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Fabrication and Characterization of Porous Nanohydroxyapatite/Chitosan-Cellulose Composite Scaffold for Biomedical Application

By S.Nagalakshmi, G.M. Pavithra, R.Tharani, Vaddarapu Ashok & Fathima Azra Zainul Afker

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Strictly as per the compliance and regulations of:

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S.Nagalakshmi [α], G.M. Pavithra [σ], R.Tharani [ρ], Vaddarapu Ashok [ω] & Fathima Azra Zainul Afker [¥]

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Results: Four formulations (S1, S2, S3, S4) of the scaffold was formulated using the freeze-drying technique. The characterization studies indicated that formulated Scaffold (S4) showed the minimum loss of weight (2.7 %) in four weeks and thus had the lowest degradation. The swelling was similar in all the scaffold formulations due to constant hydroxyapatite and chitosan concentrations. The porosity of the scaffold formulations was identical to one another. From the report of the antibacterial activity of the formulated scaffold, it was found that the scaffold (S4) with various concentrations of 100µg, 200µg, and 400µg when compared with standard positive and negative control, showed a maximum zone of inhibition of 26mm, 32mm and 34mm respectively. Hence the prepared scaffold exhibited higher antibacterial activity.

Conclusion: Nanohydroxyapatite formulation has high biocompatibility and bioactive properties. The contagions allied with the implantation recurrently minimize the usage of biomaterials in humans. Thus, the developed scaffold would be a promising biomaterial for biomedical applications.

Keywords: bone grafting, scaffolds, hydroxyapatite, ofloxacin, chitosan.

I. Introduction

In the past two decades, tissue engineering by bone regeneration has become an alternative method used to overcome the shortcomings of conventional bone defect treatments [1]. Bones are upkeep and guard various organs of the body. Damage induces a significant decrease in the quality of our life. A medical technique called bone grafting substitutes lost bones to patchup bone fractures, which are very difficult, imparting substantial health hazards to a patient, or flop to cure appropriately. The grafts may be autologous, allograft, or synthetic. Many of the grafts get reabsorbed and substituted when the normal bone reconciles over some time. The doctrines in fruitful grafts include osteoconduction, osteoinduction, and osteogenesis [2].

The progress inthe medical discipline has upgraded biomaterials role in substituting injured tissue, organs and enhancing their functions. Bone tissue engineering is a novel treatable practice for bone grafting [3]. The tissue engineering research is implemented mainly in two fields: osteo and dental applications. This technique implants scaffolds, which give mechanical strength in the crackzones. The scaffold remains as a momentary medium for cell multiplication until fresh tissue is entirely revived [4].

Hydroxyapatite (HAP) is one of the apatite materials that have a significant inorganic constituent of teeth and bone, which has high biocompatible and bioactive properties and hence employed in bone tissue engineering. Its flow property strength is very little than those required for bone tissue engineering materials and has a tend to migrate from implant sites. These limitations can be overwhelmed by combining hydroxyapatite with organic constituents, thus mimicking the ECM of bone [5].The contagions allied with the implantation recurrently minimize the usage of biomaterials in humans. Bacteria trigger the patient's immune system forming a protective film by sticking onto biomaterial exterior. To avoid these complications, ofloxacin which possesses antibacterial activity, has been incorporated in this biomaterial [6]. Thus, the present research work was intended towards the formulation of nano biocomposite scaffold of hydroxyapatite-chitosan-cellulose. Five formulations namely, S1, S2, S3, S4, and S5, was developed. These five formulations are initially characterized for various properties. The optimized formulation, i.e.,. S5, was characterized by analytical techniques.

II. Materials and Methods

a) Materials

Hydroxyapatite, Chitosan, Sodium Carboxy Methyl Cellulose, Carboxy Methyl Cellulose, Hydroxy

Author α: Department of Pharmaceutics, Sri Ramachandra Faculty of Pharmacy, Sri Ramachandra Institute of Higher Education and Research, (DU), Porur, Chennai-600116.
e-mail: nagalakshmimpharm@gmail.com

Propyl Methyl Cellulose, Ofloxacin and acetic acid were purchased from Sastha Scientific Services, Chennai.

