CHAPTER-IV

CUCUMBER SAP EXTRACT PROTEASES: ROLE IN WOUND HEALING
INTRODUCTION

Wounds are injuries that consequence in an aperture of the tissue. The wound may be defined as contravention of anatomical, cellular and functional stability of living tissues. Healing is a complex biological process which was initiated in response to an injury that reestablishes the integrity and function of damaged tissues. Blood clotting and removal of debris through inflammation, replacement of damaged tissue through the proliferation of epithelial cells and maturation of scars are the essential events [Stadelmann et al., 1998]. Untreated wounds are the potential place of the infection and multiple organ failure due to chronic wounds. For instance, management of diabetic wounds and eczema are still remained challenging and the lack of knowledge on the molecular mechanism of wound healing is the major limitation in identification of the precise target for the better management. Since time immemorial, herbal extracts have been tried extensively to achieve the better cure.

To treat wounds in the Indian traditional system of medicine such as Ayurveda, Siddha and Unani several plant species had been used. Plants naturally enhance the repair mechanism, so it is best and potent wound healer. Therefore, extensive research should be carried out in the field of wound healing and their management through the plants. Herbal medicines in the area of wound healing management involve disinfection, provided that a moist environment and debridement to endorse the establishment of the apposite environment for natural healing processes. *Rafflesia hasseltii* (Rafflesiaeeae), *Clitoria ternatea* (Rafflesiaeeae), *Rubus sanctus Schreber* (Rosaceae), *Hippophae rhamnoides* (Elaeagnaceae), *Trichosanyhes tricuspidata* (Cucurbitaceae) and *Azardica indica, Lantana camara, Tridax procumbens, Hydnocarpus wightiana, Ginkgo biloba and Centella asiatica* with promising wound healing efficacy have been evaluated scientifically [Avinash Kumar et al., 2012; Rajinder et al., 2008].
Cucumber from *Cucumis sativus* L (Cucurbitaceae) commonly known as melon or gourd or cucurbit has been extensively used for external applications to treat various skin disorders such as wrinkles, chink, skin mold, freckles, sunburn, hyperpigmentation, burning sensation, acne, dark circles skin rashes, burns, wounds and bedsores [Nema et al., 2010; Yamini Dixit et al., 2010]. Thus, from ancient to modern world the cucumber is being used extensively as an external applicant in the cosmetic industry and as well as to treat wounds without having any scientific validation. Therefore, in the present study cucumber sap extract in tissue remodeling and the wound healing process have been studied in a mouse model and the results are presented.
MATERIAL AND METHODS

Materials

Cucumbers were purchased from Krishna Raja Market, Mysore, Adult Swiss Albino mice (30–35 g; either gender) were taken from the Central animal house facility, DOS in Zoology, UOM. According to the regulations for animal research and animal ethical committee of the UOM (Sanction order- UOM/IAEC/6/2012) the animal care and experimental procedure were performed. Ketamine and Xylocaine were purchased from the University Medical facility with a prescription from the University authorized medical practitioner and PDMAB (para-dimethyl amino benzaldehyde) from Sisco Research Laboratory (Mumbai, India). From Qualigens Fine Chemicals and GlaxoSmithKline Pharmaceuticals (Mumbai, India) Gelatin and Neosporin were purchased and all other chemicals were of analytical grade.

Methods

Toxicity studies:

- **Hemorrhagic activity:** The hemorrhagic activity was determined according to the method of Kondo (1995) using groups of mice (n=4). The samples in a final volume of 50 µl saline were injected intra-dermally on the back of mice. The mice were anesthetized after 3 h and sacrificed. The dorsal patch of the skin was removed, the inner surface was observed for the hemorrhage and the diameters of the hemorrhage spots were measured. The minimum hemorrhagic dose (MHD) was defined as the amount of protein required to induce a hemorrhagic area of 10 mm diameter. The group received *Vipera russellii* venom served as positive control.
**Edema Inducing Activity:** According to the Vishwanath (1987) the edema inducing activity was done using mouse models. Groups of three mice were injected in the right footpads with CSE (100µg) and positive control *Vipera russellii* (1.2 µg) of a venom sample in 20 µl saline. The left foot pads received saline, which served as controls. The legs were cut off at the ankle joint after 1 h. An increase in weight due to edema was calculated as the edema ratio, which equals the weight of the oedematous leg × 100/weight of control leg. The minimum edema dose (MED) was defined as the amount of venom/ CSE sample required to cause an edema of 120%.

