

# Synthesis and Characteristics of Chitosan from Haruan (*Channa striata*) Fish Scales

Deby Kania Tri Putri<sup>1\*</sup>, Wijayanti Diah W.H<sup>2</sup>, Beta Widya Oktiani<sup>3</sup>, Candra<sup>4</sup>, Bayu Indra Sukmana<sup>5</sup>, Priyawan Rachmadi<sup>6</sup>, Harun Achmad<sup>7</sup>

<sup>1</sup>Department of Biomedic, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin - Indonesia

<sup>2</sup>Department of Oral Biology, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin – Indonesia

<sup>3</sup>Department of Periodontia, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin – Indonesia

<sup>4</sup>Department of Fisheries Product Technology, Faculty of Fisheries, Lambung Mangkurat University Banjarmasin – Indonesia

<sup>5</sup>Department of Dental Radiology, Faculty of Dentistry, Lambung Mangkurat University, Banjarmasin – Indonesia

<sup>6</sup>Department of Dental Material, Faculty of Dentistry, Airlangga University, Surabaya – Indonesia

<sup>7</sup>Department of Pediatric Dentistry, Faculty of Dentistry, Hasanuddin University, Makassar - Indonesia

E-mail: [debykania98@gmail.com](mailto:debykania98@gmail.com)

Article History:

Submitted: 20.01.2020

Revised: 09.03.2020

Accepted: 03.04.2020

## ABSTRACT

**Background:** Haruan fish is one of the fisheries production which is quite popular in South Kalimantan. The body parts of the fish that usually become waste are scales. The lack of management of these wastes raises various issues in the environmental field that can extend to social and health problems, whereas fish scales contain chitin which can be synthesized into chitosan which is useful in the biomedical field. However, chitosan scales (*Channa striata*) have never been used optimally because there are no data available regarding the content of chitosan scales.

**Purpose:** The purpose of this study is to identify and know the characteristics of chitosan contained in the scales of haruan (*Channa striata*).

**Methods and materials:** This research used a descriptive observational method by conducting quantitative analysis to determine physical characteristics (yield, moisture content, ash content, protein content, fat content, fiber content, carbohydrate content), chemical characteristics (pH and degree deacetylation), as well as the morphological description of chitosan powder with SEM.

**Results:** Physical characteristics of chitosan from haruan scales were yield of 24.10%, moisture content of 4.92%, ash content of 0.45%,

protein content of 0.17%, fat content of 0.66%, fiber content of 0.68%, carbohydrate content of 0.11%, and dissolve in 1% acetic acid. The chemical characteristics of chitosan from haruan fish scales are pH 10,16 and deacetylation degree (DD) of 85.25%. Morphological description of haruan chitosan fishscales in the form of many layers with structures that are almost spherical, porous, fibrous, broken, and irregular.

**Conclusion:** In this study it has been proven that chitosan scales from haruan fish (*Channa striata*) have physical, chemical, and also characteristic morphology.

**Keywords:** Characteristics, chemical, chitosan, Haruan fish scales, morphology, physical.

## Correspondence:

Deby Kania Tri Putri

Department of Biomedic, Faculty of Dentistry, Lambung University Banjarmasin Indonesia

E-mail: [debykania98@gmail.com](mailto:debykania98@gmail.com)

DOI: [10.31838/srp.2020.4.04](https://doi.org/10.31838/srp.2020.4.04)

@Advanced Scientific Research. All rights reserved

## INTRODUCTION

Haruan Fish is one of the fisheries production which is quite popular in South Kalimantan. Haruan fish is known to have properties especially in helping to accelerate wound healing and anti-inflammatory effects. In addition to treatment, haruan fish is also a favorite food of the people of South Kalimantan, so it has a bright prospect to be developed in Indonesia.<sup>1</sup> The quantity of fragrant fish which is quite large makes the meat widely used as the main raw material on an industrial scale and household scale. The average portion of edible portion is 40–50%. The body parts of the fish that usually become waste are scales. The lack of management of these wastes raises a variety of issues in the environmental field that can extend to social and health problems.<sup>2</sup>

