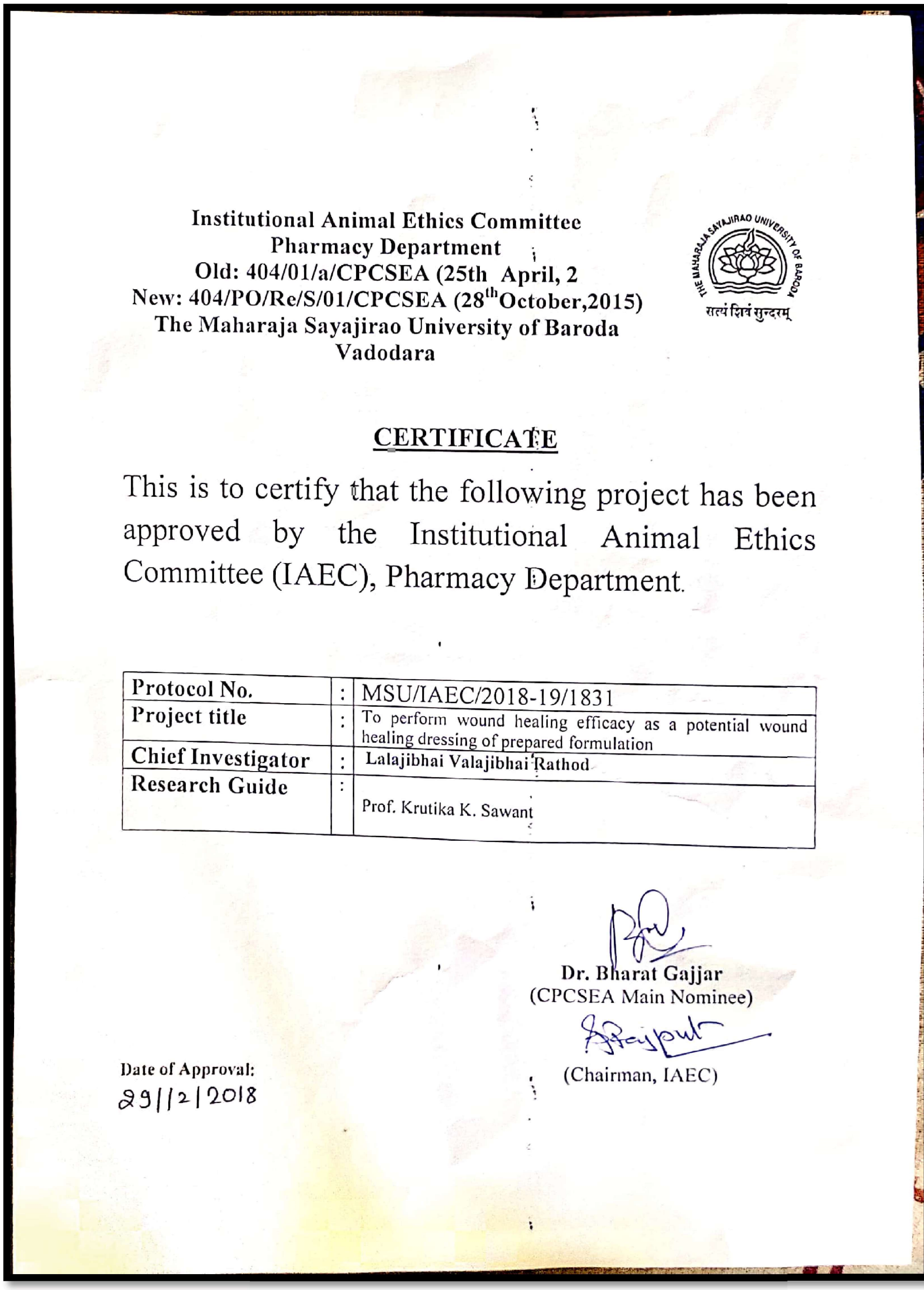
8.0 *IN-VIVO* WOUND HEALING ACTIVITY

After the scratch assay, assessment of wound healing was carried out on the experimental wound healing models in rats. Healthy male rats, weighing between 200 – 250 g were used in the study. All animals were housed in cages placed in an animal room with a constant temperature of 25°C ± 3° C and a fixed 12-hour light-dark cycle. The rats were given standard rat chow and water ad libitum. Care was taken to avoid unnecessary stress to the animals throughout the experimental period. All procedures were carried out as per the Institutional Animal Care and Use Committee (IACUC) guidelines and protocol approved by the Institutional Animal Ethical Committee (IAEC) which is mentioned in table 8.1 and certificate for them are represented into figure 8.1 & 8.2.

TABLE 8.1: Animal model and approved protocol number

Sr. no.	Animal model	Protocol no.
01	Cutaneous excision wound healing model	MSU/IAEC/2018-19/1831
02	Burn wound model	MSU/IAEC/2019-20/1916



Institutional Animal Ethics Committee
Pharmacy Department
Old: 404/01/a/CPCSEA (25th April, 2
New: 404/PO/Re/S/01/CPCSEA (28th October,2015)
The Maharaja Sayajirao University of Baroda
Vadodara

THE MAHARAJA SAYAJIRAO UNIVERSITY OF BARODA
सत्यं शिवं सुन्दरम्

CERTIFICATE

This is to certify that the following project has been approved by the Institutional Animal Ethics Committee (IAEC), Pharmacy Department.

