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Genipin as a Cross-linker in a Ciprofloxacin Delivery System Containing a Bovine Hydroxyapatite-Collagen Composite for Bone Infections

Nurul Fitriani[1,*], Aniek Setiya Budiatin[2], Esti Hendradi[3]

[1]Departement of Pharmaceutical and Pharmacy Technology, Faculty of Pharmacy, Mulawarman University, Samarinda-75119, East Kalimantan, Indonesia.
[2]Departement of Clinical Pharmacy, Faculty of Pharmacy, Airlangga University, Dharmawangsa Dalam, Surabaya-60286, East Java, Indonesia.
[3]Departement of Pharmaceutics, Faculty of Pharmacy, Airlangga University, Dharmawangsa Dalam, Surabaya-60286, East Java, Indonesia
*Corresponding author: nurulfitriani2307@gmail.com / nurulf@farmasi.unmul.ac.id

Abstract

The purpose of this research was to design an implant for a ciprofloxacin-based drug delivery system by combining bovine hydroxyapatite and collagen with genipin as the crosslinking agent. The production of ciprofloxacin implants using bovine hydroxyapatite:collagen blend (70:30). In addition, this synthetic preparation was made using three various concentrations of genipin (0.6, 0.8, and 1.0%). The pellets were created by compressing the implants. The tablets are cylindrical with a diameter of 4.0 mm and a weight of 100.0 mg. Ciprofloxacin cultures were characterized for swelling rate, porosity, density, compressive strength, morphology (SEM), dose, and drug release in vitro. The addition of genipin as a crosslinking agent may maintain ciprofloxacin release consistent with in vitro therapeutic levels of ciprofloxacin. These results are supported by compressive strength data, where the addition of genipin concentrations induces higher implant stiffness and scanning electron microscopy photomicrographs reveal small pore sizes and BHA adhere to collagen fibers so that ciprofloxacin is completely dispersed in the implant after cross-linking with genipin. As a drug delivery system for osteomyelitis, it can be concluded that the use of genipin as a cross-linking agent can sustain ciprofloxacin release commensurate with in vitro therapeutic levels of ciprofloxacin for 30 days.

Keywords: Ciprofloxacin, Collagen, Bovine hydroxyapatite, Genipin, Implant

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1 Introduction

Osteomyelitis is an infectious bone disease, characterized by the inflammation of the bone marrow and are often associated with destruction of cortical and trabecular bones [1]. Osteomyelitis occurs when microorganisms enter the bloodstream, directly from infected objects. Antibiotics can be used to reduce bacterial infection rates. In the case of a malfunction, however, the presence of devascularized tissue hinders the transport of the antibiotic to the target area. This leads to low on-target antibiotic concentrations and causes antibiotic resistance [2]. In addition, bone infections also relate to microorganisms forming biofilms which then release single cell or fragments. This allows the formation of a new colony surface [3]. Antibiotics administered intravenously or orally over an extended length of time can be used to reduce the risk of infection. [4]. Nonetheless, such limitations can not be overcome by increasing the systemic dosage as it allows toxicity due to the high concentration of antibiotic usage[1]. It is difficult to achieve the appropriate dosage by administering antibiotics intravenously or orally [5].

Drug formulation using implanted antibiotic delivery devices established for the treatment of bone infections might solve these issues. Implants are one of several local medication delivery system choices. This method has the benefit of achieving effective medication concentrations at the site of infection while maintaining safe blood levels [6]. A component in drug delivery systems (ciprofloxacin) is an implant consisting of an organic, inorganic, and cross-link phases. The organic phase is type I collagen, while the inorganic one consists of BHA, whereas the cross-link is genipin.

Ciprofloxacin is one of the most extensively distributed fluoroquinolone antibiotics and exhibits high bactericidal action against a broad spectrum of Gram-positive and Gram-negative bacteria with therapeutic significance. It has been used successfully to treat a wide range of bacterial infections, including urinary tract infections, gastrointestinal infections, sexually transmitted infections, and skin and bone diseases [7].

