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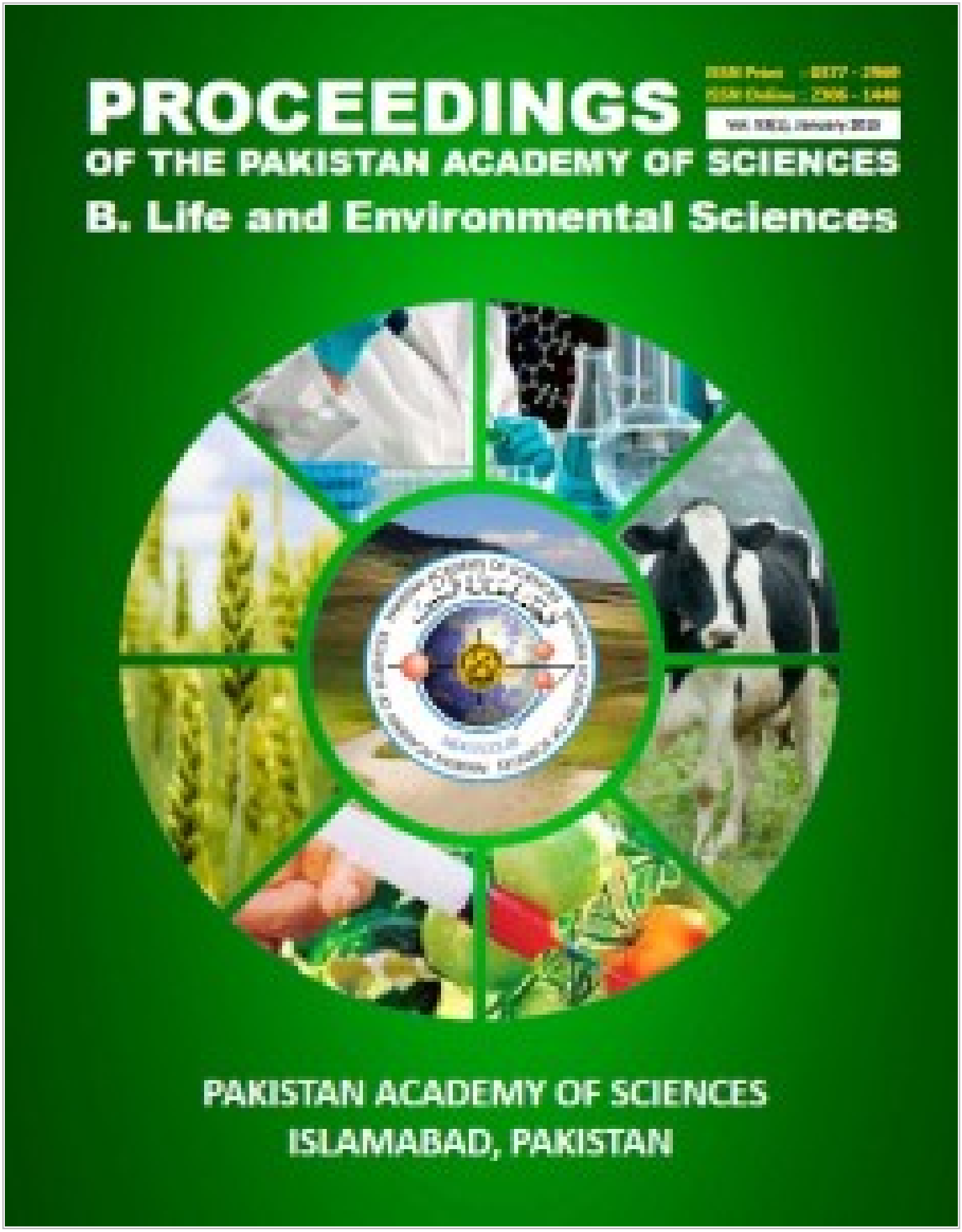

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Research Article

The Effect of Composite Composition Ratio on the Physicochemical Characteristics of Bovine Hydroxyapatite-Chitosan-Ciprofloxacin Implant

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Abstract: This study aimed to develop bone implant as either deliver ciprofloxacin to the bone or regenerate bone defects. The effect of the composite composition ratio (Bovine Hydroxyapatite-Chitosan) on the physicochemical characteristics of Bovine Hydroxyapatite-Chitosan-ciprofloxacin implant were evaluated in this study. Ciprofloxacin implants were prepared using four different compositions of composite (Bovine Hydroxyapatite-Chitosan) 20:80; 30:70; 40:60; and 70:30. The amount of ciprofloxacin in each implant was 10 %. The powder mixture was then pressed into pellets. The implants were evaluated by various parameters such as porosity, density, water absorption capacity, swelling ratio, disintegration test, compression force, drug assay, and in vitro drug release. Characterization of the implants was conducted using Fourier Transform Infrared (FT-IR), Scanning Electron Microscopy (SEM), and X-ray diffraction study. The results obtained from this work revealed that the composite composition ratio (Bovine Hydroxyapatite-Chitosan) influenced the differences in physical characteristics and the release of ciprofloxacin from Bovine Hydroxyapatite-Chitosan-ciprofloxacin implant. The iincreased in the Bovine Hydroxyapatite ratio in composite composition caused enhancement of physical characteristics (porosity, density, water absorption capacity, and compressive strength) of the implant. Meanwhile, increase in Bovine Hydroxyapatite ratio in implant caused lower release of ciprofloxacin. The best composite composition ratio to produce Bovine Hydroxyapatite-Chitosan-ciprofloxacin implant with desired physical characteristics and ciprofloxacin release was Bovine Hydroxyapatite-Chitosan (70:30).

Keywords: Composite, bovine hydroxyapatite, chitosan, ciprofloxacin, bone implant.