III. Methodology

a) Preparation of Hydroxy Apatite Nanoparticles

The orthophosphoric acid solution was added drop by drop into calcium hydroxide solution under magnetic stirring at 70°C for 3 hours. The mixture is stirred until a clear and homogenous solution formed, and then sodium hydroxide solution was added to this solution until pH value was maintained at 10. The white precipitates were left for 4 hours. The obtained nanoparticles were parted, clarified with deionized water, and dried under ambient atmosphere. It was then heated in an electric furnace at 700°C to obtain pure nanoparticles [7,8].

b) Fabrication of Scaffold

Hydroxy Propyl Methyl Cellulose(HPMC) and 100 mg of ofloxacin drug were dissolved in water using a mechanical stirrer until a homogenous solution was formed. Secondly, chitosan was solubilized in 2% acetic acid, which was instilled dropwise into the HPMC mixture. It is then mixed at 500 rpm. These mixtures were added to the above-formed nanoparticles. The stirring is kept for 24hrs and, the gel formed was then transferred into the tissue culture dish and cooled at -24°C for 24 hrs and lyophilized to form scaffolds. These scaffolds were cross-linked with $CaCl_2$ solution for 30 minutes, followed by sopping in ethanol for 10 minutes. Finally, the scaffold was clarified with water and another timely ophilized [9,10].

c) Preparation of Five Formulations of Scaffold

By experimenting with different polymers, five formulations of the scaffold was prepared namely, S1, S2, S3, S4, and S5.

IV. Characterization Studies

a) Calibration Curve of Ofloxacin

The calibration curve of ofloxacin was performed using various concentrations of ofloxacin, as given in Figure no.1 [11].

b) Fabrication of the Nanocomposite Scaffold

The scaffold was prepared as per the procedure described in Figure no.2. The quantities of the ingredients in each scaffold are described in Table no.1.

c) Weight Loss

By imbibing the scaffolds in Simulated Body Fluid (SBF), the weight losses of the five scaffold formulations are conceded.

$$\text{Weight Loss (\%)} = [(W_o - W_t) / W_o] \times 100$$

Where W_o denotes the weight of the fused scaffold, while W_t is the weight at time (t). The study recurred thrice, and the mean value is noted. The weight loss data were depicted in Table no.2 and Figure no.3.

d) Swelling Ability

The parched mass of the scaffolds was represented as W_i. Parched scaffolds were submerged in Phosphate Buffer solution at 37°C for 24 hours. Later, the scaffolds were removed from PBS solution, and its damp mass was denoted as W_f. Swelling ability data was depicted in Table no.3.

$$\text{Swelling Ability (\%)} = [(W_f - W_i) / W_i] \times 100$$

e) Porosity Measurement

W_d was used to represent the dry weight of the scaffolds, while W_l designated the mass of the scaffolds after immersing in ethyl alcohol for five minutes. After slight parching over the shallow area, W_w was recorded. The porosity data is described in Table no.4 [12].

$$\text{Porosity (\%)} = (W_w - W_d) / (W_w - W_l) \times 100$$

f) FT-IR Analysis

The spectra of the Chitosan, HPMC, Ofloxacin, and the optimized F5 formulation were documented by means of potassium bromide pellet method in the FT-IR spectrophotometer (JASCO 4100 type A) within the range of 4000cm-1 to 400cm-1 [13].