Today the use of fish scales is increasingly popular in various fields, such as in the fields of pharmaceuticals, food, and medical fields by utilizing the lower dermis layer of fish scales, namely chitin.<sup>3</sup> The nature of chitin which is non-toxic and easily degraded encourages the modification of chitin to optimize usability and expand the field of chitin application. One of the derivative compounds of chitin which is widely developed because of

its wide application is chitosan.<sup>4</sup> Chitosan is a natural product of chitin. Fawzya et al 2008 said chitosan has special properties in terms of biocompatibility, biodegradation, biological activity, is not toxic, and does not cause allergies.<sup>5</sup>

Chitosan was obtained through chemical and enzymatic reactions from deacetylated chitin so that chitosan has biodegradable polymeric properties.<sup>3</sup> Wibowo et al (2005) also added that chitosan has special properties in terms of biocompatibility, biodegradation, biological activities such as antibacterial, does not cause allergies, and its ability to form fibers and films.<sup>6</sup> According to Wardaniati and Sugiyani (2009), chitosan is very potential to be used as an antibacterial material because it contains amino polysaccharide group which can inhibit bacterial growth.<sup>7</sup> Based on the description above, it can be seen that fish scales contain chitin which can be synthesized into chitosan which is useful in the biomedical field. However, these scented fish scales (*Channa striata*) have not been used optimally because there are no data available regarding the content of scales. Therefore, this study is needed as a preliminary study to determine the extraction and characteristics of chitosan from fragrant fish scales.

Information about some of the physical and chemical characteristics of fish scales that will be obtained in this study is expected to be a bridge to find out the potential of fragrant fish scales, especially in its use in the field of dentistry. The purpose of this study is to identify and know the characteristics of chitosan contained in the scales of haruan fish (*Channa striata*).

## METHODS AND MATERIALS

This research is an observational descriptive study. The research was carried out by conducting a quantitative analysis of quantitatively identifying the physical characteristics of chitosan scales, the quantitative chemical characteristics of chitosan scales, and obtaining a morphological description of chitosan powder with SEM. The sampling technique used simple random sampling. The inclusion criteria for the study sample were haruan fish scales obtained directly from local market in Banjarmasin South Kalimantan, and haruan fish scales were scales of haruan fish that had not undergone decay with special marks/colors covered in clear mucus and not much dirt was attached. The data obtained are primary data collected directly from the results of the study. Data obtained based on the determination of yield, water, ash, protein, fat and fiber content as physical characteristics of chitosan scales, pH and deacetylation degree as chemical characteristics of chitosan scales, and SEM as morphological characteristics of chitosan scales.

**Sample preparation:** The haruan fish scales was weighed so that the weight of 3 kilograms is obtained. The next preparation was the separation of scales from haruan fish and washed of fish scales. Fish scales that have been washed with water replacement four times, wiped with a rag, are weighed so that the weight of 1003 grams is obtained. The scales of the fish to be synthesized were dried so that the fish scales become brittle to facilitate the process of shading. Drying was done by roasting and using the oven. In the stage of scooping, fish scales were inserted into the mixing machine so that the output is in the form of fine fish scale powder with a size of 50 mesh.