Collagen, as a biomaterial of natural origin, has many advantages over most polymers of synthetic origin. It exhibits superior biocompatibility, biodegradability and interlinked porous structure. collagen-hydroxyapatite synthetic scaffolds incorporate both collagen and HA into a porous scaffold matrix. These materials are two major bone components and a reasonable choice as the basis of biomimetic scaffolds capable of supporting and promoting bone regeneration [8]. Bovine bone biowaste can be use as a potential source of hydroxyapatite (BHA), thus it is important to prepare in a simple and environmentally friendly way [9].

Due of its minimal cytotoxicity, Genipin surpasses other chemical crosslinking agents. Crosslinking genipin results in improved mechanical strength and resistance to enzymatic degradation in vitro. Comparable to glutaraldehyde, it has cytotoxic potential but is approximately 5,000 to 10,000 times less hazardous [10].

Synthetic components of bovine hydroxyapatite and collagen can be formed in ratio 70:30. This is because bones are made up of organic elements (30%) and minerals compound (70%). The mineral portions of the bone provide consistent stiffness and mechanical properties. The model compound corresponding to the bone mineral phase is hydroxyapatite (HAp) and the organic phase is collagen type 1 [11].

In addition to being highly porous with a large surface-to-volume ratio to provide more space for cell adhesion and proliferation while also allowing the metabolism of nutrients and wastes, the ideal scaffold for tissue engineering must also have low cytotoxicity, exceptional biocompatibility, and biodegradability. However, collagen scaffold breakdown in vivo is also uncontrolled, particularly due to the presence of collagenase in vivo. It may be possible to overcome drawbacks by covalently

cross-linking collagen fibers inside and between constituent collagen molecules [10]. The addition of genipin as a crosslinking agent improves the physical properties of the implant and achieves the release profile of the ciprofloxacin delivery system. Optimization of genipin concentrations was performed at three various concentrations. Genipin concentration was 0.6, 0.8, and 1.0%.

2 Experimental section

2.1 Materials

Bovine hydroxyapatite (BHA) (University Hospital's Tissue Bank Dr. Soetomo, Indonesia), Ciprofloxacin (Shangyu Jingxin Pharmaceutical, Shangyu), Collagen (PT.Biochitosan). Genipin (Challenge Bioproducts), Alcohol 85%, NaCl (merck), KH2PO4 (merck), Na2HPO4 (merck), Aquabidestilata (PT. Widatra Bhakti, Indonesia). All other ingredients used are pharmaceutical grade.

2.2 Formulation of BHA-Collagen-Ciprofloxacin Implant Using Genipin as Cross-link Agent

Ciprofloxacin was dissolved in aquabidestilata, BHA was added gradually, and the mixture was stirred until it was homogenous. Collagen powder was added to the combination of ciprofloxacin and BHA. Aquabidestilata are added gradually while continually stirring until they form a mass of moist granules. To get dry pellets, wet pellets were sieved through a 1 mm sieve and dried overnight at 40°C. The seeds were steeped in 0.6%, 0.8%, and 1% genipin solutions for five days till discolouration [10]. Table 1 lists the components of the various formulations. To eliminate any remaining genipin, the beads were washed three times with 85% alcohol and once with aquabidestillata. The pellets were finally rinsed with phosphate-buffered saline (PBS) pH 7.40. The pellets were dried at 40 degrees Celsius for twenty-four hours. The 100 mg of dry granules were compressed using a tablet press with a diameter of 4 mm and a compression pressure of 2 tons.

