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In vitro study of Nano Hydroxyapatite/ Streptomycin - Gelatin-Based Injectable Bone Substitute Associated- 3D printed Bone Scaffold for Spinal Tuberculosis Case

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Abstract According to WHO (2018) Indonesia was placed in the third ranked of the highest number of TB sufferers worldwide. TB was caused by the Mycobacterium tuberculosis. This bacterial infection not only could affect the lungs, but also bone tissue, especially the spine, which then called as spinal tuberculosis. There had been many alternatives to overcome this case. One of them was by using Injectable Bone Substitutes (IBS) based on nano Hydroxyapatite (nanoHA), gelatin and streptomycin. The severe sponal tuberculosis case might cause the bone defect. Due to this bone defect, IBS alone would not be able to handle this issue. Thus, scaffold was needed to replace and support the infected bone. Besides, the combination between IBS paste and bone scaffold could help the bone tissue regeneration. The nanoHA and gelatin contents of the IBS paste provided the mineral which was need for bone tissue regeneration. While the bone scaffold would support those contents by providing the extracellular matrix for the new bone tissue attachment. The IBS associated- scaffold could be an alternative to deliver spinal tuberculosis' drug locally. This study aimed to evaluate the performance of this combination through antibacterial study and drug release test using UV-Vis spectrophotometer. As well as the cytotoxicity test to know the risk of these two materials within the body. The IBS was synthesized by mixing nano hydroxyapatite and 20 w/v% gelatin with ratio of 65:35 and 10% streptomycin addition as antibiotic agent. The mixture was added by hydroxypropyl methylcellulose as suspending agent. FTIR test showed that there was a chemical reaction occurred in the mixture. The performance of this combination showed that the released- streptomycin, qualitatively, could kill the Staphylococcus aureus trough the antibacterial test. Furthermore, it also had been proven that the IBS associated- scaffold could release an increasing streptomycin concentration over time. In addition, the combination showed a good result in cytotoxicity test where the cell viability was more than 50%. According to the results mentioned before, the IBS paste associated- scaffold was a promising breakthrough to overcome spinal tuberculosis case as a local drug deliver.

Keywords: IBS paste, nanoHA, gelatin, streptomycin bone scaffold, 3D printing, spinal tuberculosis, in vitro

1. INTRODUCTION

WHO reported, by 2017, there has been more than 1,2 billion people died because of Tuberculosis (TB). While Indonesia was ranked as the 3rd country worldwide where the sufferer of TB was mostly found [1]. This disease is caused by a kind of bacteria, called Mycobacterium tuberculosis, which commonly infect lungs through the respiratory tract. Not only lungs, Mycobacterium tuberculosis could also infect another organ, including bone, which will be known as the osteomyelitis tuberculosis. Up to 50% of all tuberculosis osteomyelitis cases took place in the spinal. It is because the plenty vascularization to the spinal could make the bacteria reactivate [2]. A severe spinal tuberculosis could cause the spinal destruction which might lead to the damage of nervous system [3]. To prevent or overcome that to happen, the surgical treatment needs to be done. This surgical operation is done by removing the infected bone tissue and replacing it with bone implant or bone graft in order to accelerate the recovery process and maintain the spinal stabilization [4]. Beside that, it is needed to concern about the bacteria which become the main cause of this disease. Hence, here we need antitubercular drugs therapy for killing the bacteria. However, this drugs therapy has to be given in the high dose in at least 6 months in order to be able killing the bacteria [2].

There are several characteristics that has to be own by the material which will be applied for bone implant. One of the most important things is the ability to regenerate a new osteoblast to grow replacing the defect of the bone. Such thing could be acquired by using Hydroxyapatite (HA). HA could encourage the mineralization of the bone because of its bioactive and osteoconductive characteristics [5]. Moreover, in the research world of nanoscience and nanotechnology, it is important to get to know the HA's formation in a scale of nanometer. It will define the physical and chemical characteristics [6]. The use of nano-HA could widen the benefit of this compound. The nano-size of this compound could make it easier to be formed as any material, including in synthesizing of IBS paste. According to the previous research, nano-HA could be successfully used for the IBS paste synthesizing. This compound could react to another material such as gelatin and streptomycin [7]. Such characteristics makes nano-HA as a promising substance for many applications, such as drug delivery, bone implant's coating, and bone graft as well [6]. Besides that, gelatin was added to improve the physical characteristics of nano-HA which is brittle. While streptomycin itself was added as an antitubercular drug which will play role for killing the bacteria.

