Cytotoxicity and mechanical properties of biphasic calcium phosphate scaffold from *Tegilarca granosa* due to its composition

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ABSTRACT

***Background**: Biphasic calcium phosphate (BCP) is graft material contained hydroxyapatite (HA) and tricalcium phosphate (TCP). Tegilarca granosa shell is a natural source that may converted into BCP. This study aims to determine the composition and cytotoxicity of BCP synthesized from Tegilarca granosa shell used various hydrothermal hours and to evaluate the mechanical properties of BCP scaffold.*

***Method**: Tegilarca granosa shell was converted into BCP using hydrothermal method at 200˚C for 6h (Group 1); 9h (Group 2); and 12h (Group 3). The composition was determined by X-Ray Diffraction (XRD) and the cell viability were evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Each group was added with 20% gelatin ratio 50:50 (w/v) and freeze-dried to form scaffold. Scaffolds (Ø6mm x 4mm) were prepared for diametral tensile strength (DTS) test (n=6) and scaffolds (Ø7mm x 11mm) were used for compressive strength (CS) test (n=6). All data were analyzed using Kruskall-Wallis followed by Mann-Whitney test.*

***Result:** The composition of BCP (HA/ TCP) at Group 1, Group 2, and Group 3 were 81.80%/14,10%; 87%/6%; and 72%/21%. The cell viabilities were good for all groups. The DTS and CS test showed there was a significant difference between Group 1 and Group 3 scaffold, meanwhile there was no significant differences between Group 2 and Group 3 scaffold. Group 3 scaffold showed the highest DTS and CS, 6.921 MPa and* 1,233 MPa*.*

***Conclusion:** The BCP composition were depent on hydrothermal hours. Although all scaffold groups were non-toxic, but BCP scaffold synthesized from Tegilarca granosa shell using hydrothermal for 12 hours showed the highest mechanical properties.*

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INTRODUCTION

Biphasic Calcium Phosphate (BCP) is a bone substitute material contained with the combination of hydroxyapatite (HA) and beta tricalcium phosphate ((β-TCP).[1,2] Bone substitute materials could derive from natural, synthetic or composite materials which are then used to fill in bone damage and improve bone healing. Bone substitute materials should facilitate bone formation and improve wound healing.[3] BCP is a bone substitute material that is in demand because it has properties that resemble bone.

BCP can be converted from derivate of shell which have a high calcium carbonate ($CaCO_3$) content. Blood cockle shells (*Tegilarca granosa*) have a high calcium carbonate content of 95.7-98.7%.[4,5] $CaCO_3$ has three types of crystal phases, namely; calcite with rhombic morphology (tilted box), aragonite with needle morphology, and vaterite with porous spheroid morphology. Calcite and aragonite have identical composition but distinct crystal structures. Aragonite transforms more easily into hydroxyapatite than calcite.[6] The hydrothermal method with time variations of 12, 24 and 36 hours could convert $CaCO_3$ from cuttlefish bone into hydroxyapatite.[7] The effect of varying hydrothermal time will affect the composition of the hydroxyapatite powder and the production of $CaCO_3$. Variations in hydrothermal time affect the structure of the powder produced and the number of crystals produced. The longer the hydrothermal time, the more crystals will form because the atomic arrangement in the material is more arranged and regulated.[7]

HA crystals and collagen matrix will form the bone structure. Bones consist of a porous cellular structure (light bone/cancellous bone) which is covered by a denser layer. The mechanical properties of bone in the form of porosity and density will vary and depend on the location of the load where the bone is located. Density determines the strength and rigidity of growing bones to support its body.[8] Therefore, bone substitute materials must provide various shapes and sizes of scaffolds with mechanical properties suitable for use in places where there is damage. The measurement of mechanical properties, especially compressive strength in cortical bone, is 110-230 MPa and in trabecular/cancellous bone is 2-12 MPa.[9] The mechanical strength of bone substitute material in a scaffold is an important and essential factor to determine the clinical success of a material. This is what causes differences in applications for various scaffolds. The mechanical strength of the scaffold can be influenced by the porosity of the scaffold. The higher the porosity, the lower the mechanical strength.[10]

