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Isolation and Toxicity Test of Chitosan from Green Mussels (*Perna viridis* L.) With Brine Shrimp Lethality Test (BSLT) Method

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Abstract

Green Mussels (*Perna viridis* L.) is one of Indonesia's fishery resources. The purpose of this study was to determine the toxicity level of chitosan from green clam shells (*Perna viridis* L.) by looking at the LC_{50} value tested on the BSLT method. This study included isolation of chitin and chitosan: deproteination, demineralization, depigmentation and deacetylation of chitin into chitosan, chitosan characterization, FTIR, and chitosan Toxicity Test using the BSLT method to see the number of deaths of *Artemia salina* L larvae obtained data (LC_{50}). The results showed that the Chitosan Green Clam Shell (Perna viridis L.) had an LC_{50} of 4369.1806 µg/mL. The results showed that green clam shell chitosan (*Perna viridis* L.) was non-toxic (LC_{50} > 1000 µg/mL) in the BSLT test.

Keywords: BSLT, Chitosan, Shellfish, Toxicity Test

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1 Introduction

Indonesia has considerable potential in the fisheries. Crustaceans and Bivalvia are one of the largest and most sought-after commodities in both local and international markets. The types of this group include shrimp, crabs, clams and so on [1]. So far, green mussel shell waste has only been used as a material for wall decoration, or even as a mixture for animal feed. The waste treatment certainly does not have a large added value because it is still limited in terms of price and amount of production. One alternative effort to utilize green clam shell waste to have the value and usability of green clam shell waste and tofu clams into products with high economic value is processing into chitin and chitosan [2]. Chitosan has been known as an additive in medicinal preparations, as a moisturizing agent, making contact lenses and food preservatives. Chitosan has an active group that can inhibit microbial growth, accelerate burn healing, and antioxidants [3]. Besides that, chitosan can also be used as a preservative, one of which is a natural preservative for crayfish [4].

Nevertheless, if chitosan is to be used it must be biocompatible, which means that it is acceptable to the human body, non-toxic, non-irritating, non-carcinogenic, and also safe without causing allergic reactions. Therefore, to reduce the adverse effects of using chitosan into the human body, researchers need to conduct toxicity tests [5].

The principle of knowing a compound is toxic or has cytotoxic abilities can be carried out a tokisity test. In vivo, the death of an experimental animal can be used as a means of monitoring the initial screening of the toxicity of a chemical active substance of a natural material against extracts, fractions or isolates. One of the natural ingredients used is ethanol extract of cocoa leaves. Based on the results of the cytotoxicity test using the Brine Shrimp Lethality Test method, it was shown that the ethanol extract of cocoa leaves (*Theobroma cacao* L.) had cytotoxicity with an LC_{50} value of 269.15 µg/mL [6]. One of the methods used to determine the toxicity of compounds is the Brine Shrimp Lethality Test (BSLT) using the larvae of *Artemia salina* Leach shrimp. This *Artemia salina* Leach is a simple organism, easy to breed and hatch under normal laboratory conditions. This toxicity test is intended to expose the presence of toxic effects and to examine the safety limits contained in the chitosan of the green clam shell.

Based on the description above, it recommends researchers to use the BSLT approach to investigate the toxicity of green mussel chitosan (*Perna viridis* L.). Green mussel shell trash that was previously discarded by the community can be utilized to manufacture chitosan, and a toxicity test can be performed to identify the hazardous effects and safety limitations present in green mussel shell chitosan.

2 Methods

2.1 Tools and Materials

The used tools in this study were : Beaker Glass (Pyrex®), Magnetic Stirrer, Hot Plate, Oven (Memmert®), Measuring Glass (Pyrex®), Stirring Rod, pH Meter, Furnace, Desiccator, Evaporator Cup, Porcelain Crust, Filter Paper, Funnel, Analytical Scales (Newtech®), FTIR Instruments (Shimadzu®). The used materials in this study were : green mussel shells (Perna viridisL.), NaOH solution, 0.315% NaOCl, HCl solution, 2% acetic acid, aquadest.