In the field of tissue engineering, a bone graft is called a scaffold. Scaffold is used as an artificial supporting structure to repair damaged organs and tissues by stimulating the regeneration of new tissue from these organs. In cases of spinal tuberculosis, scaffold is used to reconstruct the structure of the spinal geometry and to regenerate bone tissue. Scaffold used must be biocompatible and has mechanical properties similar to pore native bone, as well as the interconnective pore with shapes and sizes that could support bone cell regeneration [8].

The 3D printing method is the latest technology related to Scaffold printing in tissue engineering. The 3D printing method through additive manufacturing guarantees scaffold printing according to the geometry of bone defects with pores that can design its shape, size and interconnection. "Additive manufacturing" is a series of processes for making certain design materials, starting from making 3D designs with 3D software to computers ready to be printed through the 3D printing process layer by layer [9]. Research conducted by Gregor, et al (2017) and Gremare, et al (2018) proved that 3Dprinting printing with Fused Deposition Modeling (FDM) using polylactid acid PLA filaments can be used as a biomaterial for making bone scaffold. FDM offers fast and reproducible 3D printing for scaffold making that can be adjusted to the design needed [10].

In addition to artificial supporting structures and stimulating bone regeneration, scaffold can also be used as a drug carrier [8]. The combination of regenerative tuberculosis spinal healing therapy and drug therapy to suppress the area infected with Mycobacterium tuberculosis can be done by

administering an Injectable Bone Substitute (IBS). Research by Maulida, et al (2015) succeeded in synthesizing Injectable Bone Substitute (IBS) in the form of nano-HA, gelatin, and streptomycin-based non-toxic, biocompatible, and biodegradable and able to mix with Bovine Scaffold [7].

2. MATERIALS AND METHODS

2.1. Materials and Tools

This study used nano hydroxyapatite and gelatin which were obtained from Pusat Aplikasi Isotop dan Radiasi Badan Tenaga Nuklir Nasional (BATAN) Jakarta Indonesia. The streptomycin sulfate (powder for injection) was purchased from PT. Meiji Indonesia. While the hydroxypropyl methylcellulose (HPMC) was sourced from Sigma Aldrich H7509.

For characterization purpose, there were some materials needed, those were Phosphate buffer Saline (PBS) solution for drug release test, Staphylococcus aureus for anti-bacterial test, and human hepatocyte from Institute of Tropical Disease Universitas Airlangga for cytotoxicity test.

The tools which were used for this study were 3D printer Anycubic Kossel Linear Plus, freezer and lyophilizer, FTIR instrumentation, UV-Vis Spectrophotometer, and Elisa Reader.

2.2. Synthesis of IBS paste

The process of IBS paste synthesis was done according to the study conducted by Maulida, et al (2015). To synthesize Injectable Bone Substitute (IBS) paste, first of all, 20% (w/v) gelatin was dissolved in 40oC deionized water. After one hour, the nano hydroxyapatite (HA) powder was added to the gelatin solution with the ratio of HA: gelatin 65:35 (w/w) and stirred for one hour. Streptomycin sulfate was added to the mixture with 10% percentage of the final mass of IBS paste. Meanwhile, deionized water was heated up to 90oC to dissolve 4% (w/v) HPMC. Next, the HPMC solution, which had been cooled until 40oC, was poured to the mixture of gelatin-HA-streptomycin until producing the homogenous mixture. This mixing process needed 6 hours stirring.

To know whether the IBS paste had been successfully synthesized, the functional group test using of Fourier Transform Infrared (FTIR) was conducted. Before the IBS paste went to FTIR test, it had to be freeze-dried in order to eliminate the water content.

2.3. Synthesis of Bone Scaffold

The bone scaffold was designed using AutoCAD 2019 free-software for education. There were 5 pore size variations, i.e 600, 800, 1000, 1200, and 1400 μm. The scaffolds were respectively named as A, B, C, D, and E. All of the scaffolds were printed from Polylactide Acid (PLA) filament with 0.2 mm the diameter of nozzle's 3D printer. Each scaffold was examined its porosity using the following formula:

$$Porosity\ (\%) = \frac{\left(V_{scaffold} - {}^{W_{scaffold}}/_{\rho_{polymer}}\right)}{V_{scaffold}} \times 100\%$$

$V_{scaffold}$ was the scaffold volume as the product of the length, width, and height measured from edge to the edge of the scaffold cubes. $W_{scaffold}$ was the mass of the scaffold weighted using digital balance. While $\rho_{polymer}$ is the density of PLA [11]. It is known that the PLA density is 12.4 g/cm^3 [12].

