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Comparison of the dermal wound healing of *Centella asiatica* extract impregnated collagen and crosslinked collagen scaffolds

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ABSTRACT

The aim of this study is to hasten and improve the wound healing activity. Ensuring the physicochemical compatibility, *Centella asiatica* extract impregnated collagen and cross-linked collagen scaffolds (CAEICDS & CAEICDS) were formulated using different concentrations. The formulated scaffolds were subjected to physical, biochemical and histopathological examinations. Microshrinkage Temperature of the scaffolds containing 1%w/v & 1.5%w/v & 2%w/v of CAEICDS & CAEICDS were found to be 69°C, 71°C, 72°C & 70°C, 73°C, 76°C respectively indicating their hydrothermal stability. Wound healing studies on Male Wistar Rats were performed for a period of 7 days and it was observed that the 1.5%w/v of both CAEICDS & CAEICDS treated wistar rats possessed higher amount of Hydroxy Proline content 72.6% & 73.6% when compared to that of the existing Marketed formulation (Neu-skinTM) (60.7%). Further better wound healing activity was observed in the 1.5%w/v CAEICDS & CAEICDS treated rats (79.9% & 80.68%).

Keywords: Collagen & cross-linked collagen, *Centella asiatica* extract, Micro Shrinkage Temperature, Wound Healing.

INTRODUCTION

Wound healing is a natural retro-active response to the tissue injury restoring cellular structure & tissue layers which begins with trauma and ends with scar formation. It is related to tissue reconstitution by which body replenishes cells that are being lost by normal physiologic events[1]. The steps in the healing of the wound include inflammation, proliferation, and

migration of different cell types. Inflammation, the first phase occurs immediately after injury and is known as coagulation which results in a coordinated influx of neutrophils at the wound site. These cells which are in the primary phase possess a respiratory burst mechanism naturally synthesizing free radicals. These free radicals present causes oxidative stress to the system giving pavement to lipid peroxidation, DNA breakage and enzyme inactivation including the free radical scavenging enzymes which predominantly delay wound healing[2]. There is a substantial evidence of antioxidants playing a vital role in therapy against the pathogenesis of many diseases caused by oxidants[3]. *Centella asiatica* extract, a naturally occurring extract rich in tannins and phenolic derivative has shown to possess several biological properties including antioxidant property (free radical scavenging activity)[4,5] which play a vital role in the wound healing. However the delivery of this extract is a matter of concern. *Centella asiatica* extract incorporated Collagen in the form of Scaffold ensures slow release of drug providing the better therapy by acting as a physical support for cellular proliferation[6]. Moreover, Collagen itself acts as a wound healing agent possessing biodegradable and biocompatible properties provide the synergistic activity in significant wound healing along with the extract.

EXPERIMENTAL SECTION

Materials:

Collagen (isolated from Achilles tendon), *Centella asiatica* extract - gift sample obtained from Chemiloids –Vijayawada, India, oleic acid, 2, 2 0-azobisisobutyronitrile (AIBN) were purchased from Merck (India). All other chemicals used in this research activity were of analytical grade.

Animals:

Male Wistar rats weighing between 150-200 grams obtained from the animal house of Bapatla College of Pharmacy (1032/ac/07/CPCSEA), Bapatla, were maintained at a constant temperature of $26 \pm 2^\circ\text{C}$ and humidity at 30-40% with 12 h light and dark cycle throughout the experiment. The animals were housed in clean polypropylene cages in an air-conditioned animal house and were fed with commercial rat feed and sterile water. The experiment protocol IAEC/II/16/BCOP/2009 was approved by the Institutional Animal Ethical Committee (IAEC) of Bapatla College of Pharmacy.

Methods

Investigation of physicochemical compatibility of *Centella asiatica* root Extract and collagen:

The physicochemical compatibility between *Centella asiatica* root Extract and Collagen was studied by using Fourier Transform Infrared Spectroscopy. The infrared spectra were recorded using Perkin Elmer Fourier Transform Infrared Spectrophotometer, Shelton, USA by using KBr pellet method and spectra were recorded in the wavelength region between 4000 and 400 cm^{-1} . The spectra obtained for *Centella asiatica* root Extract, Collagen, physical mixture of *Centella asiatica* root Extract & collagen form were compared.