V. Surface Analysis

a) Scanning Electron Microscopy (SEM)

The powdered sample was taken and mounted on a double side carbon tape, which was fixed to sample specimen stub. The SEM (QUANTA FEG) instrument is used for analysis. The SEM images were described in Figure no.4 [14].

b) Optical Microscopy

MOTIC digital microscope is used to image the scaffold at 10X and 40X, as given in Figure no.5.

c) Transmission Electron Microscopy (TEM)

TEM studies were useful in examining the morphological and crystalline arrangements of the scaffold. The principle employed to view the scaffolds is high-resolution transmission electron microscopy (HRTEM). The scaffold's (20 μl) solution was taken. On the carbon-coated side of the copper lattice, the mixture was dripped. At room temperature for few hours, the lattice was dehydrated. The grid was then placed in the sample holder and mounted in the instrument. The instrument TECHNAI T20 was used for the analysis. The TEM images were given in Figure no.6 [15].

d) Electron Dispersive X-Ray Analysis

The elements present in the scaffold were estimated using EDAX analysis. It is given in Figure no.7 [16].

e) X-Ray Diffraction (XRD) Analysis

XRD is employed to determine the crystal-like nature. It was performed with a PAN analytical Xpert Pro X-Ray Diffractometer. The powdered sample for evaluation was taken on the glass slide and placed on the X-Ray diffractometer. The scanning rate was continued over a 2θ range of 10 to 90°. The XRD graph was given in Figure no.8.

VI. In-vitro Release Studies

100μg of the scaffold was pondered from each of the five formulations primed in different test tubes. To this, pH 7.4 phosphate buffer medium was added and placed in an orbital shaker. The quantity of ofloxacin expelled out from the scaffolds was assessed by amassing buffer medium from the test tubes and supplanting with fresh buffer at 30 minutes' intervals for 5 hours. The amount expelled out was recorded at 294 nm. The discharged amount was ascertained from the standard curve. From this percentage, drug release was calculated, and percentage drug release as plotted versus time. The in-vitro drug release graph was depicted in Figure no.9 [17].

VII. In-vitro Antibacterial activity

a) Agar Disc Diffusion Method

i. *Preparation of Inoculum*

On agar slant, cultures were conserved at 4°C. By relocating a coil of cells from the cultures to test tubes, lively cultures were developed. The anti-septic action was ascertained by the agar disc diffusion technique.

ii. *Antibacterial Activity*

The antiseptic activity was ascertained by the well diffusion method on Muller Hinton agar (MHA) medium. MHA was solubilized in purified water, and the medium was sterilized after the addition of agar. Then, the media was transferred into disinfected Petri plates and solidified. By using disinfected swab saturated with the bacterial suspension, the inoculums were spread on the plates. To the wells made, 100,200, 400μg of (F5), 50 μl negative control (HCl), and positive control of streptomycin suspension were added on respective wells. These plates were gestated at 37°C for a day. The area of inhibition was then recorded. The results were depicted in Table no.5 and Figure no.10 [18].

VIII. Results and Discussion

a) Calibration Curve of Ofloxacin

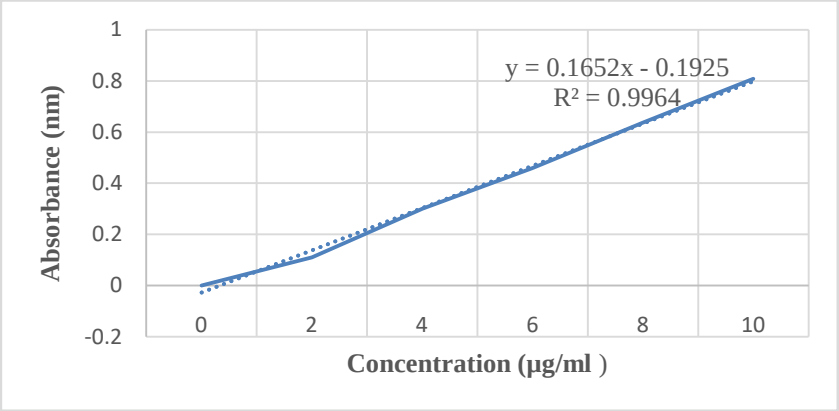

Fig. 1: Calibration curve of Ofloxacin

The calibration curve was found to obey Beers Law in the concentration range of 2-10 μg/ml as given in figure no.1.