**Chitosan preparation:** Isolation of chitin from haruan fish scales powder consists of three stages, deproteinization was using 4% NaOH (1:1 m/v) boiling for 1 hour on a hot plate. Then washed with distilled water until the pH was neutral and dried at 50°C for 24 hours. Obtained by deproteination scales powder as much as 620 grams. Then demineralized by mixed and stirred in 1 M HCL solution (1: 5 m / v) for 24 hours at 30°C. Then wash with aquadest until the pH was neutral. There were 342 grams of demineralized powder obtained. To detect the presence of chitin in fragrant fish scales samples, we could use the Van Wesslink color reaction. Chitin reacted with 1% I2-KI solution which will give a brownish yellow color,

then 1 M H<sub>2</sub>SO<sub>4</sub> is added to turn into purplish red or red violet. The color change from brownish yellow to purplish-red shows a positive reaction to chitin. Furthermore, chitosan preparation was carried out by breaking the acetyl group contained in chitin extract from fragrant fish scales. The deacetylation process was carried out by dissolving chitin in 50% NaOH and heating it at 100°C for 120 minutes on the hot plate. The results obtained were filtered and washed with distilled water to neutral pH then dried at 120°C for 24 hours using the oven. Chitosan fish scales were obtained in the form of cream colored powder as much as 82.43 grams.

### Determining the Physical Characteristics of Chitosan Fish Scales

#### a. Yield

The yield according to Kaimudin and Leonupun 2016 states that there is a correlation between molecular weight and yield. The chitosan yield decreased with increasing concentration of sodium hydroxide solution and temperature.

The yield percentage is obtained from the equation :<sup>8</sup>  
Results (%) = (Amount of chitosan obtained / chitin amount obtained) × 100%

#### b. Calculation of Water Content

Chitosan is hygroscopic so that it easily absorbs moisture from the surrounding air. The water content contained in chitosan is expressed as H<sub>2</sub>O which is bound to chitosan polymer functional groups, especially the amine group, N-acetyl and hydroxyl through hydrogen bonds. Chitosan water content depends on the relative humidity of the surrounding air storage because chitosan is hygroscopic.<sup>9</sup>

Water content =

$$\frac{\text{Initial chitosan weight} - \text{dry chitosan weight}}{\text{Dry chitosan weight}} \times 100\%$$

#### c. Calculation of Ash Content

Ash content indicates the presence of components of inorganic compounds contained in the raw material of shrimp skin, chitin and chitosan. According to Nugroho et al 2011, ash content is a measure of the success of the demineralization process in the process of isolating chitin from its raw material. Ash content can be used as a parameter of chitosan quality, because of the lower the ash content, the higher the chitosan purity level, and vice versa. Chitosan ash content indicates that the demineralization process has not been fully able to remove inorganic minerals in samples, especially calcium carbonate and calcium phosphate.<sup>9</sup>

$$\text{Ash content} = \frac{\text{Ash weight}}{\text{Early sample weight}} \times 100\%$$

**d. Calculation of Protein Content**

Protein levels obtained from the calculation of nitrogen levels multiplied by conversion factors (6.25). Nitrogen or total nitrogen content is one of the parameters that shows the good or not the quality of chitosan and determines the success of the deproteinization process where the lower the nitrogen content produced, the more effective the deproteinization process for chitosan production. Nitrogen levels determine the nature of chitosan which interacts with other groups. The presence of other compounds in chitosan, namely the form of amine groups (NH<sub>2</sub>) causes chitosan to have a high enough chemical reactivity and can bind water and dissolve in acetic acid. The high level of nitrogen is thought to be caused by a less uniform stirring process in the deproteinization and deacetylation process so that the protein in the material is not much released. Protein levels can be calculated using equations :<sup>10,11</sup>

$$\% \text{ Protein content} = \frac{(VA-VB)HCl \times N \text{ HCl} \times 14,007 \times 100\% \times 6,25}{W \times 1000}$$

Or % Protein levels = %Nitrogen content x Conversion factors

Information :

VA : ml HCl for example titration  
 VB : ml HCl for blank titration  
 N : Normality of standard HCl used 14,007: Weight of nitrogen atom  
 6.25 : Protein conversion factor for fish  
 W : sample weight (gram)

**e. Calculation of Fat Content**

The loss of minerals and oxides in the material besides causing a decrease in ash content also causes a decrease in fat content in the language. Decreasing fat content occurs in line with the decrease in ash content from fish scales into chitosan.<sup>12</sup>

$$\text{Fat Content} = \frac{C - A}{B} \times 100\%$$

Information :