1. INTRODUCTION

Bone is a composite like material which consists of organic and inorganic components such as collagen filaments, nanocrystallites, and hydroxyapatite in an orderly arrangement layered over several lengths [1]. A growing demand for bone implants is observed worldwide. Every year, approximately 2×10^6 patients sustain a bone surgical procedure to repair bone defect caused by a disease or a traumatic event. A biomaterial that has been widely applied to develop bone implant is synthetic hydroxyapatite (HA). Synthetic hydroxyapatite is a biocompatible, osteoconductive, and osteoinductive [2,3]. However, synthetic hydroxyapatite has several limitations such as high crystallinity, low porosity, poor mechanical properties, the risk of residual solvent, and relatively expensive [4]. To surmount these problems, Bovine

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Hydroxyapatite has been produced as the constituent material. Bovine Hydroxyapatite is an organic component derived from bovine bones and has adsorption ability of active factors like antibiotics, hormones, and growth factors [5]. The results from *in vitro* and *in vivo* studies indicated that the natural apatite has better osteoconductive characteristics than synthetic hydroxyapatite [6].

Application of synthetic hydroxyapatite or Bovine Hydroxyapatite as a single component to produce implant has several problems. Bovine Hydroxyapatite produces an implant with rigid characteristic and fragile [7]. Bovine Hydroxyapatite as single materials to construct an implant can act as a carrier for drugs, but the release system of the drug is hard to predict. Referable to the nonbiodegradable characteristics, these materials do not seem to be very suitable in tissue engineering perspective [8]. One path to overcome the weakness of hydroxyapatite as a single component in the bone implant is composite. Composite of polymers and ceramics such as Bovine Hydroxyapatite can be developed to produce implant with sufficient mechanical performance and promote bone development [9,10]. The combination of an inorganic material and organic material can produce an implant with desired characteristics in the bone engineering field [11].

Various kinds of synthetic and natural polymers have been applied as organic materials in composite and control drug delivery system in humans [12]. Among the natural polymers, chitosan is extensively used in the development of scaffolds and tissue-like materials which mimic body tissues. Chitosan is a natural polymer obtained from crab shell, jellyfish, coral, and shrimps [1]. Chitosan molecules built from glucosamine and N-acetylglucosamine constituent connected through the 1-4 glycosidic bonds. Chitosan has similar bioactivity like glycosaminoglycans and hyaluronic acid, which present in articular cartilage [9]. Chitosan is a natural cationic polymer that is biologically renewable, biodegradable, biocompatible, nonantigenic, nontoxic, and bifunctional. Chitosan is a useful material in bone reconstruction application and tissue engineering due to its hydrophilic surface, biocompatibility with human tissue, biodegradability by lysozyme, and the ability to promote cell growth [13].

Morphology and physicochemical characteristics of the implant are influenced by composite composition ratio. An ideal implant must have a porous structure, clear pores, and interconnected pores [14]. According to the outcomes of the previous survey, it can be concluded that Chitosan-hydroxyapatite composite produces a macroporous interconnected structure that supports human osteoblast attachment and proliferation, enhance bone mineralization, and extracellular matrix impeachment [15]. Moreover, hydroxyapatite-chitosan composites, which have been loaded with the drug can improve tissue regeneration. The drugs which are entrapped in the structure of the implant will leach out and release in a controlled pattern around the implantation sites. This process will decrease the postoperative complications incidents and encourage tissue regeneration [16]. Nowadays, several research groups have developed composites

consist of hydroxyapatite-chitosan as a drug delivery system in the fields of hard tissue engineering [17].

The development of chitosan-hydroxyapatite composites to control the delivery of the drugs to the bone has been researched. Chitosan and hydroxyapatite built a porous structure where the drug will be entrapped inside the pore. In a study, tetracycline hydrochloride has been used as a drug model. The concentration of tetracycline was 10 % w/w. The hydroxyapatite composition in this composite was 20 % and 40 %. The composite ratios which have been studied were Chitosan 80 % - hydroxyapatite 20 % and Chitosan 60 % - hydroxyapatite 40 % [16]. During the initial period, approximately 30 % of tetracycline was dissolved in the media for 2 h. This period revealed the rapid drug release or initial burst of the drug when implant contact with the media. Burst effect phenomenon caused by the release of the drug which attaches at the superficies of the composite. The sustained release period took place for 72 h. The outcomes from this study indicated that increased hydroxyapatite content in scaffolds caused decreasing tetracycline hydrochloride release rate [16].

In other research, hydroxyapatite-Chitosan composites have been produced in several ratios by a one-step co-precipitation method. The ratio of hydroxyapatite-Chitosan composites which have been studied were, 15:85; 30:70; and 70:30 [18]. It can be reasoned that the optimal concentration of Chitosan in bone composites was 15 % to 80 % in this study [16,18]. In the present study, ciprofloxacin hydrochloride is selected as drug models to overcome bone infection. The objective of this research was to prepare a bone composite designed as an implant using hydroxyapatite-Chitosan composite in several ratios (20:80; 30:70; 40:60; and 70:30) to control ciprofloxacin release. The effect of composite composition ratio (Bovine Hydroxyapatite - Chitosan) to the physical characteristics and the release of ciprofloxacin from Bovine Hydroxyapatite - Chitosan - ciprofloxacin implant had been considered.

2. MATERIALS AND METHODS

2.1 Materials

Ciprofloxacin (Shangyu Jingxin Pharmaceutical, Shangyu, China, CO., LTD), Chitosan (PT. Biotech Indonesia, Cirebon, Indonesia), Bovine Hydroxyapatite (Tissue Bank Departement of Dr. Soetomo Hospital, Surabaya, Indonesia), Glacial acetic acid (p.a) (Merck Millipore®, USA), Disodium hydrogen phosphate (p.a) (Merck Millipore®, USA), Potassium hydrogen phosphate (Merck Millipore®, USA), Sodium Chloride (Merck Millipore®, USA) and Distilled water. Instruments which were used in this study include grinder (Keenwood® AT320A), hydraulic tablet press machine and waterbath (Memmert® GmbH).

2.2 Methods

2.2.1 Preparation of Homogenous Chitosan Powder

The homogenous chitosan powder was made by neutralization of chitosan solution in acetic acid (2 %) w/v using 1 M NaOH until neutral condition reached (pH = 7). This step produced chitosan gels.