2.2 Sample Processing

5 kg of green mussel shells that have been washed and Then, they were dried using the oven at the temperature of 100°C. After drying, the samples were ground and sieved. The resulting powder is 100 mesh in size.

2.3 Chitosan isolation

2.3.1 Deproteination

Green clam shells have been mashed to 100 mesh in size 200 g each plus a 3.5% NaOH solution in a ratio of 1: 10 (w/v) between the sample and the solvent. The mixture introduced into a beaker, heated on a hot plate at a temperature of 60-70°C for 2 hours while stirring at a speed of 50 rpm using a magnetic stirrer. The obtained solids washed with aquadest several times until a neutral pH. The obtained solids dried in the oven at a

temperature of 60°C for 4 hours then cooled in a dexicator and weighed [7].

2.3.2 Demineralization

The demineralization stage used a deproteinated sample plus a 1N HCl solution in a ratio of 1:10 (w/v). The clam shell powder and 1N HCl solution mixed in a beaker and then heated in a hot plate at a temperature of 60-70°C for 1 hour while stirring at a speed of 50 rpm using a magnetic stirrer. The obtained solids washed with aquadest several times until a neutral pH. Solids dried in the oven at a temperature of 60°C for 4 hours, the clam shell powder obtained without minerals then cooled in a dexicator then weighed [7].

2.3.3 Depigmentation

Demineralized chitin residue was added with 0.315% NaOCl (1:10 b/v). The two mixtures are mixed in beaker glass then heated on a hot plate at a temperature of 40°C then, stirred for 1 hour. then filtered and the residue obtained is washed with aquadest to a neutral pH and then dried in an oven with a temperature of 80℃, then cooled in a dexicator, after which the results weighed [8].

2.3.4 Deacetylation

The residue obtained from the depigmentation process (chitin) was continued by adding 60% NaOH in a ratio of 1: 20 (w/v) then stirred at a temperature of 100°C for 1 hour. After cooling in the filter the obtained precipitate washed with aquadest until a neutral pH and dried in the oven at a temperature of 80°C for 24 hours. The residue obtained after drying then cooled in a dexicator and then weighed. The residues obtained were further quantitatively identified by FT-IR analysis to prove whether it was true that the chitosan compounds contained in them [8].

2.4 Chitosan characterization

2.4.1 Moisture Content

A total of 0.5 grams of chitosan was put into a porcelain dish that was known to have an empty weight. Chitosan ventilated at 100-105°C for 2 hours, then cooled in a dexicator for 30 minutes and then weighed. This treatment carried out until the weight is constant [9].

2.4.2 Ash Content

A total of 0.5 grams of chitosan was put into porcelain cruciferous cruciferous which had a known empty weight. Chitosan is incandescent in furnaces up to 500°C for 3 hours. Chitosan that had been intoxicated is put into a dexicator for 30 minutes and then weighed its weight [10].

2.4.3 Chitosan solubility

Chitosan solubility is a parameter that can be used as a reference for quality standards of chitosan. The higher the solubility of chitosan, the better the quality of chitosan. Chitosan was dissolved in acetic acid at a concentration of 2% in a ratio of 1:100 (g/ml) [11].

2.5 Toxicity Test With Brine Shrimp Lethality Test (BSLT) Method

2.5.1 Artificial Seawater Making

Artificial seawater made by dissolving 38 grams of salt without iodine in 1 liter of water, then stirring until homogeneous. Then filtered with Whatmann paper No 40 [12].

2.5.2 Hatching of Artemia Salina Larvae

Egg dropping is done by preparing a container for dripping shrimp eggs. The container divided into two parts, the dark and light parts by blocking it and given a hole in the bulkhead. Then it is added with artificial seawater in the amount of 500 ml. One room in the container illuminated with a lamp light of 40-60 watts so that the dropper temperature maintained at 25-31°C. While in the next room it given artificial sea water without lighting covered with aluminum foil or black duct tape. Shrimp eggs as much as 100 mg first washed and then sprinkled and soaked in a container containing aquadest for 1 hour, then drained then the eggs put into a container that already contains artificial seawater, left for 2×24 hours until they hatch into larvae that are actively moving then ready to be used as test animals [13].