A is the weight of an empty round flask, in grams  
 B is the sample weight, in grams  
 C is the weight of a round bottom and fat flask extracted, in grams

**f. Calculation of Fiber Content**

$$\% \text{ Crude fiber content} = \frac{C - A - (E - D)}{B} \times 100\%$$

Information :

A is the weight of filter paper (grams)  
 B is sample weight (gram)  
 C is the weight of filter paper and deposits (grams)  
 D is the weight of a porcelain cup (gram)  
 E is the weight of the cup with ash (gram)

**g. Calculation of Carbohydrate Content**

Calculation:

(Blanks) x N tio x 1.0 N is equivalent to reduced exposure. Then look at Luff School's list of mg of sugar contained for ml tio used.

$$\text{Carbohydrate content} = \frac{W1 \times fp}{W} \times 100\%$$

Where :

Carbohydrate level = 0.90 x glucose level  
 W1 = sample weight in mg  
 W = glucose contained for ml tio used in mg from the list  
 Fp = dilution factor

**h. Solubility**

Chitosan was dissolved in 1% acetic acid and then stirred using a stirring rod and then left for a while, then solubility was observed by comparing the clarity of chitosan solution with the clarity of the solvent. The higher the solubility of chitosan shows a clearer solution in 1% acetic acid, which shows that the quality of chitosan is getting better.

Determination of the Chemical Characteristics of Chitosan from Haruan Fish Scales

**a. PH value**

Calibrate the pH meter with a pH buffer solution. Do it every time you will take measurements. Dip the electrodes that have been cleaned with distilled water into the sample to be examined. Adjust the temperature of the example. Note and read the pH price on the pH meter scale that the needle shows.

**b. Degree of Deacetylation**

The degree of deacetylation (DD) of chitosan is very important because it affects the physical, chemical and biological properties of chitosan. Group N-acetyl bond chitin is difficult to remove and to eliminate it requires a high concentration of NaOH at high temperatures. Chitosan DD also depends on the preparation Method and the crustacean species used. DD chitosan scales of Haruan fish are obtained from the results of the Fourier Transform Infra Red (FTIR) spectrum. The method of analysis using FTIR is more often used and is a better method than the elemental analysis method. The range of DD chitosan ranges from 56-99%, with an average of 80% (Sugiyanti et al, 2018).<sup>13</sup> In

this study, 50% of NaOH was used to remove the N-acetyl group. The degree of deacetylation was characterized using spectrophotometric FTIR by providing infrared light with a frequency of 400-4,000 cm<sup>-1</sup> and 4 cm<sup>-1</sup> resolution in chitosan samples and then infrared absorbance was recorded. The hydroxyl group was calculated at a wavelength of 3450 cm<sup>-1</sup>, while the amide group was at a wavelength of 1655 cm<sup>-1</sup>.

1. The degree of deacetylation of chitosan can be calculated using the following equation:<sup>14</sup>

$$\% DD = 100 - \left[ \frac{A_{1655}}{A_{3450}} \times \frac{100}{1,33} \right]$$

A<sub>1655</sub> = absorbance at a wavelength of 1655 cm<sup>-1</sup> A<sub>3450</sub> = absorbance at a wavelength of 3450 cm<sup>-1</sup>

#### Determination of Morphological Characteristics of Chitosan Haruan Fish Scales with SEM

Characteristics of the morphology of chitosan from haruan fish scales was seen using Scanning Electron Microscopy with 1500x and 5000x magnification.