In the following step, chitosan gels were dried in tray dryer for 24 h in the temperature 40 °C. Dried chitosan gels, then were milled using grinder to produce homogenous chitosan powder [9].

2.2.2 Preparation of Bovine Hydroxyapatite-Chitosan-Ciprofloxacin Implants

Ciprofloxacin was dissolved in distilled water, and then Bovine Hydroxyapatite was added to this solution until produce wet mass like paste. Chitosan powder was mixed to the mass like paste. Distilled water was poured slowly as a granulating liquid to make a wet granule mass [11]. Wet granule mass, then was milled using 1 mm sieve and dry in temperature 50 °C for 24 h to produce dried granules. A composite composition which has been applied in this work can be viewed in Table 1. Dried granules were weighed 100 mg, and compressed using 2 t compression pressure using tablet press machine.

Table 1. Composite composition (Bovine Hydroxyapatite-Chitosan) to produce an implant

Formulation code	Composite composition (Bovine Hydroxyapatite-chitosan) ratio	Ciprofloxacin (gram)	Bovine Hydroxyapatite (gram)	Chitosan (gram)
F1	20:80	0.011	0.0178	0.0712
F2	30:70	0.011	0.0267	0.0623
F3	40:60	0.011	0.0356	0.0534
F4	70:30	0.011	0.0622	0.0268

2.3 Evaluation of implants

2.3.1 Density and Porosity Test

Density and porosity test was conducted to predict the compactness of the implant. Moreover, this test was also conducted to analyze the porosity structure of the implant. The density of the implant was calculated as in Equation (1) [19, 20].

$$\text{Density} = \frac{\text{Wi}}{\text{V}} \qquad (1)$$

Wi is the implant weights

V is the implant volume

Porosity test was evaluated the different weight of the implant before and after immersing in 5 mL of water for 1 min. Water penetrates the inner structure of the implant, so as the weight of the implant after immersion was higher than the initial weight. The porousness of the implant was calculated as in Equation (2).

$$\text{Porosity} = \frac{\text{Ww} - \text{Wi}}{\text{Wi}} \times 100 \qquad (2)$$

Ww is the implant weights after the immersion process,

Wi is the implant weights before the immersion process

2.3.2 Swelling and Water Uptake Test

The swelling ratio and water absorption capacity of the implant was found by computing the difference in weight of the implant before and after immersing in 5 mL phosphate buffer saline (PBS) pH 7.4 at temperature 37 °C ± 0.5 °C. The swelling ratio and water absorption capacity of the implant was analyzed utilizing the Equation (3) and the Equation (4) [19, 20].

$$\text{Swelling ratio} = \frac{\text{Ww} - \text{Wi}}{\text{Wi}} \times 100 \quad (3)$$

$$\text{Water absorption capacity} = \frac{\text{Ww} - \text{Wi}}{\text{Wi}} \times 100 \quad (4)$$

Wi is the weight of implant before immersing
Ww is the weight of the implant after immersing

2.3.3 Disintegration Test

The implants were immersed at 5 mL phosphate buffer saline, pH 7.4 at 37 °C ± 0.5 °C. The observation was directed to see the switching of the implant morphology during the water penetration process and erosion [21,22]. The time which was taken of the implant to disintegrate into small granules or particles is determined as disintegration time.

2.3.4 Hardness testing

Autograph E-10 instrument was used to evaluate the hardness of the implant. The implant was put in a compression machine; then the implant was pressed by 5 mm min^{-1}. The hardness of the implant indicated the ability of the implant to support bone growth [21].

2.3.5 Drug content

In a mortar, one implant was crushed in a mortar. After the crushing process, the implant was transferred in Erlenmeyer and diluted with 25 mL Hydrochloric acid 0.1 N. In the next step; this mixture was sonicated for 30 min, then hushed up for 24 h in room temperature. Following day, the solution was transferred into a 100.0 mL volumetric flask and diluted using phosphate buffer saline (PBS) pH 7.4. The aliquot then was filtered using the Whatman filter paper (0.45 mm diameter). Filtrate (1 mL) was pipette and exchanged into 25 mL volumetric flasks, then applied to the volumetric flasks up to 25 mL of phosphate buffer saline (PBS) pH 7.4. This solution then analyzed by spectrophotometer UV-Vis to obtain ciprofloxacin HCl content in each implant [23,24].

2.3.6 In-vitro Drug Release Study

The drug release from the implant was evaluated by vial method using 5 mL of release media. The media was phosphate buffer saline (PBS) pH 7.4 and incubated in a water bath at 37 °C ± 0.5 °C. At predetermined time intervals for 5 d, 1 mL of the aliquots were pipetted, then 1 mL of fresh buffer transferred into the vial to replace the aliquot. The aliquot then prepared by filtrating into the Millipore membrane (ø = 0.45 μm) and dilute using phosphate buffer saline (PBS) pH 7.4. The amount of ciprofloxacin which releases at a predetermined interval to the media was analyzed using a UV spectrophotometer [15,25].

2.3.7 Data Analysis

One-way Analysis of Variance (ANOVA) has been used to analyze the results of the physicochemical evaluation. The physicochemical evaluations which are analysed consist of density, porosity, swelling ratio, water uptake, disintegration time, and hardness of the implant. The results of four formulas have been tabulated and analysed using One-Way Analysis of Variance (ANOVA) with post-hoc Tukey HSD ($\alpha = 0.05$). The data would be significant with P-value < 0.05 ($P < 0.05$). Tukey post-hoc test was used to specify those datas showing significant differences with each other.

2.4 Characterization of Implants

2.4.1 Evaluation of Implant Morphology using Scanning Electron Microscope (SEM)

Scanning electron microscope study was performed to observe the morphology of the implant. The samples were coated with gold and fitted to aluminum stubs with conductive paint. The pores diameter of the implant were analyzed from the SEM micrograph. The pore size was evaluated from the different section of the implants [26].