2.5.3 Toxicity Test

Chitosan mother solution of green clam shells at a concentration of 1000 g/mL is made by weighing 0.1 g of chitosan and then dissolving it in 100 ml of CH_3COOH 0.5%. From this mother liquor, it diluted to 5 concentrations to first be used as orientation, namely

concentrations of 100, 250, 500, 750, and 1000 µg/mL. and 1 vial used for blanks, each with three repetitions.

Prepared vials for testing; each concentration of the test solution necessitates 5 vials and up to 3 replications. Each vial in each of the five groups contains 10 shrimp larvae that have been dissolved in artificial seawater to a volume of 10 ml. Group one (control) received 5 mL of simulated seawater. The second group was given a 100 µg/mL solution of chitosan shells. With a concentration of 250 g/ml, group 3. With a concentration of 500 µg/mL, group 4. Group 5 at 750 µg/mL concentration. With a concentration of 1000 µg/mL, group 6. Each vial is illuminated by a bulb rated at 40-60 watts. The death of shrimp larvae was observed over 24 hours and then compared to controls. The number of dead larvae is used to evaluate the degree of toxicity. When shrimp larvae do not move for a few seconds, this is the conventional criterion for determining shrimp larval mortality[13].

2.6 Data Analysis

The influence of chitosan on the larvae of Artemia salina Leach was carried out by calculations of probit analysis. The calculation can be carried out by comparing between the dead larvae against the overall number, so that the percentage of deaths is obtained in the value of the probit table. From this data, it will be known that the probit value is entered into the regression equation, so that the LC_{50} value can be [14].

3 Results and Discussions

3.1 Isolation of Chitin into Chitosan

Prepared clam shell powder must undergo a deproteination process which is the initial stage to obtain chitosan. In the chitosan manufacturing process, a deproteination stage is carried out which will form chitin first and aims to break the bond between protein and chitin by adding NaOH solvent. The next process, namely the demineralization process, affects the yield of chitosan, namely that in addition to the influence of high solvent concentrations, the soaking time of the clam shell in the HCl solution will affect the decrease in mineral levels in the chitosan manufacturing process. The longer the soaking time, the less chitosan yield will be. In the demineralization process, there is a process of removing the main minerals found in clam shells such as calcium carbonate ($CaCO_3$) and phosphorus using HCl solvents [15]. The next stage is the depigmentation process of green clam shells Based on the results of research that has been carried out, the release of color pigments contained in the sample is characterized by the formation of a white solution on the depigmentation resulting filtrate. The depigmentation process is the stage of removal (dye) in the sample. pigment removal aims to give an interesting appearance to the resulting chitosan product. The last stage to obtain chitosan is called the deacetylation process. In the process, the acetyl group (-$NHCOCH_3$) in chitin is removed to become an amine group. The process of gradual deacetylation of chitin has no effect on the yield of chitosan.

3.2 Chitosan Functional Cluster Analysis Results Using FTIR

The results of the FTIR test, it can be seen that there has been a disconnection of the chitin group into chitosan, the results of the chitosan FTIR analysis obtained the absorption area of the functional groups, can be seen in Figure 1.