Development of *Centella asiatica* extract Impregnated Collagen Based Dermal Scaffolds (CAEICDS):

Collagen was soaked in 0.05M glacial acetic acid at 25mg/ml concentration for 24 hrs at 4°C . The obtained viscous solution was homogenized for 5min, deaerated for 15 min by using

sonicator and squeezed through a muslin cloth to get rid of undissolved solid traces if any. Various solutions with different concentrations of *Centella asiatica* root Extract (1% w/v, 1.5%w/v & 2 % w/v were dissolved in 2ml of alcohol separately) were prepared. Each of the prepared solutions was mixed with 40ml of the above Collagen solution separately. The obtained mixture was casted in Petri plate (64 cm²) having polyethylene membrane base and placed in incubator at 37⁰C until dried. The scaffold thus obtained was sterilized under UV Radiation for a period of 18 hours and subjected to Microbiological studies

Development of *Centella asiatica* Extract Glutaraldehyde Cross linked Collagen Based Dermal Scaffolds (CAEICDS):

Collagen was soaked in 0.05M glacial acetic acid at 25mg/ml concentration for 24 hrs at 4⁰C. The obtained viscous solution was homogenized for 5min, deaerated for 15 min by using sonicator and squeezed through a muslin cloth to get rid of undissolved solid traces if any. To this 0.8 ml of 25% LR Glutaraldehyde was added. Various solutions with different concentrations of *Centella asiatica* root Extract (1% w/v, 1.5%w/v & 2 % w/v were dissolved in 2ml of alcohol separately) were prepared. Each of the prepared solutions was mixed with 40ml of the above Cross-linked Collagen solution separately. The obtained mixture was casted in Petri plate (64cm²) having polyethylene membrane base and placed in incubator at 37⁰C until dried. The scaffold thus obtained was sterilized under UV –Radiation for a period of 18 hours and subjected to Microbiological studies.

Microbiological Test[7]:

The presence of micro organisms in the scaffold was tested by the direct inoculation method. For this, Nutrient Agar Media and Czapek's Dox Media were prepared, sterilized and transferred to 16 Petri plates, containing 25ml separately. The Petri plates were numbered from 1 to 16 respectively. Plate 1 was maintained as control for Nutrient Agar Media, Plates 2, 3, 4, 5,6,7,8 were inoculated with plain collagen scaffold and different concentrations of (1%w/v, 1.5%w/v & 2%w/v) CAEICDS and different concentrations of (1% w/v, 1.5%w/v & 2%w/v) CAEICDS in Nutrient Agar Media respectively. Plate 9 was maintained as control for Czapek's Dox Media, Plates 10,11,12,13,14,15& 16were inoculated with plain collagen scaffold and different concentrations of (1%w/v, 1.5%w/v & 2%w/v) CAEICDS and CAEICDS in Czapek's Dox Medium respectively. All the 16 Petri plates were incubated at 37⁰C for 24 hours and observed for the growth of micro organisms

Evaluation of scaffolds:

Thickness:

The thickness of the Collagen plain and Cross-linked and different concentrations of CAEICDS & CAEICDS was measured by a screw gauge (LINKER-20 X 1/100 mm). The mean of 3 observations were calculated.

Folding Endurance:

Folding endurance was measured manually for the prepared scaffolds. For this a strip of film (2x2 cm²) was cut evenly and repeatedly folded at the same place until it broke. The number of times the film could be folded at the same place without breakage gave the exact value of Folding Endurance. The mean of 3 observations were calculated.

Water Vapor Transmission Test[8]:

For this study Glass vials of equal diameter were used as transmission cells. These cells were washed thoroughly and dried in an oven. About 1 gram of fused calcium chloride was placed in the cells and the scaffold measuring 2.836 cm^2 was fixed over the brim with the help of an adhesive. The cells were weighed accurately and initial weight was recorded and then kept in closed desiccators containing the saturated solution of potassium chloride (200 ml). Then the cells were taken out and weighed after 6, 12, 24, 48 and 72 hours. From the increase in the weights the amount of the water vapor transmitted and rate at which water vapor transmitted was calculated using the formula,

$$Q = WL/S$$

Q = Water vapor transmission coefficient (gm/cm/ 24hrs)

W = Weight of water vapor transmitted (gm / 24hrs)

L = Thickness of the patch (mm)

S = Exposed surface area of the patch (cm^2).

