

# Formulation & Evaluation of *Centella asiatica* extract impregnated Collagen Dermal Scaffolds for Wound healing

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**Abstract:** The aim of this work is to design and formulate a collagen based dermal scaffold containing *Centella asiatica* extract for the improvement in the quality of wound healing. For this purpose Collagen was isolated from bovine Achilles tendon and tested for its confirmation of presence, purity and sterility. The physicochemical compatibility between collagen and *Centella asiatica* extract was studied by FT-IR and results obtained suggested no interaction and they were compatible. Collagen based Scaffolds were formulated using different concentrations (1%w/v, 1.5%w/v & 2%w/v) of *Centella asiatica* extract (CAEICDS- *Centella asiatica* extract incorporated collagen dermal scaffolds). The prepared 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 were found to be 69°C, 71°C & 72°C respectively indicating their hydrothermal stability. Wound healing studies on Male Wister Rats were performed for a period of 7 days and it was observed that the 1.5%w/v CAEICDS treated rats possessed higher amount of hydroxyl proline content (71.3%) when compared to that of the existing Marketed formulation (Neu- skin<sup>TM</sup>) (59.7%). Further better wound healing activity was observed in the 1.5%w/v CAEICDS treated rats (79.9%) when compared to others which included the Marketed formulation (55.2%). When examined histopathologically, more production in the collagen content was clearly observed in 1.5% w/v CAEICDS treated groups which resulted in the epithelial gap reduction. Further rise in fibroblasts (69 in 100μ in the wound during the healing process) in 1.5% w/v CAEICDS treated groups suggested that it is a feasible and productive approach in the improvement of dermal wound healing process.

**Keywords:** Collagen, *Centella asiatica* extract, Micro Shrinkage Temperature, Wound Healing, Histopathological Examination.

## 1. INTRODUCTION:

Wound Repair is a multifactorial task involving several events finally restoring to normal conditions<sup>1</sup>. 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 have a natural tendency of respiratory burst mechanism and produce free radicals. Certain non phagocytic cells of the wound generate radicals by the non-phagocytic NAD(P)H oxidase mechanism making the wound site rich in oxygen and nitrogen. 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<sup>2</sup>. There is a substantial evidence of antioxidants playing a vital role in therapy against the pathogenesis of many diseases caused by oxidants<sup>3</sup>. *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)<sup>4,5</sup>. However the delivery of this extract possessing the wound healing properties becomes 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<sup>6</sup>. 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 drug.

## 2. MATERIALS AND METHODS

### Materials:

Collagen (isolated from Achilles tendon), *Centella asiatica* aqueous extract - gift sample obtained from Chemiloids - Vijayawada, 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 of Bapatla College of Pharmacy.

## Methods:

### 2.2.1. Investigation of physicochemical compatibility of *Centella asiatica* extract and polymer:

Collagen was isolated from Bovine Achilles tendon using 0.5M acetic acid and 5% w/v NaCl solution following the previously reported procedure<sup>6</sup>. The physicochemical compatibility between *Centella asiatica* extract and collagen was studied by using Perkin Elmer Fourier Transform Infra Red (FTIR) Spectroscopy. The infrared spectra were recorded using Perkin Elmer Fourier Transform Infra Red (FTIR) Spectrometer, Shelton, USA by using KBr pellet method and spectra were recorded in the wavelength region between 4000 and 400  $\text{cm}^{-1}$ . The spectra obtained for *Centella asiatica* extract, collagen and physical mixture of *Centella asiatica* extract with collagen were compared.

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

Collagen was soaked in 0.05 M glacial acetic acid at 25mg/ml concentration for 24 hrs at  $4^\circ\text{C}$ . The obtained viscous solution was homogenized for 5 min, deaerated for 15 min by using sonicator and squeezed through muslin cloth to get rid of undissolved solid traces if any. Various solutions with different concentrations of *Centella asiatica* extract such as 1%w/v & 1.5%w/v & 2%w/v previously dry heat sterilized were separately solubilized in 3ml of absolute ethanol. The prepared solutions were mixed with 18ml of collagen solution (concentration of collagen adjusted to 11mg/ml, with 0.05 m acetic acid) with constant stirring for 24 h, at  $4^\circ\text{C}$ . The suspension was then squeezed through muslin cloth to remove any precipitate formed during the process. The viscous dispersion thus obtained was deaerated by sonication and casted in Petri plate (64  $\text{cm}^2$  diameter) having polyethylene membrane base and placed in incubator at  $37^\circ\text{C}$  until dried. The scaffold thus obtained was sterilized under UV radiation for a period of 18 hours and subjected to microbial studies.