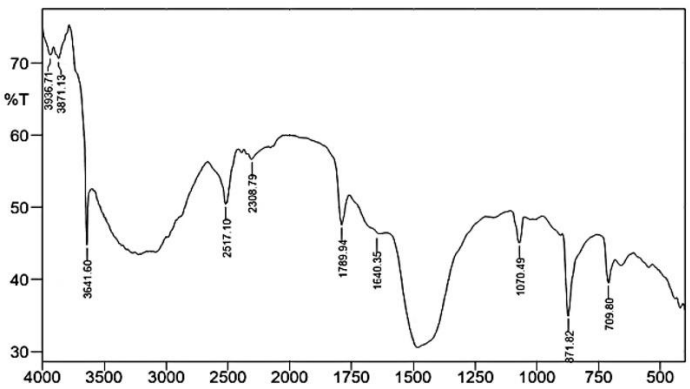

Figure 1 Green Clam Shell Chitosan Spectra Results

From the FTIR spectra data above, it can be seen that there is an absorption band that shows the vibration of the functional groups contained in chitosan. Based on the spectra above, the wave number in chitosan is 3641,60 cm^{-1} in green mussels chitosan as a result the O-H bending vibrations overlap the N-H stretching

vibrations. The stretching vibration of the C=O groups at wave number 1789.94 cm^{-1}. The stretching vibration of the C-H groups of the alkanes appears to be responsible for the absorption at wave number 1423,41 cm^{-1}. The appearance of an absorption band at wave number 1070,49 cm^{-1} suggests that the C-O group is stretching. The stretching vibration of the C-O-C stretching vibration 1070.49 cm^{-1} and β-1,4-glycosidic stretching vibration at wave number 871.82 cm^{-1}. Based on a comparison of the IR spectra of standard chitosan and isolated chitosan, it shows that the functional groups at each wave number are not much different from the standard, so it can be concluded that the compound isolated from feather clam shells produces chitin and chitosan that meet the standards [10].

3.3 Chitosan characterization

The chitosan obtained is characterized to determine the quality of the chitosan produced. The characterization carried out includes organoleptic examination (texture, color, and odor), water content test, ash content test, solubility in acetic acid 2%. The results of chitosan characterization obtained from the study were compared with the quality of SNI No. 7949 of 2013, can be seen in Table 1.

3.4 Toxicity Test

Toxicity test is a test that is carried out to determine the level of toxicity of a compound. This research uses the Brine Shrimp Lethality Test (BSLT) method because this method is one of the widely used methods, cheap, fast, easy (does not require aseptic conditions) and reliable.

The Brine Shrimp Lethality Test (BSLT) method can also be seen the effect of toxicity on green clam chitosan (*Perna Viridis* L.) on *Artemia salina* larvae in an interval of 24 hours with the concentrations used being 100, 250, 500, 750, and 1000 µg/mL. This difference in concentrations is intended to look at the level of activity of the chitosan of the green mussel (*Perna Viridis* L.) against the death of artemia salina larvae.

The result obtained is that the higher the concentration, the greater the percent mortality of shrimp larvae. In addition, negative controls were made in the form of seawater and shrimp larvae without the addition of chitosan to test the influence of seawater and other factors that affect larval death. So it can be ascertained that the death of the larvae is only due to the influence of the added chitosan, can be seen in Table 2.

Table 1 Chitosan Characterization Results

Parameters	SNI (No. 7949, Year 2013)	Vaname Shrimp Skin Chitosan
Organoleptic Examination Texture, Color, Smell	Light brown to white	Powder, white, odourless
Moisture content	≤ 12 %	1,57 %
Ash Level	≤ 5%	1,8 %
Chitosan is 2% soluble in glacial acetic acid	Soluble	Soluble
Degree of Deacetylation	≥ 75 %	90 %

Table 2 Percentage of Larval Mortality In Chitosan Green Mussels

Number of Larval Deaths Per Concentration						
Number of Replications	P1	P2	P3	P4	P5	Control
	100 µg/mL	250 µg/mL	500 µg/mL	750 µg/mL	1000 µg/mL	0
1	0	1	2	3	2	0
2	1	0	1	2	2	0
3	0	1	1	1	3	0
Total Deaths	1	2	4	6	7	0
Avarage	0,33	0,66	1,33	2	2,33	0
Percentage of Deaths (%)	3,3	6,6	13,3	20	23,3	0

Based on the data above, the number of larvae used for each treatment is 10 larvae with 3 treatments, making a total of 30 heads for one concentration. In table 2, it can be seen that the percentage of mortality from the lowest concentration of 100 µg/mL and the highest concentration is found at a concentration of 1000 µg/mL has a percentage in chitosan green

mussels which is 23.3% While in blanks it does not provide mortality to larvae. This shows that each concentration has a different influence on the mortality of A. salina larvae, the higher the concentration made, the higher the larval mortality [16].