b) Fabrication of the Nanocomposite Scaffold

Table 1: Fabrication of Scaffolds

Formulation Code	S1 (mg)	S2 (mg)	S3 (mg)	S4 (mg)
Hydroxyapatite	100	100	100	100
Chitosan	30	30	30	30
SCMC	-	10	-	-
CMC	-	-	10	-
HPMC	-	-	-	10
Ofloxacin	100	100	100	100
2% Acetic Acid	50ml	50ml	50ml	50ml
Water	100ml	100ml	100ml	100ml

Note: Details the composition of S1, S2, S3, and S4 formulations.

Fig. 2: Shows the spongy-like appearance of the scaffold.

c) *Weight Loss*

From the above shown Fig. 3 and Table 3, Scaffold S1 has a maximum weight loss of 8 % during the study. The scaffold S3 showed less weight loss compared to S2. Scaffold S4 showed the minimum loss of weight (2.7 %) in four weeks and had the less degradation [19].

Table 2: Weight loss data of scaffold formulations

Time (d)	S1 (%)	S2 (%)	S3 (%)	S4 (%)
1	1.5	0.2	0.1	0.0
3	2.7	1.2	0.9	0.0
7	3.9	2.2	1.7	0.5
15	5.4	3.1	2.4	1.2
21	6.9	4.2	3.3	1.9
28	8.0	5.1	4.0	2.7

Note: Represents the loss of weight in % of scaffolds at predetermined time intervals for 28 days.

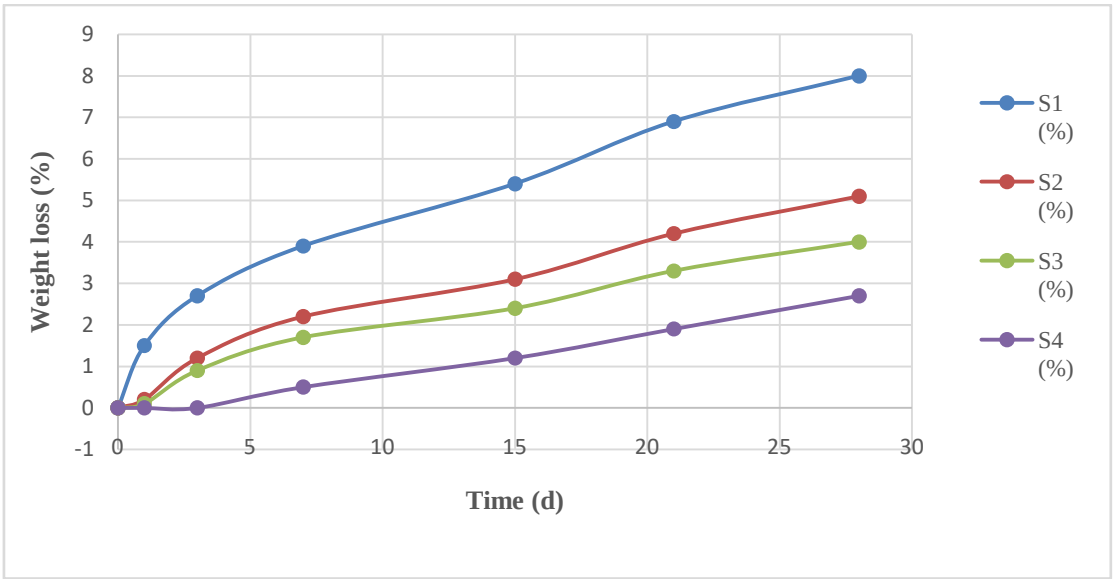

Fig. 3: Weight loss graph of scaffold formulations

From the above results, Scaffold S2 has a maximum weight loss of 8% during the study. The scaffold S4 showed minimum weight loss compared to S3. Scaffold S5 showed the least loss of weight (2.7%) in four weeks.

d) Swelling Ability

The swelling was similar in all the scaffold formulations due to constant hydroxyapatite and chitosan concentrations, as given in table no.3 [20].