## RESULT AND DISCUSSION

Table 1. Characteristics of Chitosan from Haruan Fish Scales (*Channa striata*)

No	Parameter	Chitosan from	
		Haruan Scales (%)	FishChitosan SNI 7949 (2013)*
1	Yield	24,10%	-
2	Water Content	4,92%	≤12%
3	Ash Content	0,45%	≤ 1%
4	Protein Content	0,17%	≤0,5%
5	Fat Content	0,66%	-
6	Fiber Content	0,68%	-
7	Carbohydrate Content	0,11%	-
8	Solubility in Acetic acid 1%	Soluble	Soluble
9	Ph	10,16	-
10	Deacetylation Degree	85,25%	≥75%
11	Morphology	Almost spherical, porous, fibrous, broken, and irregular	-

The results of the characterization test of chitosan from

haruan fish scales (*Channa striata*) from table 1. show that the characteristics of haruan chitosan scales include yield, proximate levels (water, ash, protein, fat, fiber, carbohydrate), solubility in 1% acetic acid, pH, degree of deacetylation (DD), and morphology. Chitosan haruan fish scales meet chitosan standards set by the National Standard Agency (BSN), namely chitosan scales of Haruan fish have quality and quality that are not less good than chitosan quality set by BSN.

#### Physical Characteristics of Chitosan from Haruan Fish Scales (*Channa striata*)

The results showed that the deproteination, demineralization, and deacetylation processes were effective. Because the three processes have resulted in the loss of minerals and proteins found in the waste of Haruan fish scales. The chitosan yield percentage was influenced by the concentration of sodium hydroxide solution and temperature. Lesbani et al (2011) said that the water content of chitosan was low because of the transformation process of chitin to chitosan using sodium hydroxide which is a hygroscopic compound so that the water content of chitosan becomes low.<sup>15</sup> Low water levels can reduce or reduce damage to chitosan, for example, avoid the activity of microorganisms due to moisture. Chitosan ash content decreased with the demineralization process of haruan fish scales into chitosan. Metal minerals and oxides contained in Haruan fish scales are decreasing. Inorganic minerals can be removed by acid and alkaline treatment. With the loss of minerals and oxides in the material besides causing a decrease in ash content, it also causes a decrease in fat, fiber and carbohydrate content in these ingredients. The deproteination process of haruan fish scales into chitosan causes the protein contained in Haruan fish scales to decrease. The higher NaOH concentration and temperature, the protein separation process is more effective.

#### Results of Chemical Characteristics of Chitosan Fish Scales Haruan (*Channa striata*)

The deacetylation rate was determined based on the absorption wavelength of 3450 cm<sup>-1</sup> for hydroxyl and 1655 cm<sup>-1</sup> for each amide group. The absorption of wavelength is calculated based on the FTIR absorbance graph obtained from chitosan scales of haruan fish (Figure 1). Based on FTIR charts, it can be seen that the deacetylation degree (DD) of haruan chitosan scales is 85.25%, this DD is higher **than SNI chitosan which is ≥75% this shows that the** deacetylation process with 50% sodium hydroxide at 80oC for approximately 2 hours it effectively removes the acetyl group and replaces it with an amine group. This is in accordance with the statement of Mursida et al (2018) which states that chitosan purity is largely determined by the degree of deacetylation (DD), the more the acetyl group can be removed and replaced by the amine group,

the higher the value of the degree of deacetylation.<sup>16,17,18,19</sup>

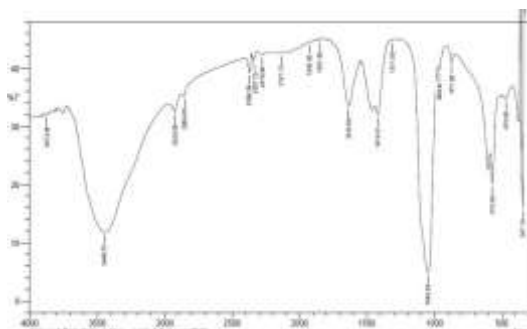


Figure 1. Chitosan FTIR Graph of Haruan Fish Scales (*Channa striata*)

#### Morphological Characteristics of Chitosan from Haruan Fish Scales with SEM

Characterization of chitosan using electron microscopy, scanning was carried out at 1500x magnification (Figure 2) and 5000x (Figure 3). Based on SEM characterization, it is known that the steps of deproteinization, demineralization, and deacetylation show an influence on chitosan produced. The figure below shows that chitosan from haruan fish scales has many layers with a structure that is almost round, porous, fibrous, broken, and irregular.