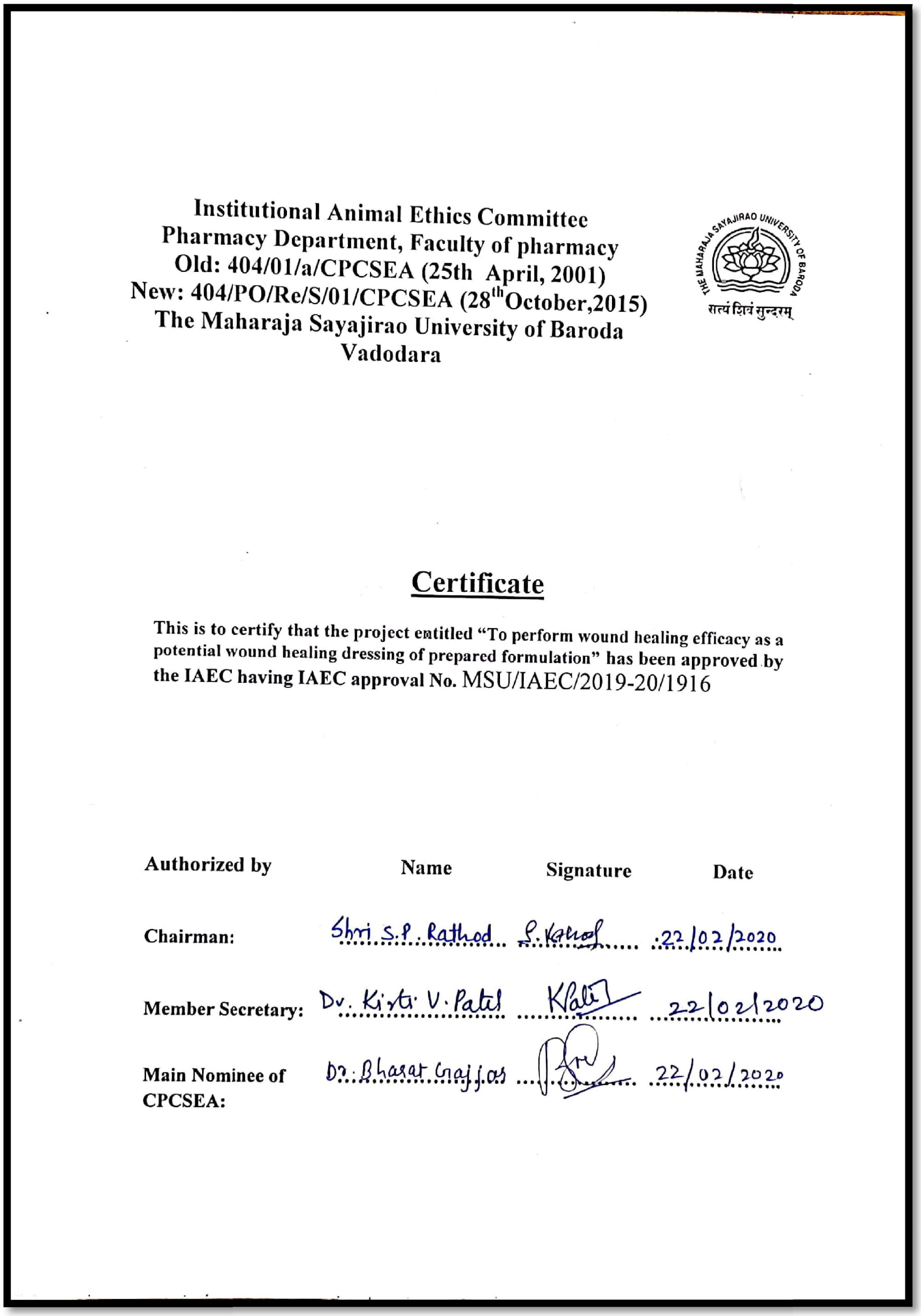
Protocol No.	:	MSU/IAEC/2018-19/1831
Project title	:	To perform wound healing efficacy as a potential wound healing dressing of prepared formulation
Chief Investigator	:	Lalajibhai Valajibhai Rathod
Research Guide	:	Prof. Krutika K. Sawant

Dr. Bharat Gajjar
(CPCSEA Main Nominee)

(Chairman, IAEC)

Date of Approval:
29/12/2018

FIGURE 8.1: Animal study certificate from IAEC for the cutaneous excision wound healing model



Institutional Animal Ethics Committee
Pharmacy Department, Faculty of pharmacy
Old: 404/01/a/CPCSEA (25th April, 2001)
New: 404/PO/Re/S/01/CPCSEA (28th October,2015)
The Maharaja Sayajirao University of Baroda
Vadodara

Certificate

This is to certify that the project entitled "To perform wound healing efficacy as a potential wound healing dressing of prepared formulation" has been approved by the IAEC having IAEC approval No. MSU/IAEC/2019-20/1916

Authorized by	Name	Signature	Date
Chairman:	Shri S.P. Rathod	S. Rathod	22/02/2020
Member Secretary:	Dr. Kirti V. Patel	KPatel	22/02/2020
Main Nominee of CPCSEA:	Dr. Bharat Gajjar	[signature]	22/02/2020

FIGURE 8.2 : Animal study certificate from IAEC for the burn wound healing model

CHAPTER 8.0: ANIMAL STUDIES

8.1 CUTANEOUS EXCISION WOUND HEALING MODEL

8.1.1 GROUPING OF ANIMALS: 24 Male SD rats were randomly allocated to 4 groups that contained 6 rats in each group as mentioned in table 8.2. Each rat was housed individually in a separate standard cage.

TABLE 8.2: Grouping of animals and treatment given in cutaneous excision wound healing model

Groups	Details of Treatment
Group I	Control group, 0.9% saline; Topical
Group II	Collagen film without CFE; Topical
Group III	CFE loaded Collagen film; Topical
Group IV	Type I collagen dressing (Lyophilized)

8.1.2 SURGICAL PROCEDURE:

1) The rats of all groups were anesthetized by injection of 0.8 cc ketamine (5%) and 0.2 cc xylazine intramuscularly in the hamstring muscles. Before making incisions, the dorsal aspect of the cervical to lumbar area was shaved and washed with a scrub solution of povidone-iodine (figure 8.3).

FIGURE 8.3: Rat with shaved back side

2) Under sterile conditions, a skin incision was made in a square shape 2x2 cm using sterile scalpel blade and skin was removed (figure 8.4).

FIGURE 8.4: Rat having skin incision (2cm x 2 cm)

3) After incision and treatment as per the group, the wound area was dressed with secondary dressing (figure 8.5).

FIGURE 8.5: Rat's wound covered with secondary dressing

8.1.3 TREATMENT REGIMENS:

1) In Group I, rats were not given any treatment on the incision.
2) The rats from Group II received collagen film without calendula flower extract.
3) In Group III, the rats received prepared CFE loaded collagen film.
4) In Group IV, the rats received marketed product of type I collagen dressing (Lyophilized).
   - Proper care was taken when changing the dressings to minimize introduction of pathogens to the wound site.

8.1.4 OBSERVATIONS:

8.1.4.1 Morphometric analysis of wound: Daily observations were performed by taking the photographed images of wound with a standard reference ruler and any wound fluid or any evidence of infection or other abnormalities were noted. Wound area was measured by tracing the wound on a millimeter scale graph paper. % wound size contraction was calculated using equation (1).

$$\% \text{ Wound contraction} = (Ai-At)/Ai * 100 \quad \text{…. Equation (1)}$$

Where,

Ai = The initial wound area and

At = The area of the wound at time t

8.1.4.2 Biochemical analysis: After 21 days post injury, the rats were euthanized by IV injection of 30 mg/kg thiopental sodium (nesdonal) via tail vein. The shaved dorsal skin sample was carefully dissected free. Tissue specimens of the skin from all groups were thoroughly rinsed using saline solution. One gram of skin tissue sample was weighed up and homogenized. The tissue homogenate was centrifuged at about 8000 rpm, 4° to 6° C & 10 min for estimation of total hydroxyproline content.