Micro Shrinkage Temperature Studies

The Micro Shrinkage Temperature measurements were carried out for the Plain Collagen, Cross-linked Collagen and different concentrations of CAEICDS & CAEICDS. For this, the Collagen scaffolds were stage fitted to an optical microscope. A small piece of Collagen scaffold was moistened with a drop of water on a glass slide and heated constantly with the help of a tungsten lamp. The temperature at which the scaffolds started to shrink was viewed through the microscope and was noted as the Micro Shrinkage Temperature.

Equilibrium Swelling Ratio Determination[9]

The equilibrium swelling ratio (E_s) was measured by the conventional gravimetric method. The dry weight of different scaffolds was measured before immersing in 0.05 M Phosphate buffer saline (PBS) pH 7.4 at a temperature of 37°C and excess surface Phosphate buffer saline was blotted out with absorbent paper. The wet weight (W_s) of the scaffold was determined after being incubated for 24 hours. The equilibrium swelling ratio of the scaffolds was defined as the ratio of weight increase ($W_s - W_d$) with respect to the initial weight (W_d) of dry samples. Each value was averaged from three parallel measurements. E_s was calculated using the following equations:

$$E_s = \frac{W_s - W_d}{W_d}$$

Where W_s and W_d denote the weights of swollen and dry samples, respectively.

Antioxidant Efficiency[10]:

Cellulose paper was dipped in a boiling tube containing oleic acid in hexane (0.1 M) solution. After adding the initiator AIBN into the above boiling tube, the oxidation of Oleic acid was monitored for the absorbance at λ_{234} for 30 min, and the tube was plugged tightly to prevent the evaporation of hexane. The CAEICDS & CAEICDS were placed over the cellulose paper separately containing Oleic acid by repeating the experiment for different concentrations.

Wound Healing Studies on Male Wistar Rats[11]:

Male Wistar rats weighing 180-200g obtained from the animal house of the Bapatla College of Pharmacy (1032/ac/07/CPCSEA), Bapatla, were maintained at constant temperature of $26 \pm 2^\circ\text{C}$ and humidity at 30-40% with 12hrs light and dark cycle through out the experiment. The animals were housed in clean polypropylene cages in an air-conditioned animal house were fed with commercial rat feed and sterile water. The experiment protocol IAEC/II/16/BCOP/2009 was approved by Institutional Animal Ethical Committee (IAEC) of Bapatla College of Pharmacy. Animals were divided into thirteen groups, each group comprising of six rats and the following groups were made.

Group 1: Rats treated as control.

Group 2: Rats treated with Marketed Formulation (Neu- skinTM)

Group 3: Rats treated with Plain collagen scaffolds

Group 4: Rats treated with 10 mg *Centella asiatica* extract only

Group 5: Rats treated with 15 mg *Centella asiatica* extract only

Group 6: Rats treated with 20 mg *Centella asiatica* extract only

Group 7: Rats treated with 1%w/v *Centella asiatica* extract impregnated collagen scaffolds

Group 8: Rats treated with 1.5%w/v *Centella asiatica* extract incorporated collagen scaffolds

Group 9: Rats treated with 2% w/v *Centella asiatica* extract incorporated collagen scaffolds

Group 10: Rats treated with 1%w/v *Centella asiatica* extract incorporated cross-linked collagen scaffolds

Group 11: Rats treated with 1.5%w/v *Centella asiatica* extract incorporated cross-linked collagen dermal scaffolds

Group 12: Rats treated with 2%w/v *Centella asiatica* extract incorporated cross-linked collagen dermal scaffolds

For this purpose the area was cleared off from hair by using a depilatory and anaesthetized using chloroform. A metal template measuring 1x1 cm (0.785cm^2 area) was placed on the stretched skin and an outline of the template was traced on the skin using a fine tipped pen. The wound was made by excision wound technique. The plain collagen scaffold, Collagen cross-linked scaffold, Marketed (Neu-SkinTM), CAEICDS & CAEICDS of different concentrations were applied separately on the excised wounds of the healthy animals of different groups.