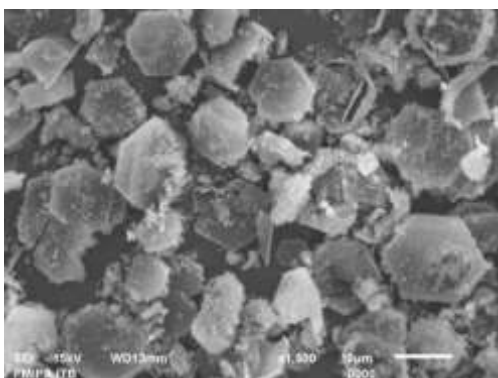


Figure 2. Morphological characteristics of chitosan haruan (*Channa striata*) fish scales using SEM 1500x magnification

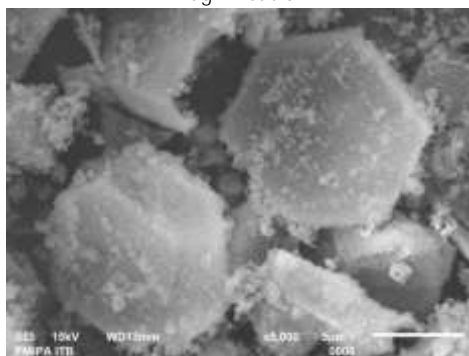


Figure 3. Morphological characteristics of chitosan

haruan (*Channa striata*) fish scales using SEM 5000x magnification

#### CONCLUSION

In this study it has been proven that chitosan scales (*Channa striata*) have physical, chemical and morphological characteristics that are unique, these characters also meet the standards of the National Standard Agency of Indonesia (BSN).

#### SOURCE OF FUNDING

The source of funding in our research comes from private funds.

#### CONFLICT OF INTEREST

The authors declare no conflicts of interest.

#### REFERENCES

1. Agustin R, Dewi N, Rahardja SD. (2016). Efektivitas Ekstrak Ikan Haruan (*Channa striata*) Dan Ibuprofen Terhadap Jumlah Sel Neutrofil Pada Proses Penyembuhan Luka Studi in Vivo pada Mukosa Bukal Tikus (*Rattus norvegicus*) Wistar. Dentino Jur. Ked.Gigi. 1(1): 68 –74.
2. Aziz N, Gufran MFFB, Pitoyo WU, Suhandi. (2017). Pemanfaatan Ekstrak Kitosan dari Limbah Sisik Ikan Bandeng di Selat Makassar pada Pembuatan Bioplastik Ramah Lingkungan. Hasanuddin Student Journal.1(1): 56-61.
3. Faridah F, Khafidzoh A, Mustikawati D, Anggraeni N, Dharmawan Y. (2012). Chitosan Pada Sisik Ikan Bandeng (*Chanos Chanos*) Sebagai Alternatif Pengawet Alami Pada Bakso. Jurnal Ilmiah Mahasiswa. 2(2):76-79.
4. Roberts GAF. (2008). Thirty Years Of Progress In Chitin And Chitosan. Progress On Chemistry And Application Of Chitin And Its Derivatives. 13: 7-15.
5. Fawzya YN, Novianty R, Wibowo S. (2008). Effect Of Deacetylation And Alkalinization On The Characteristic Of Carboxymethyl Chitosan. Jurnal Perikanan J Fish Sci. 10 (1): 64-75.
6. Wibowo S, Oktavia D.O, Fawzya Y.N. (2005). Pengaruh Jumlah Monokloro Asetat terhadap Karakteristik Karboksimetil Kitosan dari Kitosan Cangkang dan Kaki Rajungan. Jurnal Penelitian Perikanan Indonesia. 11(4):79-88.
7. Wardiani R.A dan Sugiyani S. (2009). Pembuatan Chitosan dari Kulit Udang dan Aplikasinya untuk Pengawetan Bakso. Semarang: Universitas Diponegoro.
8. Srivastav A, Mishra SS, Debnath S, Datta D. (2018). Extraction And Characterization Of Chitosan From Waste Scales Of *Labeo rohita*. Journal of Emerging Technologies and Innovative Research (JETIR). 5(6): 540-544.