**Myotoxicity and Myonecrosis:** According to the Gutierrez (1990) the myotoxicity was determined using an albino mouse model. The cytoplasmic markers such as creatine phosphokinase (CK; EC 2.7.3.2) and lactate dehydrogenase (LDH; EC 1.1.1.28) levels were determined in the serum. CSE (200-500µg) and half the LD$_{50}$ value of a venom sample (1.6 mg/kg body weight) in 50 µl saline as positive control was injected intramuscularly into the right thigh of three groups of mice. The group receiving 50µl saline alone served as an additional control experiment. The mice were anesthetized after 3 h by ketamine injection. The abdominal cavities were opened and blood was drawn from the abdominal vena cava. The serum which diluted in the ratio of 1:25 was assayed for LDH and CK activities using AGAPEE diagnostic kits. Activities were expressed as units/l. The thigh muscle tissues dissected from the site of CSE and venom sample injection were fixed in Bouin’s solution and subjected to dehydration by processing the tissue through different grades of alcohol and chloroform: alcohol mixture. The processed tissue was embedded in the paraffin cut into 4 µm thick sections. The sections were stained with haematoxylin-eosin staining for microscopic observations. The sections were observed under Leitz Wetzlar, Germany type-
307-148.002 microscope and photographs were taken using a Photometrics Colorsnap CF camera (made Roper Scientific Photometrics) attached to the microscope.

**Wound healing activity**

The wound healing activity of CSE was determined using the excision wound model [Frank and Kampfer, 2003]. About 10 cm diameter area was shaved and sterilized with 70% alcohol on the dorsal side of the mouse. Further, a full thickness excision wound of 1 cm diameter was made using sharp scissor and samples were applied over the entire wound twice a day up to 21 days starting from day of wounding. Four groups (n=3) of mice were used for the study. **Group Ia-d:** wounds were treated with saline (negative control). **Group IIa-d:** wounds were treated with CSE (10 mg/kg body weight), **Group IIIa-d:** wounds were treated with heat denatured CSE and **Group IVa-d:** wounds were treated with Neosporin (positive control) respectively for 3, 7, 14 and 21 days. Wound contraction (diameter of the wound calculated by tracing margins of the wound on a graph sheet) was monitored daily and the percentage of wound closure was calculated using the formula, \[ \% \text{ wound contraction} = \left( \frac{\text{Healed area}}{\text{Total wound area}} \right) \times 100 \] (Healed area= original wound area - present wound area). Mice were anesthetized by intraperitoneal injection of ketamine-Xylocaine mixture [Angely and Coppola, 2010] on the respective days. The healing skin from each group was taken and fixed in Bouin’s solution for a period of three days for histopathology.

**Estimation of Granulation Tissue Free Radical and Antioxidant:**

The wet granulation tissues (wound healing tissue and positive and negative control) of albino mice on day 3 to 21 were homogenized in a glass, Teflon Homogenizer (10% w/v)
at 4°C in phosphate buffer saline (PBS, pH 7) used for the estimation of antioxidants, free radicals and protein levels carried out as below.