Table 3: Swelling ability of scaffold formulations

Formulation Code	W_i (g)	W_f (g)	Swelling Ability (%)
S1	1.00	2.40	140
S2	1.00	2.60	160
S3	1.00	2.50	150
S4	1.00	2.90	190

Table 3 shows the Parched mass (W_i in g), damp mass (W_f in g) and swelling ability (%) of scaffold formulations.

e) Porosity Measurement

The porosity of the scaffold formulations was similar to one another, as given in table no.4.

Table 4: Porosity measurement data of scaffold formulations

Formulation Code	W_w (g)	W_d (g)	W_l (g)	Porosity (%)
S1	0.58	0.25	1.15	57.89
S2	0.59	0.25	1.19	56.66
S3	0.62	0.25	1.20	63.79
S4	0.65	0.25	1.24	67.79

Table 4 reveals the parched mass (W_w in g), dry weight (W_d in g), dipped mass (W_l in g) and porosity (%) of the scaffold formulations.

f) FT-IR Analysis

The results of the analysis showed various stretching, bending, and rocking vibrations based on the groups present. All the spectra indicated that there are no significant drug-excipient interactions.

IX. Surface Analysis

a) Scanning Electron Microscopy (SEM)

The images exhibit that the scaffold has an elongated surface which is shown in figure no.4.

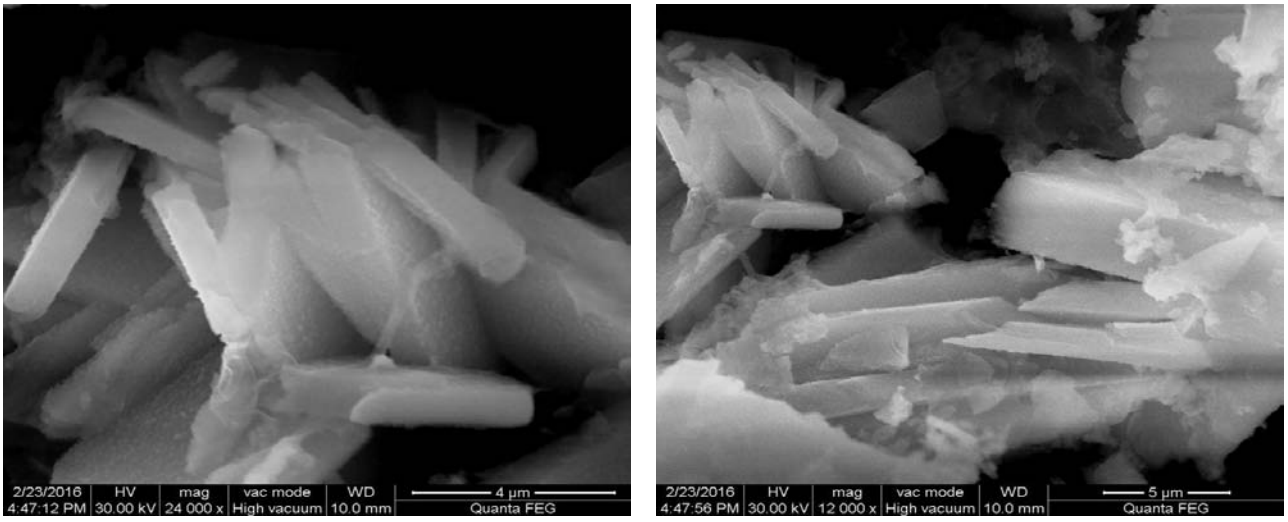

Fig. 4: SEM image

Fig. 4: Portrays the SEM image of the S4 scaffold.

b) Optical Microscopy

The images exhibited that the scaffold was found to have a flat structure with smooth surface morphology, as shown in figure no.5.