Figure: 1: FT-IR of *Centella asiatica* extract

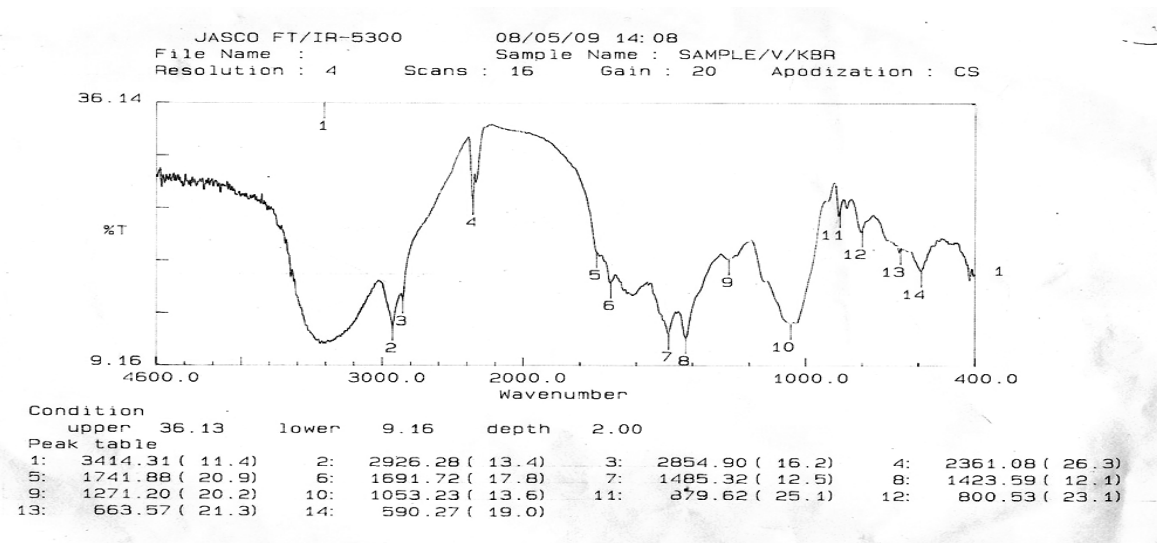
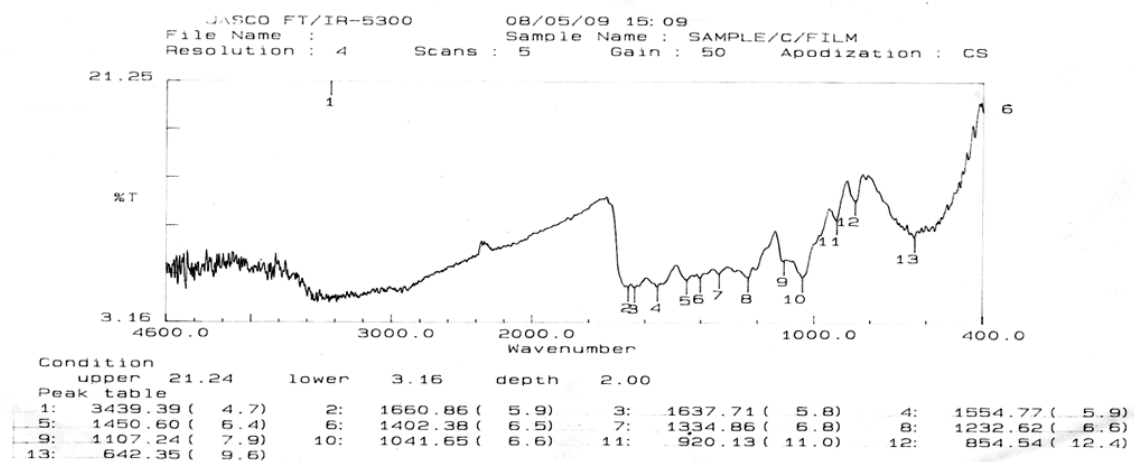
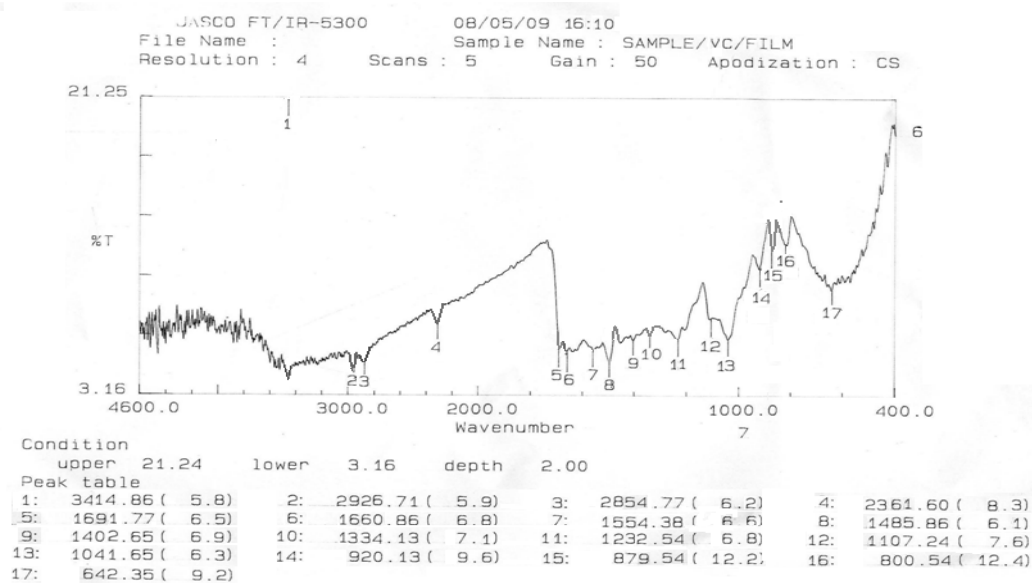