2.4. IBS Associated- Scaffold Performance

To test the scaffold performance regarding to the ability of the scaffold to release drug, the anti-bacterial test and drug release test using UV-Vis spectrophotometer were conducted. First, the IBS paste was injected to the scaffold. The IBS associated- scaffold was then soaked in 10 ml PBS. The sample of PBS as the result of soaking IBS associated- scaffold was taken every 1 hour for 4 hours long. During the soaking, streptomycin would be released from the IBS associated- scaffold. These 4 samples were used for both anti-bacterial test and drug release test using UV-Vis spectrophotometer.

The anti-bacterial test was done by using Staphylococcus aureus (SA). The inhibition zone diameter as the result of this test would show the ability of the released-streptomycin to inhibit the

growth of SA. 9 ml of Tryptic Soy Broth (TSB) was used to suspend SA. The bacteria were then cultured in the incubator at temperature of 60°C for 24 hours. Nutrient agar was prepared and placed in the petri dish as the medium of the bacteria. Every PBS sample was dropped on the disc paper. After 24 hours of incubation, the inhibition zone diameter was observed.

The drug release test using UV-Vis spectrophotometer was used to know how much the concentration of streptomycin released every one hour within 4 hours. Before testing the PBS samples the standard curve of relation between streptomycin concentration and absorbance was made first [13]. It could be done by testing the streptomycin-PBS solution with concentration 1, 2, 3, and 4 w/v %. From the standard curve, we could obtain the regression formula which would be used for determining the concentration of the released-streptomycin at every hour.

2.5. Cytotoxicity Test

The cytotoxicity test was conducted to examine whether the materials used in this study was toxic or not for the human body. MTT assay method was performed for this test. This method used 3- (4,5-dimethyl-2-thiazolil)-2.5-diphenil-2H-tetrazolium bromide (MTT) as the reagent to indicate the viability cell. This reagent would change into violet color- formazan which is caused by mitochondria's activity of the living cell [14]. The cell used in this test was human hepatocyte. The viability cell could be calculated using the formula below:

$$\%viability = \frac{(A_{560} - A_{760})}{A_{medium}} \times 100\%$$

The consideration was if the materials had more than 50% cell viability, it meant that the materials was non-toxic [15].

3. RESULT

3.1. Synthesis of IBS Paste

For about 10 hours the IBS paste could be made from the nano-HA, gelatin, and streptomycin. The materials was mixed well which could be seen by the presence of homogenous white paste mixture. IBS would be in its paste form when it was located in the temperature of 35 – 40 ^{o}C. The FTIR of the IBS showed that this mixture consisted from several functional group which related to some specific functional groups of its ingredients. The peak at 2949.89 cm^{-1} showed the wavenumber of the stretching vibration of C-H. It is the specific wavenumber range from HPMC. Three peaks at 1489.63 cm^{-1}, 1239.03 cm^{-1}, and 1136.81 cm^{-1} showed the functional group as results of carbonyl and amine group originated from gelatin and streptomycin. Moreover, the peak at 604.45 cm^{-1} is the specific wavenumber from nano-HA's functional group. The FTIR spectrum of IBS paste could be seen at the **Fig 1**.

3.2. Synthesis of Bone Scaffold

Five pore size variations of 3D printed scaffolds were tested their porosity using Equation (1) in 3 repetitions. **Table 1** showed the results of the test.

Table 1. Porosity Test Results of Scaffolds

No	Scaffold	Porosity (P) ± ΔP (%)
1	A	57.227 ± 0.488
2	B	62.095 ± 0.476
3	C	55.586 ± 0.536
4	D	65.348 ± 0.321
5	E	68.017 ± 0.417

The data showed that it had a positive trend relation between the pore size and the porosty of the scaffold

3.3. IBS Associated- Scaffold Performance

The inhibition zone which was produced by the samples of IBS associated- scaffold soaked in PBS could be seen in Fig 2.

As the qualitative data, the Fig 2 showed that the IBS associated scaffold could really release streptomycin. The inhibition zone was produced because streptomycin inhibited the growth of SA.