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

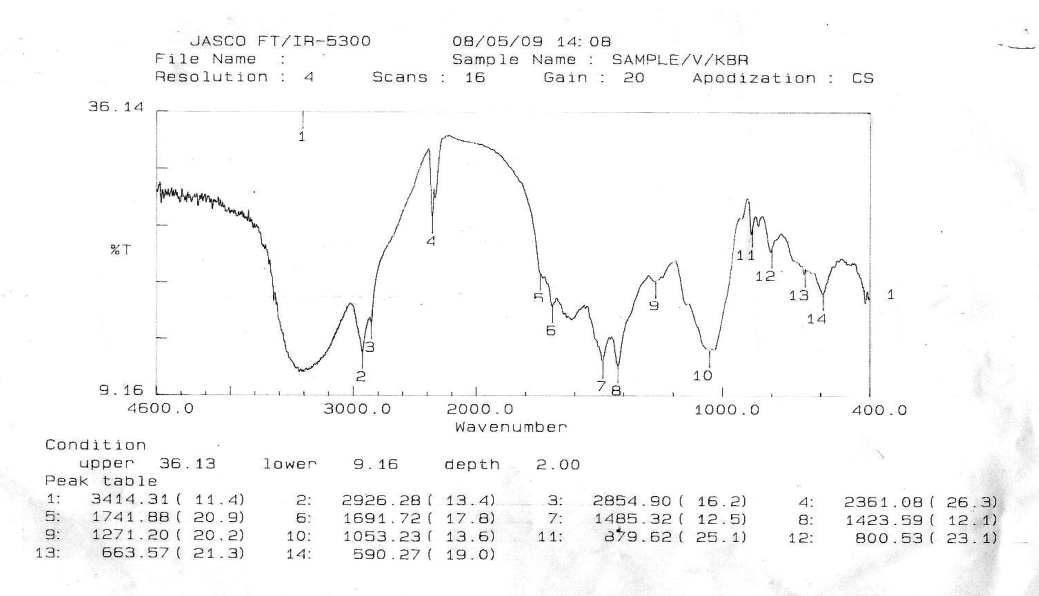
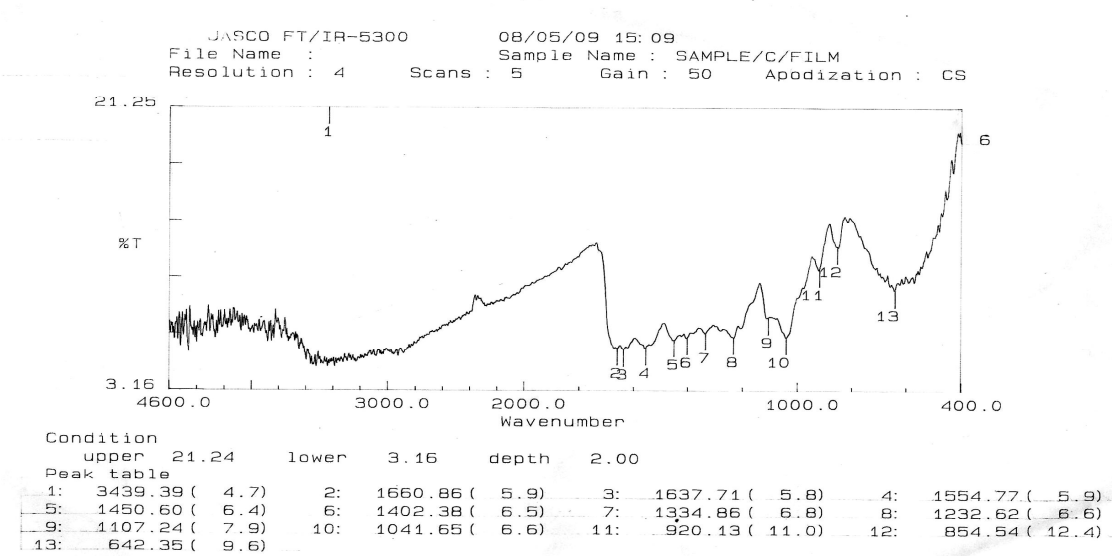
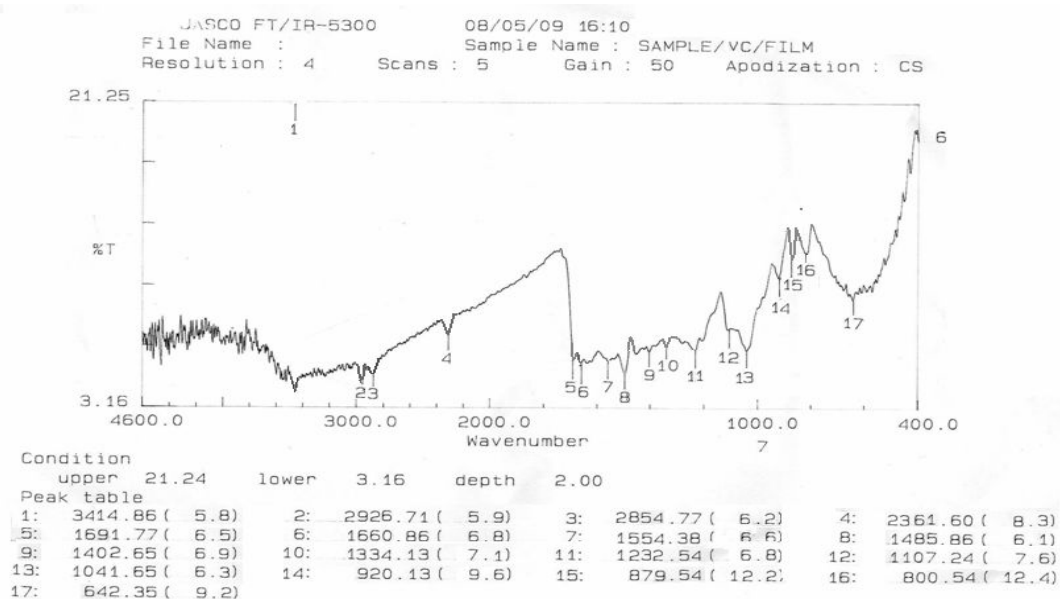