2.4.2 Fourier Transform Infrared (FT-IR) Spectroscopy

The implant was crushed and combined with KBr; then the implant pressed into a pellet. The sample was analyzed in the wave number range (4 000 to 400) cm^{-1} using FT-IR spectroscopy [26].

2.4.3 X-ray Diffraction Pattern

Crystallographic phases of the implant were observed through X-ray diffractometer using monochromatic Cu Kα radiation (40 KV, 30 MA). The x-ray diffraction pattern of the implant was analyzed in the 2θ scan range 5º to 50º [26].

3. RESULTS AND DISCUSSION

3.1 Preparation of Bovine Hydroxyapatite-Chitosan Implants using Different Composite Composition

Implants were prepared by the wet granulation method. The wet granulation method was taken to produce dry granules with homogenous drug distribution, spherical shape, good compressibility, and free-flowing characteristic.

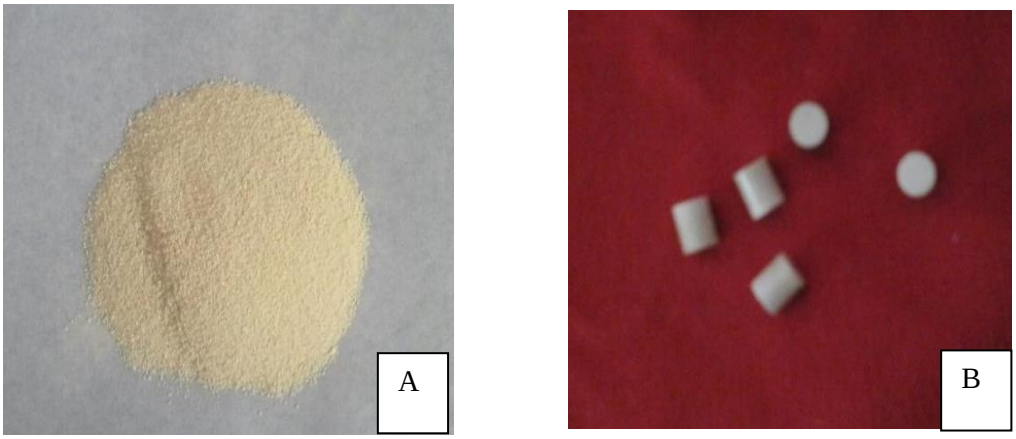

Fig. 1. The results of Bovine Hydroxyapatite-chitosan composite, A) slightly yellow granules, B) implants (cylindrical pellets)

Wet granule mass was made using four different compositions of Bovine Hydroxyapatite-chitosan (20:80; 30:70; 40:60; and 70:30). Ciprofloxacin (10 % w/w) were added to the mixture of the composite. Wet granule mass first passed through a sieve, and then dried using the oven (50 °C) for 24 h. In the last phase, slightly yellow granules were obtained. Dry granules then compressed using a single punch tablet compression machine to produce pellet (4 mm diameters).

3.2 Evaluation of Implants

3.2.1 Density and Porosity

The results of density measurement showed that the density of Bovine Hydroxyapatite - chitosan - ciprofloxacin implant decreases along with the decline of Bovine Hydroxyapatite composition in formulas. This is due to the decreased of calcium ratio in the implant along with the reduction of Bovine Hydroxyapatite composition [27]. The density of the implants with four different composite compositions can be seen in Fig. 2.

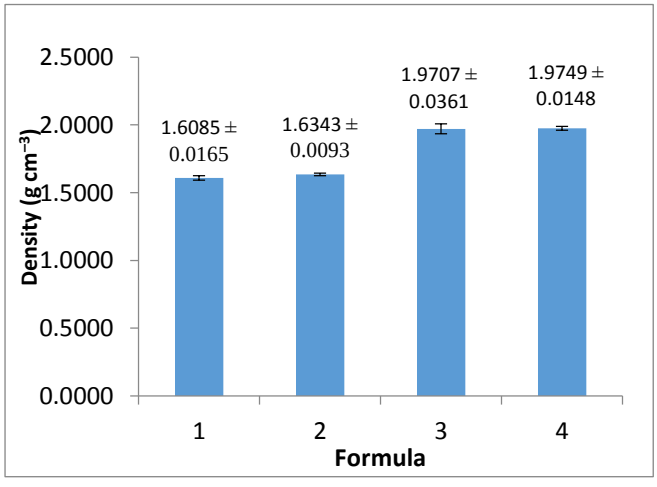

Fig. 2. The density of F1 to F4

The porosity of the implant, using four different composite compositions, can be viewed in Fig. 3. The data displayed in Fig. 3 was mean ± SD (n=3). Established along with the outcome of the porosity test, it could be concluded that the highest porosity was shown by F4. Moreover, the low porosity was observed by F1.

The number of macropore size was found to increase with decreasing apparent density, consequences the number of microstructures was limited. This phenomenon demonstrates that the significant reduction of porosity was observed in the formula with the lowest proportion of Bovine Hydroxyapatite.

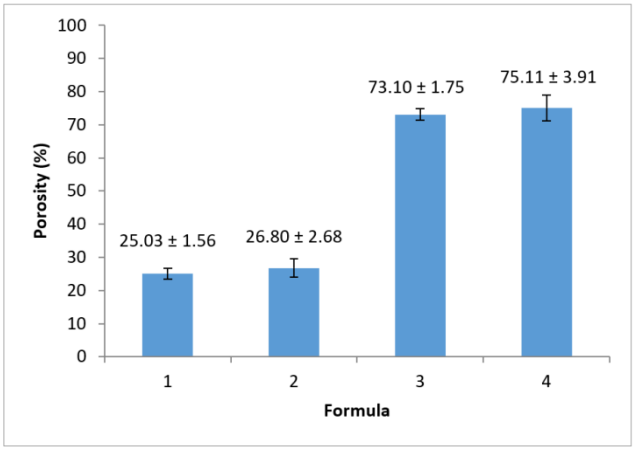

Fig. 3. The porosity of F1 to F4

3.2.2 Swelling and Water Uptake Study

The swelling ratio of F1 to F4 are tabulated in Fig. 4. The data displayed in Fig. 4 was mean ± SD (n=3). There was a significant difference in porosity between F1 to F4 (*P < 0.05). F4 had the highest swelling ratio because this formula had the highest porosity among four formulas that had been established.