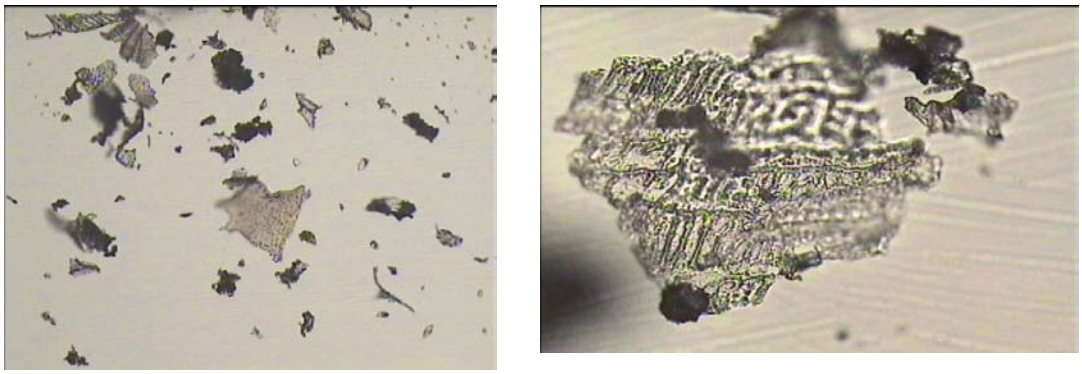

Fig. 5: Image of optical microscopy

Fig. 5 portrays the optical microscopy image of the S4 scaffold where a) 10X image b) 40X image

c) Transmission Electron Microscopy (TEM)

TEM report analysis revealed the presence of internal morphology of nanocomposite scaffold with the sizes of 0.1μm, 0.2μm, and 0.5μm. The internal morphology shows elongated flakes of the scaffold as depicted in Figure no.6 [21].

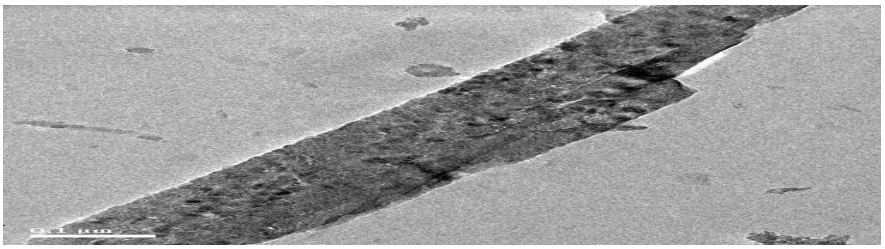

Fig. 6: TEM image

Fig. 6 portrays the TEM image of S4 scaffold.

d) Energy Dispersive X-Ray Analysis

The scaffold contains oxygen(O), carbon(C), calcium(Ca), phosphorus(P), magnesium (Mg) and chlorine(Cl) at 50.80%, 24.93%, 24.64%, 8.19%, 0.50% and 0.36% respectively as shown in Figure no.7 [22].

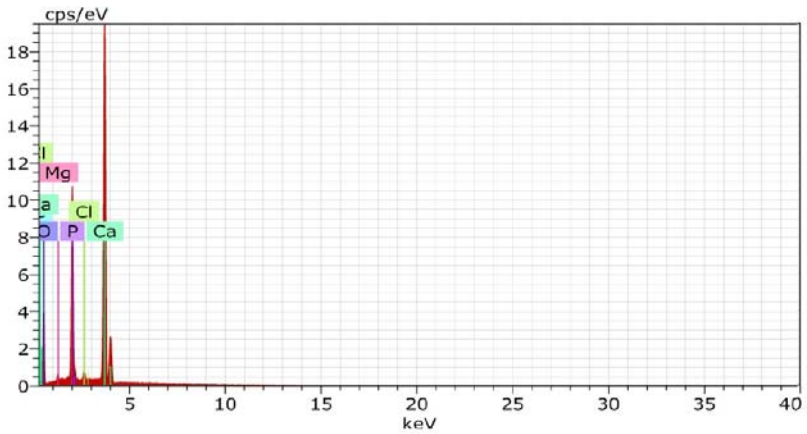

Fig. 7: EDAX analysis

Fig. 7 shows the presence of various elements and their composition of S4 scaffold.