• **Determination of Superoxide dismutase (SOD) activity:** According to the method of Kakkar (1984) the SOD assay was carried out. It is based on the inhibition of the formation of NADH-phenazine methosulphate-nitro blue tetrazolium formazan. Briefly 1.2 ml of 0.025M sodium pyrophosphate buffer pH 8.3, 0.1 ml of 186µM phenazine methosulphate (PMS) and 0.3 ml of 300µM nitroblue tetrazolium (NBT) were added to 0.2 ml of tissue homogenate sample and mix well. Then 0.2 ml of 780µM NADH solution was added to it. After incubation for 90 sec at 30°C the reaction was abolished by adding 1 ml of glacial acetic acid and shake well. Add 4 ml of n-butanol and the mixture was centrifuged at 4000 rpm for 10 min and the absorbance of the upper butanol layer recorded at 560 nm. For the comparison, corresponding blank was prepared in the same way except the addition of the test sample. One unit of SOD was defined as that amount of enzyme that inhibits the rate of reactions by 50% under specified conditions.

• **Determination of Catalase (CAT) activity:** The 0.1 ml of the different tissue homogenates was a pipette into the cuvette containing 1.9 ml of 50 mM phosphate buffer, pH 7.0 independently. The reaction was started by the addition of 1.0 ml of freshly prepared 30% (v/v) hydrogen peroxide (H₂O₂). The rate of decomposition of H₂O₂ was measured spectrophotometrically by changes in absorbance at 240 NM. The activity of the enzyme was expressed as units/mg of protein. One enzyme unit was calculated as the amount of an enzyme necessary to decrease the absorbance at 240 nm by 0.05 [Aebi et al., 1983].
• **Determination of reduced glutathione (GSH) Activity:** About 0.1 ml of the different tissue homogenate was added to 0.9 ml of sodium phosphate buffer (0.2 M, pH 8) independently. Add 2 ml of freshly prepared DTNB solution (0.6 Mm in 0.2m phosphate buffer) and incubate for 30 min at room temperature. After incubation the reaction mixture was centrifuged and the observance of the supernatants was read against a reagent blank at 412 nm and expressed as nano moles GSH per gram of sample. The main principle was the reduced glutathione on reaction with DTNB (5, 5'-dithiobis Nitro benzoic acid) produce a yellow colored product and it was read at 412 nm [Sedlak et al 1968].

• **Determination of nitric oxide (NO) scavenging activity:** According to the methods of Kumar (2008) the assay was carried out. From the sodium nitroprusside (SNP) the nitric oxide (NO) was generated it was measured by the Griess reagent. SNP in aqueous solution at physiological pH generates NO instinctively and then it interacts with oxygen to produce nitrite ions that can be determined using Griess reagent. Scavengers of NO compete with oxygen leading to reduced production of NO and then 10mM SNP in PBS (phosphate buffer saline) was mixed with different tissue homogenates independent and incubated for 180 minutes at 25 °C and finally add 0.5ml of Griess reagent (1% sulphanilamide, 0.1% naphthylethlenediamine dichloride and 3% phosphoric acid) and the total volume of reaction mixture should be 1ml. The absorbance of the chromophores formed during the diazotization of nitrite with sulphanilamide and subsequent coupling with naphthylethlenediamine dichloride was read at 546 nm and referred to the observance of ascorbic acid used as a positive control treated in the same way with Griess reagent. **Nitric Oxide scavenged (%) =control-test/control*100.** Where, **control** = Absorbance of control reaction and **test** = Absorbance in the presence of the samples of extracts.
• **Determination of Lipid peroxidation (LPO) activity:** According to the methods of Ohkawa (1979) the assay was carried out. In this experiment the lipid peroxides present in animal tissue react with TBA (thiobarbituric acid) which was mainly dependent on the pH of the reaction mixture as was the case for linoleic acid hydroperoxide and the optimum pH was found to be 3.5. The 0.1ml of tissue homogenate (10%w/v) was mixed with 0.2 ml of 8.1% sodium dodecyl sulfate (SDS), 1.5 ml of acetate buffer (20%, pH 3.5) and 1.5 ml of an aqueous solution of TBA (0.8%) and the total volume of the reaction mixture was made up to 4 ml using double distilled water. After heating at 95 °C for 60 min in oil bath, the red pigment produced was extracted with 5 ml of n-butanol-pyridine (15:1) and shake well. The mixture was centrifuged at 4000 rpm for 10min and organic layer was read at 532 nm. As an external standard, tetramethoxypropane (TMP) was used, and lipid peroxide levels were expressed in terms of normal malondialdehyde released against blank.