One of the mechanical strengths of materials that is important in the mastication process in the oral cavity is compressive strength (CS) and diametral tensile strength (DTS).[11] CS is the maximum compressive strength given to a material until the material fractures. The compression strength test is usually used to characterize cancellous bone and included porous scaffolds.[12] Meanwhile, DTS is a measure of the strength of a loose material which does not allow for a flexural tensile strength test.[13] The average DTS value of glass ionomer cement (GIC) materials ranges from 5.54 to 13.72 MPa.[14] Research performed using biocomposites bHA/gelatine with polyvynil alchohol (PVA) coating stated that the mechanical strength of DTS at a concentration of 50% w/v was 6,219 MPa.[15]

One of the ideal requirements for a biomaterial to be applied clinically is non-toxicity. The cytotoxicity test could be performed with in vitro methods through BHK-21 fibroblast cell culture with the MTT Assay test.[16,17] Fibroblasts perform and carry through in bone regeneration, especially in the reparative phase, where fibroblasts will synthesize collagen and form new granulation tissue which will covering wounds, Fibroblasts have the ability to grow quickly in wound tissue, are easy to culture,

are stable, and are able to live on their own which is the reason for fibroblast cells to be the most popular subject for biological research.[17,18]

In this research, we developed a conversion method for BCP synthesized from *Tegilarca granosa* shells using the hydrothermal method with shorter time variations, viz. 6, 9 and 12 hours to produce BCP with the optimum preferred HA and TCP ratio composition to be used as a scaffold with mechanical strength equivalent to cancellous bone and is not toxic.

RESEARCH METHODS

The procedure of creating BCP included two stages, namely the $CaCO_3$ synthesis as the first stage and the BCP synthesis as the second stage. The first stage begun with the preparation of *Tegilarca granosa* shell by boiling, washing and brushing the outer and inner shells using water and soap without bleach, then drying at room temperature. Subsequent, the calcination process was carried out by crushing the clean shell using mortal and pastle into powder and placing it in a cruissible at a temperature of 100°C for 3 hours to remove organic components and produce $CaCO_3$ powder. The progress on second stage, the $CaCO_3$ solution mixed with $NH_4H_2PO_4$ solution using a magnetic stirrer for 30 minutes, then the mixed solution was transferred to the reactor. Accordingly, the reactor was put into a vurnace oven for sintering at a temperature of 200°C with varying times of 6, 9 and 12 hours. The results obtained were cooled to room temperature. The samples were washed with distilled water repeatedly until the pH was neutral (pH=7), then a final wash was carried out with methanol. The samples were dried in an electric oven at 50°C for 4 hours, persist of the progress by sintered again at 900°C for 3 hours. BCP powder samples obtained from the hydrothermal method with a time variation of 6 hours is called Group 1 powder, while BCP obtained from the hydrothermal method with a time variation of 9 hours is called Group 2 powder and BCP obtained from the hydrothermal method with a time variation of 6 hours is called Group 3 powder. Then, the three groups underwent an X-Ray Diffraction (X-RD) examination to determine the content of each sample.

The X-RD tool characterized the composition of Biphasic Calcium Phosphate for each group. The synthesized powder was put into a block-shaped container. Advanced on, the sample was placed in the X-RD test equipment. The X-rays produced from the tube containing the cathode heat the filament, producing electrons. The difference in voltage caused the acceleration of electrons to shoot at the object. An X-ray beam was produced when electrons have a high energy level and collide with electrons in an object. The object and detector rotated to capture and recorded the intensity of the X-ray reflection. The detector recorded and processed the X-ray signal in graphic form.

Futhermore, BCP powder proceed on cytotoxicity test which was carried out for each group using the colorimetric *3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide* (MTT) assay method by calculating the percentage of viability of BHK-21 fibroblast cells. BHK-21 fibroblast cell cultures and sterile 96-well microplates were prepared in laminar flow. Column 1 contains media control and column 12 is used as a positive control which only contains fibroblast cells. In the first column the microplate is filled with *Eagle's Minimum Essential Medium* (MEM), Kanamycin, Penstrep 1%, *Foetal Bovine Serum* (FBS) 10% Fungizone 100 units/ml, 100 µl. Columns 2 to 5 on the microplate were filled with BHK-21 cells at a density of 3 x 103 in *Eagle's Minimum Essential Medium* (MEM) culture medium, Kanamycin, Penstrep 1%, FBS 10%, Fungizone 100 units/ml, as much as 100 µl in each well. HA powder resulting from the synthesis of blood cockle shells using the hydrothermal method with varying sintering times of