Figure 2: FT-IR of plain collagen

Figure 3: FT-IR graph of physical mixture of *Centella asiatica* extract and Collagen.

RESULTS AND DISCUSSION

Physicochemical compatibility of *Centella asiatica* extract and collagen

The FT- IR of *Centella asiatica* extract alone showed the principal peaks at wave numbers 3414 cm^{-1} , 2926 cm^{-1} , 1230 cm^{-1} , 1691 cm^{-1} , 1485 cm^{-1} confirming the purity of the *Centella asiatica* extract. In the FT-IRS of physical mixture of *Centella asiatica* extract and collagen the major peaks of *Centella asiatica* extract were observed at wave numbers 3414 cm^{-1} , 2926 cm^{-1} , 1696 cm^{-1} , 1485 cm^{-1} . However, some additional peaks were observed with the physical mixture, possibly because of the presence of collagen. These results suggested that the *Centella asiatica* extract and collagen were compatible.

Microbiological studies:

The microbial tests conducted on various collagen scaffolds by direct inoculation method showed no growth of microorganisms in Nutrient Agar Medium & Czapek's Dox Medium indicating that the extract lodged scaffold was sterile and safe to use.

Evaluation of scaffolds**Table 1: Physicochemical Properties of CAEICDS and CAEICDS**

Type of Formulation	Thickness (μm)	Folding Endurance	W.V.T. Coefficient (Q, g/cm/day)	M.S.T. ($^{\circ}\text{C}$)	E.S.R [(mg/mg)/24 hrs]	A.O.E (%)
Plain scaffold	36.61 \pm 0.4	410 \pm 0.81	3.90 $\times 10^{-4}$	64	4.85 \pm 0.42	89.72
1% CAEICDS	36.98 \pm 0.5	393 \pm 0.43	3.94 $\times 10^{-4}$	69	4.92 \pm 0.32	96.31
1.5% CAEICDS	37.12 \pm 0.6	390 \pm 0.38	4.20 $\times 10^{-4}$	71	4.98 \pm 0.32	98.79
2% CAEICDS	37.29 \pm 0.4	388 \pm 0.28	4.73 $\times 10^{-4}$	72	5.04 \pm 0.38	98.05
1% CAEICDS	37.84 \pm 0.5	409 \pm 0.56	2.89 $\times 10^{-4}$	70	4.01 \pm 0.38	97.00
1.5% CAEICDS	38.31 \pm 0.7	405 \pm 0.43	3.06 $\times 10^{-4}$	73	4.03 \pm 0.38	98.94
2% CAEICDS	39.01 \pm 0.8	399 \pm 0.27	3.15 $\times 10^{-4}$	76	4.09 \pm 0.38	98.52

* All Values are expressed as mean \pm SD (n=10). CAEICDS indicates *Centella asiatica* extract incorporated collagen dermal scaffolds; CAEICDS indicates *Centella asiatica* extract incorporated crosslinked collagen dermal scaffolds; W.V.T, Water Vapour Transmission; M.S.T, Microshrinkage Temperature; E.S.R, Equilibrium swelling ratio; A.O.E, Antioxidant Efficiency