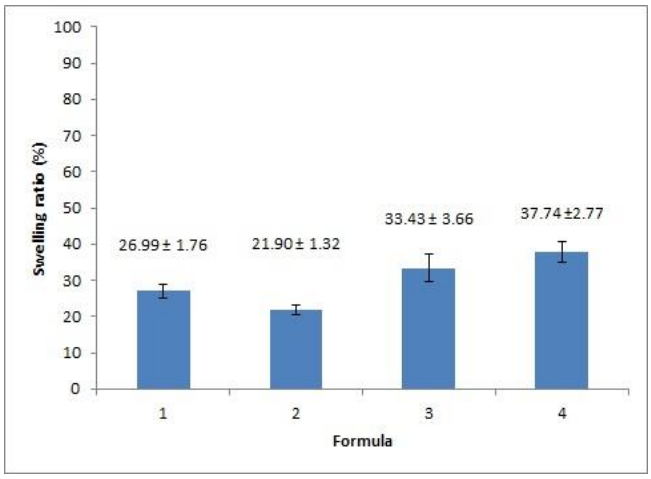

Fig. 4. The Swelling ratio of F1 to F4

3.2.3 Disintegration Test

Disintegration test was conducted to the implant for 5 d. From the results of this study, it can be observed that all the formulas showed swelling mechanism when contact with phosphate buffer saline (pH 7.40). The results of disintegration test have been tabulated in Table 2.

Table 2. The results of disintegration time of implant F1 to F4

Formulation code	Disintegration time (minutes)
F1	10.12 ± 1.02
F2	9.45 ± 1.11
F3	7.10 ± 0.41
F4	5.03 ± 3.12

3.2.4 Hardness

The hardness of the implants (F1 to F4) is as shown in Fig. 5. The data displayed in Fig. 5 was mean ± SD (n=3). Based on the outcome of statistical analysis using one way ANOVA, it can be concluded that the hardness of F4 was significantly different from F1, F2, and F3.

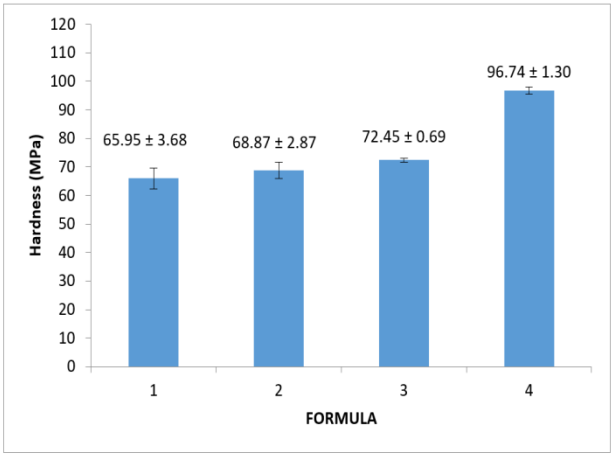

Fig. 5. The hardness of F1 to F4

3.2.5 Drug content

The analysis of ciprofloxacin in the implant was conducted using UV spectrophotometer instruments. The drug content was analyzed using three-wavelength methods, and the results are tabulated in Table 3. All the implants contain ciprofloxacin between 90 % to 100 %.

Table 3. The results of ciprofloxacin content in implant F1 to F4

Formulation code	Drug content (%)
F1	93.84 ± 1.05
F2	94.62 ± 2.06
F3	96.10 ± 0.96
F4	99.81 ± 3.37

3.2.6 In vitro Drug Release Study

The cumulative amount of drug release from four formulations (F1 to F4) is tabulated in Fig. 6. The release of ciprofloxacin from the implant was compared to the therapeutic level of ciprofloxacin for osteomyelitis, regarding the in vitro study. The therapeutic level of ciprofloxacin to produce

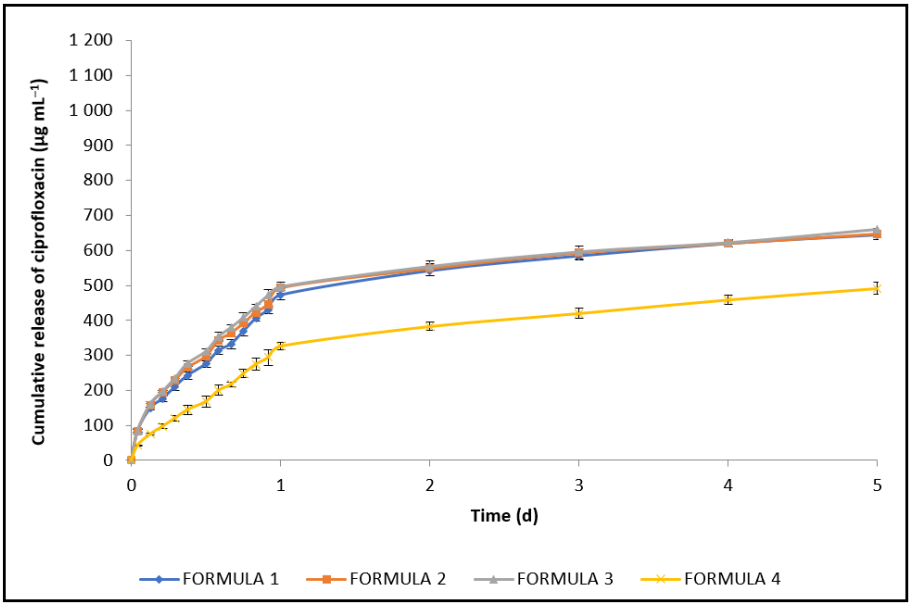

Fig. 6. The ciprofloxacin release profile of F1 to F4

antibacterial activity is > 2 µg mL^{-1} [29]. However, ciprofloxacin concentration higher than 50 µg mL^{-1} caused toxicity in chondrocyte cell [30]. In the initial period (for 24 h), the release of ciprofloxacin was quite high (burst release). F1, F2, and F3 released ciprofloxacin more than the therapeutic level (2 µg mL^{-1} to 50 µg mL^{-1}). F4 showed the release of ciprofloxacin between therapeutic level, but nearly reached the upper limit of the therapeutic level.

