Chapter 4 – Result and Discussion

4.1Development of ciprofloxacin loaded collagen-chitosan scaffold for skin tissue engineering

4.1.1. Physical and chemical characterization

4.1.1.1 Morphological characterization

The morphology of the prepared scaffolds is shown in (figure.4.1.1). SEM image of prepared scaffold has shown a vivid range of interconnected micro and macropores. Coexistence of macro and microporous structure is suitable not only for cell growth but also for the exchange of nutrients and removal of the waste [236]. Pore size of the developed scaffolds ranges from 48 μm to 169 μm. but average pore size of scaffolds was calculated using Image J software were found to be around ~ 71 μm, ~ 125 μm and ~ 142 μm for SC-1, SC-2 and SC-3 respectively. Pores are long and interconnected that are essential for cell nutrition, proliferation migration for tissue vascularization for new tissue formation [237]. As the chitosan, percentage increases the thickness of the pore wall increases and porosity decreases. Porosity is important physical design parameter of scaffold. High porosity of scaffold is necessary as it enable the effective release of biofactors, antibiotics, cell attachment and cell migration; however the mechanical strength is often compromised in highly porous structure therefore a balance between the porosity and mechanical strength need to achieve [238]. Prepared scaffold shows highly porous structure having the porosity of scaffold above 90 % for the entire set of fabricated scaffolds. Porosity of scaffolds is SC-1, SC-2 and SC-3 is 93 ± 0.25 %, 91 ± 0.56 % and 87 ± 0.25 % respectively.

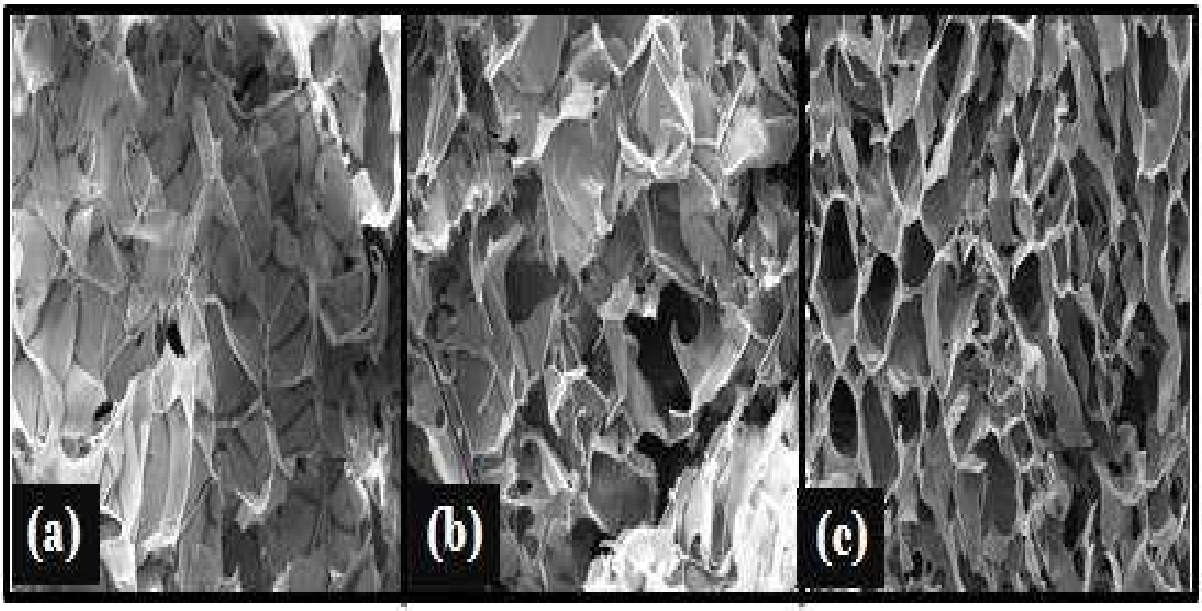

Figure 4.1.1. SEM images of developed scaffold SC-1 (a), SC-2 (b), and SC-3 (c) respectively showing morphology and distribution of pores.

4.1.1.2. Swelling

Percentages of swelling of COL/CHI/CPX based scaffolds were shown in (figure.4.1.2). Scaffold should maintain its structural integrity under wet condition in order to support cell growth, migration and proliferation. Since collagen and chitosan both are highly hydrophilic polymer and thereby scaffolds were cross-linked to improvise the structural integrity and stability of the scaffold [239]. Furthermore, cross-linking results in reduction of free hydrophilic group and allows scaffold to maintain its three dimensional structure. It has been observed that cross-linked matrix of chitosan based scaffold shows controlled swelling in the range of 40 – 300 % as compared of previously reported chitosan based matrix with higher degree of swelling behavior in the range of 500 % [138, 240-241]. Thus, the developed

scaffold shows controlled swelling behavior and might be suitable for the skin tissue regeneration.

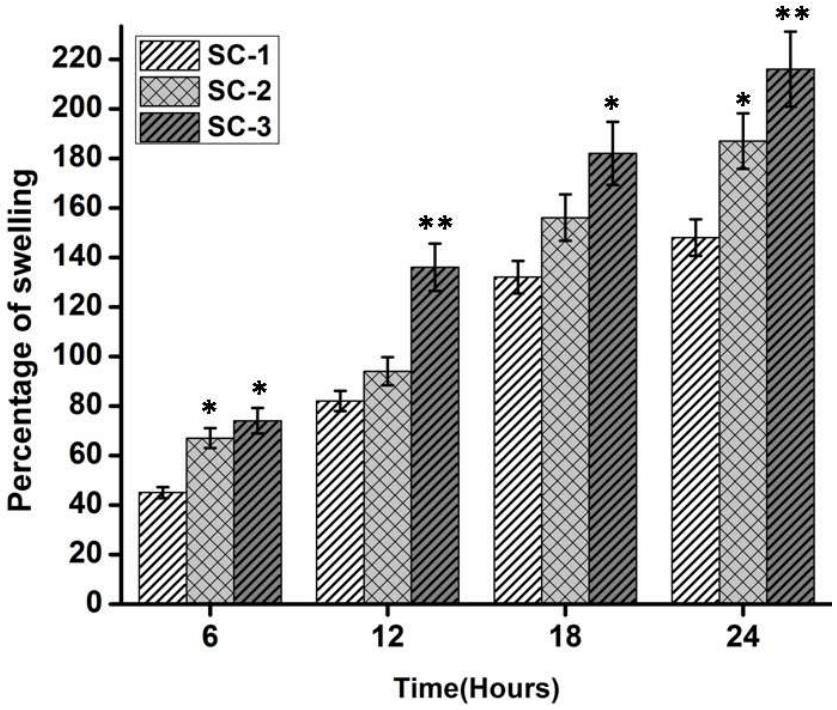

Figure 4.1.2. - Depicting the percentage of swelling of developed scaffolds at different time intervals. Data are expressed in terms of mean ± SEM (n=3), ($^{*}p<0.01$), (**$p<0.001$).

4.1.1.3. Contact angle

The measured contact angle of the composites was shown in figure 4.1.3. The hydrophilic or hydrophobic property of scaffold surface is an important factor that affects the cell affinity towards the scaffold. In most cases, the capacity for cell attachment and proliferation on a hydrophilic surface is better than on a hydrophobic surface. The contact angles for SC-1, SC-2, and SC-3 was found to be 74 ± 0.42^0, 63 ± 0.54^0, and 59 ± 0.24^0 respectively. It was found that the contact angle of scaffold decreased with increased chitosan amount which might be due to higher chitosan in scaffold. It was observed that the contact angle of scaffold

decreases with increasing chitosan content and thus scaffold with higher content of chitosan shows lower contact angle value indicating its higher hydrophilicity.

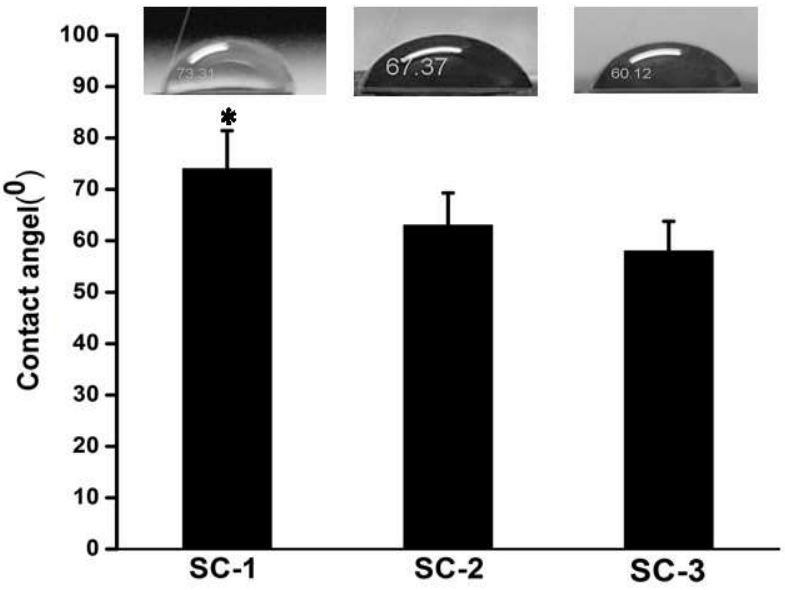

Figure 4.1.3. Measurement of contact angle of developed scaffolds. Data are expressed in terms of mean ± SEM (n=3), ($^{*}p<0.01$), (**$p<0.001$).

4.1.1.4. Biodegradation

Biodegradation is an important parameter taken into consideration while designing of scaffold for tissue regeneration. Degradation of scaffold should match the regeneration of tissue at the implant site. An optimum degradation rate of scaffold was evaluated using lysozyme. Lysozyme is exclusively present in human blood serum at high concentration at wound site produced by human immune system as direct reflection of host immune response [141, 242-243]. Therefore, lysozyme was used for biodegradation study. Lysozyme hydrolyzes the β(1-4) glycosidic bond between *N*-acetylglucosamine and glucosamine in chitosan [244]. Biodegradation pattern of developed scaffolds were depicted in figure 4.1.4.

From the graph it can be observed that SC-1 has least rate of degradation in comparison to SC-2 and SC-3. This is due to low percentage of chitosan compare to the collagen. SC-1 also loses its structure integrity and form gel like structure due to which degradation process goes slow in SC-1 c compare to SC-2. SC-3 shows very high rate of degradation in comparison with SC-2 and SC-1 which might be due the presence of the chitosan in higher amount and presence of beta-glycosidic bond in the complex scaffold. These higher concentrations of chitosan leads to high rate of degradation in SC-2 and SC-3 compare to SC-1. Also, pH of solution during the degradation remains in the range of 6.8 - 7.4. This range is very near to the physiological range of p^H and does not interfere with growth, proliferation and migration of cells.

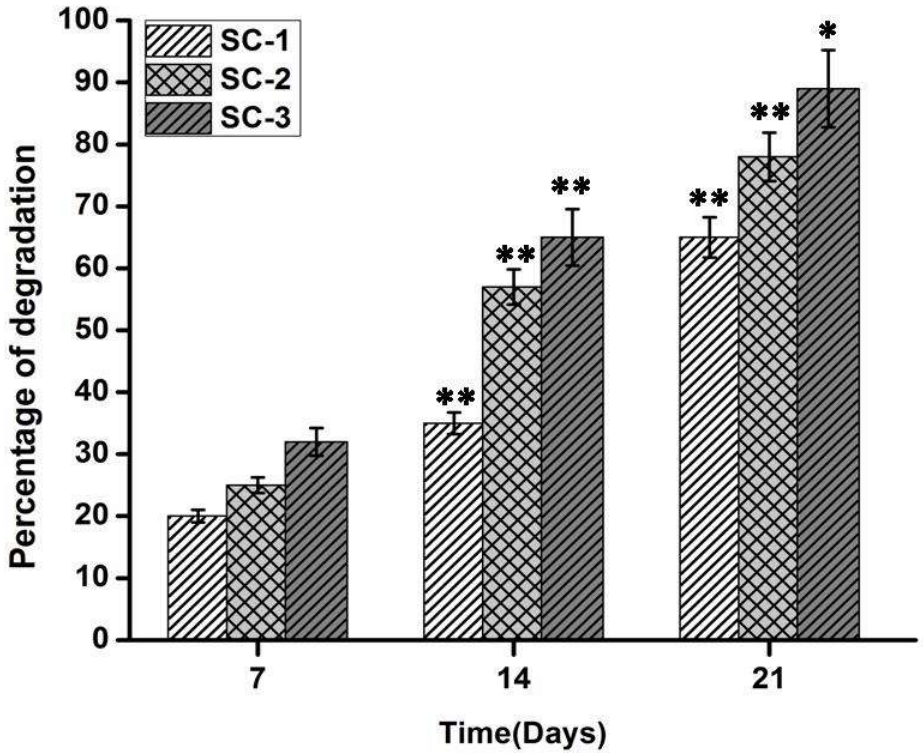

Figure 4.1.4. Representing the percentage of degradation of developed scaffolds at different time intervals. Data are expressed in terms of mean ± SEM (n=3), ($^*p<0.01$), (**$p<0.001$).

4.1.1.5. Water Vapor Transmission Rate (WVTR)

The ability of skin scaffold to reduce the loss of water vapor or moisture from the body extrude is an essential feature of scaffold. Scaffold must provide efficient transfer of gases between the wound and environment and it should absorb exudes of wound. Therefore, WVTR is measured in the fabricated scaffolds in the present study. WVTR of scaffolds was found to be 152 ± 8.5, 184 ± 10.4, 274 ± 14.6 g/m^2 /h. Gradual increase in the WVTR is due to the gradual increase in the pore size. WVTR of commercialized wound dressings vary from 33 (Op site) to 208 (Omiderm) g/m^2 h[245]. This indicates that all the developed scaffolds are in the suitable range for wound healing. Very high value of WVTR result in the drying of wound that lead to the lower moisture in healing wound. Lower values of WVTR lead to wound exudates accumulation. High accumulation of exudates leads to higher risk of bacterial growth that may lead to the sepsis of wound. Water vapor transmission rate of developed scaffold were expressed in figure 4.1.5.

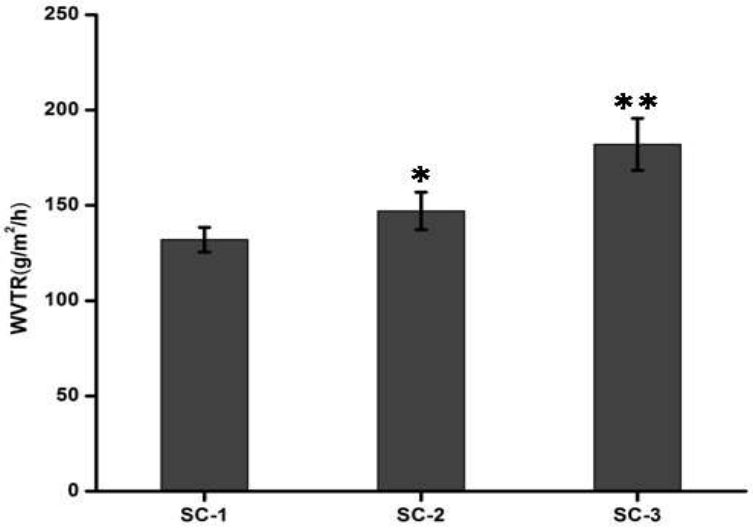

Figure 4.1.5. Depicting the Water Vapor Transmission Rate of different scaffolds at 24 hour. Data are expressed in terms of mean ± SEM (n=3), (*p<0.01), (**p<0.001).

4.1.1.6.FTIR analysis

Fourier transform infrared spectroscopy (FTIR) is used to illustrate the functional chemical group characteristics. FTIR spectrum shows the mix characteristics of collagen and chitosan as reported earlier by various investigators (figure.4.1.6.). In cross linking process various chemical groups react together to form a stable cross-linked structure. Glutaraldehyde reacts with the amine or hydroxyl functional group of proteins and polymers, respectively through a Schiff-base reaction and connects the biopolymeric chains *via* intra or intermolecular interactions [246]. EDC/NHC activates carboxyl groups and forms an amine reactive O-acylisourea intermediate that spontaneously reacts with primary amines to form an amide bond and isourea as a by-product. From the FTIR spectrum it can be inferred that characteristic peak of collagen and chitosan are present in the scaffolds. Characteristic peak of collagen can be seen in 1650 cm^{-1} which represents the amide I bands (1659 cm^{-1}) originated from C=O stretching vibrations coupled to N–H bending vibrations, 1560 cm^{-1} that attributed to the amide II band arise from the N–H bending vibrations coupled to C–N stretching vibrations and 1260 cm^{-1} which correspond to represented the combination peaks between N–H deformation and C–N stretching vibrations [247]. 1080 cm^{-1} and 3450 cm^{-1} are characteristic peaks of chitosan represent glycosidic bond and –OH group respectively [247-249]. Characteristic intense peak (1080 cm^{-1}) of chitosan, such as glycosidic linkages, appeared more clearly when the composition of chitosan was increased. For example, the intensity of amide I peak at 1650 cm-1 decreases gradually with increase in the proportion of chitosan [250]. Furthermore, the wave number difference between amide I and amide II bands was lower than 100^{-1} whichindicated that the triple helix structure of collagen was maintained upon mixing of collagen with chitosan [251].

The difference in the relative intensity between FTIR bands suggested interactions between collagen and chitosan, probably by hydrogen bonding among carboxyl, amino, and hydroxyl groups present in the components of the scaffold forming formulation [249].