- **Principle:** Hydroxyproline is oxidized by chloramine-T (sodium p-toluene sulfonchloronide) to give pyrole carboxylic acid, which is further oxidized with p-dimethyl amino benzaldeyde (PDAB) and form a cherry red color. The intensity of the colour is recorded at 577 nm using UV Spectrophotometer.
- **Procedure:** 2 -10 ppm hydoxyproline solutions were taken and the total volume was made upto 2.0 ml with distilled water. Hydroxyproline oxidation was

initiated by adding 1 ml of chloramines-T (0.05 M) to each tube. The tube contents were mixed thoroughly and allowed to stand at room temperature for 20 min. The chloramine-T was then destroyed by adding 1 ml of perchloric acid (3.15 M) to each tube. The content were mixed and allowed to stand for 5 min. Finally, 1 ml of PDAB solution (20%) was added and mixture was shaken for 2 min. The tubes were incubated for 20 min at 60° C and then cooled at room temperature. The intensity of the formed cherry red color was recorded at 577 nm using UV Spectrophotometer.

8.1.4.3 Histopathological study of collected sample: Skin samples taken from both the wound and adjoining normal skin were fixed in 10% neutral buffered formalin. After fixation, the tissues were embedded in paraffin, and 5 micrometer thickness sections were stained using hematozylin and eosin. Five zones were examined from the sample morphometrically through Nikon light microscope. The criteria that were studied in the histopathological sections consisted of hemorrhage, mononuclear cell infiltration, re-epithelialization of the epithelium, firbroblast content, present of fibrocytes, collagen content and neovascularization.

8.1.5 RESULTS AND DISCUSSION

- Photographs of wound contraction area taken during study are represented in figure 8.6. Re-epithelization was evaluated by measuring the %wound contraction during study which is represented in figure 8.7. The placebo and control group showed less re-epithelization than the formulation group and marketed sample group.
- Many wounds increased in size on the 2nd day due to inflammation. This is mostly attributed to the high activity of proteases and subsequent tissue degradation that results as a consequence of compounds being released from inflammatory cells during phagocytosis of the pathogens and senescent cells. If the inflammatory stimulus is indeed persistent, high concentrations of proteolytic enzymes are produced, which stimulates oxidative stress, impairing the healing process. Animals treated with CFE loaded collagen film had minimum inflammation and

absence of edema due to anti-inflammatory and anti-oedematous activities of CFE.

- On the 7th day, all groups showed an improvement in wound re-epithelialization relative to the 2nd day. There was clear difference in % wound contraction between the groups.
- After the 14th day, the re-epithelialization was improved in all groups except control group.
- In control, wounds were not totally re-epithelialized on the 14th and 21st day.
- The rate of wound contraction was significantly ($p<0.001$) high in CFE loaded collagen film treated wounds (92.18 ± 1.2%) when compared to control group (56.25 ± 4.1%), placebo treated group (62.5 ± 5.1%), Type-I collagen dressing® treated group (68.75± 6.1%) on day 21 (figure 8.7). Treated animals showed significant reduction in period of epithelization and wound contraction (fig 8.4 & 8.5).
- The enhanced wound healing activity may be due to collagen and phytoconstituents present in it which speed up the process of wound healing. The active components of CFE are triterpenoids, in particular faradiol monoesters which are responsible for anti-inflammatory activity.
- Triterpenes may positively influence the wound healing effect by stimulating the proliferation rate and to a higher extent, the migration of fibroblasts. Collagen helps in the wound healing process by deposition and organization of freshly formed fibers (1).
- Collagen film promotes angiogenesis, inhibits the action of matrix metalloproteinase and also enhances body's repair mechanism (2-4). Collagen film acts as mechanical support which reduces oedema, stops bleeding, facilitates migration of fibroblast and enhances the metabolic activity of the granulation tissue (2, 5, 6). Although the wound healing ability of CFE has been proven by the researchers globally but its enhancement with collagen biomaterial has been investigated for the first time in present work.