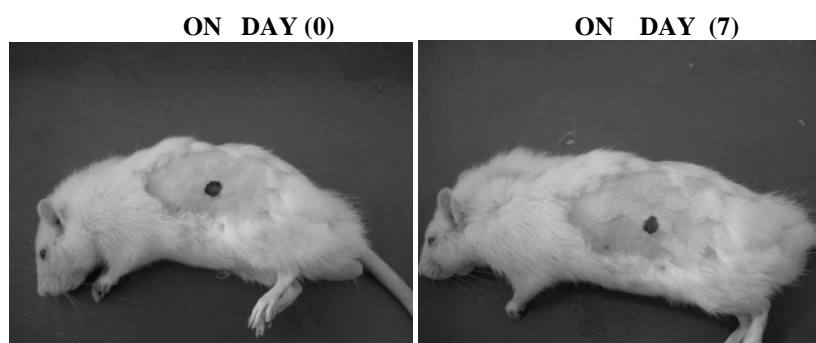
Physicochemical characterization of scaffolds:

The results of the physicochemical characterization of the scaffolds are tabulated (Table 1). The thickness of the scaffolds was found to be slightly increased with the increase in concentration. Folding endurance study indicated that the scaffolds could withstand rupture. Swelling index study results revealed that the scaffolds had a significant impact on the absorption of wound exudates. The increased hydrophilic concentration of the extract in the scaffold increased the water vapor transmission rate. The higher shrinkage temperature of different CAEICDS scaffolds suggested increased hydrothermal stability when compared to plain collagen scaffold.

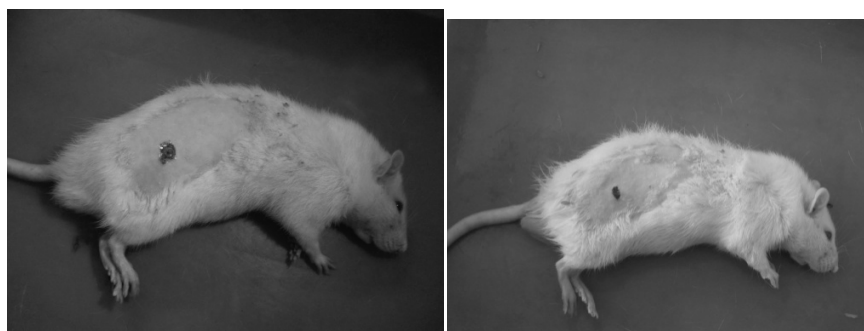
Antioxidant efficiency:

The scavenging action of *Centella asiatica* root extract was well established against peroxy radicals when subjected to time dependent absorbance study. When CAEICDS were placed on cellulose paper, sudden decrease in absorbance value was observed. This might be due to the reaction of *Centella asiatica* extract and collagen with free radicals preventing them from further peroxidation. The slight increase in the antioxidant efficiency value of CAEICDS over the CAEICDS suggested the more controlled action of the cross-linked scaffolds in releasing the extract which gradually increased the antioxidant efficiency

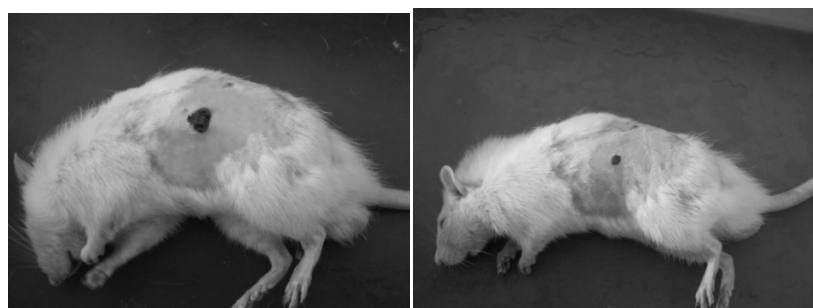
Figure 4: Wound Healing Studies (After 7 Days)