6, 9, and 12 hours, was added to each well as much as 25 mg each in columns 2, 3, and 4. Persist in, the microplate was incubated in 5% CO_2 at temperature 37°C for 20 hours. The microplate is removed from the incubation device, the culture medium and blood clam shell HA powder in the well are taken using a syringe, fibroblast cells will be left in the well. Each well was refilled with 100 µl of culture media. MTT in phosphate buffered saline (PBS) that has been filtered using 0.20 µl millipore for each well, hence incubated again for 3 hours thus that MTT can carry out metabolic activity. Total incubation time in an incubator at 37°C for 24 hours. After the incubation period is complete, the MTT and culture media are taken using a syringe. In order to dissolve the formazan crystals, 50 µl of dimethyl sulfoxide (DMSO) was added to each well, then the microplate was shaken for 5 minutes. Formazan optical density values were read with an *Elisa reader* with a wavelength of 620 nm. The result of toxicity test was carried out by calculating the percentage of fibroblast cell viability using the formula:[16]

$$Cell\ Viability\ (\%) = \frac{OD\ of\ treatment\ group + OD\ of\ media\ control\ group}{OD\ of\ cell\ control\ group + OD\ of\ media\ control\ group}\ 100\%$$

Next, the process of creating the BCP scaffold was carried out using the freeze-drying method. The freeze-drying method is a method that consists of two process sequences, which are freezing followed by drying.[19] Scaffold Group 1 is a scaffold combination of BCP group 1 powder and gelatin. Scaffold Group 2 is a scaffold combination of BCP group 2 powder and gelatin. Meanwhile, Group 3 scaffolds are scaffolds with a combination of BCP group 3 powder and gelatin. BCP powder from each group was made into a suspension, formed by stirring BCP powder and 20% gelatin (w/v) in a ratio of 50:50 for 24 hours. In the DTS test, the BCP-gelatin suspension was placed in a Teflon mold measuring Ø6 mm x 4 mm. Meanwhile, in the CS test, the BCP-gelatin suspension was placed in a Teflon mold measuring Ø3 mm x 6 mm. Continued from, the BCP-gelatin suspension was frozen at -80° C for 5 hours. Accordingly, the frozen BCP-gelatin suspension was freeze dried for 30 hours to obtain a scaffold formation. The CS test and DTS test were carried out using a Universal Testing Machine (Shimadzu Autograph AG 10TE, Japan) with a crosshead speed = 5 mm/minute.[20] The specimen was placed in the middle of the jig and then a load was applied until the specimen experienced plastic deformation. The diametral tensile strength (DTS) formula is:[14]

$$\frac{2P}{\pi\ DT}$$

P= load applied on fracture (N)

π= 3.14

D= diameter of the specimen (mm)

T= thickness of the specimen (mm)

The compressive strength (CS) formula is:[21]

$$\frac{P}{\pi\ r^2}$$

P = load applied on fracture(N)

π = 3.14

r = radius of the specimen (mm)

Data was analyzed using *Kruskall-Wallis followed by Mann-Whitney test.* with SPSS software (Version 26.0, SPSS Japan Inc., Tokyo, Japan). The significant difference was accepted at p <0.05.

RESULTS

The XRD test results in Figure 1 shown the composition of the BCP content for each hydrothermal time of 6, 9 and 12 hours. Table 1 showed the percentage composition of BCP content including HA and β-TCP. The highest HA content was shown in Group 2, namely 87%. Meanwhile, Group 3 showed the lowest HA content, namely 72%. Group 1 showed a HA content of 81.80%. The highest β-TCP content was shown in Group 3, namely 21%. Meanwhile, Group 2 showed the lowest β-TCP content, namely 6%. Group 1 showed a β-TCP content of 14.10%.