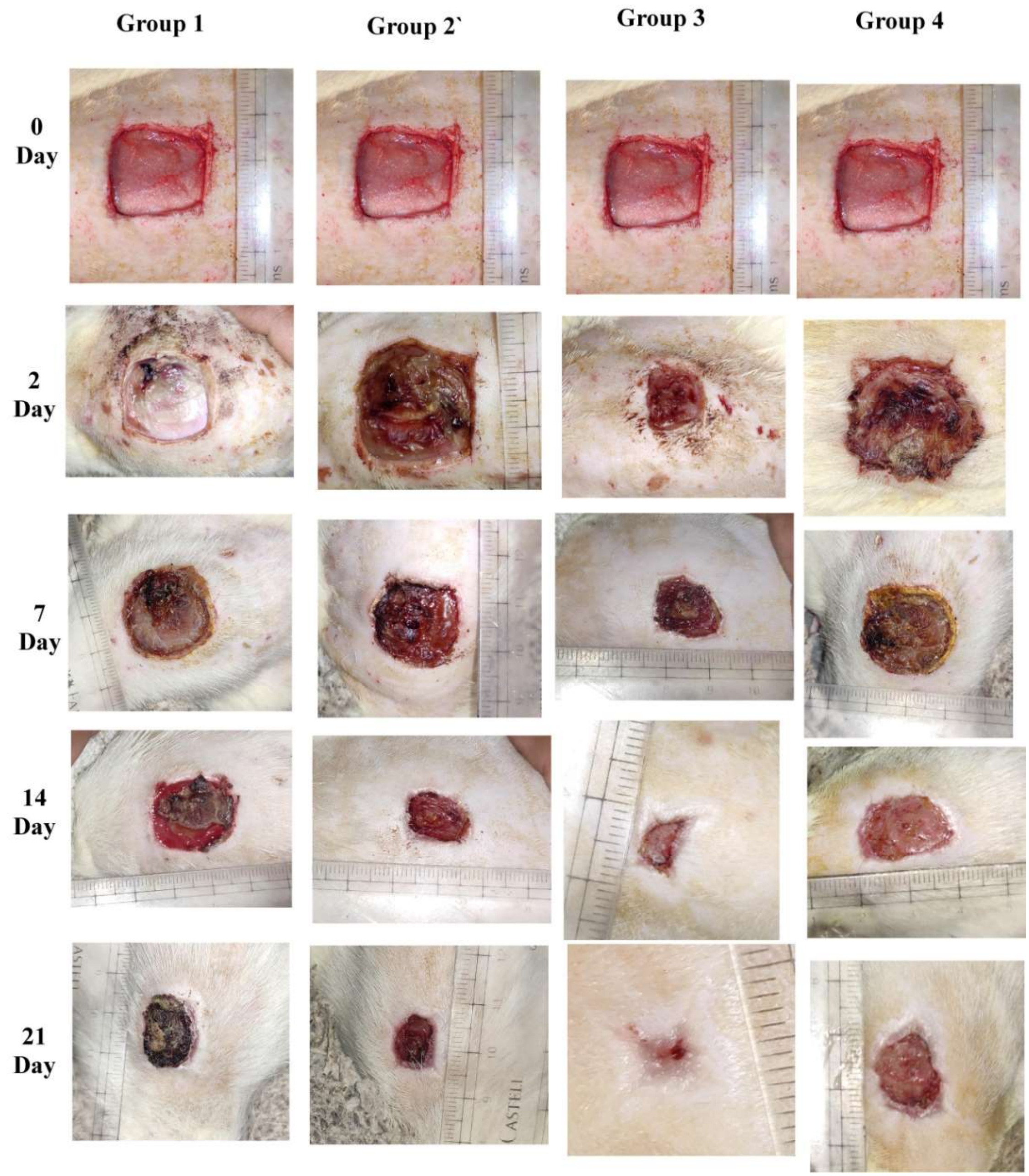


FIGURE 8.6: Macroscopic changes of wound over different post-excision days of Group I (non treated animal), Group II (collagen film without CFE treatment), Group III (CFE loaded collagen film treatment) and Group IV (market product treatment)

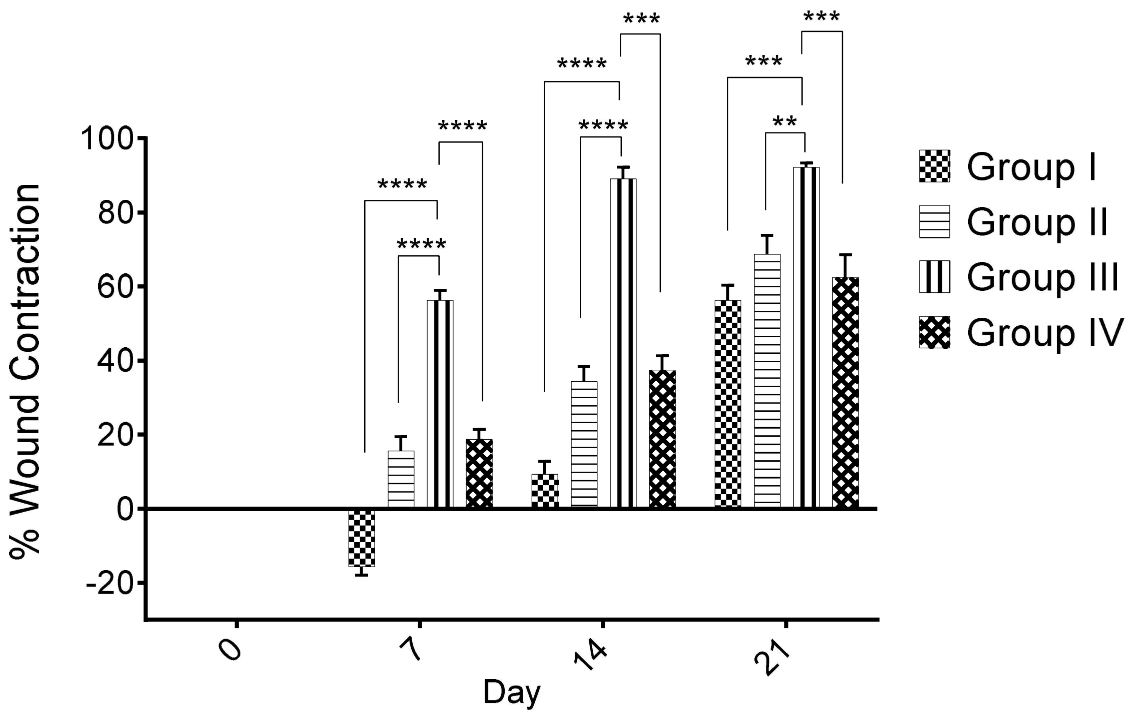


FIGURE 8.7: % Wound contraction in rat during 21 days *in-vivo* animal study: I (non treated animal), Group II (Collagen film without CFE treatment), Group III (CFE loaded collagen film treatment) and Group IV (market product treatment)

- The histopathological observation on degree of epithelialization, fibroblastic deposition and early histological maturation of collagen in treated groups as compared to control animals were in agreement with the earlier reports (7). Animals of treated groups had thicker and denser collagen fibers in granulation tissue compared to other group's animals (fig. 8.8). Better epithelialization and neovascularization resembling normal skin was observed in treated groups compared to other groups (fig. 8.8). The total collagen content of the tissues was determined by the estimation of hydroxyproline. There was significant increase in hydroxyproline content of treated group (81.31 ± 2.7 mg/g tissue) compared to placebo group (67.31 ± 1.9 mg/g tissue), untreated group (43.31 ± 2.4 mg/g tissue) and marketed sample treated group (65.31 ± 1.4 mg/g tissue) which indicates that the collagen deposition in skin was more in CFE loaded collagen film treated animal compared to other groups. CFE loaded collagen film enhanced wound healing activity in full thickness excision wound by increasing cellular proliferation, formation of granulation tissue, neovascularization, synthesis of collagen epithelization and early histological maturation in skin.