3.3 Characterization of Implants

3.3.1 Implant Morphology Study using Scanning Electron Microscope (SEM)

Scanning Electron Microscope (SEM) study was directed to examine the morphology of Bovine Hydroxyapatite-chitosan-ciprofloxacin implants. Morphology of the implants (F1 to F4) is presented in Fig. 7.

3.3.2 Fourier Transformed Infrared Spectroscopy (FT-IR) Study

The infrared spectroscopy study was conducted to analyze the spectrums of Bovine Hydroxyapatite-chitosan-ciprofloxacin implants with four different compositions of composite (F1 to F4). The spectrum of the implant was compared to the infrared spectrum of ciprofloxacin, Bovine Hydroxyapatite, and chitosan. The infrared spectrums are shown in Fig. 8.

3.3.3 X-ray Diffraction Study

X-ray diffraction study has been performed to evaluate the crystallographic phase of the implants. The results of the x-ray diffraction spectrum of the implant and the initial component (Bovine Hydroxyapatite, chitosan, and ciprofloxacin) is shown in Fig. 9. A wide peak at $2\theta \approx 20°$ was the specific peak of chitosan [16]. This peak indicated the crystallinity of chitosan. When the composition of Bovine Hydroxyapatite increased in the implant, this peak became wider and flatter. It can be concluded that Bovine Hydroxyapatite decreased the crystallinity of chitosan. Bovine Hydroxyapatite decreased intermolecular interaction between chitosan chains so that the degree of crystallinity fell down [15].

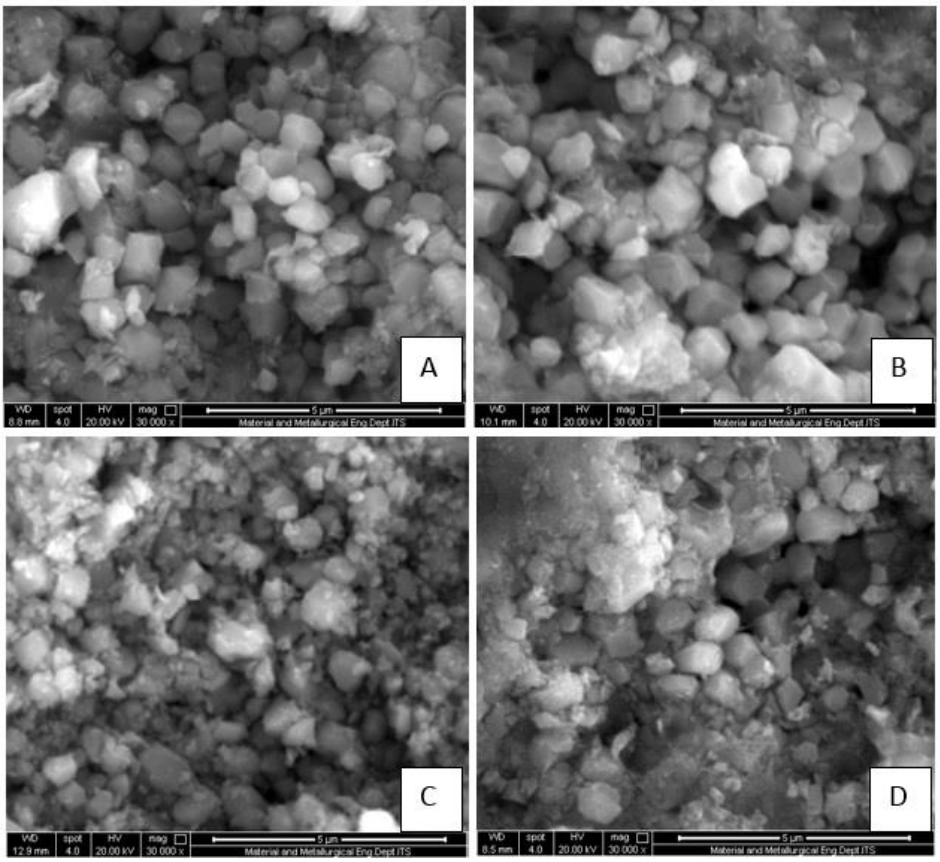

Fig. 7. SEM micrograph of the implants, A): F1 (BHA-chitosan = 20:80), B): F2 (BHA-chitosan= 30:70), C): F3 (BHA-chitosan = 40:60), D): F4 (BHA-chitosan = 70:30).