• **Determination of Myeloperoxidase (MPO) activity:** For Myeloperoxidase (MPO) estimation the granulation tissue (5% w/v) was homogenized in 0.5% hexadecyltrimethylammonium bromide (HTAB) with 50mM potassium phosphate buffer (pH 6). The previous homogenate was freeze-thawed three times, sonicated for 10 seconds, and then centrifuged at 12000 rpm for 45 minutes at 4 °C and the resulting supernatant were used for estimation of MPO [Bradley et al., 1982]. A unit of MPO activity is defined as that converting 1μmol of H$_2$O$_2$ to water in 1min at 25 °C. It is a quantitative measure to quantify the extent of neutrophile accumulation in tissue. MPO activity in the supernatant was determined in 2.9 ml of potassium phosphate buffer (pH 6.0, 50 mM) containing 0.157mg/ml ortho-dianisidine and 0.0005% H$_2$O$_2$. The assay was started by adding 0.1ml of the different tissue homogenate. A change in absorbance at 460 nm every 15 seconds for 5 minutes.
Estimation of connective tissue components in granulation tissue

Approximately 250mg of wet granulation tissue from day 3 to 21 was defatted with acetone till four days (change the acetone twice a day) independently and dried at 50 °C for 24h. It was weighed and kept in glass stopper test tubes. To each tube containing 40 mg of the dried granulation tissue, 1000µl of 6N HCl was added. The tubes were then kept in boiling water bath for 24 h (12 h each day for two days) for hydrolysis. The hydrolyzate was then cooled and excess of acid was neutralized by 10N NaOH using phenolphthalein as indicator. The volume of neutral hydrolyzate was diluted to a concentration of 20 mg/ml with distilled water. The final hydrolyzate was used for the estimation of hydroxyproline [Newman et al., 1950], hexosamine [Dische et al., 1950] and hexuronic acid [Bitter et al., 1962] following the standard curve prepared using the proper substrate as below.

- **Estimation of Hydroxyproline (HPR):** The different tissue hydrolyzate (300µl) were mixed with 1ml of 0.01M CuSO$_4$ followed by the addition of 1ml of 2.5N NaOH and then 1ml of 6% H$_2$O$_2$. The solution was mixed and shaken intermittently for 5 min. All the tubes were incubated at 80 °C for 10min with frequent vigorous shaking. Upon cooling, 1.2 ml of 3N H$_2$SO$_4$ was added with agitation. Finally, 0.6 ml of freshly prepared 5% p-dimethylaminobenzaldehyde (PDMAB) was added. The samples were incubated at 75 °C for 15 min, cooled by placing the tubes in running stream of water and the absorbance was measured at 540 nm against the blank. The amount of hydroxyproline in the samples was calculated using a standard curve prepared with L-hydroxyproline.

- **Estimation of Hexosamine (HXA):** The different tissue hydrolyzate (500µl) was diluted with distilled water (500µl) independently and add 0.5ml of acetyl acetone reagent (15.4 g of ammonium acetate, 0.2 ml of acetyl acetone, 0.3 ml of glacial acetic acid and make up to 100ml with reagent water and stored in brown bottles in refrigerator) and heated in boiling
water bath for 20min then cooled under tap water. To this add 1.5ml of 95% ethanol and 0.5ml of Ehrlich’s reagent [1 g of para-dimethylamino benzaldehyde (PDMAB) in 95 ml of 95% ethanol and add 20 ml of conc. HCl and mix well]. The reaction was allowed for 30 minutes to complete at room temperature. Color intensity was measured at 530nm against the blank. The Hexosamine content of the samples was determined using the standard curve by using D (+) Glucosamine hydrochloride (HiMedia Laboratories Pvt. Ltd., Mumbai, India), from 5 to 50μg/0.5mL using the 100μg/mL working solution.