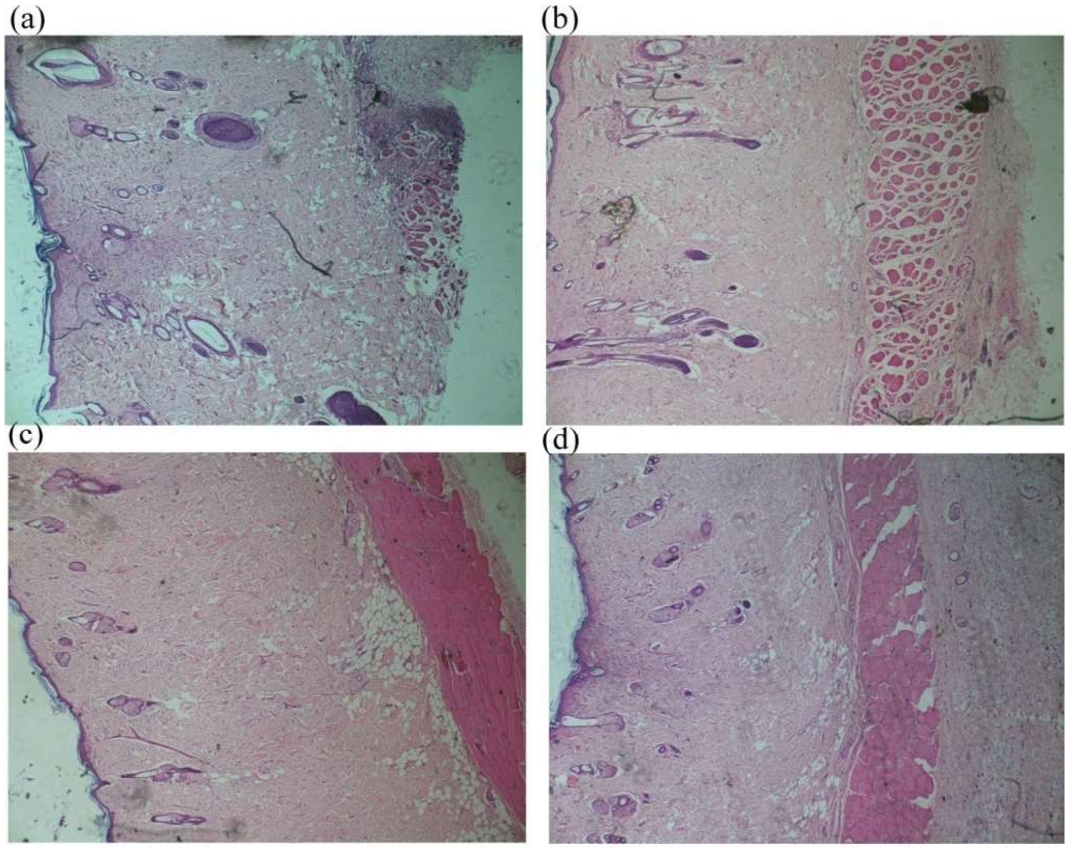


FIGURE 8.8: Photomicrograph of histopathological wound tissue section: (a) Non treated group, (b) Placebo collagen film treated group, (c) Market product treated group and (d) collagen film containing CFE treated group

8.2 BURN WOUND MODEL:

8.2.1 GROUPING OF ANIMALS

24 Male Wistar rats were randomly allocated to 4 groups that contained 6 rats in each group as mention in table 8.3

TABLE 8.3: Grouping of animals and treatment given in burn wound model

Groups	**Details of Treatment**
Group I	Control group, 0.9% saline; Topical
Group II	Hydrogel sheet without CFE; Topical
Group III	CFE loaded hydrogel sheet; Topical
Group IV	1% framycetin sulphate skin cream: Topical

8.2.2 SURGICAL PROCEDURE

1) The rats of all groups were anesthetized by injection of 0.8 cc ketamine (5%) and 0.2 cc xylazine intramuscularly in the hamstring muscles.

2) Before making burn lesion, back side of rat (the dorsal aspect of the cervical or cervical to lumbar area) was shaved (figure 8.9) and cleaned with povidone-iodine solution.

FIGURE 8.9: Rat having shaved back side

3) Burn wound was created by placing the heated and moistened aluminum template (about 2.5 cm diameter x 3 cm length, a handle measuring 24 cm, and total weight 400 g) at right angles perpendicular to the dorsum of the rat on the pre-marked location for 5 seconds using an analogue stopwatch (figure 8.10) (1, 2, 4-6). Aluminum template was heated in a water bath at a constant temperature at 80°-85° C for 3 hr prior to inflicting burns on the skin.

FIGURE 8.10: Rat having burn wound (Day 0)

4) All wounds in the treatment groups were dressed with either marketed product or formulation followed by transparent adhesive dressing as a secondary dressing.

8.2.3 TREATMENT REGIMENS

1) In Group I, Rats were not given any treatment on the incision.
2) The rats from Group II received hydrogel sheet without calendula flower extract.
3) In Group III, the rats received prepared CFE loaded hydrogel sheet.
4) In Group IV, the rats received marketed product of 1% framycetin sulphate skin cream

Proper care was taken when applying the dressings to minimize introduction of pathogens to the wound site.

8.2.4 OBSERVATIONS

- Daily observations were performed by taking the photographed images of wound with a standard reference ruler and any wound fluid or any evidence of infection or other abnormalities were noted. Wound area was measured by tracing the wound on a millimeter scale graph paper. % Wound size contraction was calculated using equation (2).

$$\% \text{ Wound contraction} = (Ai-At)/Ai * 100 \quad \text{.... Equation (2)}$$

Where,

Ai = The initial wound area and

At = The area of the wound at time t

- After 21 day, skin tissues were examined for hydroxyproline content. Skin tissues were also examined morphometrically through Nikon light microscope. The criteria that were studied in the histopathological sections consisted of hemorrhage, mononuclear cell infiltration, reepithelialization of the epithelium, firbroblast content, present of fibrocytes, collagen content and neovascularization.

8.2.5 RESULTS AND DISCUSSION

- In this study, the burn healing potential was evaluated through 21 days experimental period by monitoring regularly the percentage of wound areas reduction. The daily behaviors of rats were observed as normal.
- The macroscopic images of the burn area on days 2,4, 7,14 and 21 presented in fig 8.11
- On day 1, necrosis and edema were observed on the wound surface area in all groups. Exudates were seen, and the edges of the wounds started to pronounce. Edema was also present around the wound surface area.
- In first 5 days after treatment, there was high inflammation with edema in all wounds except wound treated with CFE loaded hydrogel sheet. There was a greater inflammatory reaction in the wounds in non treated group compared to others. There was less inflammation and absence of edema wound treated with CFE loaded hydrogel sheet compared to other groups. There was high inflammation and edema in wounds treated with placebo hydrogel sheet compared to wounds treated with CFE loaded hydrogel sheet. It proves that the *Calendula officinalis* flower extract improves the efficacy of hydrogel sheet by its anti-inflammatory and anti antiodeteomous activity. This is due to the presence of a potent anti-inflammatory agent (triterpene glycoside) in the extract of *Calendula officinalis*. Previous studies evaluated and evidenced the anti-inflammatory activity, antiodeteomous activity of *C. officinalis* and related its activity to the presence of triterpenes, especially to faradiol esters and taraxasterol (8-10). Anti-inflammatory activity is essential for the wound healing process because long period of inflammation phase may result in retardation of healing (11).
- The crusts started to form 2nd day in all group, but they were thinner and wetter crusts in Group III compared to the other groups in which they were thicker and drier. Until the 5thday, the entire area of the burned skin was covered with a solid layer of crust. On the 7th day, the crust layer of CFE loaded hydrogel sheet treated wound was fallen off which was observed in marketed treated group on 10th day. The crusts began to detach showing signs of epithelialization of the

wound. On the 14th day, all groups except non treated group showed an improvement in wound re-epithelialization relative to the 7nd day. Reaching day 21, the wound area was small but the complete epithelialization had not yet achieved in any group.