e) X-Ray Diffraction (XRD) Analysis

The peaks were obtained at 2Θ level at positions 27.21, 29.15, 30.65, 34.35, 37.03, 41.61, 45.44, 48.45, 50.42, 53.60, 59.59, 64.05, and 68.77. Hence, the formulated scaffold was found to exhibit the crystalline structure of the scaffold as depicted in figure no.8.

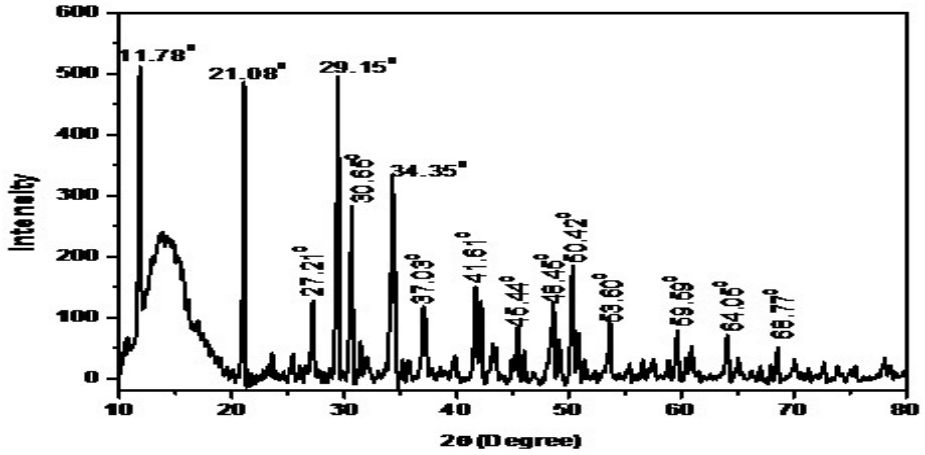

Fig. 8: XRD graph

Fig. 8 shows the 2θ (degree) vs intensity XRD graph of S4 scaffold.

X. *In-vitro* Release Studies

From the Figure no.9, the scaffold S4 showed an initial burst release succeeded by a persistent release and the release rate was found to be 100% at the end of 8hours, whereas scaffolds S2 and S3 showed release of 62%, and 82% at the end of 8 hours study. However, S5 showed a sustained release profile over an extended period of study of upto 24 hours. Hence, the formulation S5 has been optimized for characterization [23].