Table 1: Formulation of implant using genipin as cross-link agent

Formulation code	Composite composition (BHA:collagen)	Genipin concentration (%v/v)
F1	70:30	0.6
F2	70:30	0.8
F3	70.30	1.0

2.3 Evaluation of Implant

2.3.1 Porosity and density test [8]

Fluid displacement is used to determine an implant's porosity and density. Water is the liquid utilized in this investigation. A sample of known weight (W) is submerged for 10 seconds in a graduated cylinder containing a known volume of water (V1). V2 is the total volume of water contained within the submerged cylinder and scaffold. The residual water volume was measured when the water-soaked scaffold was removed from the cylinder (V3). Each specimen was measured thrice. Density of porous samples and porosity of scaffold represented as show in equation 1 and equation 2.

$$\text{Porosity} = \frac{V1-V3}{V2-V3} \text{ x } 100\% \quad \text{(equation 1)}$$

$$\text{Density} = \frac{W}{V2-V3} \quad \text{(equation 2)}$$

2.3.2 Determination of the swelling ratio [10]

After weighing the dry scaffolds (w0), they were hydrated in PBS for 10 seconds at room temperature. The wet scaffold was reweighed after filter paper was used to extract excess surface water (w). The expansion rate of the scaffold was calculated by comparing the increase in wet weight (w - w0) to the original weight (w0).

2.3.3 Hardness test [12]

Using the E-10 signature equipment, the implant's hardness was evaluated. At a rate of 5 mm/min, the implant was compressed with a load cell compressor. The hardness of the implant is determined by dividing the force (F in

newtons) indicated on the device by the implant's contact surface (in mm).

2.3.4 Scanning electron microscopy (SEM) [10]

Using a scanning electron microscope, the morphology of the created scaffolds was analyzed. The mold is attached to copper rods with mutually conductive tape and coated with gold spray paint. Using a SEM microcomputer, the pore diameter was determined.

2.3.5 In vitro drug release study [13,14]

The vial approach was used to study drug release from the implant. In order to conduct a drug release research, the inoculum was immersed in a vial containing 5 ml of phosphate-buffered saline (PBS) with a pH of 7.4. The vials were put on a rack and incubated at 37±0.5°C in a water bath. Sampling was performed by pouring 1 ml of the eluate at predetermined intervals (1^{st}, 3^{rd}, 5^{th} , 7^{th} , 9^{th} , 12^{th} , 14^{th} , 16^{th} , 18^{th} , 20^{th} , 22^{th} , and 24^{th} h on first day and every 24 h for 30 days) and replace with new gasket to maintain sink condition. The sample solution was filtered via a 0.45-m-thick Millipore membrane. Phosphate-buffered saline (PBS) pH 7.4 was used to provide a suitable diluent. At three wavelengths, the absorbance of the solution was measured using a UV spectrophotometer (260, 270, and 280 nm). Using a standard curve, the drug concentration in the sample was determined. Percentages of cumulative drug release found at each time period. In triplicate, the release of ciprofloxacin HCL from the implant was evaluated.

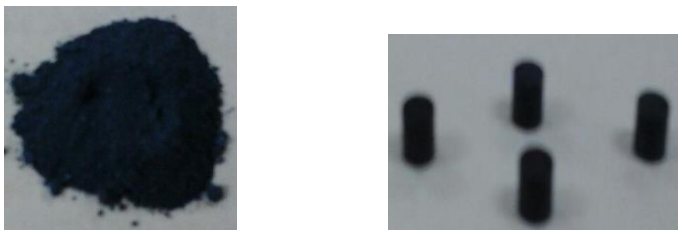
2.3.6 Statistical analysis [10]

The data are provided as the mean standard deviation, and the levels were analyzed using one-way analysis of variance (ANOVA). P value less than or equal to 0.05 was considered statistically significant.

3 Results and Discussion

3.1 Formulation of BHA-Collagen-Ciprofloxacin Implant Using Genipin as Cross-link Agent.

The construction of the implant begins with the production of dry granules. Genipin can react spontaneously with amino groups in amino acids or proteins to produce a dark blue colour. The color change in scaffolds crosslinked with genipin indicates microstructural changes upon crosslinking [10]. The crosslinking process has been described as a nucleophilic substitution reaction involving the replacement of the ester group on the G molecule with a secondary amide bond [15]. The dried pellets are dark green in color and about 1 mm in diameter. The dried granules are then pressed by a pellet machine to produce cylindrical particles with a diameter of 4 mm.