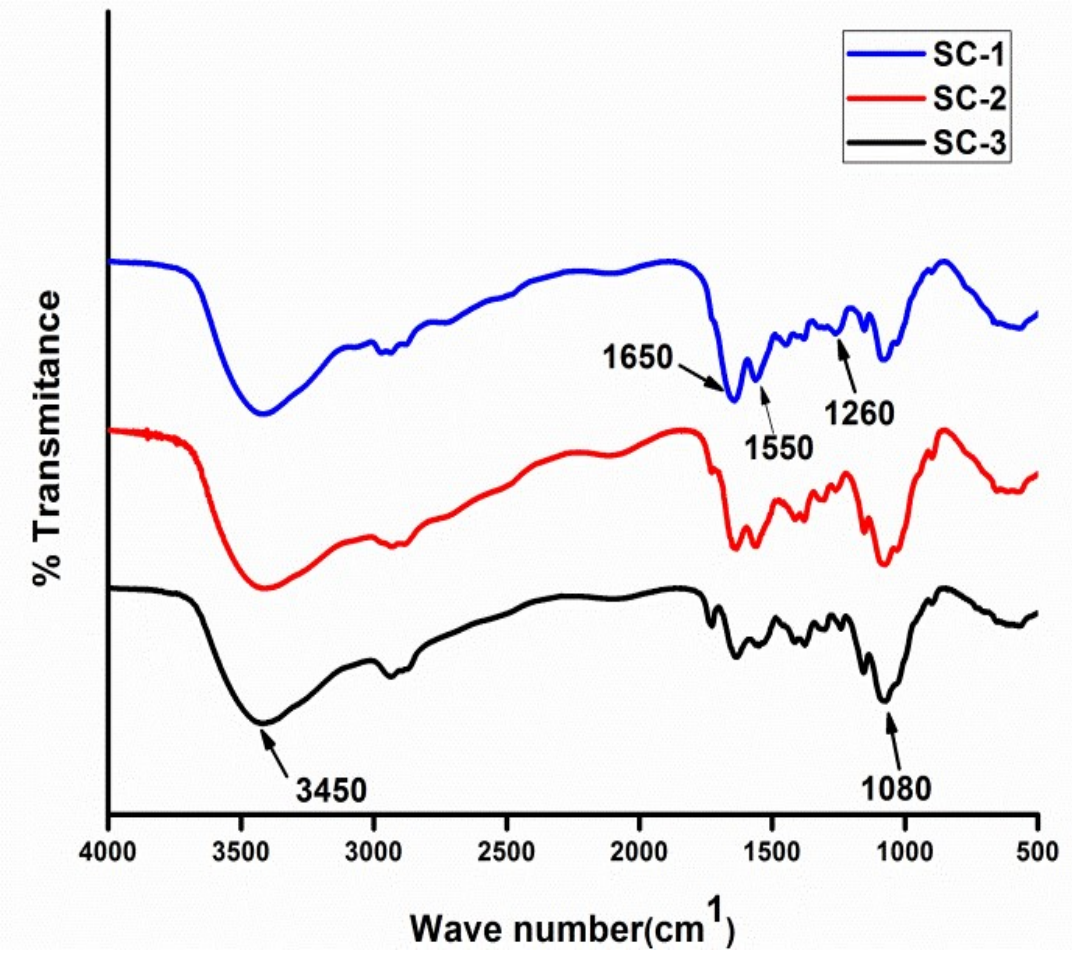

Figure 4.1.6. FTIR Spectrum of Collagen-Chitosan scaffoldsdepicting the characteristic peaks of scaffolds.

4.1.1.7.Mechanical strenght study

The mechanical strength of scaffold is very important design parameter.The tensile strength of scaffolds was measured in dry and wet condition (figure 4.1.7). Tensile strength of SC-2 was found to be 7.12MPa in comparison to SC-1and SC-3 which having tensile strength of 6.70MPa and 2.8MPa respectively at dry condition (figure.4.1.7.A). The tensile strength pattern was found similar to dry condition with visible reduction in the tensile strength of the scaffold in wet condition (figure. 4.1.7.B). In wet condition the tensile strength of scaffolds was found to be 1.58 MPa, 2.4 MPa and 2.62 MPa respectively. Moreover, collagen has poor mechanical strength therefore it was mixed with chitosan in order to improvise the mechanical strength behavior in wet condition. Chitosan has inherent property of enhancing the mechanical strength with increasing concentration however it makes the scaffold brittle in nature. Chitosan helps to stabilize the open triple helix structure through hydrogen bonding. In (figure.4.1.7A) SC-3 has depicted high tensile strength but low elasticity whereas SC-1 shows low mechanical strength due to the less amount of the chitosan. SC-2 shows optimum tensile and strain bearing capacity compared to SC-1 and SC-3. In wet condition scaffold absorbs water and get swelled due to which the tensile strength of scaffold get reduced and brittleness of SC-3 also gets compromised. Thus, appropriate blending of chitosan and collagen might be suitable to fabricate natural biopolymer based scaffold with improvised mechanical properties for skin tissue engineering applications.

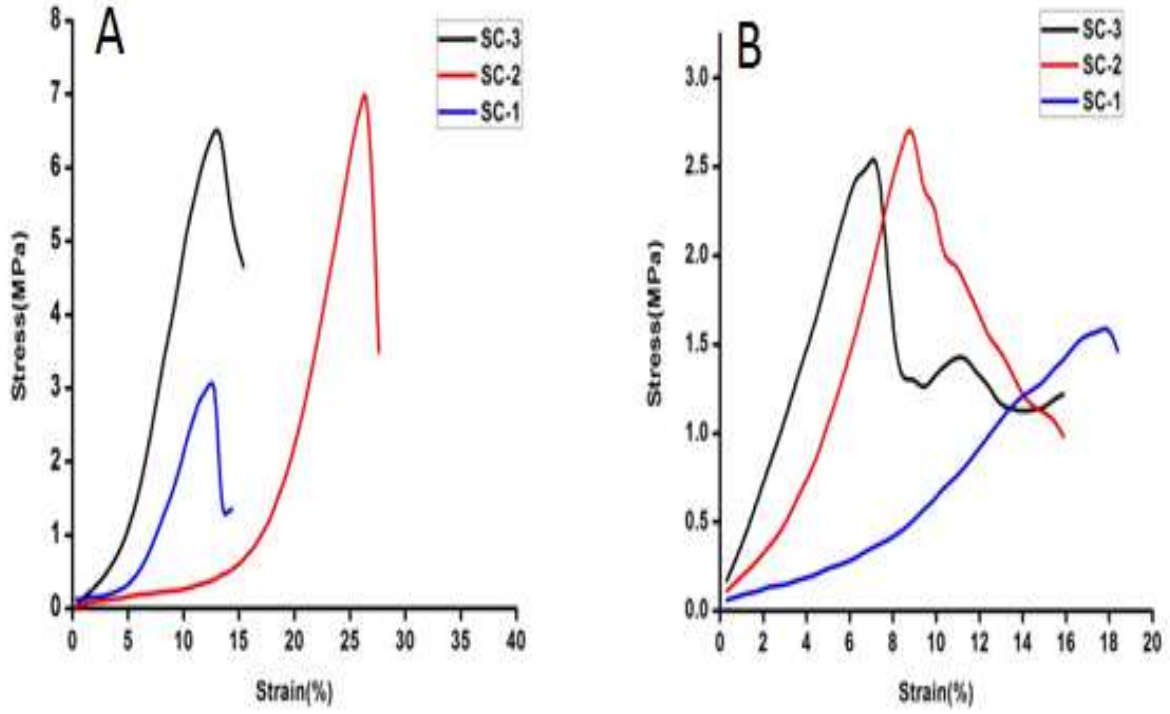

Figure 4.1.7. (A) and (B) representing the mechanical strength of developed scaffolds under dry and wet condition respectively.

4.1.1.8.Thermal property study

The weight loss in the scaffold samples occurred at various stages, the first one refers to the loss of water molecule associated with the scaffold at (25−200°C) followed by thermal degradation of the polymers at (200−400°C) and carbonization of material (400−700°C). [252]. Moreover, the obtained thermal graph (figure.4.1.8.) showed the direct relationship between chitosan concentration and water loss. As the percentage of chitosan increase the percentage loss of water was also increased. Thus, it can be concluded that at the initial heating phase (25-200°C) SC-2 and SC-3 showed high loss of structural water molecules as compare to SC-1. Hence, thermal degradation was observed less in SC-2 and SC-3 compared

to SC-1. This might be due to interaction of collagen and chitosan that resulted into stabilization of collagen triple helical peptide structure and higher chitosan proportion in the blend might added the thermal stability of SC-2 and SC-3 [248]. Thereby, scaffolds were stable up to 200 °C without any kind of alteration or change in its chemical and physical properties hence it can be efficiently used for the tissue engineering.

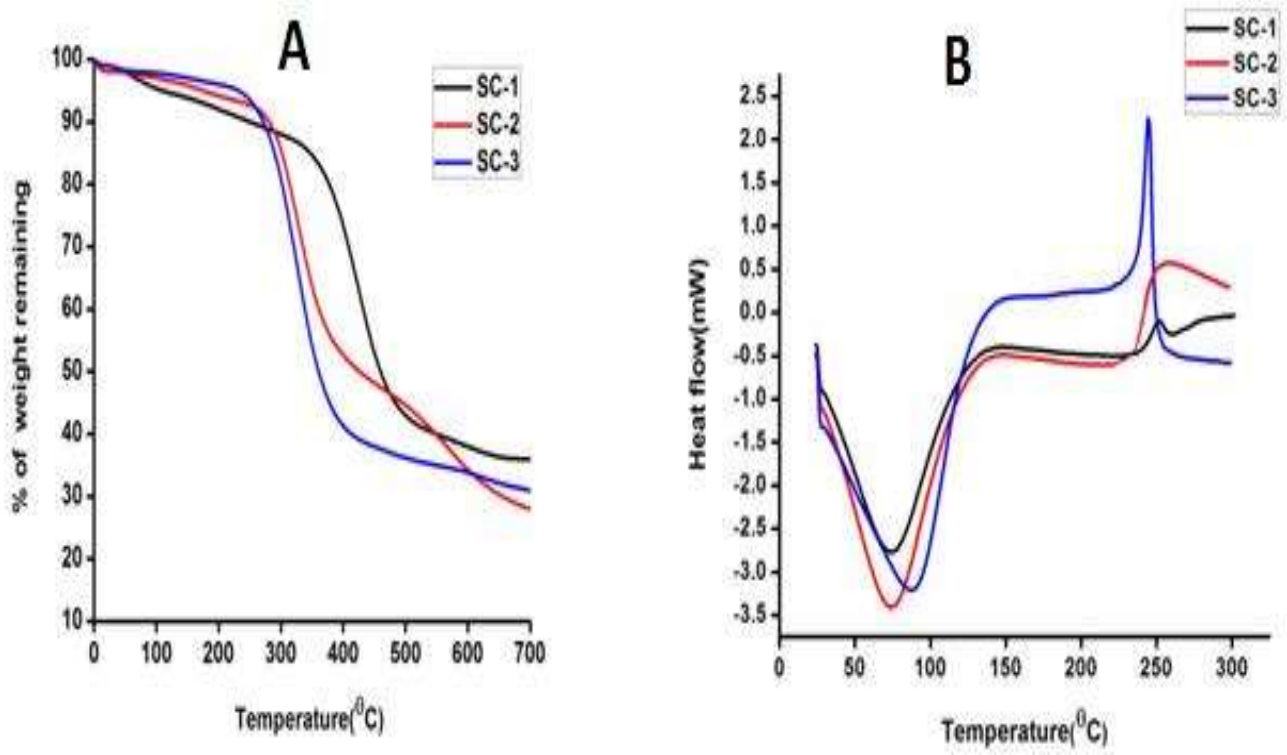

Figure 4.1.8. Thermal behavior of developed scaffolds. (A) Representing TGA curve showing the percentage of weight loss with temperature. (B) Showing DSC pattern of scaffolds representing the endothermic peaks.

	Peak maximum temperature(^{0}C)	Percentage of weight loss		
		35-200^{0}C	**200-400^{0}C**	**500-700^{0}C**
Scaffold-1	373.6	8.9	35.56	8.65
Scaffold-2	303.7	5.35	40.26	16.20
Scaffold-3	286.9	3.62	47.45	6.65

Table 4.1. Representing the thermal degradation of scaffolds with temperature.

DSC pattern of scaffold was depicted in (figure.4.1.8.B). The characteristic endothermic peaks represent the temperature of dehydration (T_D) of individual scaffold in the presence of nitrogen gas. The T_D values along with the enthalpy of dehydration (ΔH_D) are listed in Table 4.2.

Scaffold	**SC-1**	**SC-2**	**SC-3**
T_D(^{0}C)	74	76	96
ΔH_D(J/g)	272	332	352

Table 4.2. Depicting the thermal properties of scaffolds (T_D-Temperature of dehydration, ΔH_D-Enthalpy of dehydration)

These DSC results clearly indicates T_D value of scaffold which increases with increasing chitosan concentration and this might be due to the stabilization of the collagen super coiled structure when comes in contact with chitosan. This was perfectly coordinated with the increase in the ΔH_D of the scaffold. Moreover, increases in the ΔH_D shows consistency with the greater stabilization of the intermolecular interaction between the collagen and chitosan molecule.

4.1.1.9.*In vitro* drug release

One of the objectives of our study was to fabricate ciprofloxacin loaded collagen-chitosan scaffold that can release antibiotic to provide the necessary protection against any kind of bacterial infections. There are many factors that affect the drug release including water solubility, permeability, and degree of crystallinity of antibiotic. In addition, nature of polymer (hydrophilicity and hydrophobicity) used for the fabrication of scaffold, method of scaffold preparation and cross-linking, nature of cross linker are other parameters that affect the drug release.

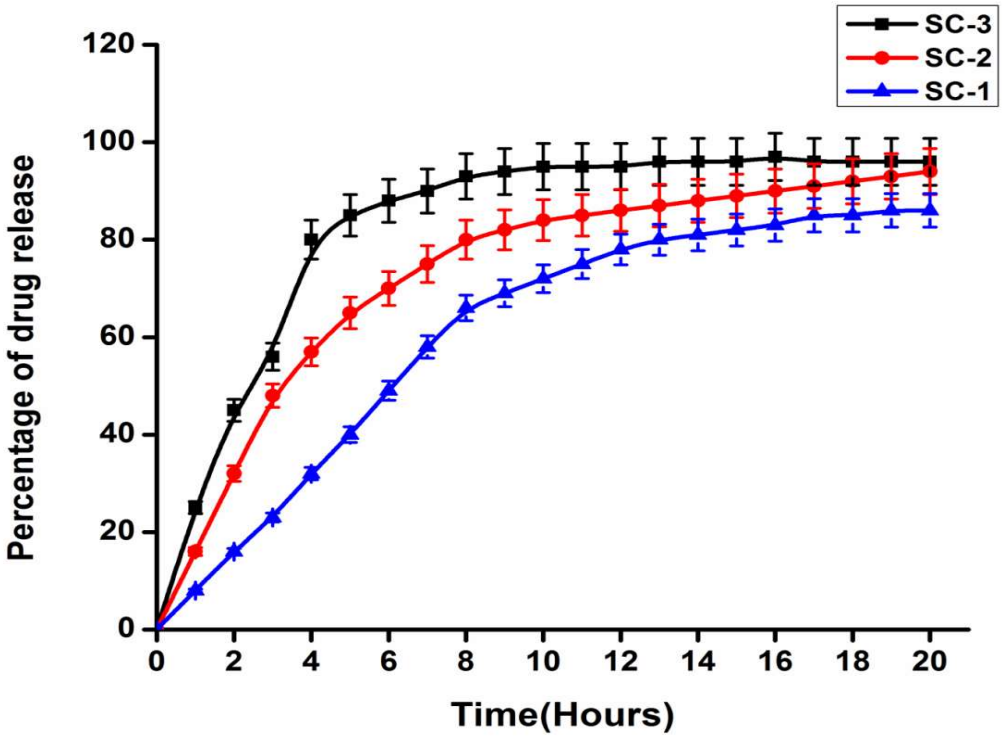

Figure 4.1.9. Timedependent release pattern of ciprofloxacin from the developed scaffolds.

For active wound healing management, suitable and appropriate release properties of the antibiotics must be taken in account, which depend heavily on type of wound and the antibiotics. Based on type of wound such as fresh wound which need burst or quick release of antibiotic whereas epithelialized wound need stable release of antibiotics [253].

Such rapid antibiotic release in initial 4 -6 hr followed by stable antibiotic release is desirable especially in early stage of wound healing (inflammation stage). it has been reported that collagen and chitosan based hydrogels shows rapid release of antibiotics while employed for wound healing.[138, 254-257] Thus, the developed matrix designed specifically for active wound need to prevent infection thereby rapid drug releasing scaffold was designed.

In present study, freeze drying method was used for the scaffold preparation which provides highly porous structure. This high porosity provides high surface to volume ratio that leads to the rapid and high release of drug ciprofloxacin. The high drug release efficiency varied from 78% to 94% as depicted in (figure.4.1.9.). Such rapid release of antibiotic is desirable as it provides protection against microbes that may infect the wound form the surrounding.

SC-1 initially showed slow and sustained release, while SC-2 and SC-3 showed the rapid release of antibiotic. This difference is due to the change in the architecture of scaffold after coming in contact with PBS. SC-1 has high collagen content that makes it mechanically unstable and it also forms gel like structure that reduces its surface to volume ratio therefore, reduces its drug release efficiency. SC-3 is mechanically stable and has high swelling percentage that has lead to the enhancement in surface to volume ratio which leads to the rapid release. SC-2 has maintained the structural integrity and has optimum and intermediate swelling percentage therefore, shows rapid release in initial phase and sustained and continuous release in later phase.