- The wound contraction ratio was assessed as the percentage reducing in wound size during study which illustrated in figure 8.12. CFE loaded hydrogel sheet showed a significant ($p<0.001$) burn healing progression on the day 4, 7, 14 and 21 compared to untreated group. The healing percent of lesion area ranged from 4.72% to 25.41% in the period from 2 to 21 days in the control groups (Group I), 6.21% to 72.63% , with Placebo hydrogel sheet, 7.69% to 87.01% with CFE loaded hydrogel sheet and 4.86% to 84.0% with marketed product. CFE loaded hydrogel sheet treated wound recovered quickly and the burn area rapidly decreased in size by the 21st day. The main objective in burn injury healing is the early wound closure and rapid replacement of normal skin with minimal scarring which was achieved with developed formulation.

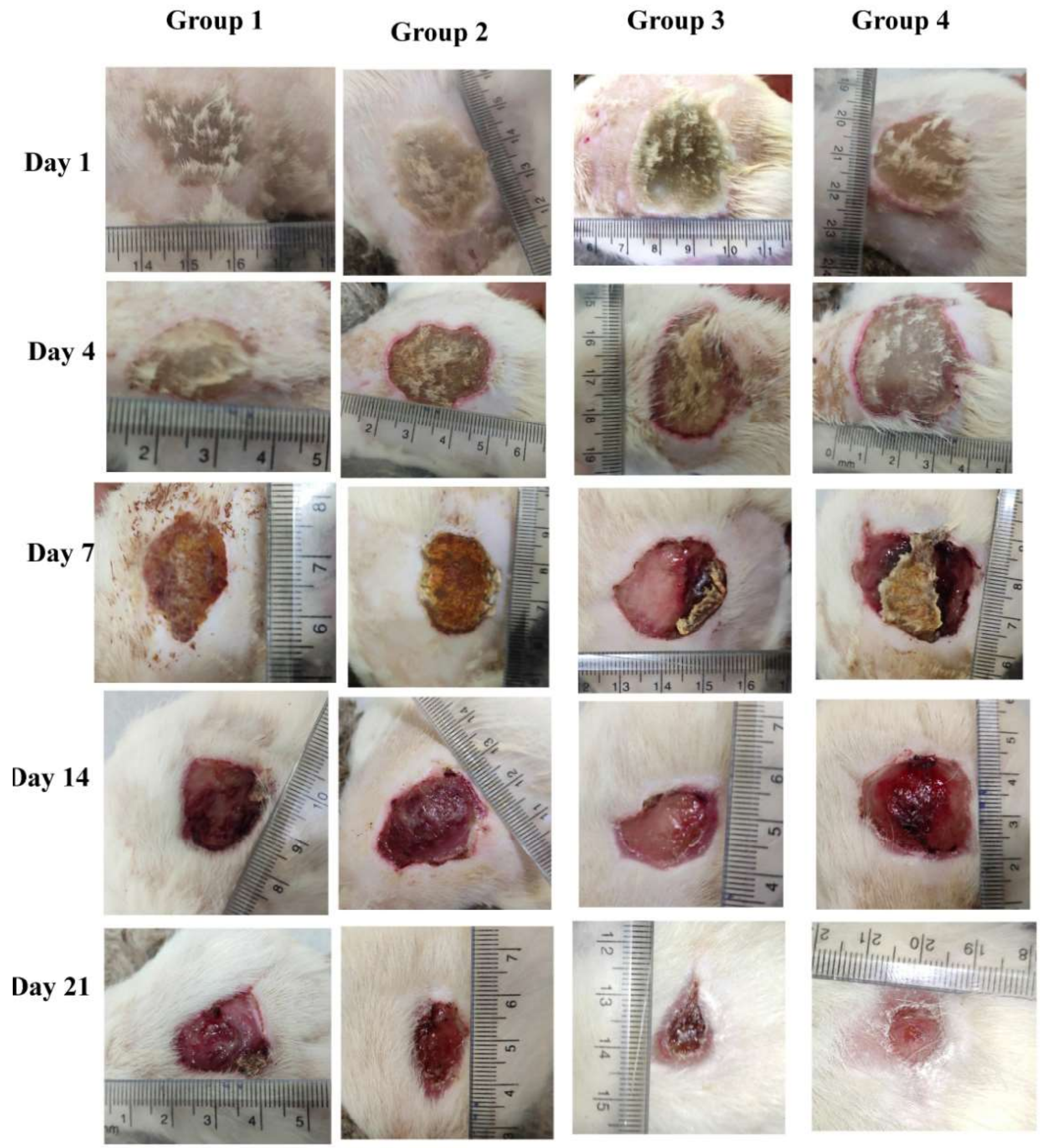


FIGURE 8.11: Macroscopic changes of wound over different post-excision days of Group I(non treated animal), Group II (Hydrogel sheet without CFE treatment), Group III (CFE loaded hydrogel sheet treatment) and Group IV (market product treatment)

- Re-epithelialization was estimated by measuring the % wound contraction with time. The placebo and non-treated group showed less re-epithelization than the formulation group and marketed sample group. The enhanced wound healing activity of CFE loaded hydrogel sheet is due to the phytoconstituents present in CFE which enhanced the process of wound healing. The active components of CFE responsible for anti-inflammatory activity are triterpenoids, in particular faradiol monoesters. Also, triterpenes may positively influence the wound healing effect of CFE by stimulation of proliferation and to a higher extent, the migration of fibroblasts. Efficacy of Calendula extracts has been previously demonstrated in some in vivo studies (12, 13), and also in a phase III trial in the prevention of acute dermatitis during irradiation for breast cancer (14). Many studies have described the wound re-epithelialization effect by calendula flower extract. Furthermore, the wounds treated with hydrogel sheet were considerably moist compared to control group. In general, it has been proposed that the hydration of the wound stimulates epithelialization, granulation, tissue formation, angiogenesis, fibroblast migration, collagen synthesis, and remodeling of injured tissue, thus reducing the possible trauma during changes of the dressing. This suggested that the hydrogel sheet might have influenced the maintenance of moisture in the wound and enhanced total wound re-epithelization.