(A) (B)

Fig. 1: Formulation process of BHA-collagen-ciprofloxacin implant using genipin, A) dry granules, B) implant (cylindrical pellets)

3.2 Evaluation of Implant

3.2.1 Porosity and density

Figure 2 depicts the porosity of the implant at three different genipin concentrations. There was no statistically significant difference in porosity between BHA-collagen-ciprofloxacin implants with three different concentrations of genipin, as determined by a one-way anova ($p > 0.05$). On the basis of these findings, it was determined that the variation in genipin concentration had no effect on implant porosity.

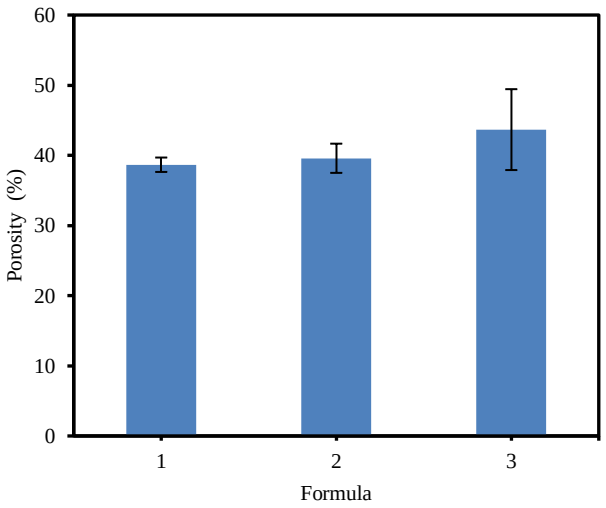


Fig. 2: Porosity of F1 to F3 formulations

Figure 3 depicts the density of the implant at three different genipin concentrations. There was no statistically significant difference in porosity between BHA-collagen-ciprofloxacin implants with three different concentrations of genipin, as determined by a one-way anova ($p > 0.05$). On the basis of these findings, it was determined that the variation in genipin concentrations had no effect on the implant density.

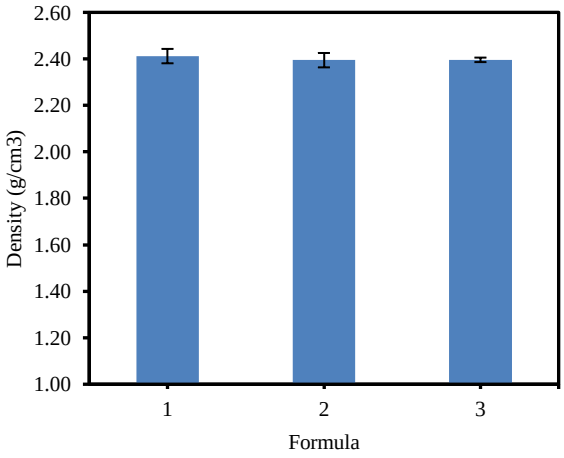


Fig. 3: Density of F1 to F3 formulations

3.2.2 Swelling ratio

Figure 4 depicts the proportion of implant enlargement with three different concentrations of genipin. There was no statistically significant difference in porosity between BHA-collagen-ciprofloxacin implants with three different concentrations of genipin, as determined by a one-way anova ($p > 0.05$). On the basis of these data, it was determined that the variation in genipin concentrations had no effect on the implant's swelling rate.