Figure 2: FT-IR of plain collagen scaffold.



**Figure: 3: FT-IR graph of physical mixture of *Centella asiatica* extract and Collagen.****2.2.3 Microbial Test<sup>7</sup>:**

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 10 Petri plates, containing 25ml separately. The Petri plates were numbered from 1 to 10 respectively. Plate 1 was maintained as control for Nutrient Agar Media, Plates 2, 3, 4 and 5 were inoculated with plain collagen scaffold and different concentrations of (1%w/v & 1.5%w/v & 2%w/v) CAEICDS in Nutrient Agar Media respectively. Plate 6 was maintained as control for Czapek's Dox Media, Plates 7, 8, 9 and 10 were inoculated with plain collagen scaffold and different concentrations of (1%w/v & 1.5%w/v & 2%w/v) CAEICDS in Czapek's Dox Medium respectively. All the 10 Petri plates were incubated at 37°C for 24 hours and observed for the growth of micro organisms.

**2.2.4 EVALUATION OF SCAFFOLDS:****2.2.4.1 Thickness:**

The thickness of the different concentrations of CAEICDS was measured by using a screw gauge (LINKER-20 X 1/100 mm).

**2.2.4.2 Folding Endurance:**

Folding endurance was measured manually for the prepared scaffolds. For this a strip of film (2x2 cm<sup>2</sup>)

was cut evenly and repeatedly folded at the same place until it broke. The number of times the scaffold could be folded at the same place without breakage gave the exact value of Folding Endurance.