Furthermore, the qualitative data which was obtained from antibacterial test would be clarified quantitatively by the data from UV-Vis spectrophotometer. The specific wavelength of streptomycin absorbance was around 200 nm [13]. Fig 3 showed the graph of streptomycin absorbance with various concentration (1% until 4 %) over the wavelength. The peaks which were detected using UV-Vis spectrophotometer took place at 274.00 nm and 324.50 nm.

Based on the reference [13], only the wavelength of 274 nm that would be used to make the standard curve. The standard curve of streptomycin concentration over its absorbance would be shown in Fig 4. Using the graph, we could know the regression formula which then named as Equation (2). This equation below could be used for determining the concentration of streptomycin released by IBS associated- scaffold within 1 until 4 hours. The x variable referred to the streptomycin concentration, while the y variable was for absorbance result.

$$y = 65.08x + 0.159$$

Fig 5 presented an increasing concentration of streptomycin which had been released from the IBS associated- scaffold over time.

3.4. Cytotoxicity Test

After knowing the performance of IBS associated-scaffold, we ought to know how it would affect our body cell, whether it is toxic or not. According to the cytotoxicity test, all the materials didn't show any chance of being toxic. The following Fig 6 presented the viability of the cell.

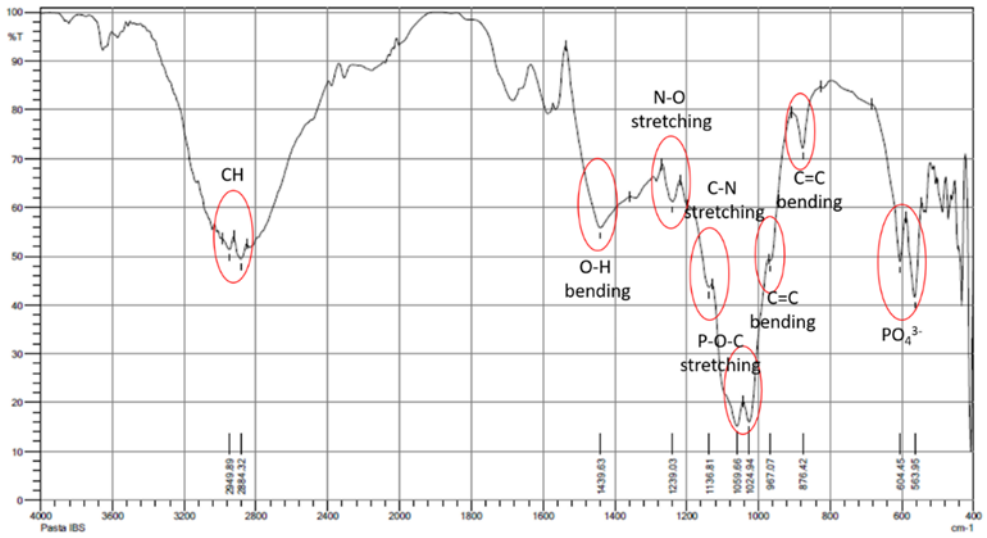

Figure 1. FTIR spectrum of IBS paste

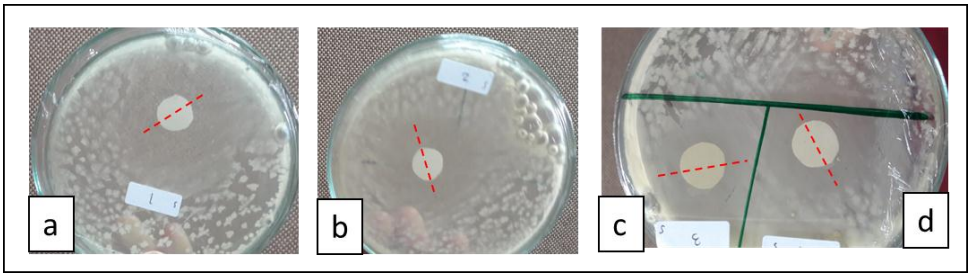

Figure 2. The Inhibition Zone of IBS associated- scaffold soaked in PBS in 1 hour (a) 2 hours (b) 3 hours (c) and 4 hours (d)