4.1.1.10. Haemocompatibility study

Haemocompatibility test was performed to evaluate the percentage of erythrocytes broken or ruptured when scafold comes in contact with blood. Evaluation of haemocompatibility is necessary before proceeding for any *in vivo* study. Our result showed the percent haemolysis (%) of scaffold was found to be 1.25, 1.32 and 1.42 for SC-1, SC-2 and SC-3 respectively. Haemolysis percentage was found to be less than 5% which clearly indicates the higher degree of haemocompatibility that make it suitable for further for *in vivo* study.

4.1.2. Cell culture studies and in vivo studies of ciprofloxacin loaded collagen chitosan scaffold

4.1.2.1 Cell isolation and cell attachment study

Cellular biocompatibility of developed COL/CHI/CPX scaffolds was investigated *in vitro* by evaluating the attachment, spreading, proliferation, and migration of fibroblast over the scaffolds. Cell attachment, proliferation and migration over the scaffold are the essential features of scaffold in order to support, promote and facilitate the function of wound healing. SEM images of cell seeded scaffold showed in (figure.4.1.10.) have clear indication that fibroblast cells were attached over the surface of all the scaffolds and started to proliferate. It has been also shown that scaffold after 10 and 15 days form monolayer like structure where cells were interconnected and attached over the scaffolds. Cultured scaffolds (SC-1, SC-2 and SC-3) stained with DAPI were shown in figure. 4.1.11. for the duration of 5, 10 and 15 days respectively. Fluorescent image revealed the successive increase in the cell number over the scaffolds with time. It was observed that SC-2 showed better cell growth as compare to rest that also been further supported by the SEM image of the mentioned scaffold. This might be due to suitable scaffold architecture, mechanical properties and swelling behavior of SC-2 as compared to SC-3 which has very high swelling percentage that leads to destabilization of scaffold architecture. All these factors make SC-2 more suitable than rest for cell attachment, growth, and proliferation.

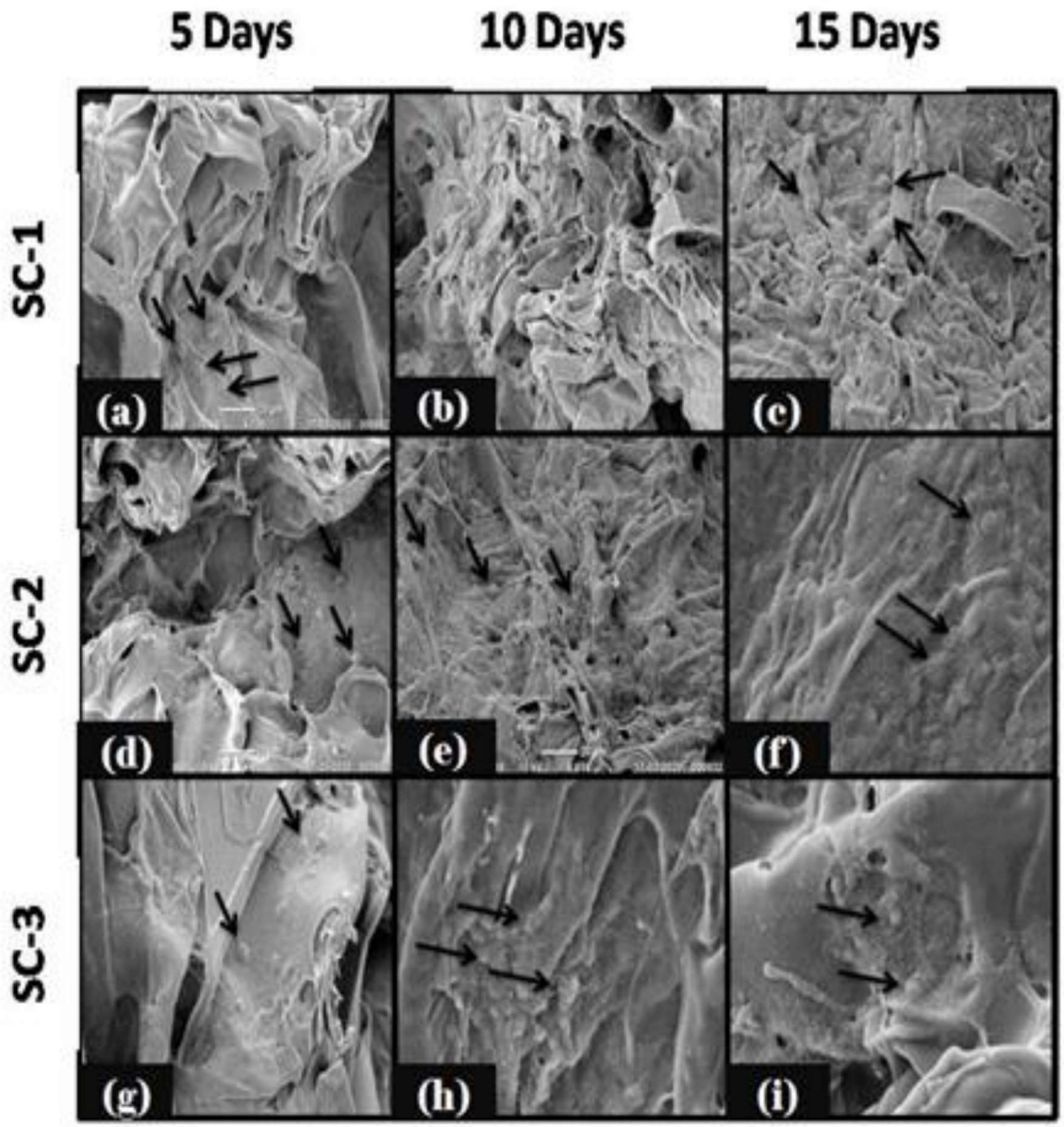

Figure 4.1.10. SEM images of cell seeded scaffolds depicting the attachment of fibroblast after 5th, 10th, and 15th day. SC-2 showed more compatibility than rest for the attachment, growth, and proliferation of fibroblast.

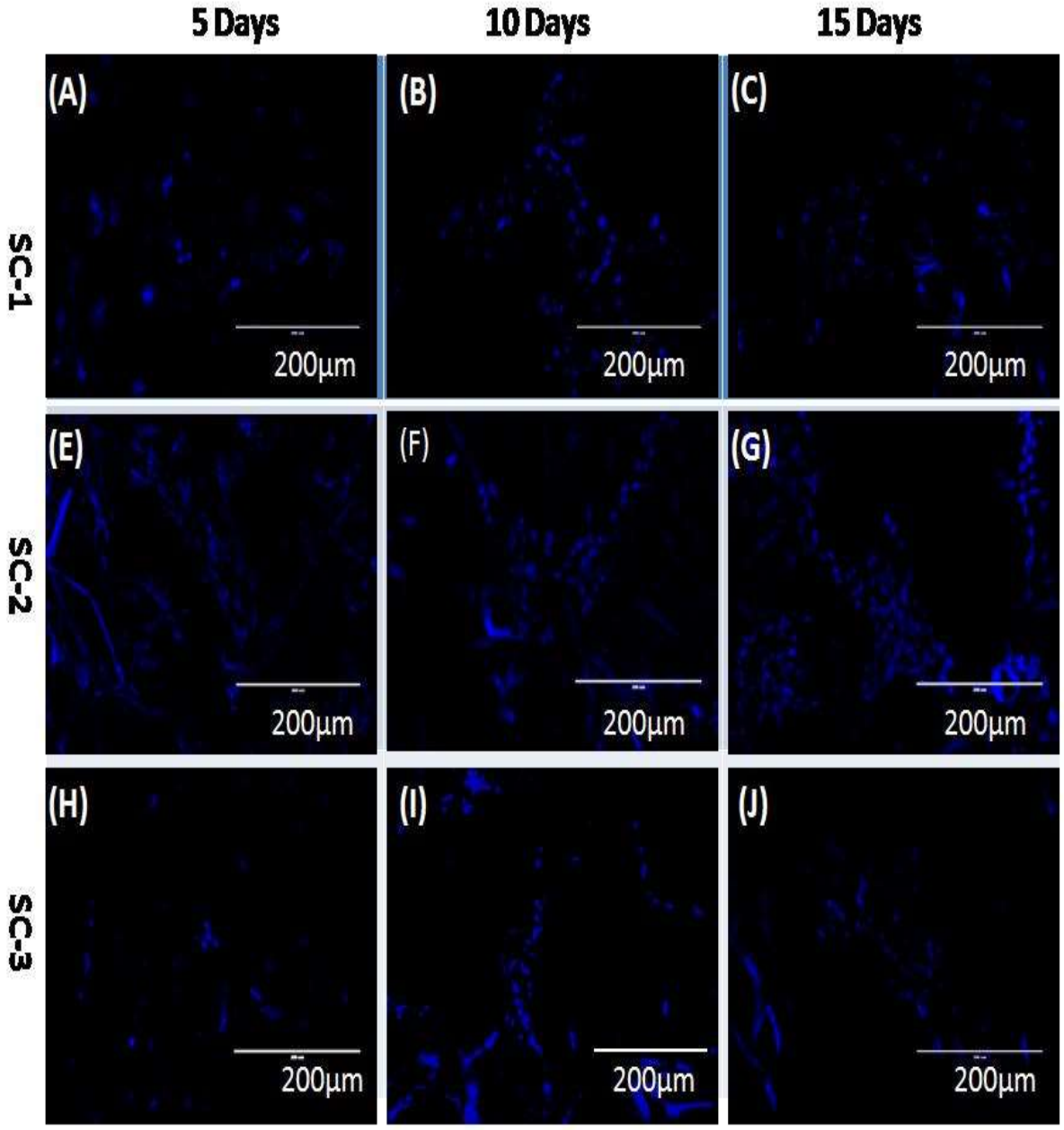

Figure 4.1.11. Fluorescence microscopy images of cell seeded scaffolds stained with DAPI showing the growth and proliferation of fibroblast after 5th, 10th, and 15th day.

4.1.2.2. DNA estimation

Cell proliferation on the scaffolds was quantified through estimation of total DNA content. The amount of DNA was quantified after 5 days and was found to be 1805.24 ± 0.54 ng/scaffold, 2089.21 ± 0.47 ng/scaffold and 2214.52 ± 0.84 ng/scaffold for SC-1, SC-2, and SC-3 respectively. After day 5, the cell number increases and the DNA content double (figure.4.1.12). After 10 days the cells proliferate and DNA content was found to be 3589.25 ±.52 ng/scaffold, 4710.78 ± 0.85 ng/scaffold and 4187.87 ± 0.24 ng/scaffold for SC-1, SC-2, and SC-3 respectively. After 10 days the cell has migrated throughout scaffold and the process of cell division tends to slowdown. Thereby, observed DNA content on day 15 was 5545.52 ± 28, 7432.54 ± 57 and 6546.87 ± 0.54 ng/scaffold for SC-1, SC-2 and SC-3 respectively. Moreover, chitosan in high concentration swells more than in low concentration and ultimately leads to higher porosity and larger pore size due to which cellular growth was higher in scaffold 2 and 3 in comparison to the scaffold 1. While SC-2 showed higher growth in comparison to the SC-3 because it swells and degrades faster in comparison to the SC-2.

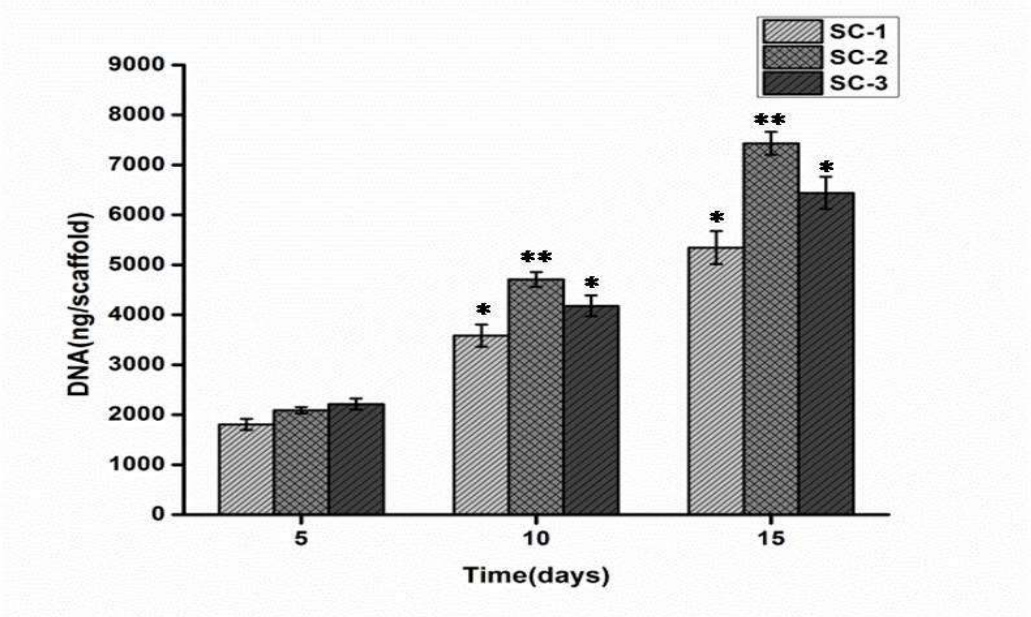

Figure 4.1.12. Total DNA content of fibroblast present inside the scaffold was quantified at different time intervals.Data are expressed in terms of mean ± SEM , (n=3), ($^{*}p<0.01$), (**p<0.001).

4.1.2.3. MTT assay

The result of MTT assay showed (figure.4.13.) that the metabolic activity of SC-2 was higher comparison to other scaffolds, which might be due to suitable pore size, mechanical integrity and optimum swelling of SC-2. Previous studies have reported that the structural integrity of the scaffold is necessary for the cellular growth and migration. Also, pore size of scaffold in between 80 and 150 μm was reported to be suitable for fibroblast growth over the porous scaffold.

SC-1 has pore size of ~71 μm which was in lower range that makes it less suitable for the growth of fibroblast compare to the other set of scaffolds. As previously discussed

mechanical integrity of SC-1 get compromised in wet condition, which may lead to collapse of pore architecture during cell culture that may have resulted in low cell proliferation and low cell migration.

Besides SC-2, SC-3 also showed better growth of fibroblast over the scaffolds, which might be due to suitable mechanical strength and development of better pore architecture to support cell attachment and growth. Many studies have shown that scaffold with good mechanical strength and higher pore connectivity shows higher rate of cell growth, cell migration, and proliferation [258].

However SC-3 has greater degree of porosity compared to SC-2. Very high porosity is not suitable for cell growth because it may damage the internal architecture of scaffold that ultimately leads to the adverse affects in cell growth and migration.

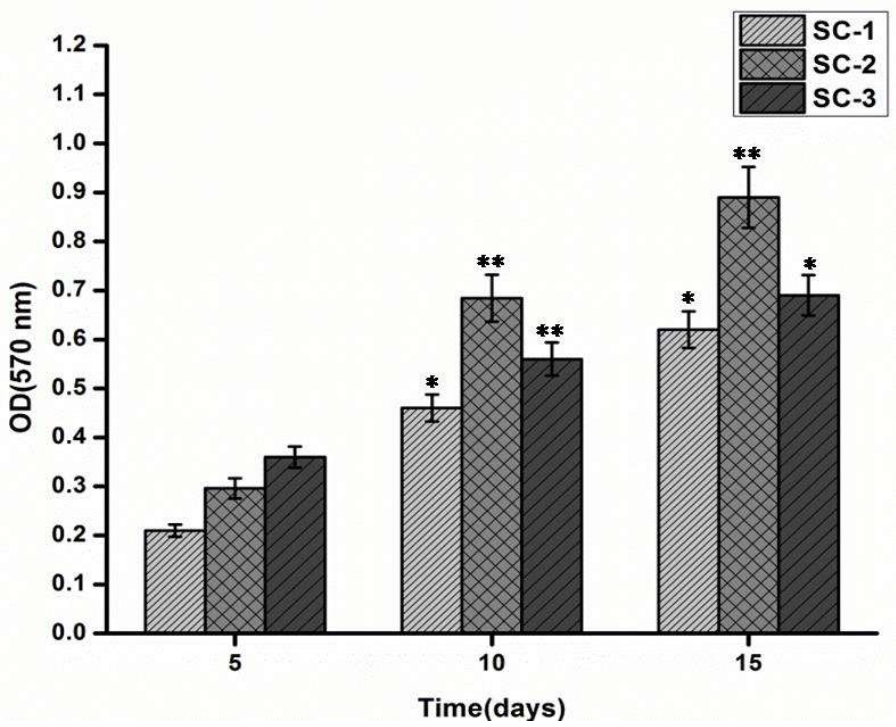

Figure 4.1.13. Time dependent effect of developed scaffolds on viability of fibroblast. Data are expressed in terms of mean ± SEM (n=3), ($^{*}p<0.01$), (**$p<0.001$).

4.1.3. Antimicrobial study

The antibacterial effect of selected scaffold (SC-2) was examined against the two types of bacteria that usually involved in wound infections, i.e., *E. coli* (gram negative) and *S. aureus* (gram positive). Control samples shows no zone of inhibition and thus shows poor antimicrobial activity against any of the two bacteria while antibiotics loaded scaffold has a clear zone of inhibition in agar plate against both the bacteria. Zone of inhibition produced in both agar plates was about 20 ± 1.8 mm and 18 ± 1.4 mm in diameter against *E. coli* and *S. aureus* respectively. Hence, scaffold has antibacterial properties which can be used for wound healing applications to prevent bacterial infection and promote tissue regeneration.