Table 1. The percentage composition of BCP powder content

	HA (%)	β-TCP (%)
Group 1	81,8	14,1
Group 2	87	6
Group 3	72	21

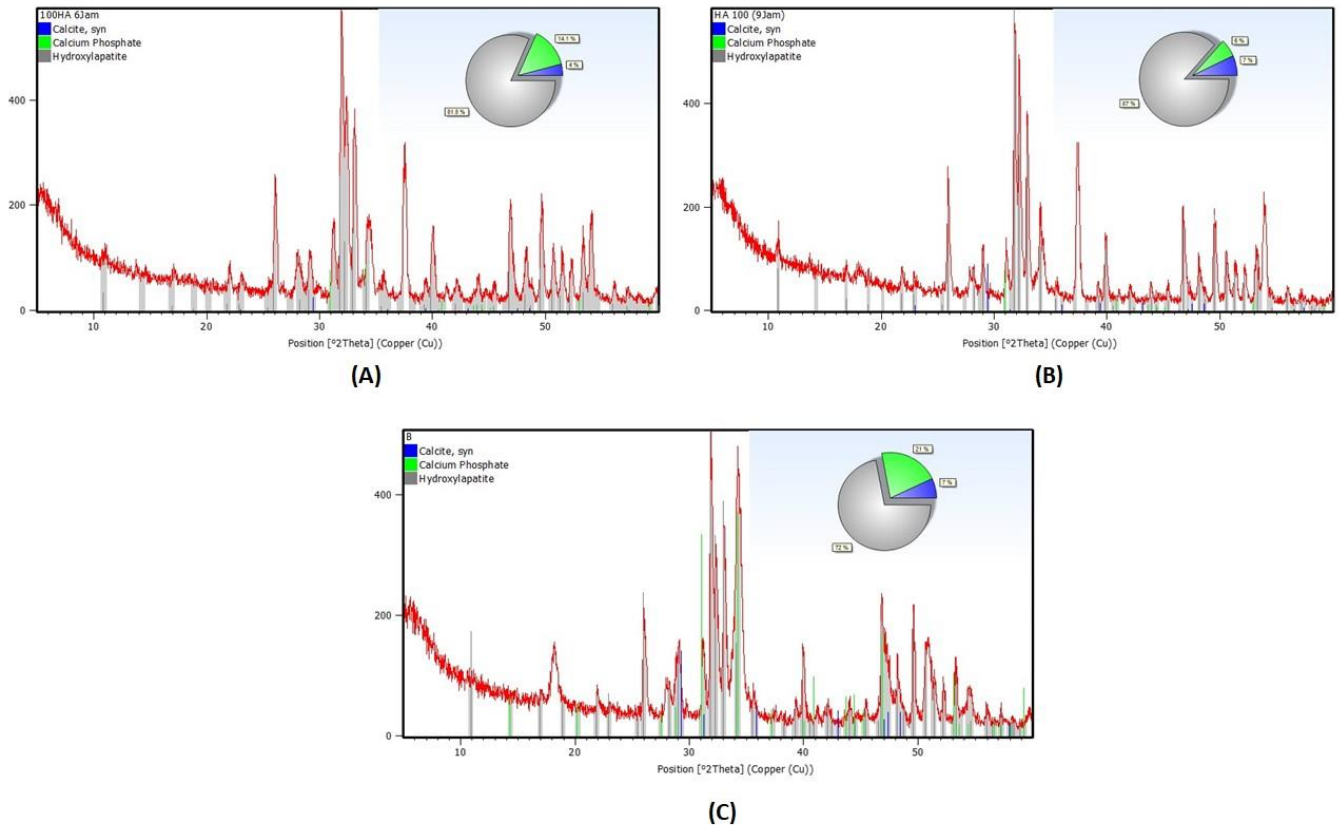

Figure 1. The XRD test results. (A) The composition of the BCP powder content for hydrothermal time of 6

hours. (B) The composition of the BCP powder content for hydrothermal time of 9 hours. (C) The composition of the BCP powder content for hydrothermal time of 12 hours.

The average results of fibroblast cell viability indicated in Table 2. Group 1, Group 2, and Group 3 showed a percentage of fibroblast cell viability, viz. 57.86%; 52.64% and 55.6%, respectively. The results of statistical analysis showed that there were no significant differences between Groups 1 and 2 (p=0.674), Groups 1 and 3 (p=0.916), Groups 2 and 3 (p=1.000).

