (100 X)

Slide No.4- Group-I showing absence of reepitheliazation with immature granulation tissue formation, less blood vessels formation and poor collagen fibres. (400 X).

Wound breaking strength

In incision wound model, wound breaking strength of each group measured and compare. It was found more in groups treated with chitosan gel containing plant proteinases as compared to untreated group. However group treated with gel containing mixture of plant proteinases shows highest breaking strength.

DISCUSSION

Experimental data for assessment of wound healing activity of plant proteinases showed increase in rate of wound contraction and decrease in epithelization time in excision model similarly, good wound breaking strength in incision wound model. Histopathological studies also supports the results. Therefore, present study revealed that plant proteinases possess significant wound healing activity in excision and incision wound healing models. Hence, we propose that the additive and synergistic effect of plant proteinases with chitosan were responsible for its potent wound healing activity. The present investigations supports scientific evidence to the folkloric use of plant products in wound healing.

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