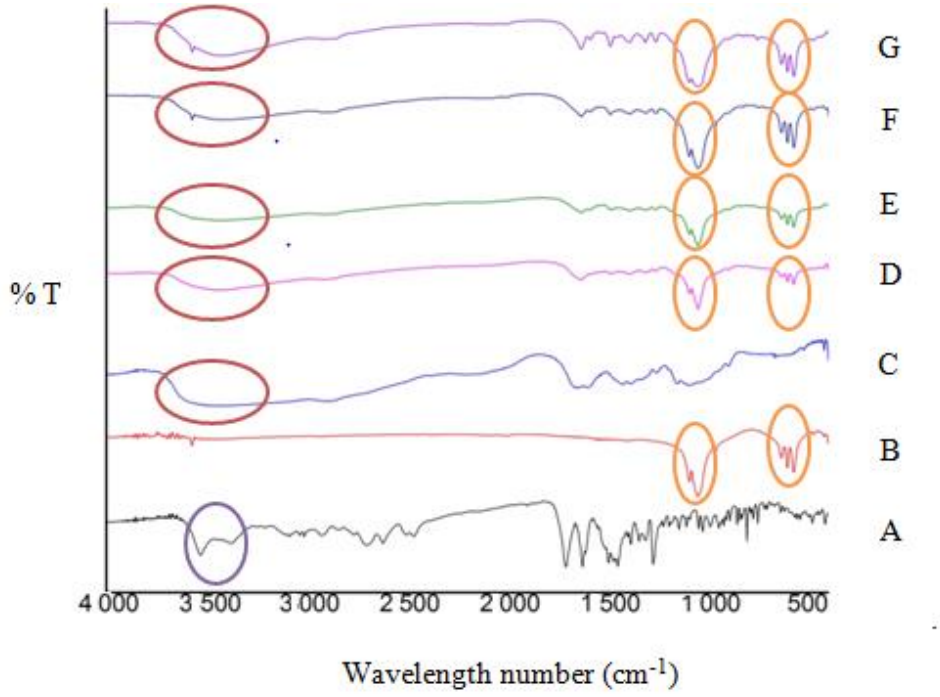

Fig. 8. The FT-IR spectrum of, (A): Ciprofloxacin; (B): Bovine Hydroxyapatite; (C): Chitosan; (D): Formula 1; (E): Formula 2; (F): Formula 3; (G): Formula 4

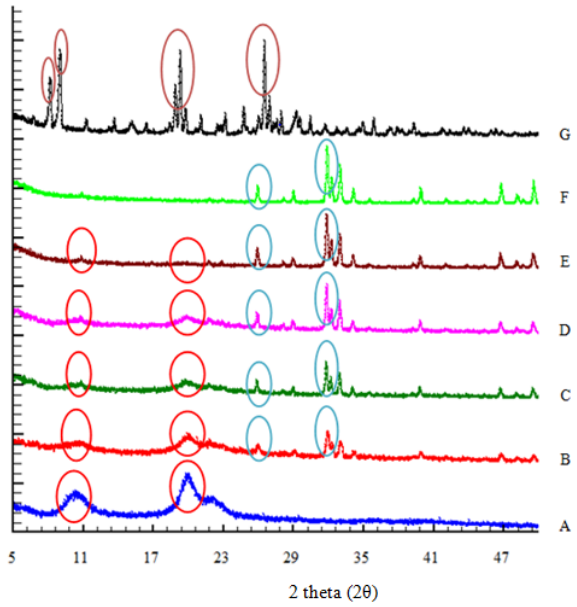

Fig. 9. X-ray diffraction spectrum of (A): Chitosan; (B): Formula 1; (C): Formula 2; (D): Formula 3; (E): Formula 4; (F): Bovine Hydroxyapatite; (G): Ciprofloxacin

4. DISCUSSION

Physichochemical characterization has been conducted to the implant including density and porosity, swelling and water uptake, disintegration time, hardness, drug content, and in-vitro drug release. The results of density evaluation revealed a significant difference in density between the implants (*P < 0.05). Post hoc Tukey-HSD test indicated that the density of F1 was significantly different with F3 and F4. Moreover, the density of F2 is also significantly different with F3 and F4. F3 and F4 showed no substantial difference in density. These results are in line with the previous study which uncovered that the Bovine Hydroxyapatite concentration did not affect linearly to the density of the implant. The microstructure arrangement of Bovine Hydroxyapatite and chitosan conducted a significant role to influence the density of the implant [27].

Based on this result, it can be seen that Formula 4 (Bovine Hydroxyapatite: chitosan = 70:30) had the highest density. The increase of Bovine Hydroxyapatite proportion in the composite was observed increase the density of implants. Bovine Hydroxyapatite was dispersed in the chitosan polymer wall, so that the more compact structure of the implant was obtained. This condition caused the pores of the implant became smaller. The results from the previous study about the nanohydroxyapatite-chitosan scaffold, also showed that the addition of Bovine Hydroxyapatite in the formula caused the density of the implant became higher comparable to pure chitosan scaffold [27].

The results of the porosity test were statistically analyzed using one-way ANOVA. The porosity of the implant, using four different composite compositions was significantly different (*P < 0.05). Post hoc Tukey-HSD test indicated that the porosity of F1 was significantly different with F3 and F4 (*P < 0.05). The porosity of F2 also significantly different with F3 and F4 (*P < 0.05), but the porosity of F3 and F4 were not different (*P > 0.05). Slightly difference in Bovine Hydroxyapatite composition of F3 and F4 did not influenced the microstructure arrangement of the implant. Increasing the Bovine Hydroxyapatite concentration in a composite caused the porosity of the implant higher. By the increase of Bovine Hydroxyapatite composition in composite, the composition of chitosan was reduced. The reduction of chitosan composition led the pore wall to become more flexible, and the pore became weaker. However, the number of pores increased, so that the porosity of the implant increased [27].

The porosity of the implant controlled the amount of water that will penetrate the structure of the implant. Interconnected pores which can be observed in F4 play an essential role to increase the porosity of the implant. This condition caused the amount of water which penetrate to the implant was comparatively higher to the other formulas so that water will expand the structure of implant rapidly [1]. The result of water uptake study revealed a significant difference of water uptake between F4 and F1 (*P < 0.05), F4 and F2 (*P < 0.05),F4 and F3 (*P < 0.05). Meanwhile, there was no significant difference in water uptake between F1, F2, and F3. F4 had the highest water uptake compare to the others. This may be due to an increase of Bovine Hydroxyapatite composition in the implant structure. The increased of Bovine Hydroxyapatite composition caused the surface of the implant became rougher and the structure became more porous [15]. In addition to porosity, the microstructure of the matrix also determines the ability of water absorption [27]. Water absorption capacity related to the ability of the implant to absorb body fluid, transport cell nutrient, and transport metabolite [27].

Disintegration test was conducted to evaluate the ability of the implant to have enlarged pore size when applicated in body fluid. Enlargement of the pore size can facilitate cell attachment and bone tissue growth [27]. F4 showed the fastest disintegration rate among the four formulas, whereas F1 showed the slowest disintegration time. This is due to the dissolution of hydroxyapatite from the matrix was greater in the formula which used high hydroxyapatite composition [27]. Moreover, high porosity in the structure of the implant also promoted water penetration to the implant. This condition caused the implant to swell quickly.