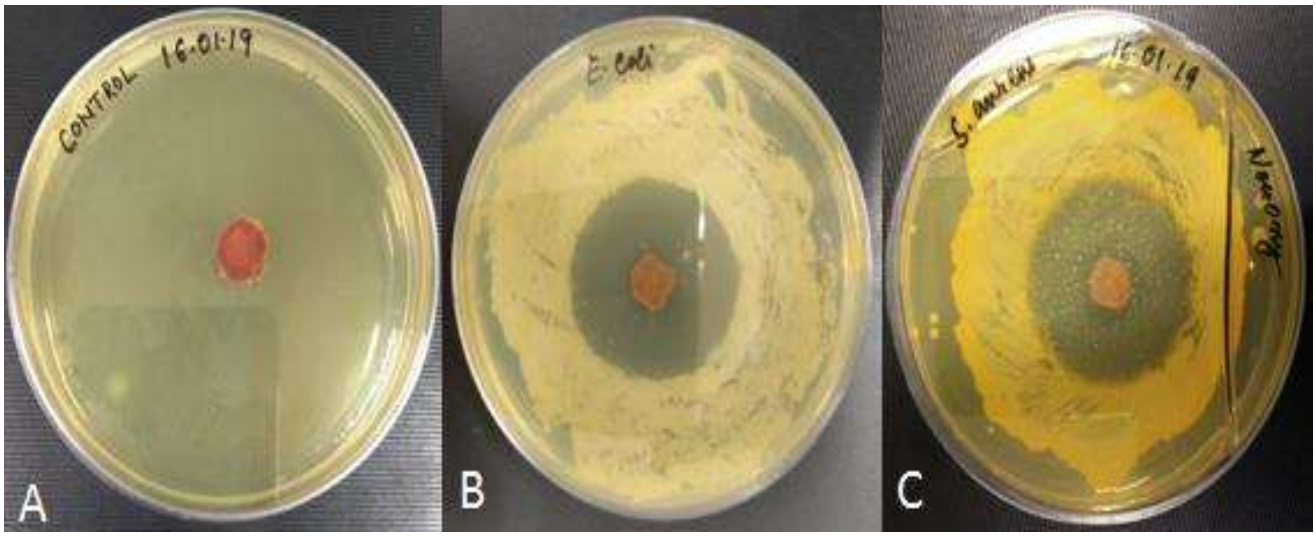

Figure 4.1.14. Antibacterial effect of scaffold (A) Control (B) Against the growth of gram negative bacteria *E. coli*. (C) Against the growth of gram positive bacteria *S. aureus.*

4.1.4. *In vivo* and histological study

4.1.4.1. *In vivo* studies

During the *in vivo* study, wound healing rate in control and test group was compared at different time intervals. Images of wound were captured at 5^{th}, 10^{th}, and 15^{th} day and the surface area was calculated to evaluate the rate of wound healing and closure. (figure.4.15.A) image representing the wound healing with time, (figure.4.15.B) and (figure.4.15.C) showing rate of wound closure and wound healing respectively. On day 0 the wound having area of 225 mm^2 was treated with SC-2 and control was devoid of scaffold. On day 5 day, healing was observed with reduction in wound area from 225 mm^2 to 164 ± 7.2 mm^2 and 130 ± 4 mm^2, which shows 27 ± 2.5% and 42 ± 2.7% of wound healing in control and scaffold treated groups respectively.Wound healing on both the case was significant as $p<0.05$.After day 5 the rate of wound healing accelerated and on 10^{th} day area of wound remained 103 ± 4.5 mm^2 and 63 ± 0.45 mm^2 that represents 54.15 % and 72.5% of wound healing in control and scaffold treated groups respectively. Statistical analysis indicates that wound healing in SC-2 was more significant than control as value of $p<0.005$ & $p<0.05$ respectively. On 15^{th} day, scaffold treated wound showed almost complete recovery while control group showedless wound healing. There are two main factors which escalated the rate of healing and recovery. One is the utilization of collagen and chitosan in the process of wound healing and second is the Ciprofloxacin, an antibiotic in the scaffold that protected the wound against the bacterial infection and inflammation thus helped to accelerate the process of healing

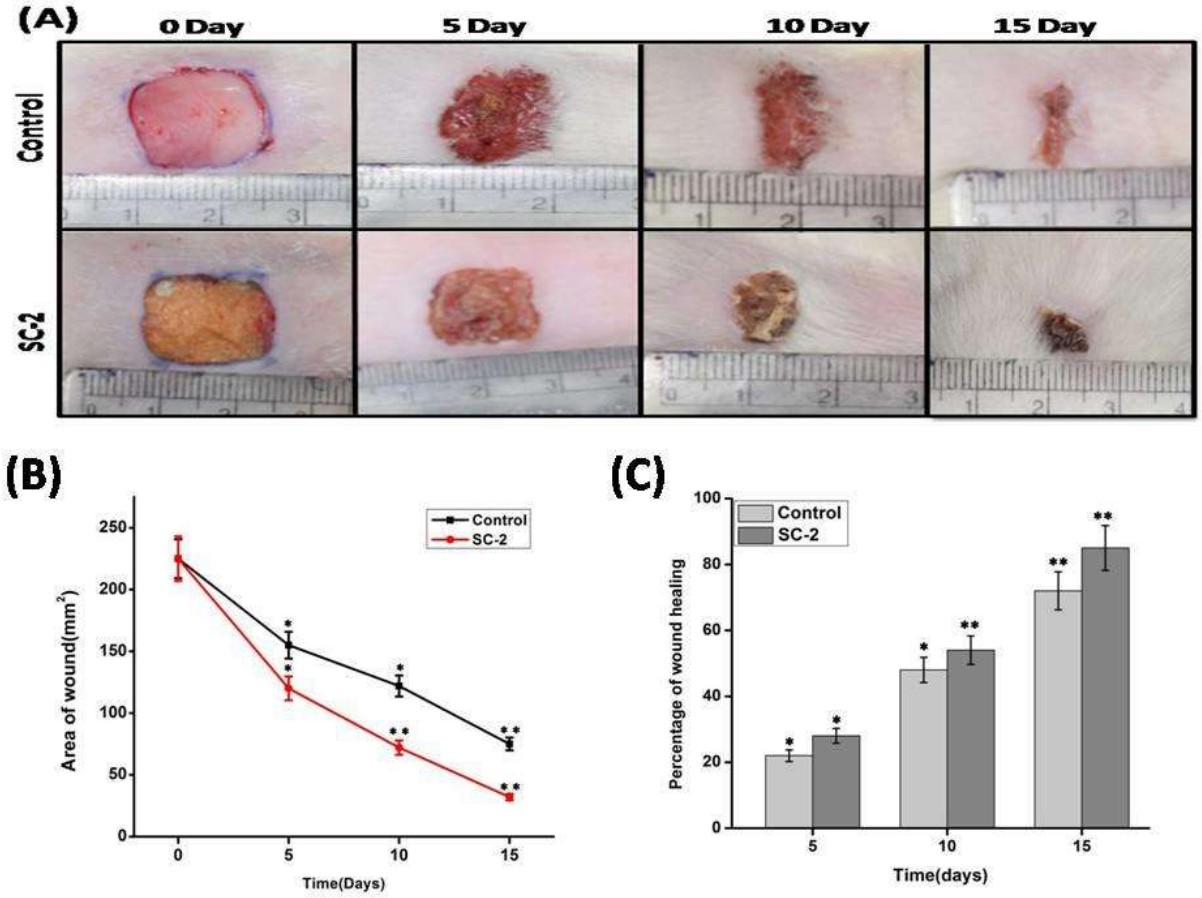

Figure 4.1.15. (A) Representing wound healing potency of control and SC-2 treated groups at different time intervals and images were captured at 0, 5th, 10th and 15th day. (B) Representing rate of wound closure. (C) Representing the rate of wound heling. . Data are expressed in terms of mean ± SEM (n=3), (*p<0.01), (**p<0.001).

4.1.4.2. Histological study

Histological stuies were performed using Hematoxylin and Eosin staining on 5th, 10th, and 15th day of wound healing in test and control groups. Microscopic studies revealed the macroscopic appearances along with some differences in the quality of wound healing. Process of epidermis and dermis formation was observed to be slowed in control as compared to scaffold treated wound which showed well developed and thick dermis compared to control (figure. 4.16.). Architecture of connective tissues below the dermis is irregular and haphazard in control. Scaffold treated wound show better differentiation into outer and inner zones, which is the result of better remodeling of extracellular matrix compare to control. Besides, all these differences well organized granulation was seen at the latter stage of healing in scaffold compare to control that lacks organized granulation.

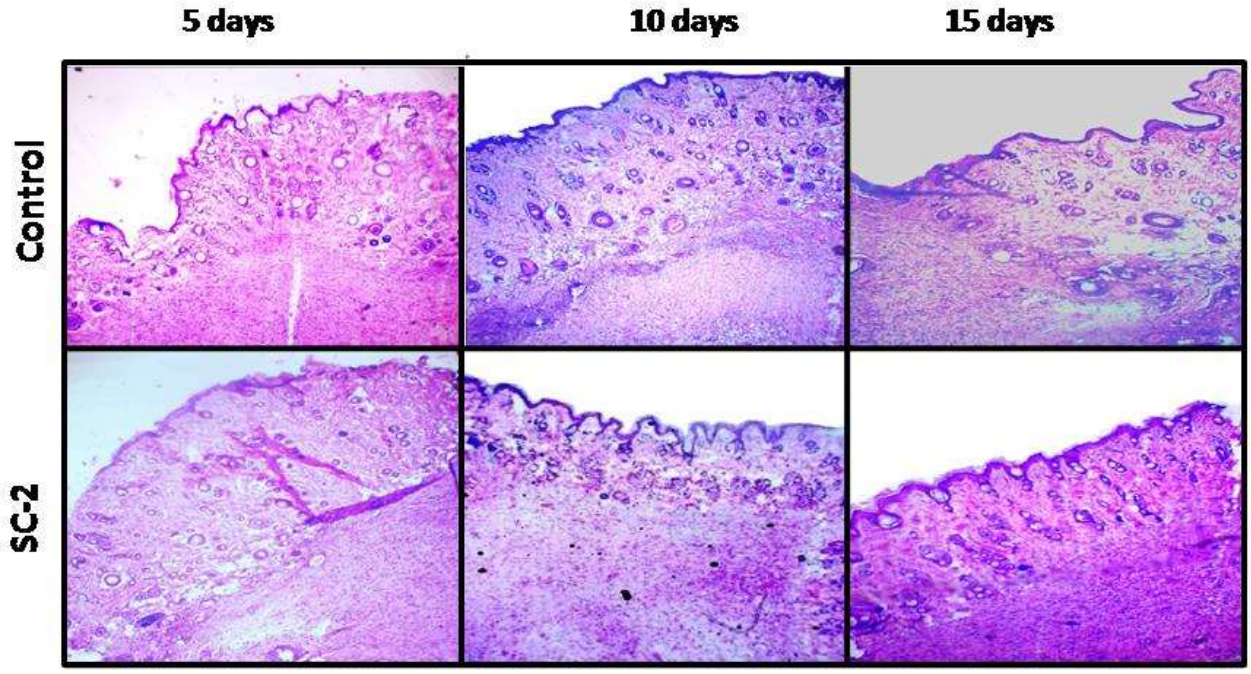

Figure 4.1.16. Histological examination of control and SC-2 treated groups at different time intervals and images were captured at 0, 5th, 10th and 15th day at 10X magnification.

4.2. Development of ciprofloxacin loaded oxygen releasing co llagen chitosan scaffold for skin tissue engineering

4.2.2. Physical and chemical study

4.2.1.1. Morphological study

The surface morphology of developed scaffolds was evaluated using a scanning electron microscope (SEM) (figure. 4.2.1.) and it was observed that the pores were uniformly distributed and well connected. Higher resolution showed the presence of the CPO over the surface of the scaffolds. SC-0 is devoid of CPO coating and density of the CPO on the surface is highest in the SC-4 and lowest in the SC-1 depending upon the concentration of CPO used during the coating of the scaffold. The EDX data (figure-4.2.2.) shows that concentration of calcium successively increased as the concentration of the CPO increased. It is evident from the figure-4.2.1 that the designed scaffolds have good porosity and interconnectivity. The porosity was found to be more than 85 % for all the scaffolds, and highest porosity was for SC-0 and least was for the SC-4. The SC-0, SC-1, SC-2, SC-3, and SC-4 have porosity 95.2±1.2, 93.3±0.4, 91±0.5, 89.6±0.7 and 87.42±0.4 respectively. The porosity decreases due to the high concentration of CPO and PCL on the surface of the scaffold, which was also evident from the SEM micrograph and elemental analysis through EDX. In general porosity higher than the 85 % is suitable for the tissue engineering applications as the higher porosity results into higher cell migration and proliferation with increased gaseous exchange, inflow and outflow of toxic products for the scaffold[237]. EDX profile of developed scaffold was performed to evaluate the elemental composition of scaffolds. EDX data showed an increase in percentage of calcium from 0, 3.63, 8.58, 13.79, and 17.51 for SC-0, SC-1, SC-2, SC-3 and SC-4 respectively that indicates the increase in

deposition of CPO on the surface of scaffolds. Increasing deposition of CPO is also visible at higher magnification of SEM in figure 4.2.1.

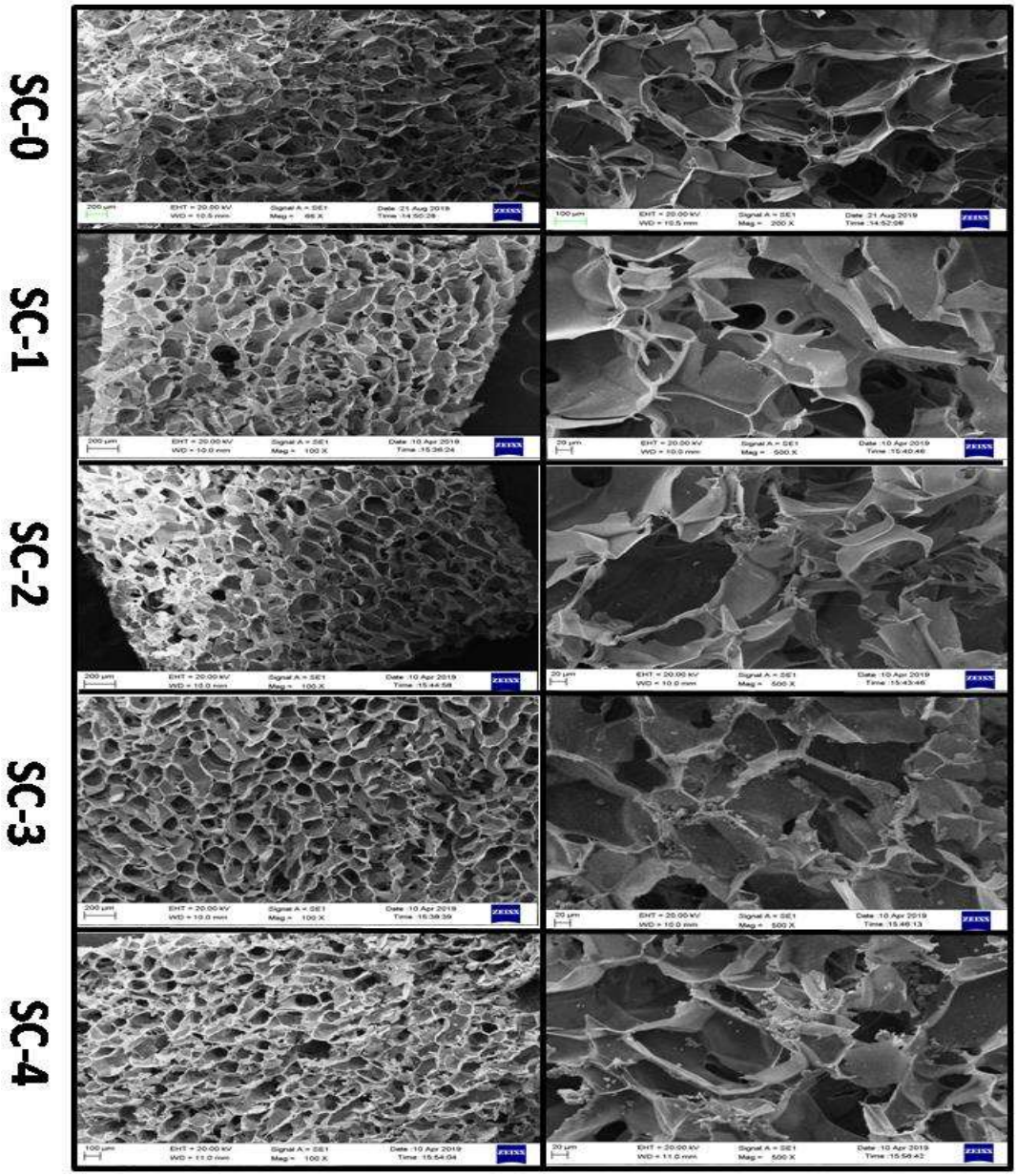

Figure 4.2.1. Scanning electron microscopic images of developed porous scaffolds.