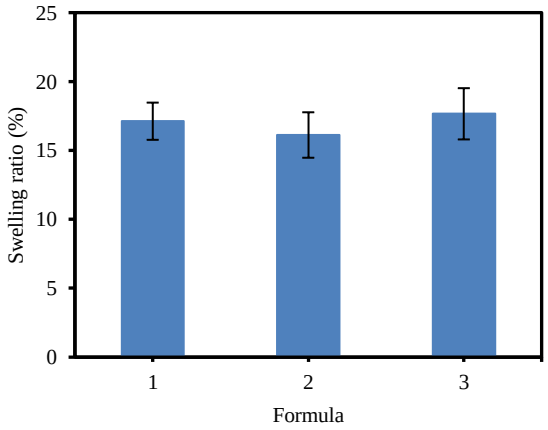


Fig. 4: Swelling ratio of F1 to F3 formulations

3.2.3 Hardness test

Figure 5 depicts a test of implant stiffness with three different doses of genipin. A one-way anova revealed a statistically significant difference in compressive strength between 1%-Gp(1) and 0.6%-Gp(2). Based on these results, one can conclude that the addition of a higher concentration of Genipin results in higher rigidity of the implant. When the crosslinking reagent was present at lower concentrations (0.1 to 1%), the gain in mechanical strength outweighed the impediment caused by the enlarged pore size. Consequently, the ultimate compressive strength increased [15].

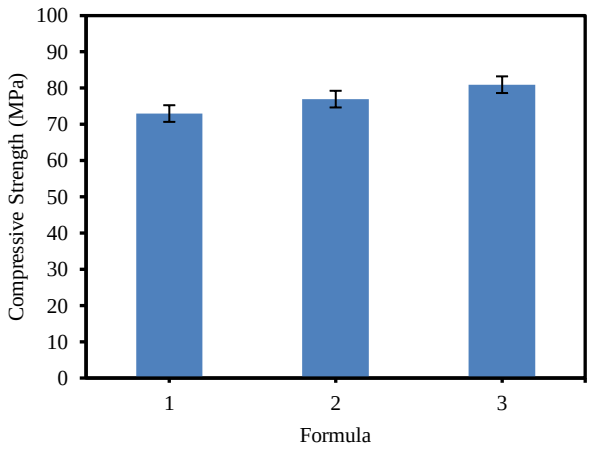


Fig. 5: Hardness test of F1 to F3 formulations

3.2.4 Morphological characterization

Scanning electron microscopy was used to examine cross-sections of reticular formations. Figure 6 depicts the reticle's tiny pictures. The structure of the implant is porous and dense. This disease causes the implant's structure to grow dense and its pores to shrink.

3.2.5 In vitro drug release study

Cumulative drug release from the three formulations (F1 to F3) was determined and shown in Fig. PBS pH 7.40 showed that in vitro release of ciprofloxacin after cross-linking was at therapeutic levels of ciprofloxacin (2-50 µg/ml) for 30 days [15,16]. This profile suggests that genipin can maintain ciprofloxacin release consistent with in vitro therapeutic levels of ciprofloxacin. On the basis of statistical analysis employing a one-way anova, the following conclusion may be drawn there was no significant difference in AUC BHA-collagen-ciprofloxacin inoculated with three different concentrations (0.6 %-Gp(1); 0.8%-Gp (2);1%-Gp(3)) Genipin. The variation in genipin concentrations has no effect on the AUC value of the implant, it may be inferred.