**2.2.4.3 Water Vapor Transmission Test<sup>8</sup>:**

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<sup>2</sup> 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 a closed desiccator 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 (g/cm<sup>2</sup> / 24h)

W = Weight of water vapor transmitted (g / 24h)

L = Thickness of the patch (mm)

S = Exposed surface area of the patch (cm<sup>2</sup>)

**Table 1: Physicochemical Properties of CAEICDS\***

Type of Formulation	Thickness (μm)	Folding Endurance	W.V.T. Coefficient (Q,g/cm/day)	M.S.T. (°C)	E.S.R[(mg/mg)/24 hrs]	A.O.E (%)
Plain scaffold	36.33±0.47	410±0.81	$3.90 \times 10^{-4}$	64±0.11	4.85±0.42	90.58
1%w/v CAEICDS	36.98±0.5	393±0.43	$3.94 \times 10^{-4}$	69±0.13	4.92±0.36	96.31
1.5%w/v CAEICDS	37.12±0.5	390±0.38	$4.20 \times 10^{-4}$	71±0.24	4.98±0.32	98.79
2%w/v CAEICDS	37.29±0.5	388±0.59	$4.73 \times 10^{-4}$	72±0.21	5.04±0.38	98.05

\*All Values are expressed as mean ± SD (n=10). CAEICDS indicates *Centella asiatica* extract incorporated 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;

#### 2.2.4.4 Micro Shrinkage Temperature Studies

The Micro shrinkage Temperature measurements were carried out for the plain scaffold and different concentrations of 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 Micro shrinkage Temperature.

#### 2.2.4.5 Equilibrium Swelling Ratio Determination<sup>9</sup>

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.

#### 2.2.4.6 Antioxidant Efficiency<sup>10</sup>:

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 234nm for 30 min, and the tube was plugged tightly to prevent the evaporation of hexane. The CAEICDS were placed over the cellulose paper separately containing Oleic acid. The experiment was repeated and the absorbance was measured at 234nm.

#### 2.2.4.7 Wound Healing Studies on Male Wistar Rats<sup>11</sup>:

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/14/BCOP/2009 was approved by Institutional Animal Ethical Committee (IAEC) of Bapatla College of Pharmacy. Animals were divided into nine 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-skin<sup>TM</sup>)

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 dermal scaffolds

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

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

For this, the area was cleared off from hair by using a depilatory and anaesthetized using chloroform. A metal template measuring 1x1 cm (0.785cm<sup>2</sup> 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, Marketed (Neu-Skin<sup>TM</sup>) and CAEICDS of different concentrations were applied

separately on the excised wounds of the healthy male animals of different groups.

#### 2.2.4.8 Histopathological Examinations:

Serial sections of paraffin embedded tissue (1mm<sup>2</sup> area) of 3-5µm thickness were cut with a Rotary Microtome (SIPCON<sup>®</sup>) and stained under light microscope (OLYMPUS CKX41<sup>®</sup>) whose stage micrometer of 100 µm was calibrated with 96µ of eyepiece micrometer. The tissue was focused and the number of fibroblasts were counted at 40X x 10 magnification and presented in number per 100 µm. To evaluate re-epithelization the epithelial gap was measured at 10X x 10 magnifications.

Wound Healing Data		Treated Groups								
		G <sub>1</sub>	G <sub>2</sub>	G <sub>3</sub>	G <sub>4</sub>	G <sub>5</sub>	G <sub>6</sub>	G <sub>7</sub>	G <sub>8</sub>	G
Wound area (cm <sup>2</sup> )	Day 0	0.785	0.785	0.785	0.785	0.785	0.785	0.785	0.785	0.785
	Day 7	0.458±0.05	0.351±0.04	0.350±0.02	0.414±0.04	0.381±0.02	0.383±0.01	0.275±0.03	0.20±0.04	0.212±0.02
% Wound Reduction		41.0	55.7	55.4	53.0	55.7	55.6	72.24	79.99	79.21