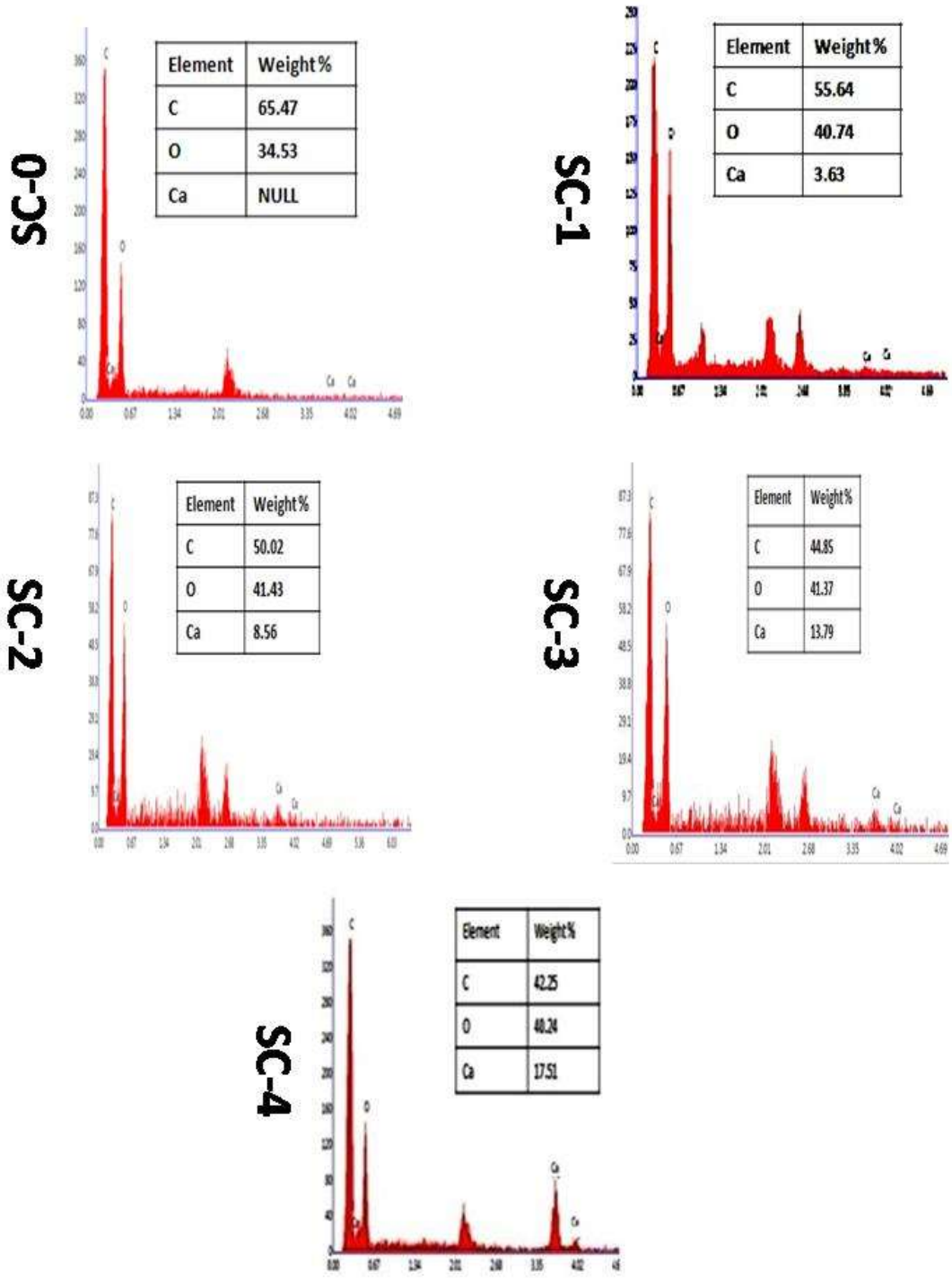

Figure 4.2.2. EDX of developed porous scaffolds.

4.2.1.2. Swelling

The ability to absorb water is an important property of scaffold used in skin tissue engineering. Both collagen and chitosan are highly hydrophilic hence, it is necessary that the developed scaffold should maintain its structural integrity after getting contact with water. Therefore, cross-linking of developed scaffold was performed to prevent any type of structural deformation and to enhance the structural integrity of the developed scaffold to prevent gel-type structure formation. It was found that cross-linked scaffolds have a higher degree of swelling than the uncross-linked scaffolds. It has also been observed that with a higher degree of cross-linking swelling ratio is reduce [136, 259]. In general, the swelling ratio is decreased as the degree of cross-linking is increased because of decrease in the hydrophilic group[260]. After cross-linking the scaffolds were coated with PCL solution. Being hydrophilic in nature, PCL coating further decreases the swelling ratio. Figure 4.2.3. shows the swelling behavior of developed scaffolds. It was observed that, SC-4 and SC-0 exhibited the lowest and highest swelling respectively. SC-0 is devoid of PCL and CPO therefore it does not have hydrophobic coat on the surface that will lead to higher swelling. Scaffolds form SC-1 to SC-4 has gradual increase in CPO and PCL that lead to gradual decrease in swelling rate.

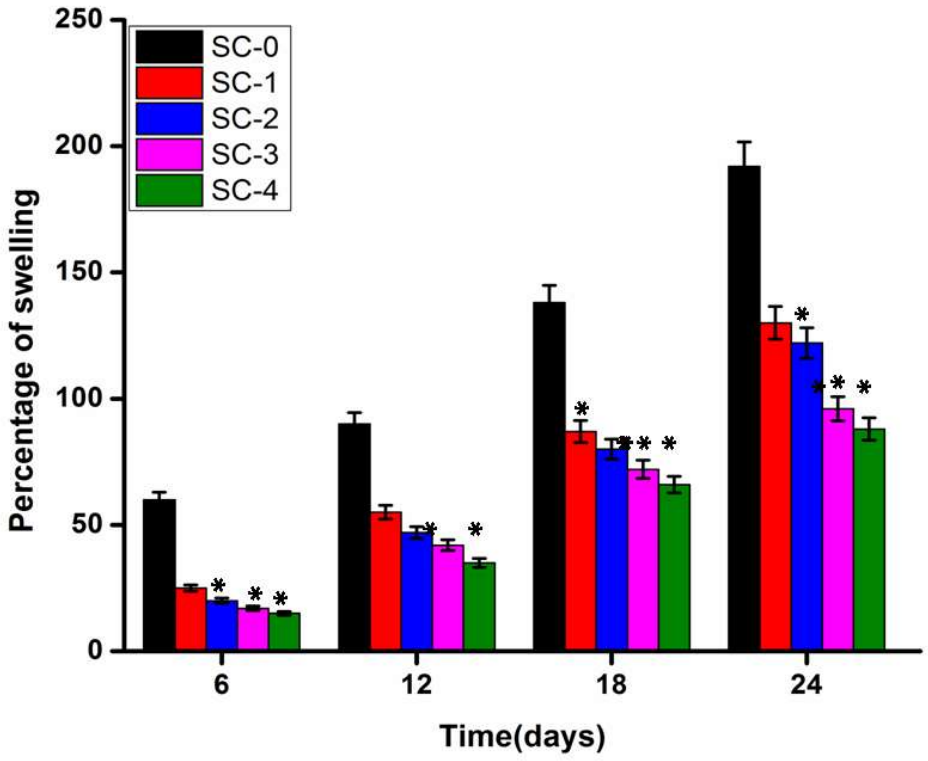

Figure 4.2.3. Percentage of swelling of developed scaffolds at different time intervals. Data are expressed in terms of mean ± SEM (n=3), ($^{*}p<0.01$), ($^{**}p<0.001$).

4.2.1.3.Biodegradation-

Biodegradation is another essential hallmark needs to be considered while designing of scaffold for tissue regeneration. Rate of scaffold degradation should meet the rate of tissue formation so that it can be integrated during tissue regeneration. Lysozyme is present in the extracellular matrix and body fluids that hydrolyses the β(1-4) glycosidic bond between *N*-acetyl glucosamine and glucosamine in chitosan [261-262]. *In vitro* degradation behavior of scaffolds has been evaluated and showed in figure 4.2.4. It is observed that SC-0 has maximum rate of degradation compared to rest. Maximum degradation rate is due to direct access of enzyme to substrate during degradation process. Other scaffolds from SC-1 to SC-4

have gradual increases in CPO that has led to accumulation of PCL in the scaffold; therefore, a lower rate of degradation has been observed form SC-4 to SC-1. It was observed that its biodegradation is slow in the early phase which may be due to the presence of hydrophobic PCL coating in the surface of the scaffolds. After seven days the biodegradation rate of scaffold increased as the permeability of water into scaffolds increased with time and hence the degradation of scaffold is accelerated. It is observed that SC-1 has the high rate of degradation throughout the study and SC-4 has a lowest rate of degradation because of the higher concentration of CPO, which leads to the higher accumulation of the PCL in the scaffold.

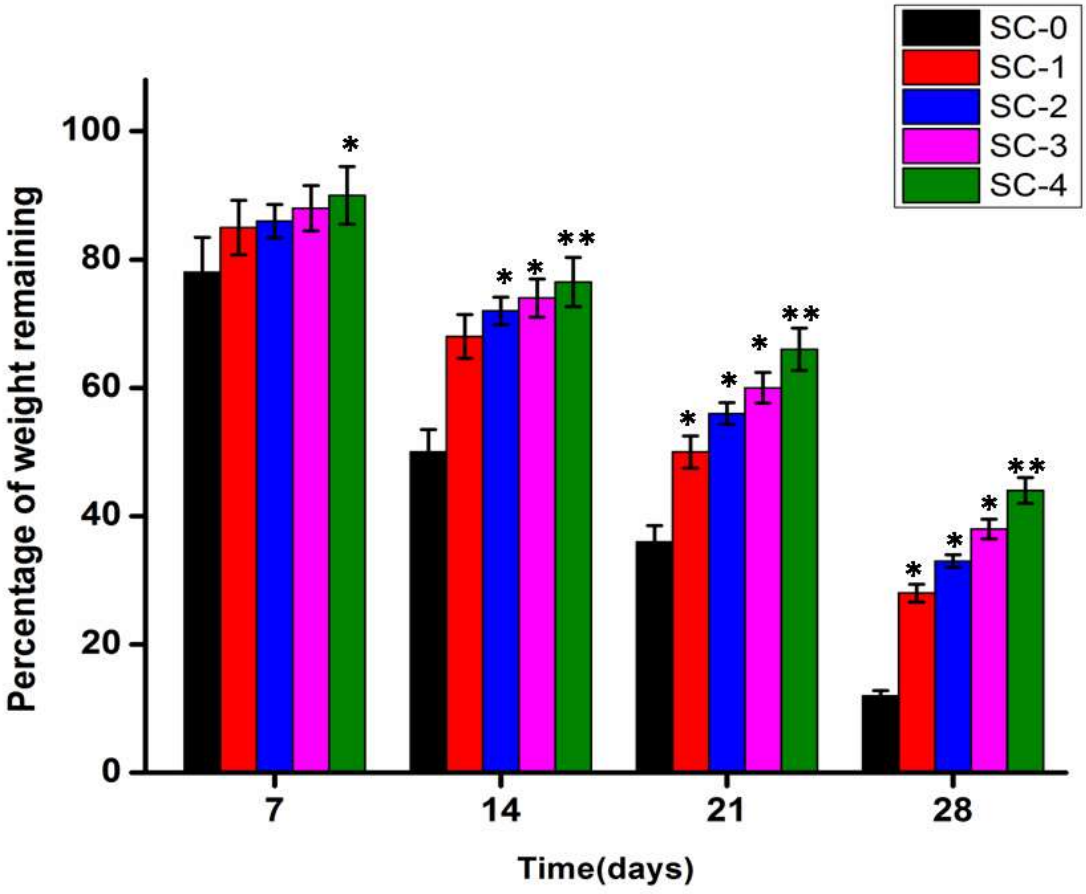

Figure 4.2.4. Degradation profile of developed scaffolds. Data are expressed in terms of mean ± SEM (n=3), ($^{*}p<0.01$), ($^{**}p<0.001$).

4.2.1.4.Water Vapor Transmission Rate

Optimum WVTR is an important factor to be considered during scaffold designing for wound healing[263]. Scaffold should control the evaporation of water from the wound surface. A health skin has WVTR 204 gm/m^2/day. While injured skin, it can reach from 279 gm/m^2/day for first degree burn to 5,138 gm/m^2/day for a granulating wound[264]. It is good to have a WVTR from 2500 gm/m^2/day which is a mid range of water loss rates. WVTR of commercialized wound dressings vary from 33 (Op site) to 208 (Omiderm) g/m^2 h[245]. WVTR of SC-0, SC-1, SC-2, SC-3 and SC-4 was found to be 152 ± 10.5,137 ± 12.5, 126 ± 6.8 , 106 ± 6.5 and 92 ± 8.2 gm/m^2/h respectively. This decrease in WVTR is due to successive increase in the percentage coating of CPO. This increase leads to decrease pore size, this decrease pore size will lead into low WVTR. These ranges are well in the range of commercially available tissue engineered products. This mid rates provides an adequate level of moisture with risking wound dehydration. Very high value of WVTR result in the drying of wound that lead to the lower moisture in healing wound. Lower values of WVTR lead to wound exudates accumulation. High accumulation of exudates leads to higher risk of bacterial growth that may lead to the sepsis of wound[264].

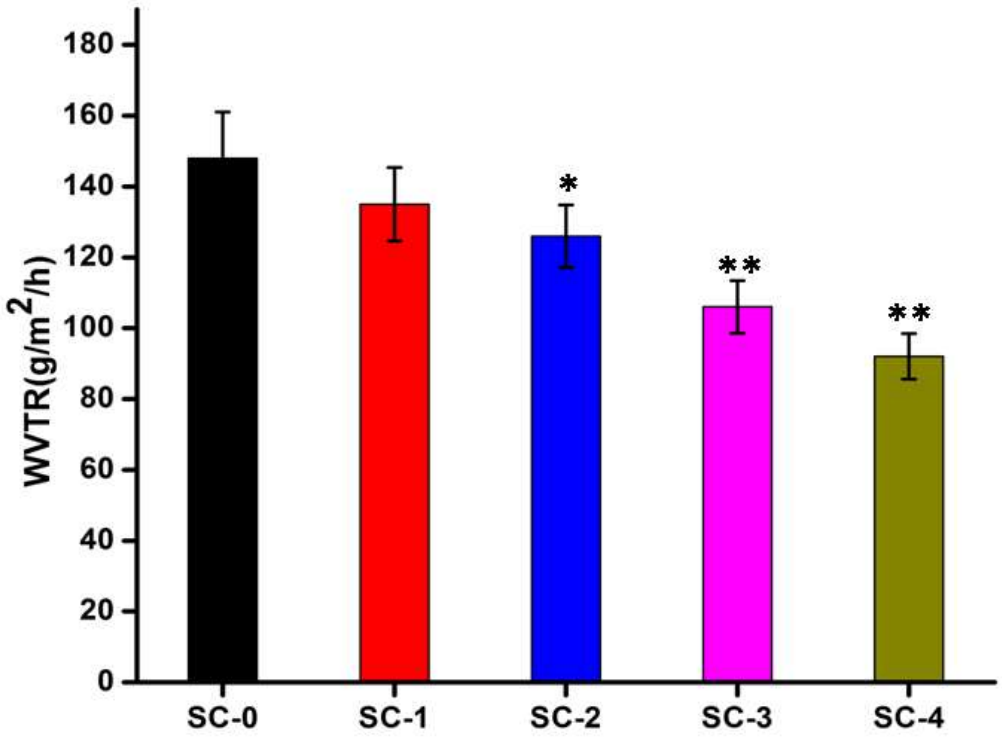

Figure 4.2.5. Depicting the Water Vapor Transmission Rate of different scaffolds at 24 hour. Data are expressed in terms of mean ± SEM (n=3), (*p<0.01), (**p<0.001).

4.2.1.5.FTIR study

FTIR spectrum of scaffolds was depicted in figure 4.2.1.6 and results showed that the peak at 1640 cm $^{-1}$ was due to the presence of the amide bond at C=O stretching and N-H bending. Peak at 1560 cm $^{-1}$ was due to the presence of the secondary amide, and the presence of amide III was confirmed with the peak present at 1235 cm $^{-1}$[248]. The bands at1560 cm $^{-1}$ and 1640 cm $^{-1}$indicate the presence of amide (CO-NH_2)group[265]. A band at 1053 cm $^{-1}$ is due to presence of -OH stretching[266] and band at1152 cm $^{-1}$ is due to C-O-C bond

stretching due to the presence of the glycosidic bonds[267]. The peaks at 2950 cm $^{-1}$ and 2865 cm^{-1}are due to the presence of–CH_2 group, which is also present in PCL and 1765 cm^{-1} is characteristic peak for CPO[268], increasing peaks intensity may contribute to increased amount of PCL and CPO. Hydrogen bond present due to the electrostatic interaction involves the -NH_2 group of chitosan and -COOH group of collagens.