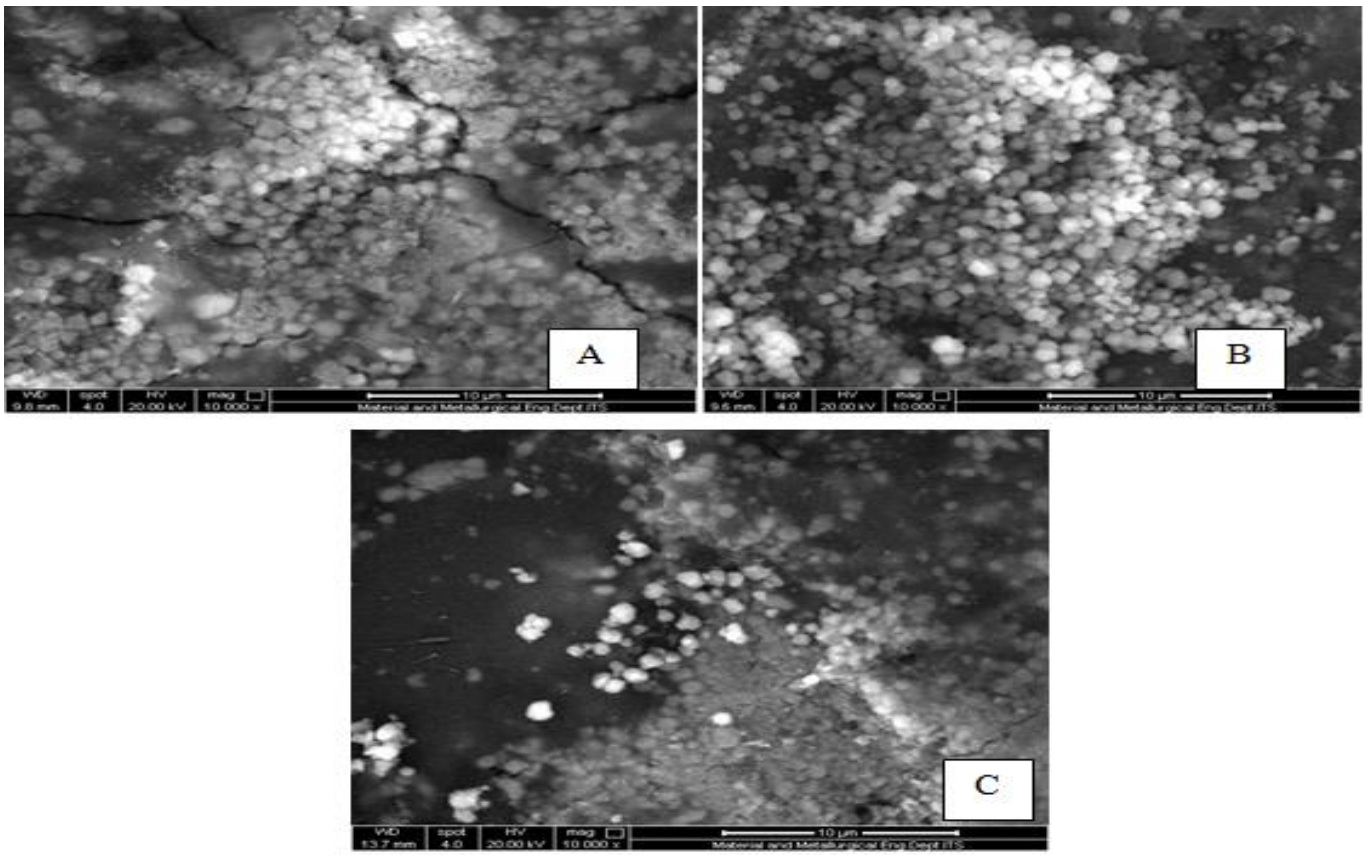


Fig. 6 SEM Images of BHA-Collagen-Ciprofloxacin Implants with Genipin as cross-link agent (Magnification 20.000X). A. 0,6%-Gp; B. 0,8%-Gp; C. 1%-Gp.

