MOLECULAR CHARACTERIZATION, ANTIOXIDANT, AND TOXICITY ACTIVITY OF CHITOSAN ISOLATED FROM *Lissahatina Fulica* SHELL WASTE USING HOT PLATE MAGNETIC STIRRER TECHNIQUE

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ABSTRACT

*Lissahatina Fulica* shell waste contains chitosan with the potential for antioxidants and hypercholesterolemia. This study, therefore, reports on the analysis of molecular characterization, antioxidant assay, and acute lethal dose 50 (LD50) toxicity in *Lissahatina Fulica* shell waste chitosan rats using the hot plate magnetic stirrer technique. Chitosan was performed to analyze water content, protein, functional groups, molecular weight, particle size, morphology, antioxidants, and acute toxicity test. The researchers found that the hot plate magnetic stirrer treatment affected the water content, molecular weight, particle size, morphology, antioxidant, and non-toxicity. In *Lissahatina Fulica* shell waste chitosan, the water content was 0.345%, the protein content was 1.87%, the molecular weight was 2.297 kDa, the deacetylation degree value was 92.37%, and the particle size was 59 µm. Surface roughness had an Rp value of 249, Rv value of 0, Ra value of 81.5, Rq value of 97.8, and IC₅₀ value of 2.35 µg/mL. In addition, the chitosan dose of 3000mg/kg BW did not cause death in the sample rats. In conclusion, chitosan from *Lissahatina Fulica* shell waste has the potential to be developed in the pharmaceutical industry for further development.

Keywords: Snail Shell Waste, Hot Plate Magnetic Stirrer, Characterization, Antioxidant, Acute Toxicity.

INTRODUCTION

In Indonesia, *Lissahatina Fulica* is categorized as a field pest that eats cultivated plants, such as rice. *Lissahatina Fulica* is Mollusca with a hard shell. *Lissahatina Fulica* shell is also a domestic waste disposal problem because only meat is used. In fact, the *Lissahatina Fulica* shell can be a valuable product for industrial use. Moreover, the abundance of *Lissahatina Fulica* shell waste is a serious problem, and there needs to be an immediate solution. In this case, the *Lissahatina Fulica* shell contains chitosan.¹ Chitosan is applied as an antioxidant, adsorbent of Hg, and membrane material.⁴ The effectiveness of chitosan as an antioxidant is determined mainly by the hydroxyl group and amine group, which can be identified from the value of the degree of deacetylation (DD) and molecular weight (MW). Furthermore, the DD and MW values can be influenced by base concentration, temperature, time, and isolation method chitosan.⁵ On *Scylla Serrata*, the chitosan isolation method using the conventional magnetic stirrer obtained a DD value of 23.6%.⁶ Meanwhile, the DD value of *Achatina achatina* and *Achatina marginata* is 72-79% using the magnetic stirrer technique.⁷ In this study, isolation of chitosan by a modified isolation method with a hot plate magnetic stirrer. This technique was chosen to mix samples with other solutions with a moving process equipped with speed and time.⁸ It is a finding of a new method for chitosan isolation, so it is appropriate to be applied. This scientific finding aims to prove the claim of snail shell chitosan by using the hot plate magnetic stirrer method to obtain molecular characterization that meets chitosan quality standards, antioxidant, and non-toxicity to be applied in various fields.
EXPERIMENTAL

*Lissahatina Fulica* shell waste was obtained from rice fields in Kediri, East Java. The chemical materials used in this research comprised DPPH, Methanol, acetic acid, HCL, NaOH, NaOCl, distilled water, and *Rattus norvegicus*. For the standards of conduct, the committee on the code of ethics for experimental animal care and use, Brawijaya University, Malang, Indonesia, has approved this in vivo research (Approval No. 112—KEP-UB-2021).

Modified Isolation of Chitosan from *Lissahatina Fulica* Shell Waste

*Lissahatina Fulica* shells derived from rice plants were cleaned, dried, blended, and sieved with 100 mesh. Snail shell powder was weighed at 100 grams and added solvent HCl 6% solution, with a ratio of 10:1 (v/b). The deproteinization was accomplished by adding 4% NaOH in a 10:1 (v/b) ratio. The depigmentation involved adding a 0.315% NaOCl solution to the powder from the deproteination process in a 10:1 (v/b) ratio. In addition, the deacetylation involved adding 50 % NaOH (1:10 v/b) to the chitin isolation powder and heating it while stirring it. The solid material was then dried in an oven at a temperature of 100°C for 24 hours until a constant weight was obtained. Then, it was heated while stirred using a hot plate magnetic stirrer for seven hours at a temperature of 121°C and 400 rpm.