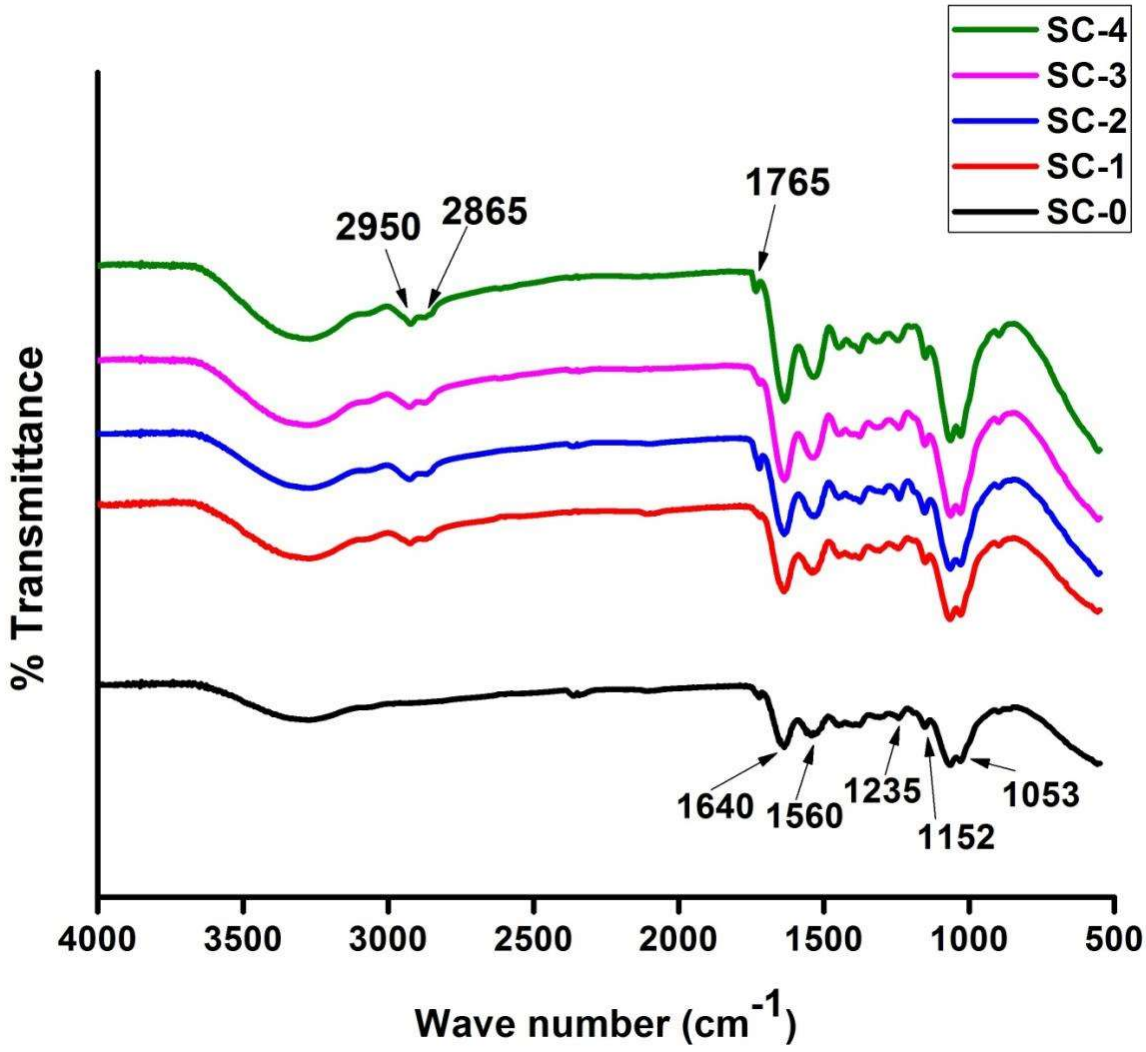

Figure4.2.6. FTIR spectra of developed scaffolds

4.2.1.6.XRD analysis-

Crystalline nature of the scaffolds was detected using XRD. XRD pattern as showed in figure-4.2.7. , represented a prominent peak at around 2θ of 21° and 36° which can be attributed to PCL[269]. Collagen-chitosan complex has distinct peaks which are attributed to 24.7°, 27.1°, and 30.90°. CPO has a characteristic peak at 41.31° and the peak gradually increases with increase in concentration of CPO over the coating of scaffold[217]. The XRD peak intensity defines the quantity of constituent material and it can be inferred from the graph that the amount of PCL associated with scaffold increases with increase in concentration of CPO[270].

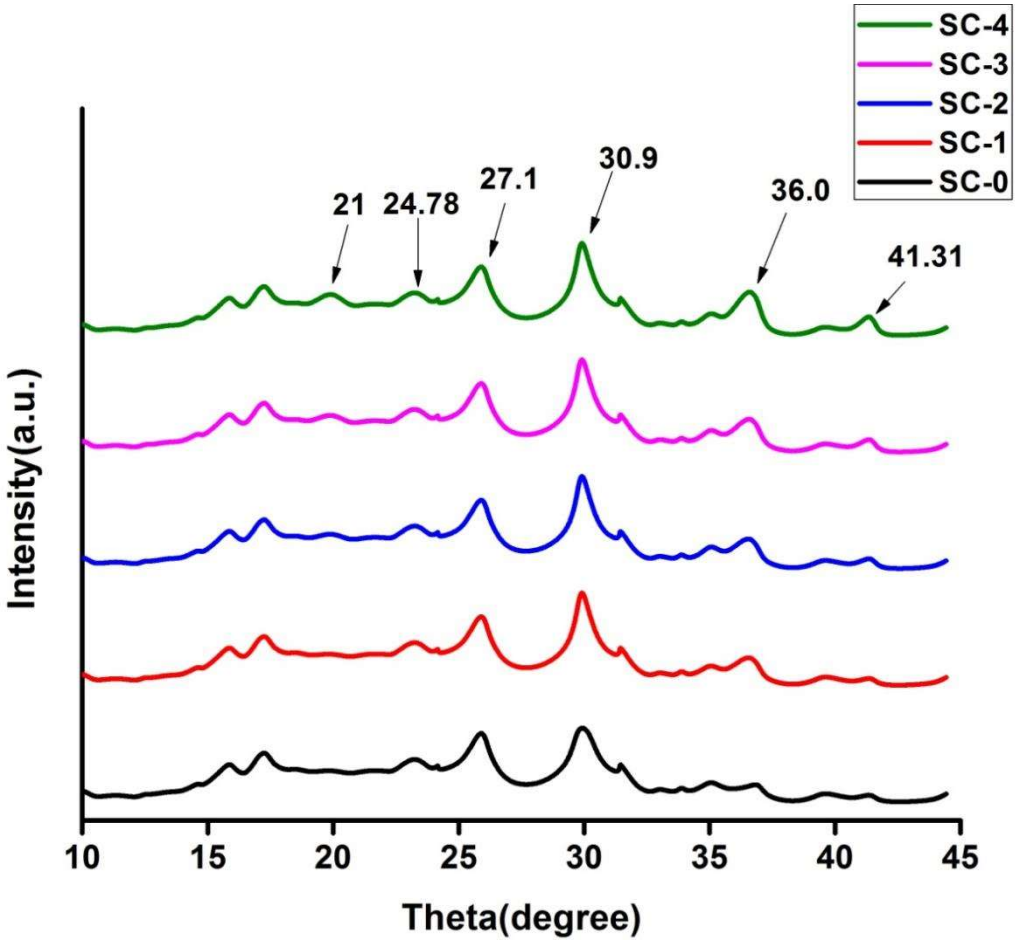

Figure 4.2.7 XRD pattern of CPO developed scaffold.

4.2.1.7. Oxygen release behavior

In the present study an attempt has been made to eliminate the hypoxic condition by adding CPO in scaffold which releases oxygen in the surrounding environment. In the first step hydrogen peroxide (H_2O_2) is formed while in the second H_2O_2 is decomposed to release oxygen.CPO produces burst of oxygen after getting contact with the aqueous media. Therefore, it has to be coated with a hydrophobic polymer to get prolonged and sustained release of the oxygen for over a long period.

Solid peroxide was encapsulated with PCL coatings at four different concentrations varying from 1-4 %. The oxygen was released in dose dependent manner. It has been observed that there is no burst release of oxygen in the medium from the scaffolds. Oxygen released from the SC-4 is significantly higher than the rest over ten days. After first day the oxygen concentration in SC-0, SC-1, SC-2, SC-3 and SC-4 was found to be 3.1 ± 0.21, 6.8 ± 0.28, 7.01±0.41, 7.52±0.22 and 8.5± 0.32 mg/mL that has reached to level 7.41±0.45, 8.02±0.24, 8.42±0.25, 9.62±0.27, mg/ml respectively. After two days the oxygen release was found to be constant. SC-0 is devoid of CPO therefore, the oxygen level is constant nearly about 3.2±0.14 mg/ml and no increase in oxygen concentration was observed. The obtained oxygen level was optimal to overcome the hypoxic condition. It was also found that the pH varies among 7.0 to 7.5 for ten days. However, this slight fluctuation in pH does not have negative effect on the cell culture and *in vivo* applications.

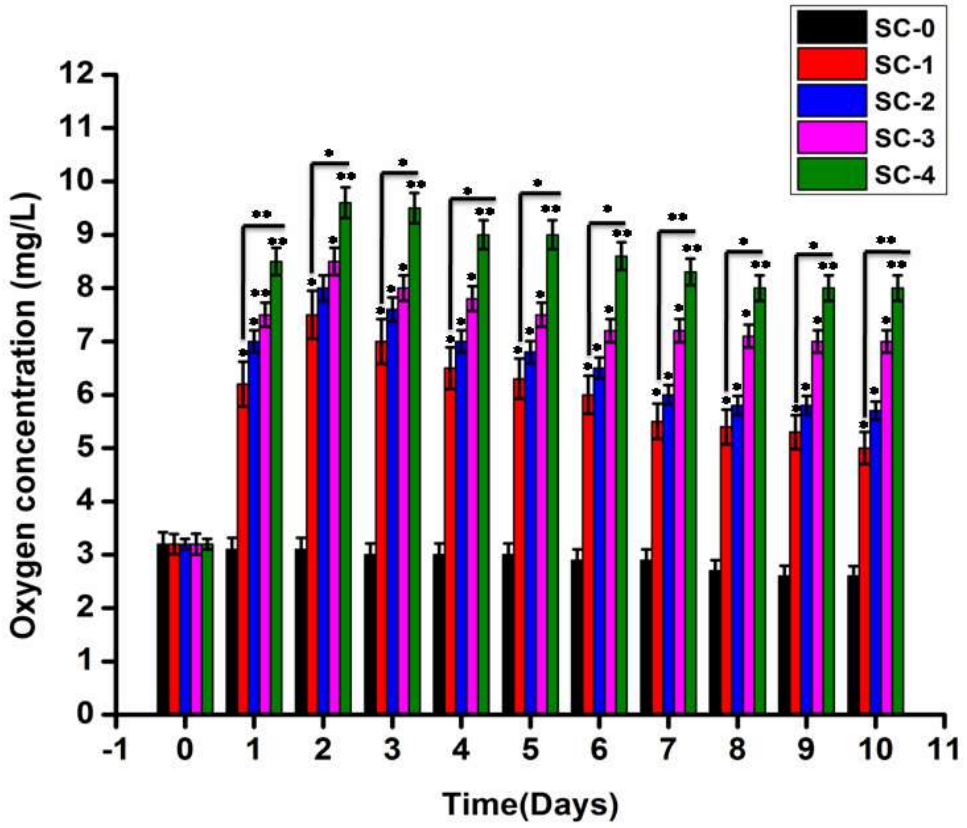

Figure 4.2.8. Oxygen-Releasing Kinetics of the developed scaffolds at different time intervals. Data are expressed in terms of mean ± SEM (n=3), ($^{*}p<0.01$), (**$p<0.001$).

4.2.1.8. Drug Releases study

Scaffold is loaded with antibiotic to provide the protection against the microorganism. Antibiotic is released after getting with water. Drug release kinetics of drug loaded scaffold depends upon the various factors including solubility, permeability and crystallinity of drug, structure of scaffold, porosity, degree of cross-linking, composition of scaffold, polymer ratio, preparation methods, incorporation of coating with polymer and the nature of the polymer used in the coating etc. Since all scaffolds have the same composition of the polymer (collagen and chitosan) loaded with the same amount of the ciprofloxacin, they differ only in the concentration of CPO and the amount of PCL in the scaffold. It has also

been shown, that CPO at higher concentration, surrounds the higher amount of the PCL and higher PCL concentration leads to the higher hydrophobicity in the scaffold that resulted in prolonged, sustained and continuous release of ciprofloxacin as compared to the other scaffolds as shown in the figure4.2.9.

Drug release pattern shown in figure6.1 indicates that SC-0 has rapid release of ciprofloxacin and releases more than 70% of total drug in initial 20 hrs. After rapid release it attains its maximum peak and gets stabilized. Other scaffold have hydrophobic PCL coating, therefore it reduces the accessibility of water to reach the entrapped ciprofloxacin inside the scaffold, thus slows and prolong the release of ciprofloxacin[271]. PCL and CPO content associated with scaffold has successively increased form SC-1, SC-2, SC-3 and SC-4 from 1 % to 4 % that has led to progressively slows and prolongs the release of ciprofloxacin as explained earlier in figure6. Such constant, sustained and prolonged release of antibiotics is required for efficient wound healing process. Therefore, an antimicrobial effect on the scaffold is necessary during the whole process of wound healing, especially in the early phase of healing[271].In SC-0 drug transport mechanism is super case II transport which is associated with stresses and state-transition in hydrophilic polymer. These hydrophilic polymers swell when comes in contact with water or biological fluids. PCL coated scaffold control (SC-1, SC-2, SC-3 and SC-4) the swelling of scaffold is controlled which results in non-fickian diffusion of drug. The drug release kinetics studies were evaluated using Korsmeyer-Peppas (KP) model. The data obtained from *in vitro* drug release studies were plotted as cumulative percentage drug release *versus* time. KP model was used to evaluate the n value and adjusted R-square value. n value was calculated for the scaffold SC-0, SC-1, SC-2, SC-3 and SC-4 and found to be 1.3, 0.73, 0.79, 0.82 and 0.87 respectively. From the n value, it is confirmed

that drug release pattern is fickian transport. The value is n is less than 0.445 ($n \geq 0.45$).This model describes drug release from several modified release dosage forms. $0.45 \leq n$ corresponds to a Fickian diffusion mechanism, $0.45 < n < 0.89$ to non-fickian transport, $n = 0.89$ to Case II (relaxation) transport, and $n > 0.89$ to super case II transport[272]. The kormeyer-Pappas model has been summarized in the table below-

Sample	**Adj. R-Square**	**n value**	**diffusion mechanism**
SC-0	0.9503	1.3	Super transport-II
SC-1	0.9503	0.73	Non- fickian
SC-2	0.9457	0.79	Non- fickian
SC-3	0.9463	0.82	Non- fickian
SC-4	0.9421	0.87	Non- fickian

Table 4.2.1 Diffusion mechanism, Adjusted R-Square and n value of different scaffolds.

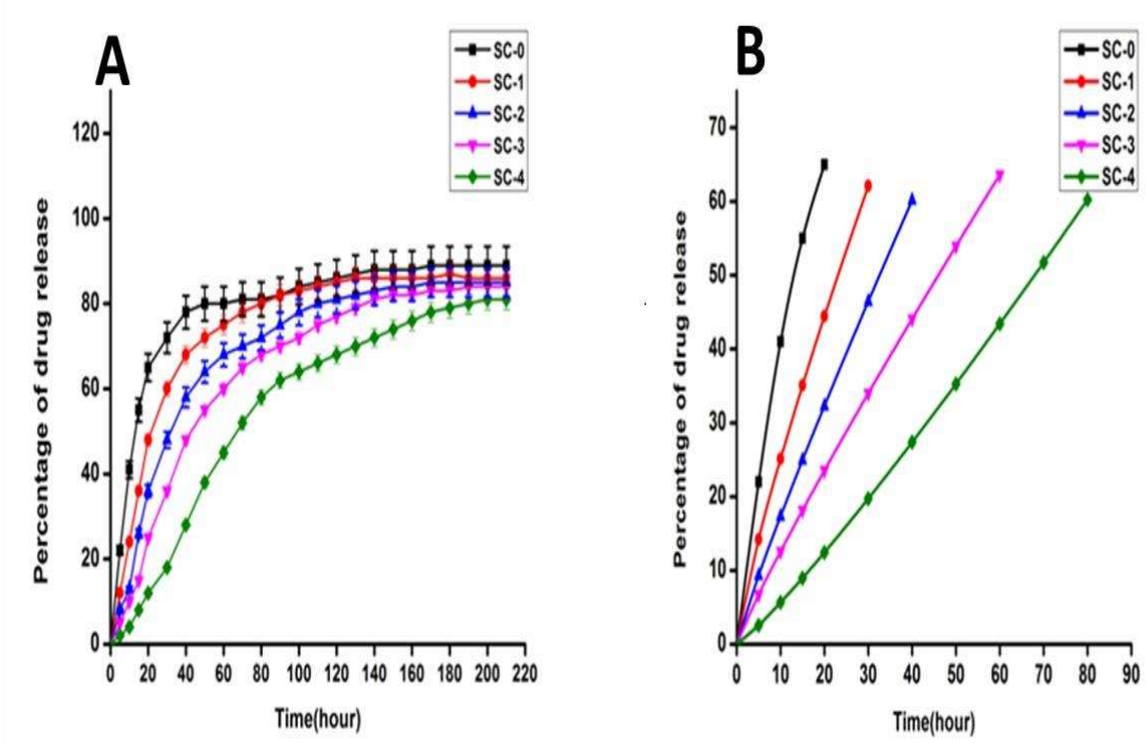

Figure 4.2.9.(A) Release of ciprofloxacin from the different scaffolds.(B) Fitting of the drug release data to the Korsmyer-Peppas model.The experiment was performed in triplicate (n=3)

4.2.1.9 Hemocompatibility study

Before executing any *in vivo* experiment, it is necessary to estimate the hemocompatibility of the developed scaffolds. Hemocompatibility test was performed to evaluate the percentage of erythrocytes broken or ruptured after getting contact with the scaffold. Our result showed that haemolysis percentage for the scaffolds was found to be 1.54, 1.52, 1.45, 1.82, and 1.75 for SC-0, SC-1, SC-2, SC-3, and SC-4respectively.The results showed higher degree of hemocompatibility of scaffolds which further emphasizes that these scaffolds are suitable for the skin tissue engineering application[273].

4.2.2. *In vitro* cell culture study

4.2.2.1 Live dead assay and cell attachment study

Fibroblasts were cultured in the developed scaffolds for the period of 14 days under hypoxic environment. The cell viability was qualitatively evaluated using live-dead assay. Figure 4.2.10.(A) shows that the retention of Calcein-AM within the live cells after the culture of fibroblast in the scaffolds for period of 7 and 14 days and live cells emit bright green color and dead cells emit red color under the fluorescence. Our results showed that under hypoxic condition viability of cells was very low and large number of dead cells can be seen in red color in SC-0 in 7- and 14-days period. CPO loaded scaffolds were capable of producing oxygen that will be utilized by cells for metabolic activities. Therefore, most of cells were viable and distributed evenly over scaffolds and only limited numbers of cells were found to be dead overSC-1, SC-2, SC-3, and SC-4.