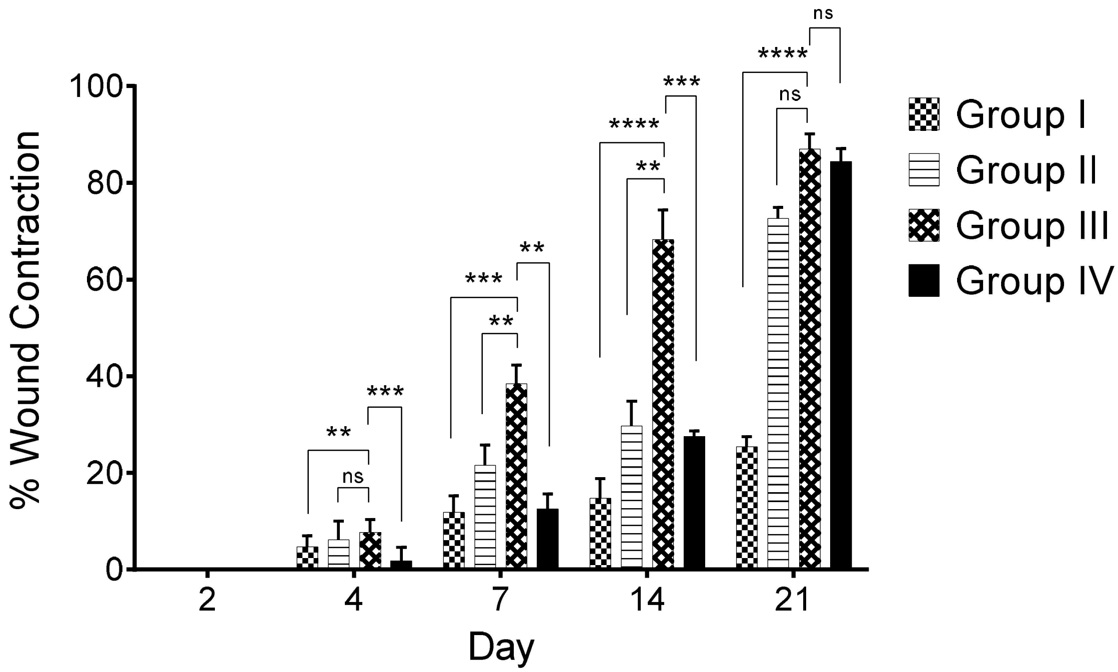


FIGURE 8.12: % Wound contraction in rat during 21 days *in-vivo* animal study: I (non treated animal), Group II (Hydrogel sheet without CFE treatment), Group III (CFE loaded hydrogel sheet treatment) and Group IV (market product treatment)

- The treated group had a higher concentration of hydroxyproline compared to other groups which is a reflection of higher collagen content. This clearly suggested that treatment with *Calendula officinalis* enhanced the production of collagen (15) which is mostly found in connective tissues. One key component of chronic wounds is an elevated level of matrix metalloproteinase (MMPs). Higher amount of MMPs degrade nonviable and viable collagen. Condition becomes worst when firboblasts cannot secrete tissue inhibitors of MMPs at an adequate level. Calendula extract inhibits MMPs (16-18) which enhances the amount of collagen in tissue. *Calendula flower* contains triterpene saponins and flavonoids which increases the collagen synthesis (19).
- Neovascularization occurs concurrently with fibroblast proliferation when endothelial cells, originating from part of uninjured blood vessels migrate to the wound (20). The aqueous extract of *Calendula officinalis* enhanced neouvascularization and promoted wound healing due to its ability to induce

hyaluronan associated with neovascularization (21). So there might be higher neovascularizaion in early phase of wound healing in treated group compared to other groups. The wound healing activity of CFE is due its triterpenoid (22), flavonoids (23) and polyphenolic compounds which have been reported to have wound healing potential (24).

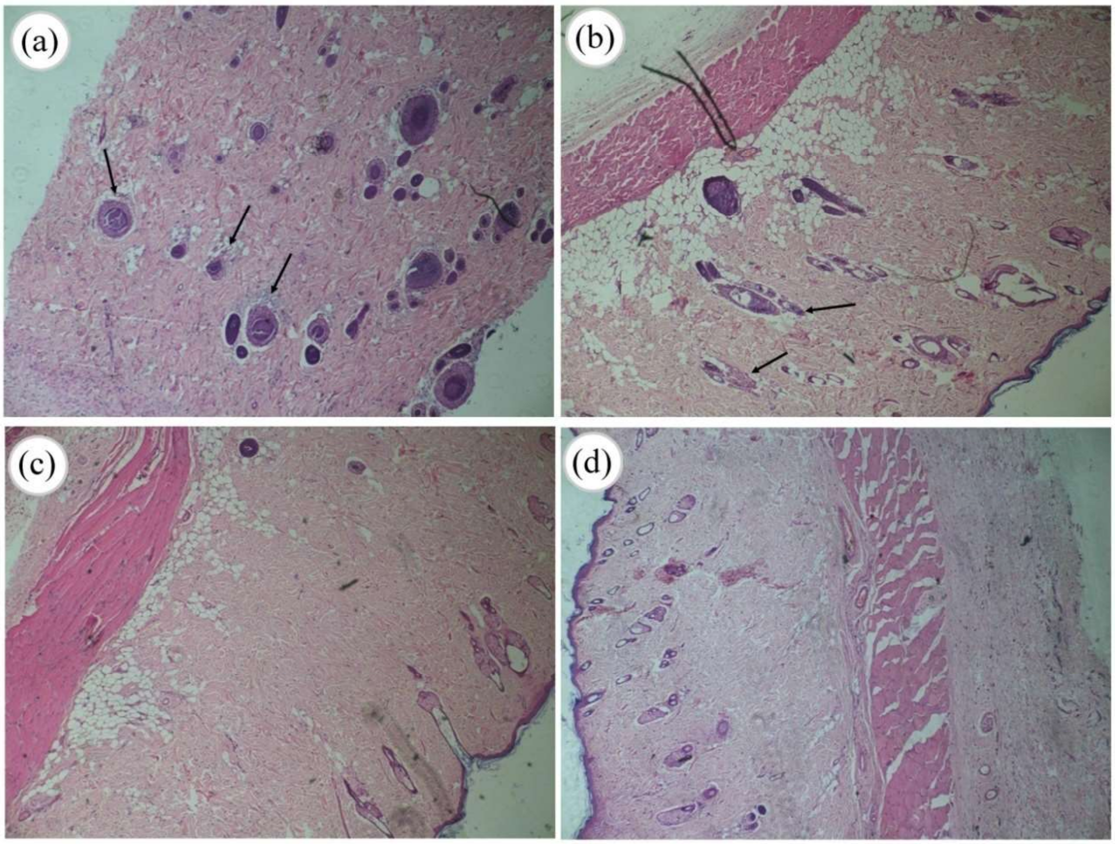


FIGURE 8.13: Histopathology study of skin after 21 days (a) Non treated group (b) Placebo hydrogel sheet treated group (c) Market product treated group (d) Hydrogel sheet containing CFE treated group

The histopathological observation pertaining to the degree of epithelialization, fibroblastic deposition and early maturation of collagen in the treated animals as compared to control animals were in agreement with the earlier reports (7). The histological analysis demonstrated thicker and denser collagen fibers in granulation tissue in the treated animals as compared to the control animals (figure 8.13). Improved epithelialization and neovascularization, resembling normal skin was observed in treated groups compared to the control group. CFE loaded Hydrogel sheet enhanced wound healing activity in full-thickness excision wound by increasing cellular proliferation, formation of granulation tissue, neovascularization, synthesis of collagen epithelization and early histological maturation in the skin.