**Table2: Observed Wound Reduction\*:**

\*All values are expressed as mean ± SD (n=10). G<sub>1</sub> indicates control group; G<sub>2</sub> indicates Marketed Formulation (Neu-Skin<sup>TM</sup>) treated group; G<sub>3</sub> Plain collagen scaffold treated groups; G<sub>4</sub>, 10 mg *Centella asiatica extract* only treated groups; G<sub>5</sub>, 15 mg *Centella asiatica extract* only treated groups; G<sub>6</sub>, 20 mg *Centella asiatica extract* only treated groups; G<sub>7</sub>, 1% CAEICDS treated groups; G<sub>8</sub>, 1.5% CAEICDS treated groups; G<sub>9</sub>, 2% CAEICDS treated group.

**Table 3: Results of Histopathological Studies (ON DAY 7)**

Parameters	G <sub>2</sub>	G <sub>3</sub>	G <sub>5</sub>	G <sub>8</sub>
Fibroblasts*	52	51	48	69
Hydroxyproline (mg/100mg tissue)	8.12±0.5	8.07±0.2	8.02±0.8	9.73±0.4

- Fibroblasts focussed at 40X x10 magnification for 100µm.
- CAEICDS- *Centella asiatica* extract impregnated collagen based dermal scaffolds. G<sub>2</sub> indicates Marketed Formulation (Neu-Skin<sup>TM</sup>) treated group; G<sub>3</sub>, Plain collagen scaffold treated groups; G<sub>5</sub>, 15 mg *Centella asiatica extract* only treated groups; G<sub>8</sub>, 1.5% CAEICDS treated groups.

### **3. RESULTS AND DISCUSSION**

#### **3.1 Physicochemical compatibility of *Centella asiatica* extract and collagen**

The IR spectra of *Centella asiatica* extract alone showed the principal peaks at wave numbers  $3414\text{ cm}^{-1}$ ,  $2926\text{ cm}^{-1}$ ,  $1230\text{ cm}^{-1}$ ,  $1691\text{ cm}^{-1}$ ,  $1485\text{ cm}^{-1}$  confirming the purity of the *Centella asiatica* extract. In the IR spectra of physical mixture of *Centella asiatica* extract and collagen the major peaks of *Centella asiatica* extract were observed at wave numbers  $3414\text{ cm}^{-1}$ ,  $2926\text{ cm}^{-1}$ ,  $1696\text{ cm}^{-1}$ ,  $1485\text{ cm}^{-1}$ . 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.

#### **3.2 Microbial 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.

#### **3.3 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.

#### **3.4 Antioxidant efficiency:**

The scavenging action of *Centella asiatica* 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.

#### **3.5 Wound healing studies**

Wound healing studies performed on various treated groups indicated that there was a significant wound healing in the *Centella asiatica* extract treated groups and highest wound healing was observed in the 1.5% *Centella asiatica* extract treated group when compared to the other groups of which included the marketed treated group. Hence 1.5% w/v concentration used in the CAEICDS could be considered as an optimized concentration, which resulted in the maximum action against free radicals by scavenging them thus hastening the wound healing process.

#### **3.6 Histopathological studies**

Results of histopathological studies revealed the presence of highest amount of hydroxyl proline content in the 1.5% w/v CAEICDS treated groups. The rise in the content of upper part indicated the collagen production which in turn reduced the epithelial gap to a markable extent when compared to the other concentrations of CAEICDS including other marketed formulation (Neu-Skin™). During this process, the granulation tissue formation takes place which is indicated by the growth of fibroblasts. These fibroblasts differentiate into myo-fibroblasts which is the characteristic feature of tissue undergoing repair. The highest number of fibroblasts in 1.5 %w/v CAEICDS treated groups confirms that these scaffolds when used could increase cellular proliferation in a quicker manner and improve the quality of wound healing.