Behavior of fibroblast attachment on scaffold was further analyzed by culturing fibroblast over scaffold at hypoxic environment for 14 days using scanning electron microscopy and fluorescent microscopy. Cell attachment, cell growth, and migration in scaffold depend upon a variety of factors such as nature of scaffold, nutrient supply, oxygen availability etc. SEM and fluorescent images of cell-scaffold construct (figure-4.2.11 & 4.2.12 respectively) showed the uniform fibroblast attachment and growth over scaffolds; however, cells differ in shape and number depending upon the nature of scaffold and availability of oxygen during cell culture. SEM image of SC-0 at 7^{th}day clearly showed the attached fibroblast on surface with slightly altered morphology with lower number when compared to other scaffolds. Scaffolds with CPO were able to form an extensive network of layered fibroblast with enhanced cell spreading and superior cell-cell interconnection. However, with the

advancement from 7^{th} to 14^{th}day showed the spreading and migration of fibroblast over and inside the scaffold. SC-0 has limited support of oxygen for the fibroblast growth in the hypoxic environment during cell culture therefore it has shown slightly altered thread like projection form the fibroblast that may be due to low availability of oxygen during cell culture. Oxygen is crucial for cell growth and in hypoxia; oxidative stress may lead to free radical formation that ultimately lead to cell shape alteration and cell death. With increasing amount of CPO in scaffold, oxygen releasing potential increases that provides oxygen during cell culture and protects against hypoxia. Therefore, cells have better spreading; migration and interconnectivity in scaffolds as can be conferred from SC-1 to SC-4.Fluorescent microscopy using DAPI dye further strengthen the observation made during SEM study. SC-0 has very limited growth due to hypoxia. SC-1 to SC-4 has continuous increasing cell number with SC-4 has highest number of cells due to their ability to generate oxygen during hypoxia that enables the scaffold to support the growth of cells inside scaffolds.

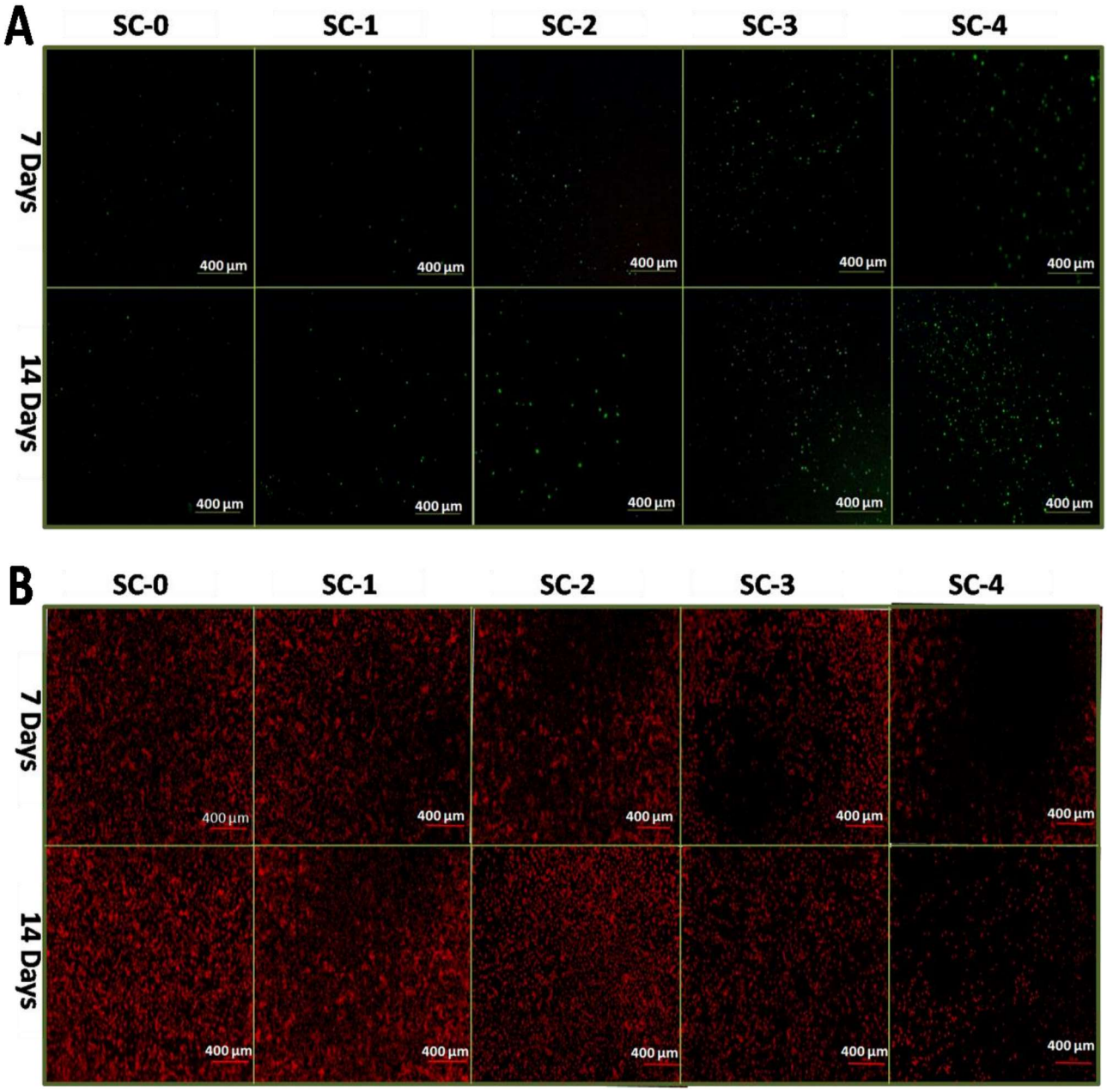

Figure 4.2.10. Dual staining assay of fibroblast on scaffold.(A) Shows the live cells in green color and B depicts the dead cells in red color.

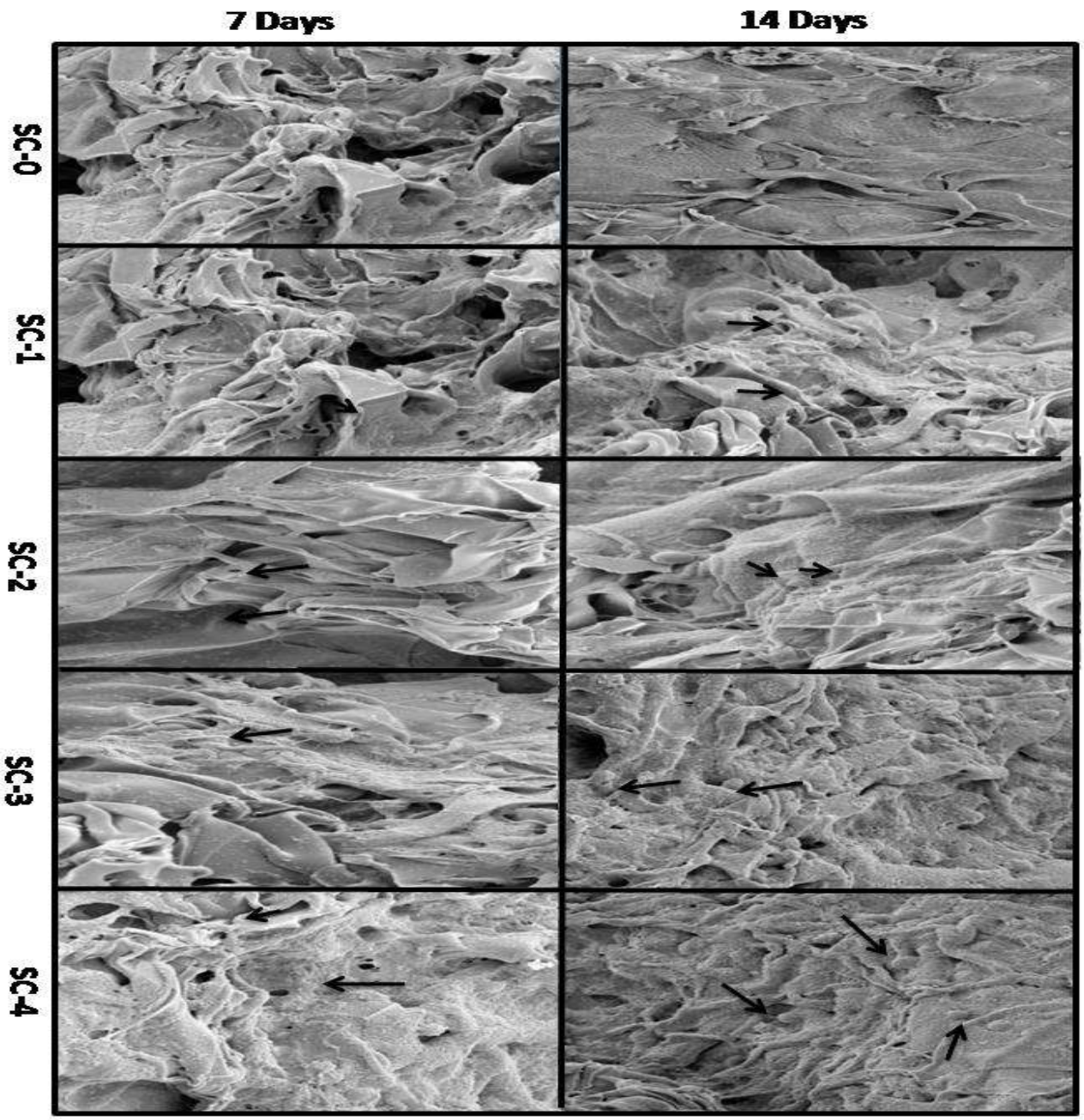

Figure 4.2.11. SEM images of cell seeded scaffolds depicting the attachment of fibroblast after 7th, and 15th day under hypoxic condition.

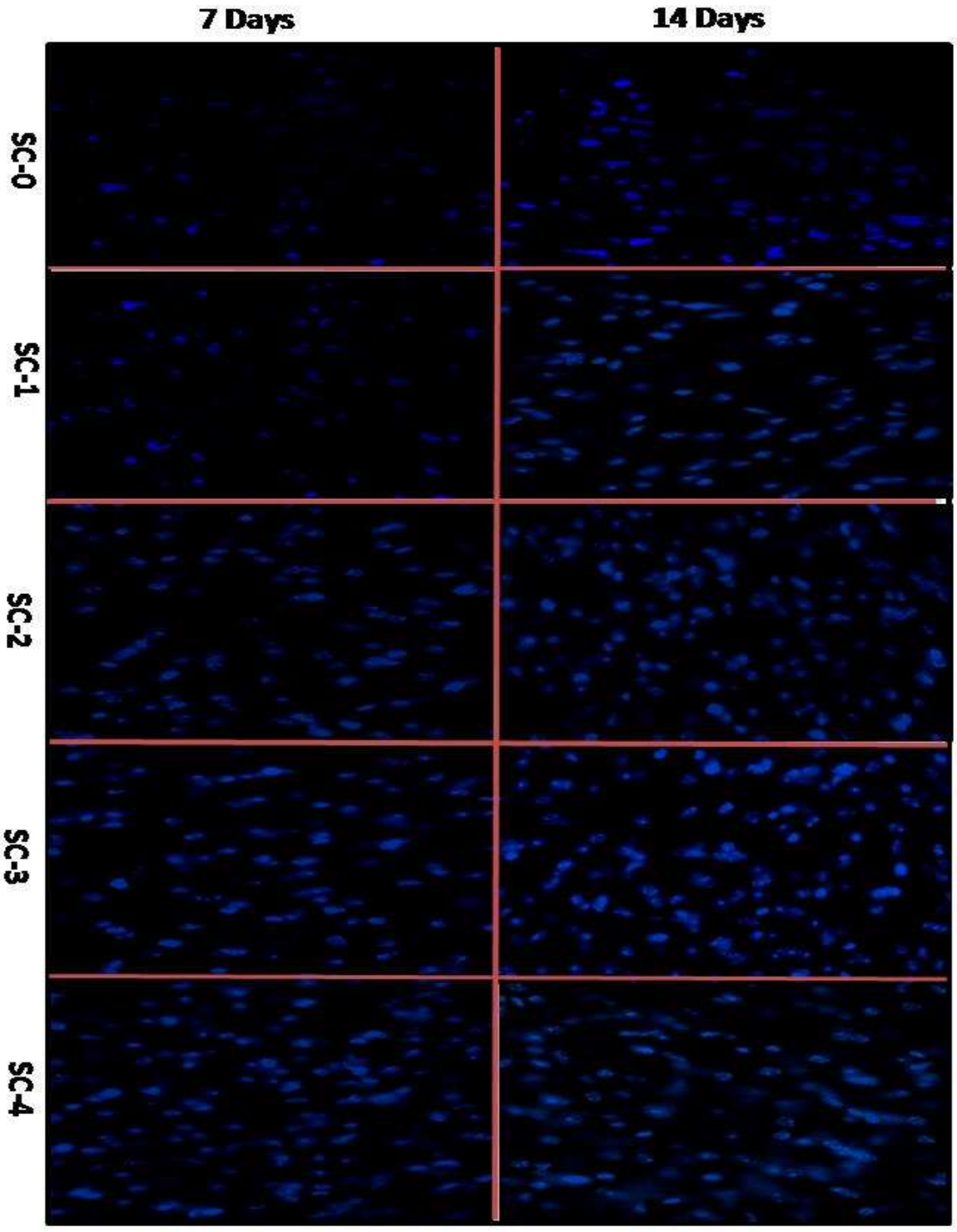

Figure 4.2.12. Fluorescence microscopy images of cell seeded scaffolds stained with DAPI showing the growth and proliferation of fibroblast after 7^{th} and 15^{th} day under hypoxic condition.

4.2.2.2 MTT assay

Metabolic activity and viability of cells on scaffolds were studied in vitro by MTT test. Because differences in the chemical composition, conformation, porosity, hydrophilicity, oxygen concentration, and nutrient availability affect cellular activities[8]. Cell viability studies were performed at different time points over the period of 7 and 14 days. Under the hypoxic condition of cell culture, it can be seen from the figure-4.2.13, the percentage viability of fibroblast value of SC-0 is significantly lower than other scaffolds. This might be due to low availability of oxygen during cell culture. Hypoxia also increases the oxidative stress, which causes cell death, narcosis and reduces cell viability[274]. Low viability in SC-0 is also evident form live/dead assay and SEM images shown in figure 5.20 and 5.21. Cell viability was significantly higher in CPO loaded scaffold that may be due to the ability to generate oxygen during hypoxic condition of cell growth. Increasing amount of CPO in scaffolds generates huge amount of oxygen (figure5) that has led to higher cell viability in accordance with scaffold with higher CPO.

From the above studies, it can be concluded that SC-4 has highest level of cell growth, good hemocompatibility, morphological characteristics and suitable physical parameters such as optimum swelling and degradation. Therefore SC-4 was further used during *in vivo* studies and SC-0 was taken as negative control.

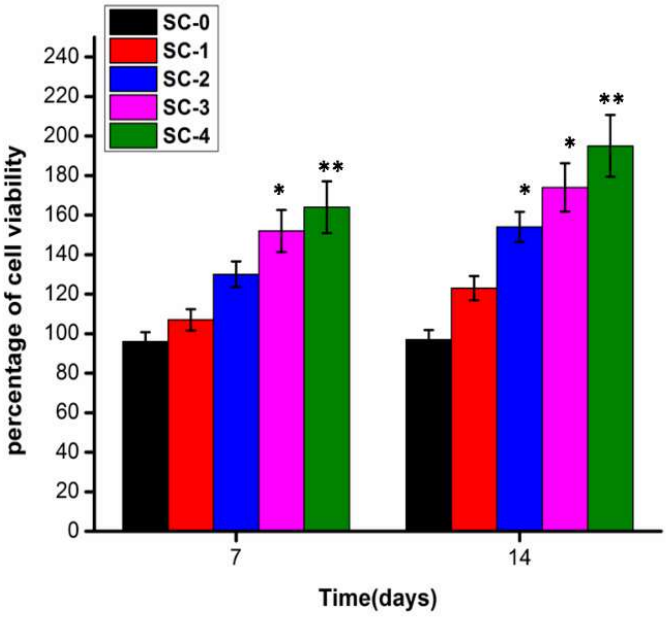

Figure 4.2.13. Cell viability study using MTT in cell seeded scaffold. Data are expressed in terms of mean ± SEM (n=3), ($^{*}p<0.01$), (**$p<0.001$).

4.2.2.3. DNA content

Cell proliferation in scaffolds was quantified through estimation of total DNA content (figure-4.2.14.). Fibroblasts were cultured under hypoxic condition for the period of 14 days. The DNA content was estimated that has shown the effect of hypoxic condition in cell growth and proliferation. Total DNA content per scaffold was quantified after 7 days that was found 421.30±33 ng, 1252±110 ng, 1664±98ng, 2611±131 ng, 3925±196.15 ng and after 14 days 510±41 ng, 1774±158 ng, 3668±212 ng, 5421±271.05 ng and 7442±376.52 ng for scaffold SC-0, SC-1, SC-2, SC-3 and SC-4 respectively. It is evident that SC-0 has limited capability to support fibroblast under hypoxic condition due to the inability to produce oxygen to support the growth of cells, which has resulted into low DNA content per scaffold. CPO loaded scaffold has shown the ability of scaffolds to support the cell growth under

hypoxic condition. The increasing amount of CPO enhances oxygen release that has supported the fibroblast growth in increasing manner. SC-4 that has highest amount of CPO generates highest amount of oxygen that support the growth of cells.