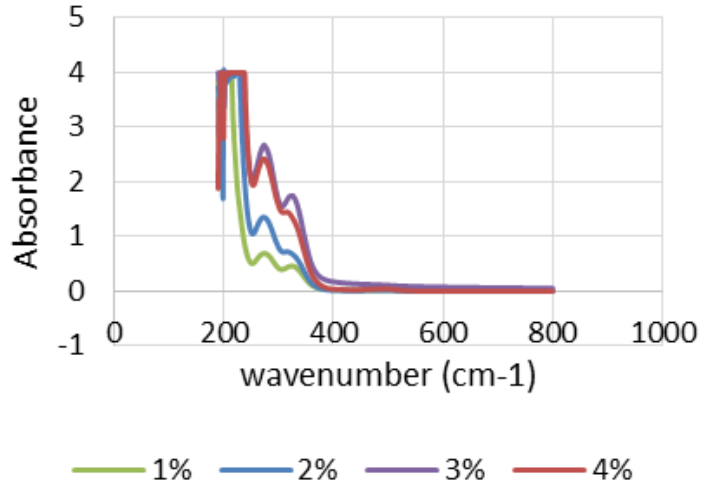

Figure 3. The Graph of Streptomycin Absorbance with Various Concentration (1% Until 4 %) over The Wavelength

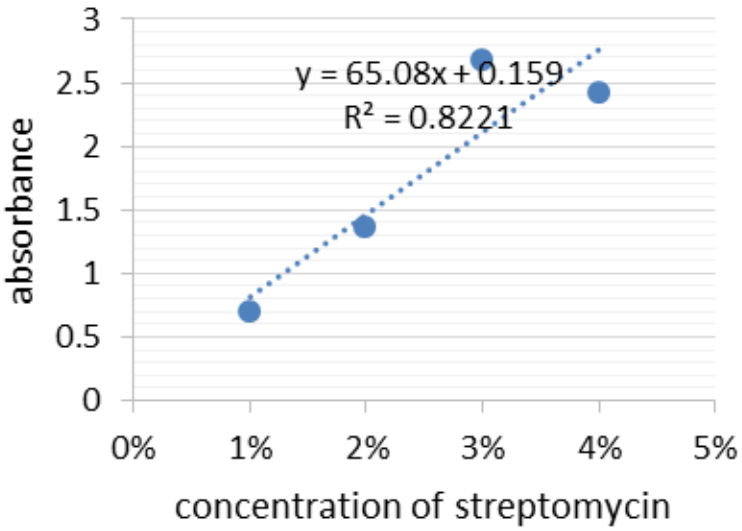

Figure 4. The Standard Curve of Streptomycin Concentration over Its Absorbance

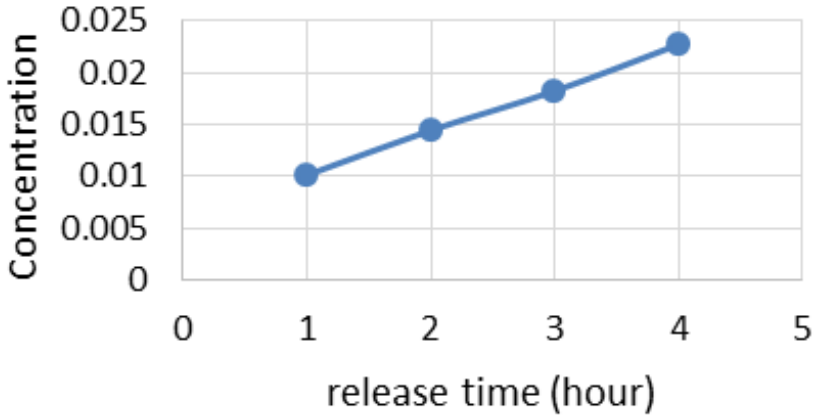

Figure 5. Concentration of Streptomycin which Had Been Released from The IBS Associated-Scaffold Over Time