Table 2. The cell viability of BCP powder

	Cell Viability (%)
Group 1	57,86
Group 2	52,64
Group 3	55,6

Table 3 and Figure 2 indicated the results of the diametral tensile strength and compressive strength tests. Diametral tensile strength scaffold Group 1, Group 2, and Group 3 are 0.401 N/mm2, 6.446 N/mm2 and 6.921 N/mm2, respectively. There was a significant difference in diametral tensile strength between Group 1 and Group 2 scaffolds (p = 0.006) and between Group 1 and Group 3 scaffolds (p = 0.002). Meanwhile, there was no significant difference in the diametral tensile strength of Group 2 and Group 3 scaffolds (p = 0.655). The highest compressive strength was shown in Group 3, 1.233 MPa. Concurrently, Group 1 showed the lowest compressive strength, that is 9 hours with 0.098 MPa. Group 2 shows a compressive strength of 1.028 MPa. There is a significant difference between compressive strength Group 1 and Group 3 (p=0.003), while between compressive strength Group 2 and Group 3 there is no significant difference (p=0.150).

Table 3. The diametral tensile strength and the compressive strength of BCP scaffolds

	DTS (N/mm2)	CS (N/mm2)
Group 1	0.401	0.098
Group 2	6.446	1.028
Group 3	6.921	1.233

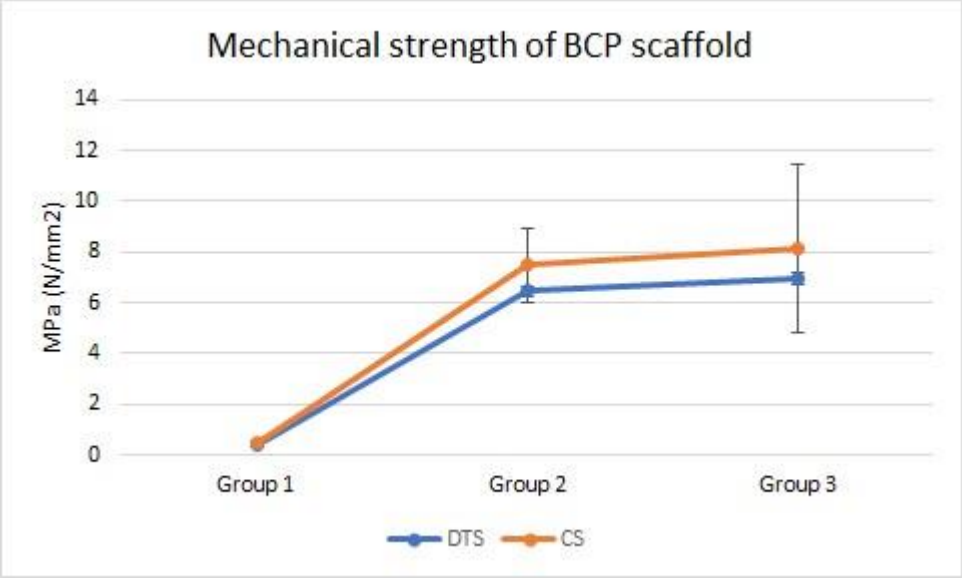

Figure 2. The diametral tensile strength (DTS) and the compressive strength (CS) of BCP scaffolds

DISCUSSION

This research developed a hydrothermal method with various variations in hydrothermal time to obtain BCP with various HA and TCP compositions from *Tegilarca granosa* shell. At a hydrothermal time of 6 hours, the HA content was 81.80% and the TCP content was 14.10%. At a hydrothermal time of 9 hours, a higher percentage of HA was produced, viz. 87% and a lower percentage of TCP, viz. 6%. The longer the hydrothermal time, the higher the HA content obtained and the lower the TCP content obtained. These results indicate that TCP transforms into HA phase and the percentage of HA increases gradually amidst with increasing hydrothermal time. Similar research also proves that with increasing length of hydrothermal time there is an increase in the percentage of HA followed by a decrease in the TCP peak and an increase in the HA peak in XRD examination.[22] On the other hand, at a hydrothermal time of 12 hours the HA content was found to be lower and the TCP content was higher than at a hydrothermal time of 6 hours and 9 hours, namely 72% and 21%. These results prove that in this study the highest peak point of the HA phase was found at a hydrothermal time of 9 hours. The decreasing HA content at a hydrothermal time of 12 hours is presumably due to hydroxyapatite having a peak point at a certain hydrothermal time and subsequently passing the peak point the hydroxyapatite will decrease. The results of this research indicate that the change in TCP to HA which occurs gradually and over a certain length of time in the hydrothermal reaction makes it potential and probable to control the composition of BCP and HA by regulating the length of the hydrothermal time. Apart from the length of hydrothermal time, the hydrothermal temperature factor also influences the formation of HA and TCP.