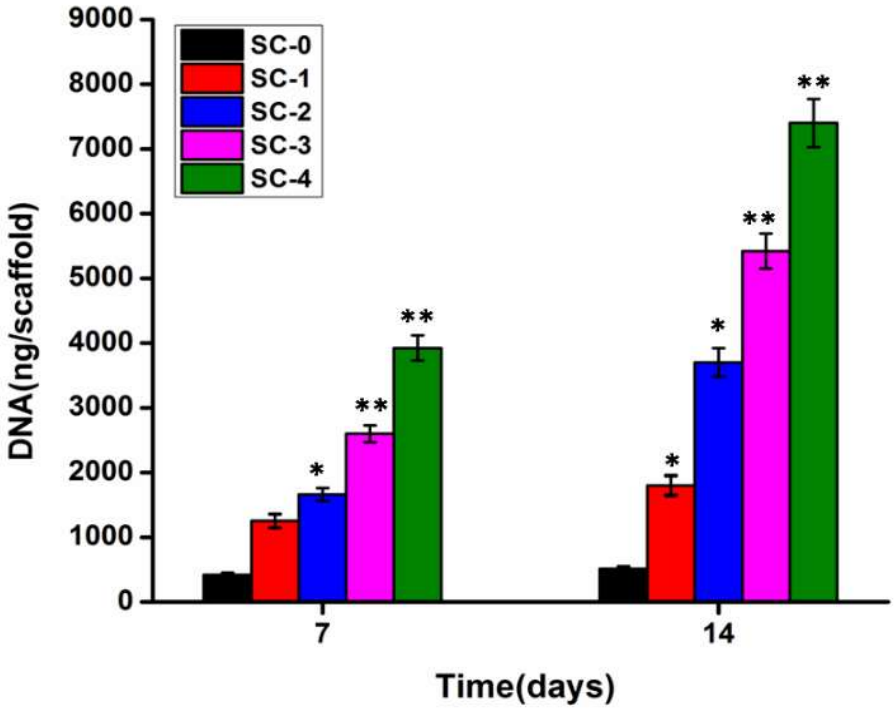

Figure 4.2.14 DNA content estimation of cell seeded scaffolds. Data are expressed in terms of mean ± SEM (n=3), (*p<0.01), (**p<0.001).

4.2.3. Antibacterial test

Based on physio-chemical and biological studies scaffold SC-4 was selected for the antibacterial study. Antimicrobial effect of selected scaffold (SC-4) was evaluated against the *S. aureus*and*E. coli.* The control showed absence of the zone of inhibition depicting the poor antimicrobial ability of scaffold while the developed scaffold has clear zone of inhibition (15 ± 1.2 mm and 18 ± 0.8 mm) against the *S. aureus* and *E. coli*bacteria respectively (figure11). Hence, from the antibacterial study, it can be conferred that the developed scaffold has antibacterial property which can be useful to prevent the infection thereby promoting wound healing[275].

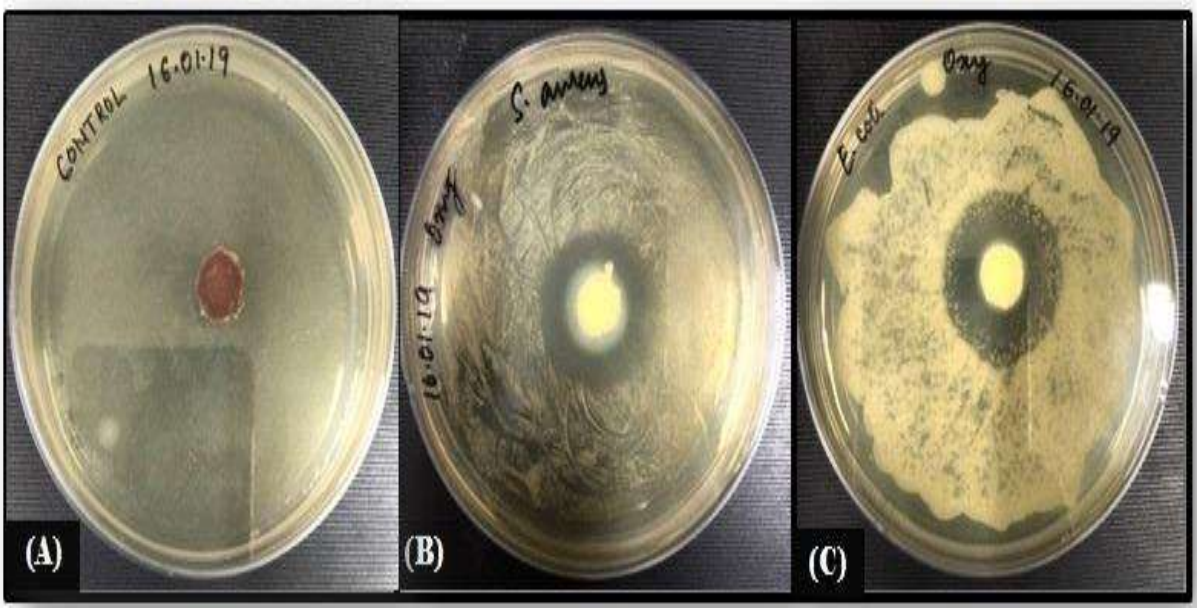

Figure 4.2.15. Antibacterial effect of scaffold (A) Control, (B) Against the growth of *S. aureus* (C) Against the growth of *E. coli.*

4.2.4 *In vivo* and morphological studies

4.2.4.1 *In vivo* studies

During the *in vivo* study the wound healing rate in control and test groups was compared at different time intervals. The developed scaffolds were applied over artificially created wound over rat dorsum to examine its effect in wound healing for the period of 15 days. All the rats implanted with scaffold were survived during the experiment. During the periodical observation, differential wound healing rate was observed among control, scaffold and oxygenating scaffold on various days after the operation as shown in the figure 4.2.16 (A). On 5^{th} day after implantation, the wound was ruddy and started to contract from the edge and area of wound was greatly reduced in CPO loaded scaffold(124mm^2) compared to oxygenating scaffold (152 mm^2) and control(192mm^2) which showed higher degree of inflammation with less wound contraction. After 5 day of wound healing $p<.001$ which indicates capacity of wound healing of oxygenating was very significant. These wound contractions are 15 ± 0.09, 33 ± 1.2 and 46 ± 1.8 % of wound healing in control, scaffold, and oxygenating scaffold respectively. After ten days the inflammatory response in control became less severe and wound was healed considerably compared to earlier.CPO loaded scaffold (SC-4) treated wound showed faster recovery compared to SC-0 and control. The area of wound remained after 10 days was 53 mm^2, 98 mm^2, and 115 mm^2, that was 48, 56 and 77.7 % of total wound healed for control, SC-0, and SC-4 respectively. Statistical analysis after 10 day wound healing indicated that healed area and percentage of wound healing for oxygenating scaffold was statistically very significant ($p<0.001$). Wound was greatly reduced and was about to heal in the same period of time in oxygenating scaffold (SC-4) compared to control and non-oxygenating scaffold (SC-0). After fifteen days, wound

was disappeared at the site of implantation of SC-4 while the wound was clearly visible in case of control and SC-0. Wound area was reduced to 58 mm^2 and 23 mm^2compared to 225 mm^2 that represent 72.8 and 84.23 % of wound healed for control and SC-0 compared to SC-4 that has achieved complete recovery. The p value for SC-O and oxygenating scaffold after 15 day of wound healing was found $p<0.01$ & $p<0.001$respectively.Statistical parameter indicates the wound healing by SC-0 and oxygenating scaffold significant and most significant respectively.Percentage of wound healing was calculated by using ImageJ software. The control has least level of skin regeneration that might be due to higher level of infection at healing site, absence of structural support, and generation of hypoxia during healing. SC-0 has intermediate range of healing between control and SC-4 because of having structural support of collagen and chitosan and presence of antibiotic but lack of oxygen (generation of hypoxia) during the wound healing process. Ciprofloxacin was released in a sustained and continuous manner that protected the wound against bacterial infections, thus inhibited prolong infection and inflammation which promotes faster wound healing. Calcium peroxide inhibited the hypoxic condition in the wound, and thereby promoted necrosis and faster healing. Wound surface area and healing rate was evaluated at different time intervals that showed that wound area was decreased in a time-dependent manner.

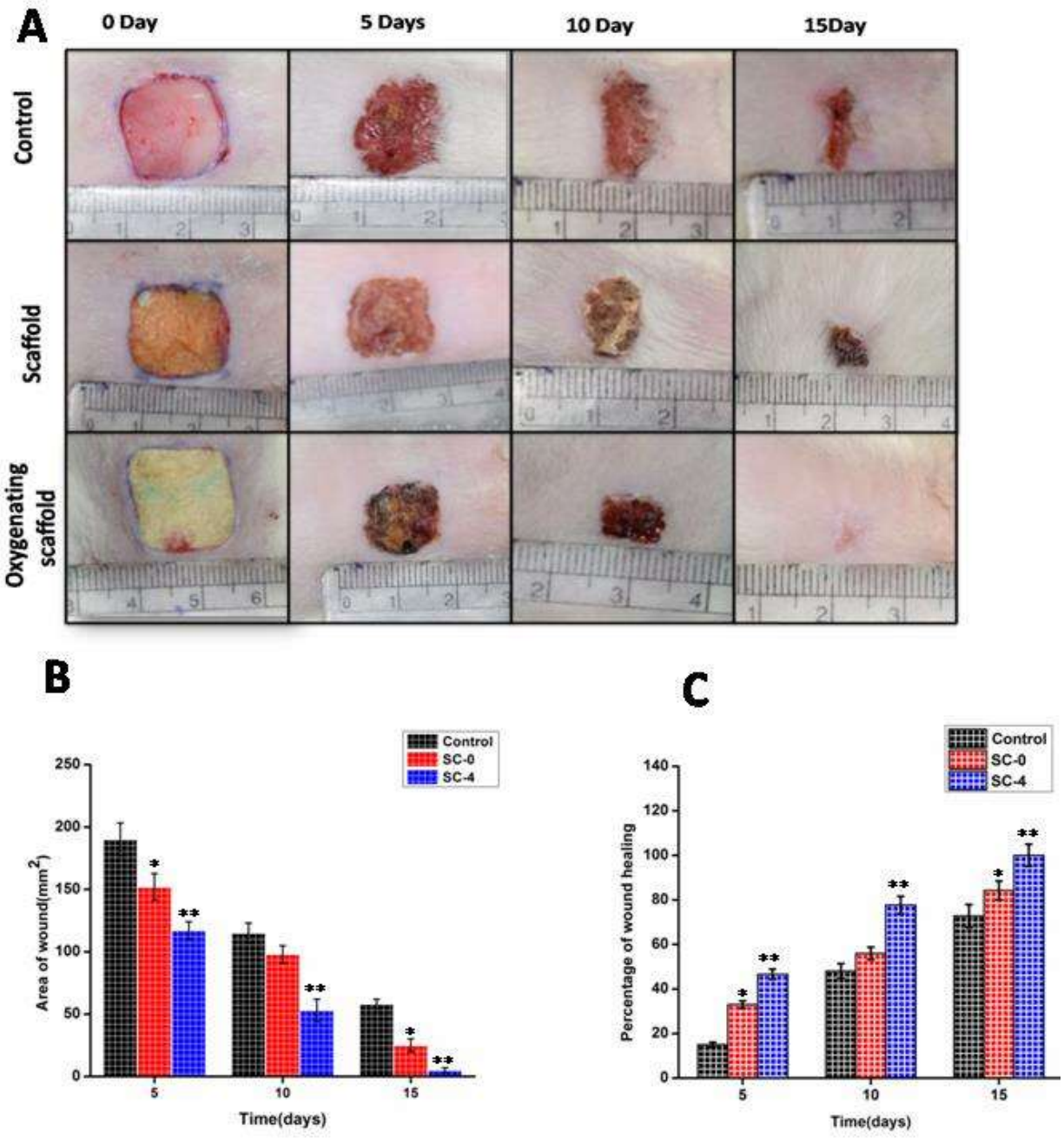

Figure 4.2.16. In vivo study using rat model. In figure 4.2.16.(A) Representative images depict the wound healing effect of scaffold at different time intervals. Figure 4.2.16.(B) represents wound closure rate; Figure 4.2.16.(C) represents wound healing rate. Data are expressed in terms of mean ± SEM (n=3), ($^{*}p<0.01$), (**$p<0.001$).

4.2.4.2 Histological examination

Histological examination of implanted scaffolds (SC-4, SC-0 and control) was performed at 5^{th}, 10^{th} and 15^{th}day. For further characterization of wound healing at cellular level histological study was performed using Haematoxylin and Eosin staining and our results showed that SC-4 was found to be effective as compare to control. Histological examination showed that developed scaffold is biocompatible and promoted the growth and migration of cell. Histological observations showed the initiation of epithelialisation and migration of cells SC-4 has higher rate of epithelisation at 5^{th} day period as compared to of SC-0 and control. At the tenth day, higher rate of granulation was observed in the SC-4, compared to control and SC-0. In higher degree of epithelisation and extracellular matrix is formed in the scaffold as compared to control which has thin layer of epidermis and poor extracellular matrix deposition. At 15^{th} day, the epidermis and dermis were found to be fully developed and extracellular matrix architecture with blood vessels and hair follicles was also observed compared to SC-0 that has loosely organized extracellular matrix and control which still has less develop epidermal and dermal component along with extracellular matrix. The study concludes that oxygen producing scaffold (SC-4) accelerated the wound healing process by providing protection against microbes, reducing the inflammatory period, and faster tissue regeneration of the epidermal, dermal, extracellular matrix, and blood vessels formation through continuous supply of oxygen for the wound healing as shown in figure 4.2.17.

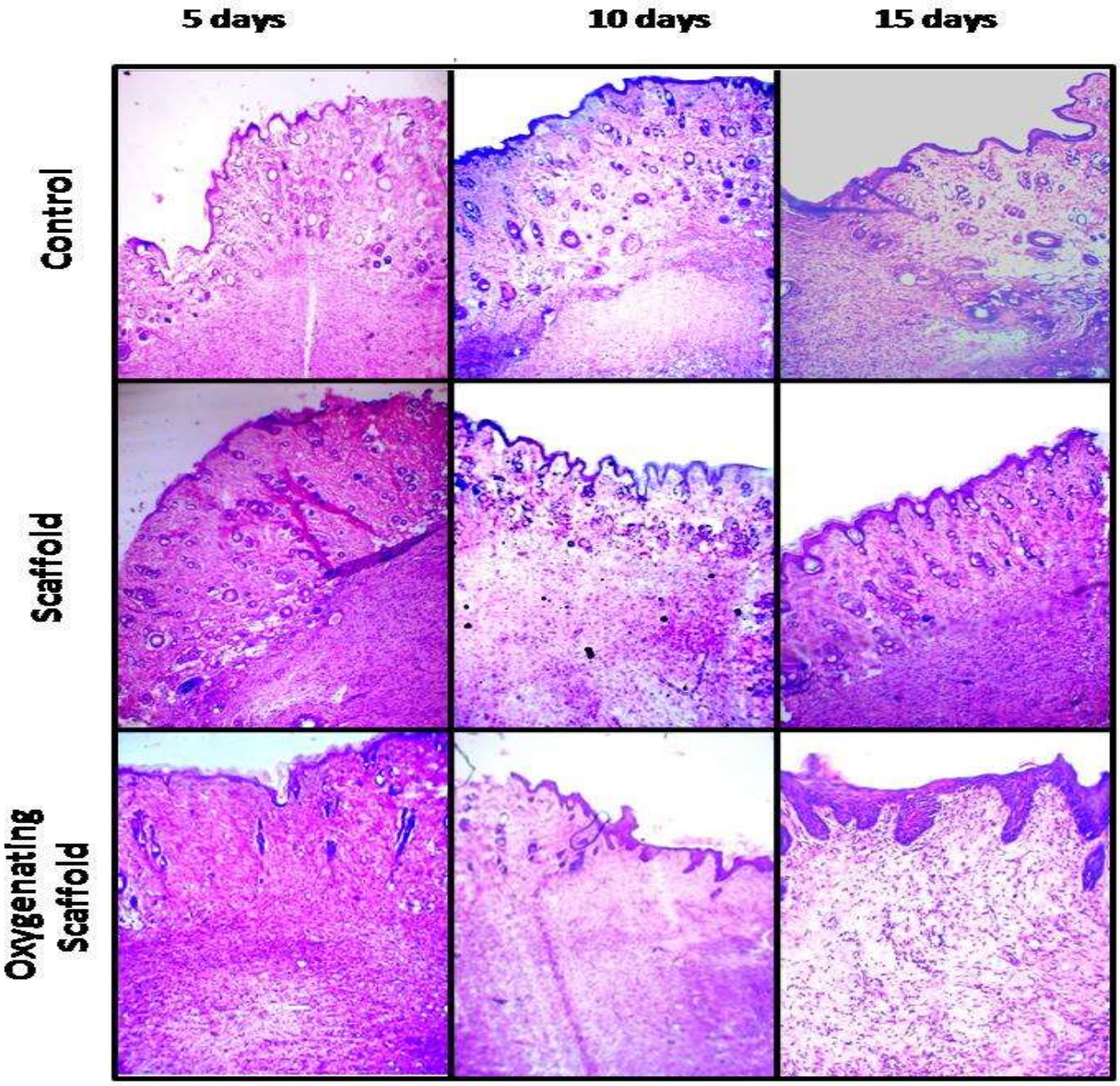

Figure 4.2.17. Histological analysis, H&E staining of skin flaps harvested at 5, 10, and15 days.Images are taken at 40X and 100X.