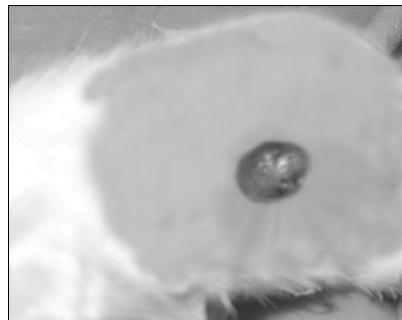
**Figure 4: Wound Healing Studies (After 7 Days)**



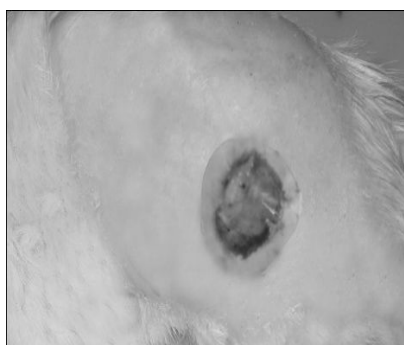
**Male Wistar Rat- Wound Site**

ON DAY (0)

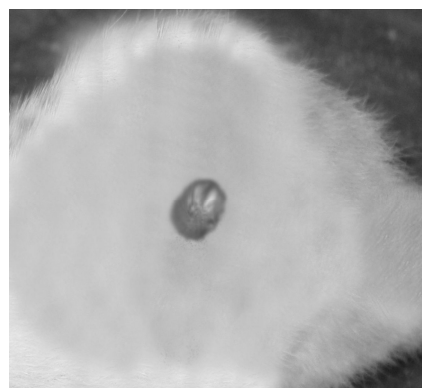
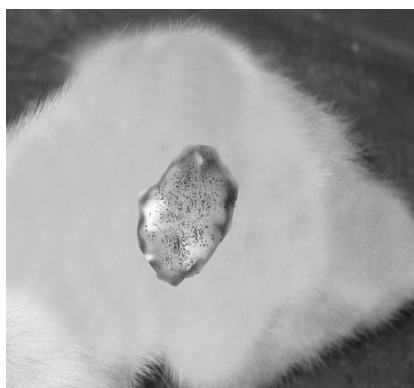
ON DAY (7)



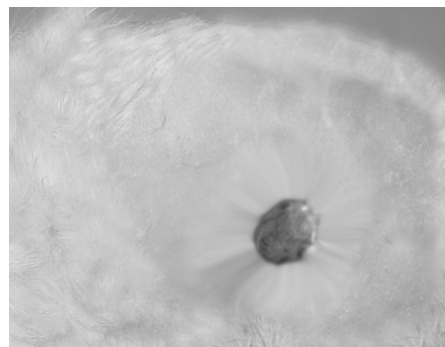
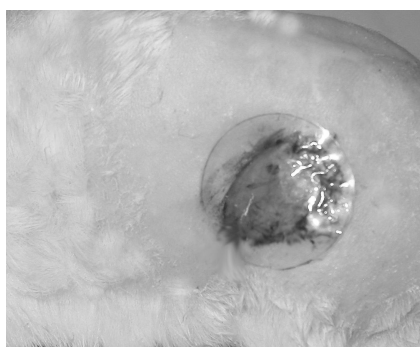
**G<sub>1</sub>- Marketed Formulation ((Neu- Skin<sup>TM</sup>) treated group.**



**G<sub>2</sub>- Plain collagen scaffold treated group**



**G<sub>5</sub>- 15 mg *Centella asiatica* extract only treated Group**



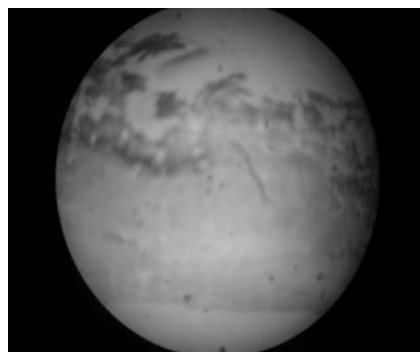
**G<sub>8</sub>. 1.5%w/v CAEICDS treated group**