- **Estimation of Hexuronic acid (HUA):** About 2.5ml of 0.025M Borax in concentrated sulfuric acid is placed in stoppered tubes fixed in a rack and cooled to 4°C. 0.125ml of the different tissue hydrolyzate was diluted with 0.5ml of distilled water. Now, this 0.5ml of hydrolyzate is layered carefully on Borax-sulfuric acid mixture kept in rack at 4°C. The tubes were closed with glass stoppers and then shaken, first slowly, then vigorously, with constant cooling by placing tubes in ice container. The tubes were then heated in a boiling water bath for 10 min, and then cooled to room temperature. Subsequently, 0.1ml of 0.125% carbazole reagent in absolute alcohol/ethanol was added to each tube, shaken and again for 15 min the samples were heated in the boiling water bath, and cooled to room temperature gradually. Color intensity was measured at 530 nm against the blank. The content of hexuronic acid in the samples was determined from the standard curve which was prepared from D (+) Glucurono-6, 3-lactone (HiMedia Laboratories Pvt. Ltd., Mumbai, India), from 5 to 40μg/0.5ml using 100μg/ml working solution.
Histopathology Granulation Tissue

The skin samples of mice were stored in Bouin’s solution and subjected to the process of dehydration using different grades of alcohol and chloroform mixture and finally the samples were kept in chloroform. The tissues were entrenched in molten paraffin wax and 5 μm thick sections were prepared by Spencer 800 microtome. Then the tissue sections were stained using hematoxylin-eosin stain for microscopic observations according to the standard protocol. Finally the tissue sections were observed under the Leitz Wetzlar, Germany type microscope and photographed.

Protein estimation

Protein content was determined by Biuret method [Harrison 1937].

Statistical Analysis

The experiments were repeated for three independent observations. Results were expressed as mean values mean ± SEM. Data were compared by analysis of variance (ANOVA) followed by Bonferroni post hoc test for multiple comparisons; significance was accepted at P < 0.05 (*), P < 0.01 (**) and P < 0.001 (***) . The results were analyzed using GraphPad Prism software.
RESULT

CSE was non hemorrhagic up to a tested dose of 200 µg in a mouse model while *Vipera russelli* venom was highly hemorrhagic and served as a positive control (Fig. 4.1), further, CSE was devoid of other toxic properties such as edema, dermonecrosis and myonecrosis.

CSE was tested for wound healing property and it was followed from day 3rd to 21st days. CSE readily promoted wound healing in a Swiss albino mouse model. The rate of wound contraction achieved in CSE, positive control (PC), negative control (NC) and B-CSE (boiled CSE) treated wounds were found to be 25 to 99%, 27 to 96%, 16 to 75%, 17 to 78% respectively (Fig. 4.2A and B). In case of CSE treated wounds, complete scar free healing was observed on the 21st day of treatment and this was highly comparable to the healing rate of Neosporin treated control (PC) wound, while the healing was achieved on the 25th day of treatment in the case of NC and B-CSE treated wounds.

Further, the tissues at the site of wound for each group of mice were studied for antioxidant markers, free radical scavenging activity and biochemical parameters. The antioxidant markers such as SOD and CAT activities and GSH levels were increased significantly from day 3rd to 14th day, however, their levels decreased drastically in CSE and Neosporin treated wound tissues at 21st day, as compared to NC and B-CSE (Fig 4.3A, B and C). Free radicals such as NO and LPO’s levels and acute inflammatory marker MPO activity were decreased gradually from day 3rd to 21st day in CSE and Neosporin treated wound tissues, while their levels remained high in NC and B-CSE treated wound tissues (Fig 4.4 A, B and C). The biochemical parameters such as hydroxyproline, hexosamine and hexuronic acid contents were significantly increased from day 3rd to 21st day of CSE and Neosporin
treated tissue sections as compared to NC and B-CSE which showed marked decrease levels (Fig 4.5A, B and C).