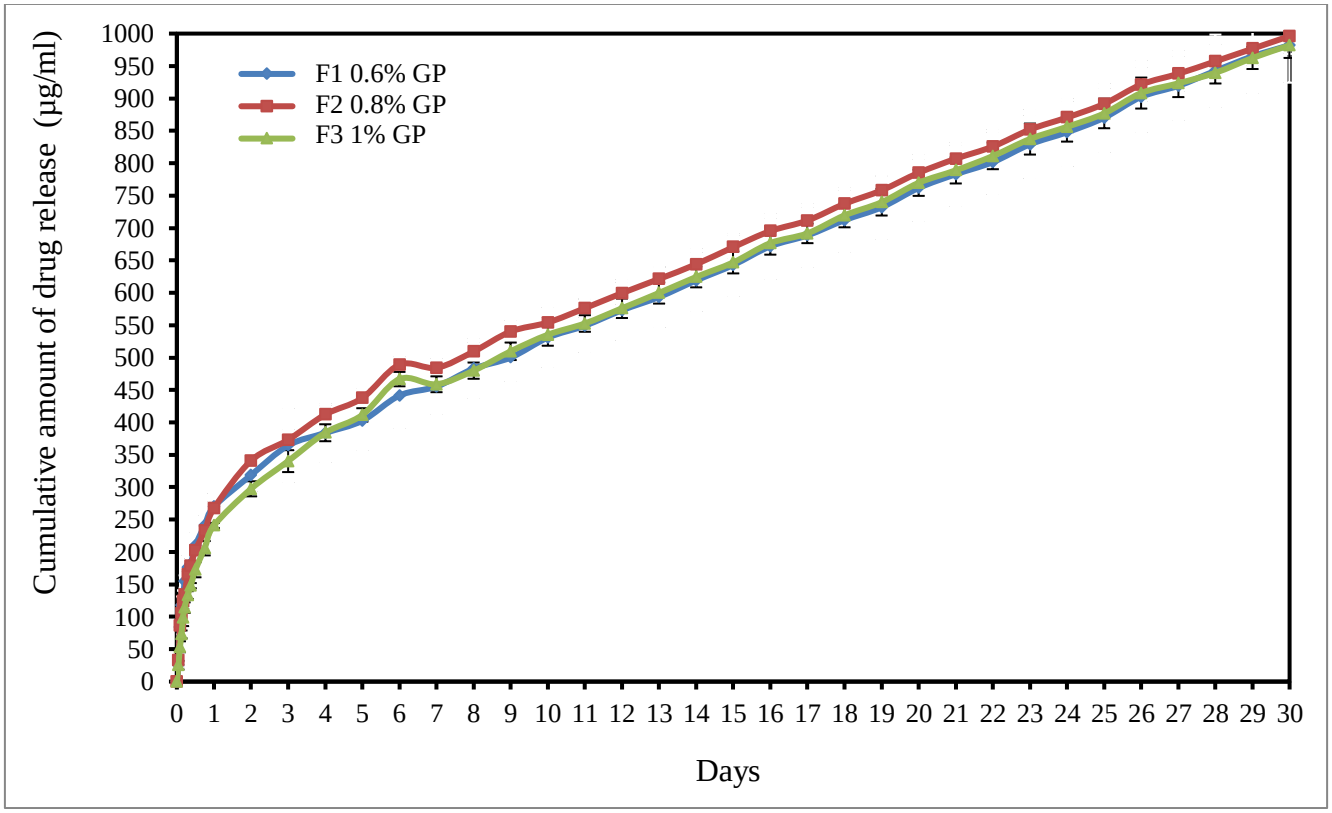


Fig. 7: Ciprofloxacin release profile of F1 to F3 formulations

4 Conclusions

The properties of hydroxyapatite-collagen-ciprofloxacin cross-linked bovine genipin can be strongly influenced by various concentrations of genipin. For optimum mechanical strength, a genipin concentration of 1.0% should be used. The release of ciprofloxacin from bovine hydroxyapatite-collagen cultures with genipin as a cross-linking agent lies between the in vitro therapeutic levels of ciprofloxacin for 30-day osteomyelitis.

5 Declarations

5.1 Author Contributions

NF, developed the concept and designed the manuscript; ASB and EH provided key information and intellectual support.

5.2 Funding Statement

This research was not supported by any funding sources.

5.3 Conflicts of Interest

The authors declare no conflict of interest.

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