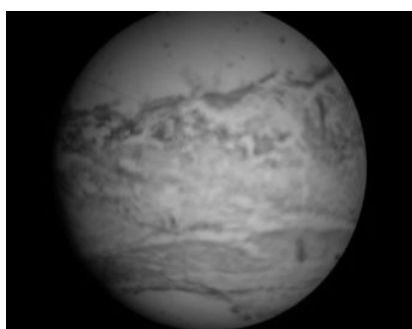
**Fig 5: Histopathological studies:**



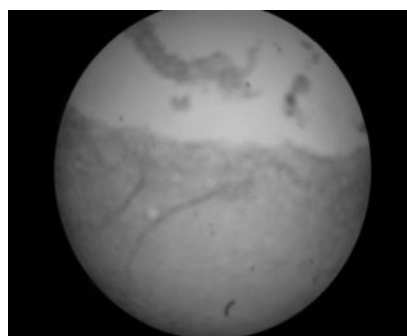
**Control group**



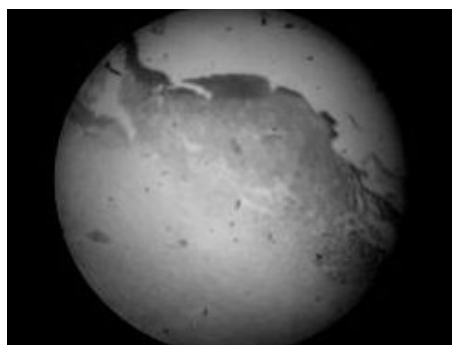
**G<sub>2</sub>-Marketed (Neu-Skin™) treated group**



**G<sub>3</sub>- Plain collagen scaffold treated group**



**G<sub>5</sub>: Rats treated with 15 mg *Centella asiatica* extract only**



**G<sub>8</sub>: Rats treated with 1.5% w/v CAEICDS**

#### **4. CONCLUSION**

The developed *Centella asiatica* extract incorporated Collagen based scaffolds enhance the wound healing process.

#### **ACKNOWLEDGEMENTS:**

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**REFERENCES:**

- 1.Hunt TK, Hopf H, Hussain Z. "Physiology of wound healing". Adv Skin Wound Care 2000; 13:6–11.
- 2.Wiseman H, Halliwell B. "Damage to DNA by reactive oxygen and nitrogen species: role in inflammatory disease and progression to cancer". Biochem J 1996; 313:17–29.
- 3.Skaper SD, Fabris M, Ferrari V, Carbonare MD, Eon A. "Quercetin protects cutaneous tissue associated cell types including sensory neurons from oxidative stress induces by glutathione depletion" Cooperative effects of ascorbic acid. Free Radic Biol Med 1997; 22(4):669–78.
- 4.Umachigi S.P, Jayaveera K.N, Ashok Kumar C.K, Kumar G.S, Vrushabendra Swamy B.M and Ashok Kumar D.V "Studies on wound healing properties of *Quercus infectoria*" , Tropical Journal of pharmaceutical research, March 2008, 7(1), 913-919.
- 5.Umachigi S.P, Jayaveera K.N, Ashok Kumar C.K, Kumar G.S, "Antioxidant potential of galls of *Quercus infectoria*", Journal of pharmacology 2008, Volume 5, No 2.
6. Pachence JM. "Collagen based device for soft tissue repair". J Biomed Mater Res (Appl Biomater) 1996; 33:35–40.
- 7.Deyl Z, Adam M. "Preparation of insoluble collagen". In: Hall DA, editor.The methodology of connective tissue research. 1st ed.Oxford: Joynson-Bruvvers; 1976:1–7.
8. Neuman RE, Logan MA. "The determination of hydroxyproline". J Biol Chem. 1950;184: 299–306.
9. Comparative Evaluation of polymeric films for Transdermal application, The Eastern Pharmacist – December, 2004, 109-111
10. D.Gopinath," Dermal Wound Healing Process with Curcumin incorporated collagen films Biomaterials, 25; 2004; 1911-1917
- 11.Ubaidulla, Transdermal therapeutic system of Carvedilol: "Effect of hydrophilic and hydrophobic matrix on in vitro and in vivo characteristics, AAPS Pharmascitech, 2007, 19, 101-106.

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