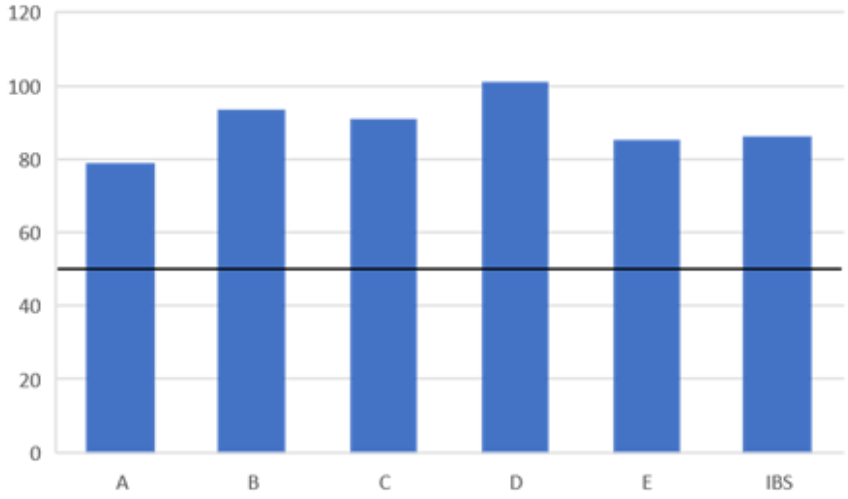

Figure 6. Cell Viability of The 5 Various Scaffold and IBS Paste

4. DISCUSSION

As a tropical country, Indonesia still has been dealing with infectious diseases caused by bacteria for many years. One of the diseases was Tuberculosis (TB). Not only infected the lungs, this bactery could infect the bone as well. Most of the tuberculosis which infected the bone occurred in the spine. A breakthrough to overcome this disease was developed by Maulida, et al (2015) using IBS paste. This study continued to develop the previous study by optimizing the IBS paste's function for handling spinal TB by combining it with 3D- printed bone scaffold.

To develop the previous IBS paste study, we chose the composition 65:35 of HA: gelatin because it was the fastest composition of IBS to set on bone scaffold. According to the FTIR test result, IBS paste for this study could successfully made by the chemical reaction between nanoHA, gelatin, streptomycin, and HPMC. The presence of nanoHA for one of the ingredients in this study helped a lot in forming the IBS into its paste form, considering nanoHA was the main composition to synthesize IBS paste.

For more serious case of spinal TB, where the bacteria caused bone defect in wide range of the bone, the IBS paste itself would not be enough. Aside from killing the bacteria, it also had to replace and help support the structure of the bone. Hence, 3D printed bone scaffold would be a right choice for this case. Besides that, the bone scaffold would also be able to regenerate new bone cell by providing artificial extracellular matrix which is useful for the new bone tissue attachment, which later will experience the proliferation and differentiation process [14]. To support a proper cell proliferation, scaffold should has sufficient and regular porosity. The recommended porosity to be bone scaffold was cca 50-60 %. Because that porosity had already tested its satisfaction regarding to the cell proliferation [15]. Based on the data in this study, the porosity of the scaffolds was around 55 – 68%. It meant that the bone scaffold was really promising to help the bone cell to regenerate.

The combination between IBS paste and bone scaffold showed a positive result int heir performance to release and then kill the bacteria. The qualitative data which was collected from antibacterial test represented that the released-streptomycin could really kill the SA bacterium. While SA itself was a kind of bacteria which could commonly found in the environment. It meant that this bacteria was more adaptable than *Mycobacterium tuberculosis*. This result could represent the capability of the released-streptomycin to kill the TB bacteria. Quantitatively, the UV-Vis spectrophotometer data showed that the IBS-associated scaffold could control the release of streptomycin. It was proven by the increasing of streptomycin concentration over time.

After considering the performance of the IBS associated- scaffold, we also wanted to know how the body would react to this material. The previous study conducted by Maulida, et al (2015), showed that IBS paste alone was non-toxic if it was tested in fibroblast from Baby Hamster Kidney (BHK-21). In this study, we used hepatocyte of human. The use of this cell hopefully could provide more credible data regarding to the toxicity of the materials in this study. Moreover, this test showed that all the materials, both IBS paste and scaffold, were not toxic.

5. CONCLUSION

The IBS paste could successfully synthesized using nanoHA, gelatin, and streptomycin proved by the result of FTIR test. NanoHA was the main composition of the IBS paste synthesis. This kind of material took a great role in the succeed of this paste synthesis. The *in vitro* study of showed that the IBS paste associated- scaffold was a promising breakthrough to overcome spinal tuberculosis case as a local drug deliver. This statement was based on the antibacterial test, drug release using UV-Vis spectrophotometer test, and the cytotoxicity test, which had shown a positively good result to this vision.

CONFLICT OF INTEREST

Author declares the submitted manuscript have no any conflict of interest.

6. Reference

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