On respective days, such as 3rd, 7th, 14th and 21st days of treatment, the animals from all the groups were sacrificed to remove skin tissue at the site of wound healing and subjected for histopathology. The CSE and Neosporin treated wound tissue sections revealed the accumulation of a large number of inflammatory cells at 3rd day, as compared to NC and B-CSE. On the 7th day there observed an increased formation and accumulation of collagen bundles in CSE and Neosporin treated tissue sections as compared to NC and B-CSE. On day 14, an increased collagen network and less numbers of inflammatory cells with a relatively intact extracellular matrix in CSE and Neosporin treated sections were prominently noticed while contrasting features were associated with NC and B-CSE treated wound tissue sections. On day 21st, the normal collagen, intact extracellular matrix and with no significant inflammatory cells characteristic of normal tissue have been restored in CSE and Neosporin treated wound tissue sections. However, the normal histological features did not restore even after day 25th in NC and B-CSE treated wound tissue sections (Fig 4.6).
DISCUSSION

The wound is generally characterized by the damage of tissues and vascular endothelium resulting in the onset of complex acute phase events such as primary and secondary hemostasis and inflammatory response which not only offer protection to the wound but also prevent spreading of wound due to the possible infection.

Millions of people worldwide suffer from the painful chronic wounds. Chronic and unhealed wounds persistently produce inflammatory mediators which induce the swelling and pain at the site of the wound. Generally the wound becomes the substrate for infection and it reduces the healing of wounds in the patients and leads to the chronic wound condition.

Wound healing is a complex biochemical process which was initiated in response to a trauma that restores the integrity and function of damage tissue. Healing involves the several steps such as hemostasis, inflammation, proliferation and maturation. However, removal of fibrin clot and debris and replacement of damaged tissue through the proliferation of epithelial cells and maturation of scars are the essential events of wound healing process. It is extremely complex and sternly regulated process involving the symphony of a variety of factors such as platelets, collagen, blood clotting factors, cytokines, chemokines, hormones, proteases and etc. Many modern medicines/drugs have been developed to treat wounds, while these have their own constraint and side effects. To overcome these problems research is going in worldwide to find out the novel drug from plant sources.

In in vivo experiments using swiss albino mice wound healing was carried out, the wound contraction/closure percentage was increased in case of wound treated with CSE compare to negative control, positive control and protease control. Histology of the excision wound also showed normal epithelization, adnexa and fibrosis within the dermis.
During the wound contraction process the healthy skin surrounds the wound area to cover the uncovered area; this involves complex and tremendously organized interaction of cells, cytokines and extracellular matrix. This centripetal movement of wound margin is believed to be due to the activity of myofibroblast. In the present study the CSE increased the rate of wound contraction, either by enhancing the contractile property of myofibroblasts or may be by increasing the number of myofibroblasts through controlled proliferation. In granulation tissue the hexosamine, hexuronic acid and hydroxyproline contents were increased gradually, which reflects the rate of wound healing. Further, the sections of granulation tissue of CSE and Neosporin treated mice showed a profusion of collagen tissue and neovascularisation with few inflammatory cells on the 14th day of treatment compared to NC and B-CSE which suggests an augmented wound healing process.

Reactive oxygen species (ROS) and non-radical oxidant plays a very important role in the wound healing process, these served as cellular messengers in many biochemical pathways. At micromolar concentration, hydrogen peroxide promotes the expression of vascular endothelial growth factor (VEGF) in keratinocytes [Fronza et al., 2009]. The compounds with free radical scavenging activity shown the promising the wound healing effect and protects the tissue from oxidative damage [Roy et al., 2012]. Over production of ROS leads to oxidative stress, which results the cytotoxicity and delayed wound healing process. Therefore the elimination of ROS is important for proper wound healing in chronic conditions [Agarwal et al., 2009]. The initial increased SOD, CAT, GSH levels and their decreased pattern after 14th day and the decreased levels of NO, LPO and MPO at 21st day in CSE treated granulation tissue suggests the significant antioxidant activity. Reduction of free radicals and MPO levels could avoid oxidative damage by free radicals and promote the
healing processes. These studies may helpful for design safe drugs for to treat wounds in the future.