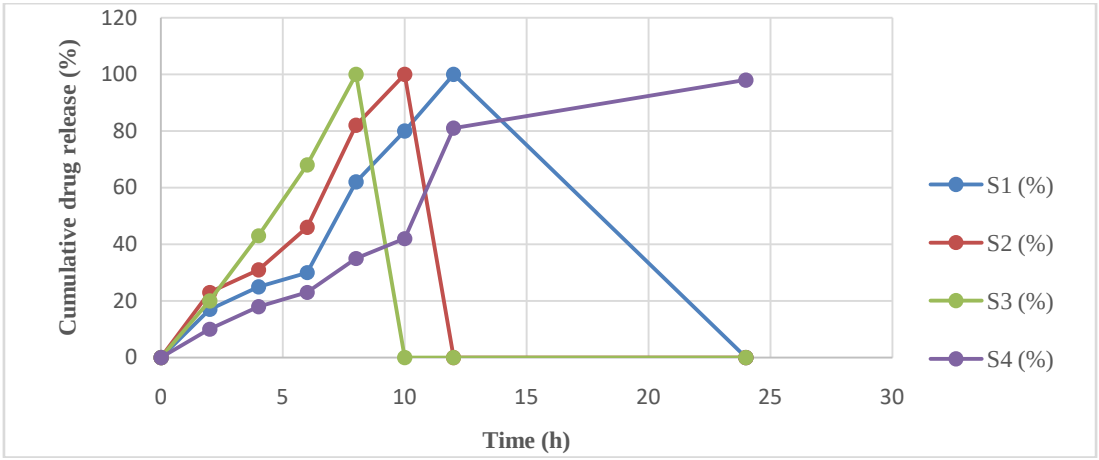

Fig. 9: *In-vitro* drug discharge profile graph

Fig. 9 shows the time (h) vs. cumulative percentage drug release (%) of the scaffold formulations.

a) In-Vitro Antibacterial Activity

Table 5: *In-vitro* antiseptic action results

S.No.	Microorganisms	Zone of Inhibition (mm)				
		100μg	200μg	400μg	HCl (negative control)	Streptomycin 15 μg (positive control)
1	*Escherichia coli*	26	32	34	23	16

From the report of the antibacterial activity of the formulated scaffold as shown in Table no.5 and Figure no.10, it was found that the scaffold with various concentrations 100μg, 200μg and 400μg when compared with standard positive and negative control, showed maximum zone of inhibition of 26mm, 32mm and 34mm respectively. Hence the prepared scaffold exhibits antibacterial activity [24].

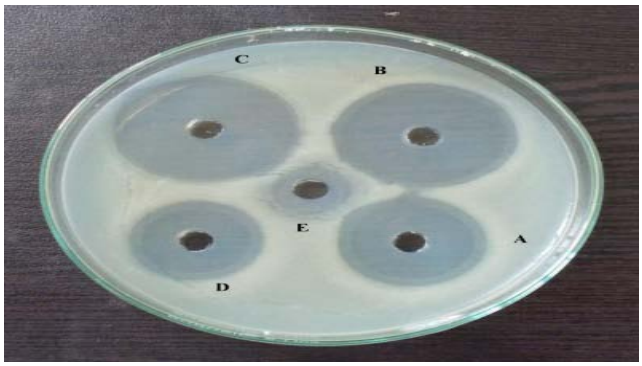

Fig. 10: *In-vitro* antibacterial activity against *E.Coli*

Fig. 10 shows the agar disc plate used for *in-vitro* antibacterial activity where A represents 100 μg of scaffold, B represents 200 μg of scaffold, C represents 400 μg of scaffold, D represents hydrochloric acid (negative control) and E represents standard (positive control).

XI. Conclusion

The scaffold is a versatile bioactive product among wound dressing materials, whose production is flexible and economical. The present work was aimed towards fabricating a scaffold containing hydroxyapatite using various polymers like chitosan, carboxy methylcellulose (CMC), sodium carboxy methylcellulose (SCMC), and hydroxypropyl methylcellulose (HPMC) by freeze-drying technique by incorporating Ofloxacin as an anti-microbial agent. Five formulations, namely S1, S2, S3, S4, and S5, were prepared using various combinations of the polymers mentioned. The prepared scaffolds were studied for their characteristic properties like weight loss, swelling ability, porosity, and *in-vitro* drug release studies. The optimized formulation (S5) was characterized by SEM, optical microscopy, TEM, EDAX, XRD, FT-IR, and *in-vitro* antibacterial activity.

Due to the greater water acceptance, sufficient porosity, improved antibacterial activity, and extended drug release, the hydroxyapatite-chitosan-HPMC-ofloxacin scaffold would be a hopeful biomaterial for bone tissue engineering. From this research, it was concluded that the nano-composite scaffold is a viable alternative to existing conventional dosage forms, which lead to improved bioactivity and a promising biomaterial for bone tissue engineering in case of administration affords resulting in better patient compliance and cost-effective therapy in the field of biomedical application.

Conflicts of Interest

Nil

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