Bovine Hydroxyapatite plays a significant role to determine the hardness of the implant. An implant, which contains higher hydroxyapatite composition, had higher mechanical strength. This was due to hydrogen bonding and metal coordination between chitosan (NH_2 group and OH group of chitosan) and ion Ca^{2+} of Bovine Hydroxyapatite. Increasing concentration of chitosan in Bovine Hydroxyapatite-chitosan composite caused the hardness of the implant to decrease. The higher chitosan composition in composite promotes a weakening of interfacial interaction between chitosan and Bovine Hydroxyapatite [28]. This phenomenon in line with the results of this study, F4 which consist of the highest proportion of Bovine Hydroxyapatite performed the highest mechanical strength (97.74 ± 1.30) MPa. The hardness of F4 nearly similar to the hardness of cortical bone filler (100 MPa to 120 MPa).

Parameter to evaluate the difference of ciprofloxacin release profile among four formulas was area under curve (AUC). The area under the curve (AUC) of F1 to F4 were analyzed to observe the release pattern of ciprofloxacin. There was a significant difference in the area under the curve between four formulas. Post Hoc test using Tukey-HSD showed that the value of the area under curve form F4 was significantly different compared to F1, F2, and F3. The results of *in vitro* drug release study for 5 d indicated that the release of ciprofloxacin from F4 was (491.65 ± 17.00) μg mL^{-1}. It was only 75 %

portion of ciprofloxacin released from F4 compare to the other formulas. This condition showed that the drug fraction which left behind from F4 higher than F1, F2, and F3. The drug fraction attached to the chitosan network of hydrogen bond and ionic interaction. The drug fraction also can be absorbed into a Bovine Hydroxyapatite particle through high affinity with calcium ions [16]. Formula 4 can be developed in the next study using crosslink agent to control the release of ciprofloxacin during range therapeutic range for osteomyelitis (4 wk to 6 wk). A cross-linking agent like glutaraldehyde, genipin, and tripolyphosphate can be added in the formula to control the release of ciprofloxacin.

Characterization study of Bovine Hydroxyapatite-chitosan-ciprofloxacin implant was conducted to evaluate the morphology structure of the implant, crystallographic state, and chemical interaction between each component. The implants had a porous structure with nanometer pores. Implants consisted of open pores canal and interconnected pores. The pores were cylindrical and distributed homogeneously in the surface of the implants. The results of SEM indicated that F1 had crowd structure and large pores. The pore wall was thick and cylindrical. The increased of Bovine Hydroxyapatite caused the pores became smaller, pore wall became thinner, and the number of pores became higher. The results of F4 showed that the implants had a porous structure. Bovine Hydroxyapatite was dispersed in chitosan matrix. Bovine Hydroxyapatite formed canal with smaller pore canal. An implant which had a porous structure enhanced cell attachment to the implant during the implantation period. This condition caused by tissue infiltration in the structure of implants [15].

Analysis of the change in functional groups of Bovine Hydroxyapatite - chitosan - ciprofloxacin implants (F1 to F4) was conducted by comparing the infrared spectrum of the implants and starting material. The infrared spectrum of Bovine Hydroxyapatite – chitosan - ciprofloxacin implants showed peaks in wavelength 955 cm^{-1} and 635 cm^{-1}. These peaks occurred because stretching vibration P-O from PO_4^{3-} group and O-H deformation from Bovine hydroxyapatite.

Furthermore, peak in wavelength 3 571.92 cm^{-1} caused widening modification of N-H group chitosan (3 394.48 cm^{-1}). This condition showed by F2 and F3, but F1 did not show the same characteristics. The infrared spectrum analysis could give an information about the possibility of interaction. The interaction which occurred among the components in formula consisted of hydrogen bond and ionic bond. This interaction involved (Ca^{2+} dan PO_3^{3-}) from Bovine Hydroxyapatite and NH_2 group of chitosan [28]. This phenomenon revealed that chitosan also acts as a binding site of Bovine Hydroxyapatite in the structure of implants. Characteristics peak of ciprofloxacin, which indicated more second amine group (-NH) (3 529.49 cm^{-1}) and OH group (3 377.12 cm^{-1}), did not observe in implant formulation. This fact explained that there were an interaction via hydrogen bound between ciprofloxacin and another component in the formula. Hydrogen bound was a weak interaction, consequence the bond between ciprofloxacin and the other components was disrupting quickly when this implant contact with

water. Therefore, ciprofloxacin did not lose its activity in implant dosage forms and still effective after it was released from the implants [29].

X-ray diffraction study was performed to evaluate the crystallinity state of the implants. The specific peaks intensity of Bovine Hydroxyapatite became higher along with the increased of Bovine Hydroxyapatite composition. The diffraction pattern of implants indicated that Bovine Hydroxyapatite was in unique crystalline phase [30]. Characteristic peaks of Bovine Hydroxyapatite in implants decreased when compared to pure Bovine Hydroxyapatite. The decreased of Bovine Hydroxyapatite crystallinity in implants caused by chitosan molecule [31]. Chitosan macromolecules inhibited the growth of Bovine Hydroxyapatite along c-axis so that the crystallinity of Bovine Hydroxyapatite decreased [15]. The diffraction peak of implant showed that the system is still in crystalline state, however there was a decreased of crystal lattice regularity compare to the initial components [32].

5. CONCLUSIONS

Composite composition ratio (Bovine Hydroxyapatite and chitosan) influenced the porosity, density, hardness, swelling index, and in vitro drug release from Bovine Hydroxyapatite-chitosan-ciprofloxacin implant. Physicochemical evaluation and in vitro drug release study revealed that Formula 4 (Bovine Hydroxyapatite: chitosan = 70:30) fulfilled the requirement of the ideal bone implant. Moreover, the release of ciprofloxacin from this formula was lower compared to the other formulas. But, at the initial period this formula still released ciprofloxacin higher than the therapeutic level of ciprofloxacin in osteomyelitis which has been declared from in vitro drug release study.

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