Probit analysis can be known graph of straight-line equation y = 1.1504x + 0.812 from the data on the results of the toxicity of chitosan of green clam shells, can be seen in Figure 2.

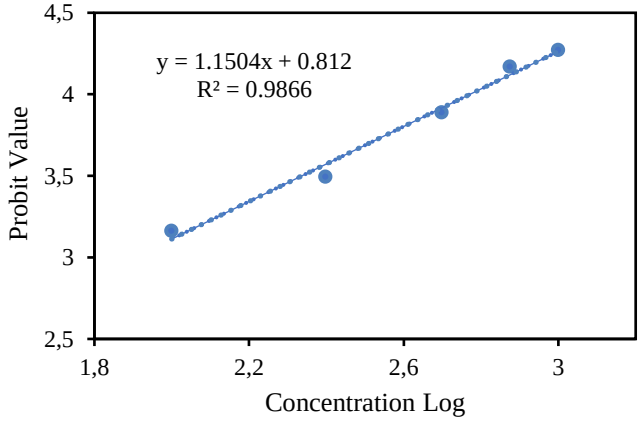

Figure 2 Correlation Curve of Percentage of Larval Mortality With Chitosan Green Clams Log Concentration

The graph above shows a log of concentrations against probit values derived from the percentage of larval mortality. After that, the value of y is entered, namely the probit value of 50% of the test animal and obtained the value of x = 3.6404, then the value of LC_{50} antilogue is 4369.180 µg/mL

4 Conclusions

Chitosan contained in the green clam shell (*Perna Viridis* L.) obtained % degree of deacetylation in green clam chitosan, which is 90.25% which states that it meets the requirements set out in the SNI. The LC_{50} value obtained from chitosan of green clam shells was 4369.180 µg/mL. The results showed that the chitosan did not have a toxic effect on the larvae of the shrimp *Artemia salina* Leach.

5 Declarations

5.1 Acknowledgements

Thank you to the Integrated Pharmacy Laboratory of the Muslim Nusantara University Al-Washliyah Medan who has assisted in the process of carrying out this research.

5.2 Author contribution

Audry Pratiwi is the researcher, collecting library data, and Zulmai Rani is preparing manuscript data. Ridwanto as director, supervisor, and manuscript coordinator.

5.3 Conflict of Interest

The authors declare no conflict of interest.

6 References

[1] L. Handayani and F. Syahputra, "Isolasi dan karakterisasi nanokalsium dari cangkang tiram (Crassostrea gigas)," *Jphpi*, vol. 20, no. 3, pp. 515–523, 2017.

[2] N. I. Sepvina, R. Ridwanto, and Z. Rani, "Uji Toksisitas Kitosan Cangkang Kerang Bulu (Anadara antiquata) Dengan Metode Brine Shrimp Lethality Test (BSLT)," *Jurnal Ilmiah Ibnu Sina*, vol. 7, no. 2, pp. 380–389, 2022.

[3] R. Ridwanto, G. I. Dalimunthe, and A. S. Daulay, "Penggunaan Kitosan Alami Hasil Isolasi Sebagai Pengawet dan Sifat Toksik Berdasarkan Uji Sitotoksisitas dengan Metode Brine Shrimp Lethality," in *Prosiding Seminar Nasional Hasil Penelitian*, 2022, pp. 482–488.

[4] N. Nurhayati, R. Ridwanto, A. S. Daulay, R. A. Syahputra, and Z. Rani, "Utilization of Chitosan As A Natural Preservative Against Catfish," *International Journal of Science, Technology & Management*, vol. 3, no. 5, pp. 1396–1401, 2022.