In conclusion, this systematic study not only uncover the beneficial properties of the proteases of cucumber sap, but also provide the scientific basis for the wide use of cucumber in the cosmetic industry and as well as in traditional medicine as a skin conditioner and cleansing agent during wound healing and in the treatment and management of acne and other skin disorders.
Fig 4.1: Hemorrhagic activity of CSE: CSE was injected intradermally into different groups of mice. After 3 h, the mice were anesthetized using ketamine and sacrificed. The dorsal patch of the skin of mice was removed and examined for the hemorrhagic spot and the diameter was measured in mm. Saline (A), CSE 50 μg (B), CSE 100 μg (C) and CSE 200 μg (D) and D. russelli venom (2 MHD, 5 μg) (E) which served as a positive control.
Fig 4.2: Effect of CSE on wound healing activity using mouse model: 10 mm diameter full thickness excision wounds were surgically made on the dorsal portion of the mice. An amount of 250 µg of CSE was applied twice daily on the wound (10 mg/kg/day). A represents the photographic representation of percentage of wound contraction from day 3 to 21 of cucumber sap extract (CSE) Neosporin, negative control (NC), and boiled extract of CSE (B-CSE). B represents the wound contraction which was monitored by measuring the diameter of the wound using a graph sheet. Results are articulated as a percentage of wound closure and are expressed in mean ± SEM and analyzed using two-way ANOVA followed by Bonferroni’s multiple comparison Test (** P<0.001, significant when compared to the negative control (NC) group of mice).
Fig 4.3: Estimation of antioxidant of granulation tissue: A, B, and C represent the SOD, CAT and GSH levels of 3 to 21 days granulation tissue of mice treated with CSE (cucumber sap extract), Neosporin, NC (negative control, without any treatment) and B-CSE (boiled extract of CSE). Results are expressed in mean ± SEM and then analyzed using two-way ANOVA test which was followed by Bonferroni post-tests (** P<0.001, significant when compared to the negative control group of mice).
Fig 4.4: Estimation of free radical of granulation tissue: A, B, and C represent the NO, LPO and MPO activities of 3rd to 21st days granulation tissue of mice treated with CSE (cucumber sap extract), Neosporin, NC (negative control, without any treatment) and B-CSE (boiled extract of CSE). Results are expressed in mean ± SEM and then analyzed using two-way ANOVA test which was followed by Bonferroni post-tests (*** P<0.001, significant when compared to the negative control group of mice).
Fig 4.5: Estimation of biochemical parameters of granulation tissue: A, B, and C represent the content of hydroxyproline, hexosamine and hexuronic acid of 3rd to 21st days granulation tissue of mice treated with CSE (cucumber sap extract), Neosporin, NC (negative control, without any treatment) and B-CSE (boiled extract of CSE). Results are expressed in the mean ± SEM and then analyzed using two-way ANOVA test and which was followed by Bonferroni post-tests (*** P<0.001, significant when compared to the negative control group of mice).
Fig 4.6: Histopathology of skin granulation tissue of mice from day 3 to 21 stained with Hematoxylin-Eosin: Tropical application of CSE (250 µg), standard cream Neosporin and boiled CSE extract twice a day for wounded skin of albino mice till 21 days. After 3\textsuperscript{rd}, 7\textsuperscript{th}, 14\textsuperscript{th} and 21\textsuperscript{st} days the mice were scarified in each group. The skin was dissected and processed for hematoxylin-eosin staining. Images were under the microscope and photographed (10X).

[Note: Cucumber sap extracts (CSE), Neosporin, Negative control (NC), Boiled extract of CSE (B-CSE) M- Macrophages, IC- inflammatory cells, C- collagen, BV- blood vessel, CB- collagen bundles, FB- fibroblast, DC- dense collagen and EC- intact extracellular matrix].