Research that was conducted using a hydrothermal temperature of 160˚C shows that the percentage of HA increases meanwhile the percentage of TCP decreases results in increasing hydrothermal time of 4h, 24h, 48h, and 72h.[22] At a temperature of 900˚C HA can change into Tricalsium phosphate (TCP) and Tetracalcium phosphate (TTCP). The longer the hydrothermal time, the higher the percentage of the TCP phase.[23] In contrast to the HA phase, the TCP phase is formed at high temperatures. The XRD phase

analysis, most HA will convert into TCP after the sintering process at a temperature of 1000°C.[23] Increasing the temperature and length of the hydrothermal reaction time are crucial and substantial parameters in the synthesis of HA and TCP using the hydrothermal method.[24]

BCP is a bone graft material contained of a combination of HA and TCP which is widely used to accelerate the healing of bone defects. HA has osteoconductive, osteoinductive, osteogenesis, and hydrophobic properties aside from it has a structure similar to bone components, and a low resorption rate. TCP has osteoconductive properties and will have better biodegradation and incorporation properties when combined with HA. Therefore, the biocompatibility properties of BCP synthesized from *Tegilarca granosa* shell using the hydrothermal method with various variations in hydrothermal time need to be known and researched. This research shows that all BCP groups synthesized using the 6 hour, 9 hour and 12 hour hydrothermal method show a percentage of fibroblast cell viability above 50%. The percentage of fibroblast cell viability in the BCP group which was synthesized using the hydrothermal method for 6 hours was 57.86%, the viability of fibroblast cells in the HA group which was sintered for 9 hours was 52.64%, while the hydroxyapatite which was sintered for 12 hours was 55.60%. Based on the lethal concentration(LC50) principle, toxicity becomes a toxic condition if the cell viability research results show a percentage of less than 50%.[25,26] Therefore, in this study it can be concluded that HA synthesized from *Tegillarca granosa* using the hydrothermal method with varying hydrothermal times of 6, 9 and 12 hours, is non-toxic because fibroblast cell viability in all groups showed results exceeding 50%.

Barring to have favourable biocompatibility properties, bone substitute material must also have mechanical properties that are equivalent to the mechanical properties of bone in the area of the material. High crystalline HA (Ca/P ratio: 1.67) shows sufficient mechanical strength, but HA is relatively insoluble in vivo. On the other hand, TCP is fragile, but TCP shows proper bio absorption in vivo. BCP as a scaffold is more effective in hard tissue regeneration than pure HA or pure TCP.[27] This research shows that Group 1 has low diametral tensile strength and compressive strength values, namely 0.401 N/mm2 and 0.098 N/mm2 compared to Group 2 which has the highest diametral tensile strength and compressive strength values, namely 6,446 N/mm2 and 1,028 MPa. Group 1 has a HA composition of 81.80% and TCP 14.10%, while Group 2 has a HA composition of 87% and TCP 6%. These results prove that the composition of HA and TCP in BCP affects the mechanical strength of the BCP scaffold. Similar research on BCP scaffolds with similar HA and TCP compositions (73%:26%) also showed the highest hardness values compared to the hardness values on BCP scaffolds with HA compositions of 2.8% and TCP 97%.[28] The other studies also proved the mechanical strength of BCP scaffold increased with the increased density of scaffold wall due to the increase in the amount of HA.[27] On the other hand, this study showed that the group which had a HA composition of 72% and TCP 21% showed diametral tensile values strength and compressive strength the highest, namely 6,921 N/mm2 and 1,233 N/mm2 compared to Group 1 which had a HA composition of 81.80% - TCP 14.10% and Group 2 which had a HA composition of 87% and TCP 6%. This study proved the initial composition of BCP scaffold have an impact and leverage on mechanical properties. Whereas, the other study explained the mechanical properties of the BCP scaffold can also be influenced by the size, morphology and distribution of pores of the scaffold.[29] The porosity of the BCP

scaffold itself was also dependent on the composition of HA and TCP.[30]

CONCLUSION

The characteristics of BCP composition that synthesized from *Tegilarca granosa* using hydrothermal method were depent on hydrothermal times. Although all groups of scaffolds were non-toxic, but BCP scaffold that synthesized from *Tegilarca granosa* using hydrothermal for 12 hours showed the highest mechanical properties.

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