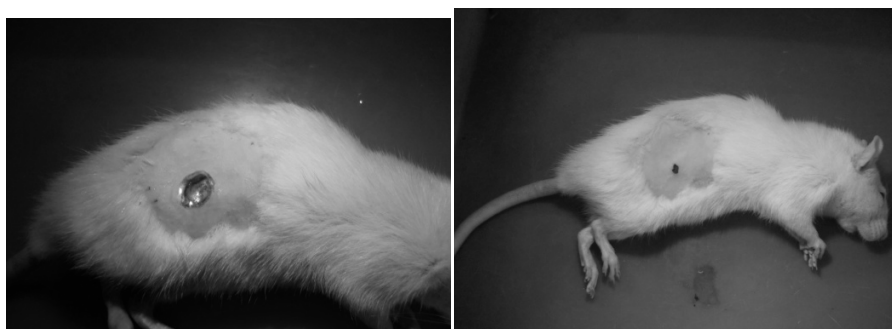
G₁- Control Group



G₂- Marketed Formulation ((Neu- SkinTM) treated group.



G₅- 15 mg *Centella asiatica* extract only treated Group



G₈. 1.5%w/v CAEICDS treated group

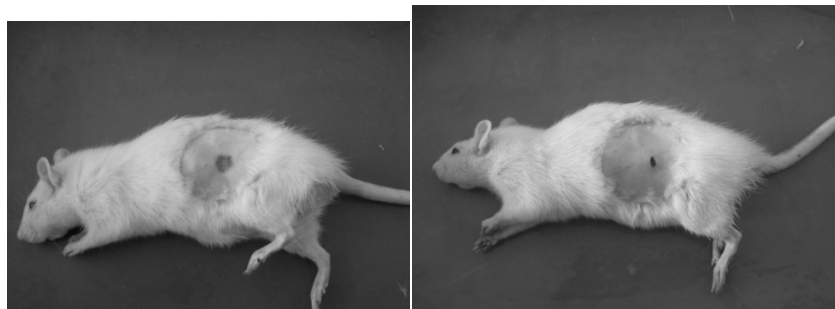
G₁₂. 1.5%w/v CAEICDS treated group

Table 2.1 Observed Wound Reduction

Group		G ₁	G ₂	G ₃	G ₄	G ₅	G ₆
Wound Area (Cm ²)	Day 0	0.785	0.785	0.785	0.785	0.785	0.785
	Day 7	0.458 ± 0.05	0.351 ± 0.05	0.350 ± 0.02	0.368 ± 0.04	0.347 ± 0.02	0.348 ± 0.01
% Wound reduction		41	55.7	55.4	53	55.7	55.6

Table 2.2 Observed Wound Reduction

Group		G ₇	G ₈	G ₉	G ₁₀	G ₁₁	G ₁₂
Wound Area (Cm ²)	Day 0	0.785	0.785	0.785	0.785	0.785	0.785
	Day 7	0.217 ± 0.03	0.157 ± 0.04	0.163 ± 0.02	0.205 ± 0.03	0.151 ± 0.04	0.152 ± 0.02
% Wound reduction		72.24	79.99	79.21	73.79	80.68	80.59

All values are expressed as mean ± SD (n=10). Group 1, indicates control; Group 2: Rats treated with Marketed Formulation (Neu- Skin™); Group 3: Rats treated with Plain Collagen Scaffolds; Group 4: Rats treated with 10 mg *Centella asiatica* Extract only; Group 5: Rats treated with 15 mg *Glycyrrhiza glabra* root Extract only; Group 6: Rats treated with 20 mg *Glycyrrhiza glabra* root Extract only; Group 7: Rats treated with 5% CAEICDS; Group 8: Rats treated with 10% CAEICDS; Group 9: Rats treated with 15% CAEICDS; Group 10: Rats treated with 5% CAEICDS; Group 11: Rats treated with 10% CAEICDS; Group 12: Rats treated with 15% CAEICDS.

Wound Healing Studies:

Wound healing studies when performed indicated (Figure.4) that there was a significant wound healing in the *Centella asiatica* treated groups and highest wound healing was observed in the 1.5% *Centella asiatica* treated group both Plain and Cross-linked when compared to the wound healing of other groups including the marketed one (Table.2). This could be where the release of the extract from the Collagen is much appropriated (optimum concentration) resulting in the maximum action against free radicals by scavenging them.

CONCLUSION

Thus the developed 1.5% CAEICDS and CAEICDS would be a feasible, productive & novel approach in improving the quality of wound healing.

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