8.3 REFERENCES

1. Nataraj C, Ritter G, Dumas S, Helfer FD, Brunelle J, Sander TW. Extracellular wound matrices: novel stabilization and sterilization method for collagen-based biologic wound dressings. Wounds: a compendium of clinical research and practice. 2007;19(6):148-56.
2. Park S-N, Lee HJ, Lee KH, Suh H. Biological characterization of EDC-crosslinked collagen–hyaluronic acid matrix in dermal tissue restoration. Biomaterials. 2003;24(9):1631-41.
3. Lazovic G, Colic M, Grubor M, Jovanovic M. The application of collagen sheet in open wound healing. Annals of burns and fire disasters. 2005;18(3):151.
4. Veves A, Sheehan P, Pham HT. A randomized, controlled trial of Promogran (a collagen/oxidized regenerated cellulose dressing) vs standard treatment in the management of diabetic foot ulcers. Archives of Surgery. 2002;137(7):822-7.
5. Nagata H, Ueki H, Moriguchi T. Fibronectin: Localization in normal human skin, granulation tissue, hypertrophic scar, mature scar, progressive systemic sclerotic skin, and other fibrosing dermatoses. Archives of dermatology. 1985;121(8):995-9.
6. Motta G, Ratto G, De Barbieri A, Corte G, Zardi L, Sacco A, et al. Can heterologous collagen enhance the granulation tissue growth? an experimental study. The Italian Journal of Surgical Sciences. 1983;13(2):101-8.

7. Chandran PK, Kuttan R. Effect of Calendula officinalis flower extract on acute phase proteins, antioxidant defense mechanism and granuloma formation during thermal burns. Journal of clinical biochemistry and nutrition. 2008;43(2):58-64.

8. Zitterl-Eglseer K, Sosa S, Jurenitsch J, Schubert-Zsilavecz M, Della Loggia R, Tubaro A, et al. Anti-oedematous activities of the main triterpendiol esters of marigold (Calendula officinalis L.). Journal of ethnopharmacology. 1997;57(2):139-44.

9. Parente LML, Júnior L, de Souza R, Tresvenzol LMF, Vinaud MC, de Paula JR, et al. Wound healing and anti-inflammatory effect in animal models of Calendula officinalis L. growing in Brazil. Evidence-based complementary and alternative medicine. 2012;2012.

10. Hamburger M, Adler S, Baumann D, Förg A, Weinreich B. Preparative purification of the major anti-inflammatory triterpenoid esters from Marigold (Calendula officinalis). Fitoterapia. 2003;74(4):328-38.

11. Midwood KS, Williams LV, Schwarzbauer JE. Tissue repair and the dynamics of the extracellular matrix. The international journal of biochemistry & cell biology. 2004;36(6):1031-7.

12. Klouchek-Popova E, Popov A, Pavlova N, Krŭsteva S. Influence of the physiological regeneration and epithelialization using fractions isolated from Calendula officinalis. Acta Physiol Pharmacol Bulg. 1982;8(4):63-7.

13. Rao S, Udupa A, Udupa S, Rao P, Rao G, Kulkarni D. Calendula and Hypericum: two homeopathic drugs promoting wound healing in rats. Fitoterapia. 1991;62(6):508-10.

14. Pommier P, Gomez F, Sunyach M, D'hombres A, Carrie C, Montbarbon X. Phase III randomized trial of Calendula officinalis compared with trolamine for the prevention of acute dermatitis during irradiation for breast cancer. Journal of clinical oncology. 2004;22(8):1447-53.

15. Nicolaus C, Junghanns S, Hartmann A, Murillo R, Ganzera M, Merfort I. In vitro studies to evaluate the wound healing properties of Calendula officinalis extracts. Journal of Ethnopharmacology. 2017;196:94-103.

16. Naeini AT, Miri R, Shafiei N, Tabandeh MR, Oryan A, Nazifi S. Effects of topical application of Calendula officinalis gel on collagen and hydroxyproline content of skin in rats. Comparative Clinical Pathology. 2012;21(3):253-7.

17. Aro A, Perez M, Vieira C, Esquisatto M, Rodrigues R, Gomes L, et al. Effect of C alendula Officinalis Cream on A chilles Tendon Healing. The Anatomical Record. 2015;298(2):428-35.

18. Okuma CH, Andrade TAM, Caetano GF, Finci LI, Maciel NR, Topan JF, et al. Development of lamellar gel phase emulsion containing marigold oil (Calendula officinalis) as a potential modern wound dressing. European Journal of Pharmaceutical Sciences. 2015;71:62-72.

19. Morikawa T, Ninomiya K, Takamori Y, Nishida E, Yasue M, Hayakawa T, et al. Oleanane-type triterpene saponins with collagen synthesis-promoting activity from the flowers of Bellis perennis. Phytochemistry. 2015;116:203-12.

20. Kuwahara R, Rasberry R. Chemical peels. Emedicine com Accessed September. 2007;15:2007.

21. Patrick K, Kumar S, Edwardson P, Hutchinson J. Induction of vascularisation by an aqueous extract of the flowers of Calendula officinalis L. the European marigold. Phytomedicine. 1996;3(1):11-8.

22. Della Loggia R, Tubaro A, Sosa S, Becker H, Isaac O. The role of triterpenoids in the topical anti-inflammatory activity of Calendula officinalis flowers. Planta medica. 1994;60(06):516-20.

23. Popović M, Kaurinović B, Mimica-Dukić N, Vojinović-Miloradov M, Ćupić V. Combined effects of plant extracts and xenobiotics on liposomal lipid peroxidation. Part 1. Marigold extract-ciprofloxacin/pyralene. Oxidation Communications. 1999;22(4):487-94.

24. Leach MJ. Calendula officinalis and Wound Healing: A Systematic Review. Wounds. 2008;20(8):236-43.