9. Dompeipen EJ, Kaimudin M, Dewa RP. (2016). Isolation Of Chitin And Chitosan From Waste Of Skin Shrimp. e jurnal Kemenperin Majalah Biam. 12 (1): 32-38.
10. Suptijah P, Jacob AM, Rachmania D. (2011). Karakterisasi Nano Kitosan Cangkang Udang Vannamei (*Litopenaeus vannamei*) Dengan Metode Gelasi Ionik. Jurnal Pengolahan Hasil Perikanan Indonesia. 14(2): 78-84.
11. Nadia LMH, Suptijah P, Ibrahim B. (2014). Produksi Dan Karakterisasi Nano Kitosan Dari Cangkang Udang Windu Dengan Metode Gelasi Ionik. JPHPI.; 17(2): 119-126.
12. Kaimudin M, Leounupun MF. (2016). Characterization Chitosan From Waste Leather With Shrimp Process Using Different Bleaching And Deacetylation. E Jurnal Kemenperin Majalah Biam. 12 (1): 1- 7.
13. Sugiyanti D, Darmadji P, Anggrahini S, Anwar C, Santoso U. (2018). Preparation and Characterization of Chitosan from Indonesian Tambak Lorok Shrimp Shell Waste and Crab Shell Waste. Pakistan Journal of Nutrition.17(9) : 446-453.
14. Kumari S, P Rath, Kumar ASH. (2016). Chitosan From Shrimp Shell (*Crangon crangon*) and Fish Scales (*Labeo rohita*) : Extraction and Characterization. Afr J Biotechnol. 15(24): 1258-1268.
15. Lesbani A, Yusuf S, Melviana RAM. (2011). Karakterisasi kitin dan kitosan dari cangkang kepiting bakau (*Scylla serrata*). Jurnal penelitian sains. 14 (3): 32-36.
16. Mursida, Tasir, Sahriawati. (2018). Efektifitas Larutan Alkali Pada Proses Deasetilasi Dari Berbagai Bahan Baku Kitosan. JPHPI. 21(2): 356-366.
17. Achmad H, Ramadhany YF. (2017). Effectiveness of Chitosan Tooth Paste from White Shrimp (*Litopenaeusvannamei*) to Reduce Number of Streptococcus Mutans in the Case of Early Childhood Caries. Journal of International Dental and Medical Research. 10 (2): 358-363.
18. Achmad H, Ramadhany YF, Ramadhany S. (2019). Resistivity of Protein Kinase-B (Akt), Nf-Kb Transduction Obstacles, and Apoptosis Induction (Caspase -3, -9) as Anti-Proliferation and Anti-Cancer of Burkitt's Lymphoma Using Flavonoid Fraction Of Ethyl Acetate From Ant Nest (*Myrmecodia pendans*). The 3<sup>rd</sup> International Conference On Science. Journal of Physics: Conference Series. p.1341.
19. Achmad H, Horax S, Ramadhany S, et.al. (2019). Resistivity of Ant Nest (*Myrmecodia pendans*) On Ethanol Fraction Burkitt's Lymphoma Cancer Cells (Invitro) Through Interleukin 8 Angiogenesis Obstacles (II-8). Journal of International Dental and Medical Research ,.ISSN 1309-100X. Vol 12 No. (2) pp.516-523.