[5] R. Lestari, D. I. Lufita, L. Yuliatun, and L. Destiarti, *Nanomaterial Dan Analisis Risiko Terhadap Lingkungan*. Deepublish, 2023.

[6] Z. Rani *et al.*, "Cytotoxicity Test of Cocoa Leaf Ethanol Extract (Theobroma Cacao L.) With Brine Shrimp Lethality Test (BSLT) Method," *Indonesian Journal of Chemical Science and Technology (IJCST)*, vol. 5, no. 2, pp. 80–87, 2022.

[7] N. Z. Arsyi, E. Nurjannah, D. Nurahlina, and E. Budiyati, "Karakterisasi nano kitosan dari cangkang kerang hijau dengan metode gelasi ionik," *Jurnal Teknologi Bahan Alam*, vol. 2, no. 2, pp. 106–111, 2018.

[8] E. J. Dompeipen, M. Kaimudin, and R. P. Dewa, "Isolasi kitin dan kitosan dari limbah kulit udang," *Majalah Biam*, vol. 12, no. 1, pp. 32–39, 2016.

[9] D. S. Saragih, R. Ridwanto, A. S. Daulay, D. Miswanda, and H. M. Nasution, "Toxicity Test of Windu Shrimp (Penaeus monodon) Skin Chitosan with Brine Shrimp Lethality Test Method," *Indonesian Journal of Chemical Science and Technology (IJCST)*, vol. 5, no. 2, pp. 88–93, 2022.

[10] Z. Rani, H. M. Nasution, V. E. Kaban, N. Nasri, and N. B. Karo, "Antibacterial activity of freshwater lobster (Cherax quadricarinatus) shell chitosan gel preparation against Escherichia coli and Staphylococcus aureus," *Journal of Applied Pharmaceutical Science*, vol. 13, no. 2, pp. 146–153, 2023.

[11] S. Agustina, I. M. D. Swantara, and I. N. Suartha, "Isolasi kitin, karakterisasi, dan sintesis kitosan dari kulit udang," *Jurnal Kimia*, vol. 9, no. 2, pp. 271–278, 2015.

[12] A. Fadli, D. Drastinawati, O. Alexander, and F. Huda, "Pengaruh rasio massa kitin/naoh dan waktu reaksi terhadap karakteristik kitosan yang disintesis dari limbah industri udang kering," *Jurnal sains materi Indonesia*, vol. 18, no. 2, p. 61, 2018.

[13] R. Widyasari, D. Yuspitasari, W. Wildaniah, and R. C. Wahida, "Uji Toksisitas Akut Ekstrak Metanol Kulit Buah Jeruk Sambal (Citrus microcarpa Bunge) Terhadap Larva Artemia salina L. Dengan Metode Brine Shrimp Lethality Test (BSLT)," *Medical Sains: Jurnal Ilmiah Kefarmasian*, vol. 3, no. 1, pp. 51–58, 2018.

[14] F. Fadli, S. Suhaimi, and M. Idris, "Uji Toksisitas Akut Ekstrak Etanol Daun Salam (Syzygium polyanthum (Wight) Walp.) Dengan Metode BSLT (Brine Shrimp Lethality Test)," *Medical Sains: Jurnal Ilmiah Kefarmasian*, vol. 4, no. 1, pp. 35–42, 2019.

[15] L. H. Rahayu and S. Purnavita, "Optimasi pembuatan kitosan dari kitin limbah cangkang rajungan (Portunus pelagicus) untuk adsorben ion logam merkuri," *Reaktor*, vol. 11, no. 1, pp. 45–49, 2007.

[16] R. Supriningrum, S. Sapri, and V. A. Pranamala, "Uji Toksisitas Akut Ekstrak Etanol Akar KB (Coptosapelta tomentosa Valeton ex K. Heyne) Dengan Metode Brine Shrimp Lethality Test (BSLT)," *Jurnal ilmiah manuntung*, vol. 2, no. 2, pp. 161–165, 2016.