Analyzing Molecular Characterization

Proximate analysis of chitosan from *Lissahatina Fulica* shells (water and protein content), using FTIR (the wavenumber spectrum generated in this research was between A1320 and A1420 cm⁻¹) was conducted at the PAU Laboratory, Gadjah Mada University. On the other hand, molecular weight and particle sizer testing were carried out at the Integrated Laboratory at Diponegoro University. Then, SEM was performed at the Laboratory of Energy and Environment DRPM, ITS, Indonesia. The surface roughness analysis and surface plot were conducted using ImageJ software.

Antioxidant Activity Test

The DPPH method was utilized in this study's antioxidant test, which used 10 mg chitosan from *Lissahatina Fulica* shell waste in 10 mL 2% acetic acid as a stock solution. Various concentrations of 62.5 µl/mL, 125 µl/mL, 250 µl/mL, and 1000 µl/mL were used to make the stock solution. Besides, 1 mL 0.1 mM DPPH in acetic acid and 0.45 buffer Tris HCl pH 7.4 were added to each 1.1 mL sample solution (50mM). In this respect, acetic acid is a negative control, while the positive control is quercetin. It was then measured with a spectrophotometer with a wavelength of 517 mm. The formula then calculated the percentage of inhibition.

Acute Toxicity LD₅₀ Test on Rats (*Rattus norvegicus*)

Rats were grouped into control and experimental groups, each consisting of a male rat, and the total amount was 12 rats. The rats were fasted (unfed) for approximately 3-4 hours before receiving treatment, but the water was provided (ad libitum) and then measured. For the P0 group (control): six rats were given distilled water. For the P1 group (experimental): six rats used 3000 mg/kg Bw dose of chitosan from *Lissahatina Fulica* shell waste. Then, the toxicity and death rate signs were analyzed every 30 minutes for the first 4 hours before the 14th day.

RESULTS AND DISCUSSION

Chitosan Molecular Characterization

Chitosan isolation in this research was obtained from a hot plate magnetic stirrer. Results of chitosan quality characterization are presented in Table-1. Table-1 shows that the water content of chitosan was in a low category of <1%. It is supported by previous studies, stating that the water content <1% is a polymer with good quality. Meanwhile, with the traditional method, the water content was obtained at 1.73%.

Moreover, the Mw value plays a significant role in the application used. In this regard, several factors affect the Mw and DD value during the isolation of chitosan, including isolation method, high temperature, a solvent used, and particle size, affecting the Mw and DD value. This study revealed that the hot plate method was appropriate for chitosan isolation since continuous stirring and heating impacted the MW value obtained in the high category and small particle size.
Table-1: Characteristics of Chitosan from *Lissahatina Fulica* Shell

<table>
<thead>
<tr>
<th>Parameter</th>
<th>International Standard</th>
<th>Chitosan from <em>Lissahatina Fulica</em> shell</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water content</td>
<td>≤2%</td>
<td>0.345%</td>
</tr>
<tr>
<td>Protein content</td>
<td>1.87%</td>
<td></td>
</tr>
<tr>
<td>Molecular weight</td>
<td>2.297 kDa</td>
<td></td>
</tr>
<tr>
<td>Particle size</td>
<td>59 µm</td>
<td></td>
</tr>
<tr>
<td>Degree of deacetylation</td>
<td>40-98%</td>
<td>92.37%</td>
</tr>
</tbody>
</table>

The chemical group of chitosan from *Lissahatina Fulica* shells scales using FTIR analysis is depicted in Fig.-1:

![FTIR Spectra](image1)

In Fig.-1, the chitosan spectrum results were recorded at 500 cm\(^{-1}\) to 4000 cm\(^{-1}\) waves.\(^{15}\) The chitosan showed that an infrared spectra absorption band was produced at a wavenumber of 753 cm\(^{-1}\), confirming the NH group's bending vibration and at 3265 cm\(^{-1}\), revealing the NH bond's stretching vibrations. Here, one of the signs of the chitosan compound is the presence of stretching vibrations at a wavenumber of 895 cm\(^{-1}\), suggesting a -1,4-glycosidic bond. In addition, the DD of chitosan from *Lissahatina Fulica* shells was 92.37%. This research finding strongly supports a commercial scale that can increase the DD value by snail shell chitosan using the hot plate magnetic stirrer method. Besides, the DD value in this study was more significant than the DD value of chitosan in mud crabs, 53.4%.\(^{16}\) This procedure included constant stirring and heating to ensure that interactions affected the DD value. On the other hand, in previous studies, isolation of chitosan without stirring and heating methods and deacetylation time for two hours resulted in the DD value being not maximal.\(^{16}\)

**SEM Chitosan from *Lissahatina Fulica* Shells**

The image of the SEM test results analysis is illustrated in Fig.-2 and Table-2.

![SEM Images](image2)

**Table-2: The Results of Surface Roughness Calculation**

<table>
<thead>
<tr>
<th>Method</th>
<th>Rq</th>
<th>Ra</th>
<th>Rp</th>
<th>Ry</th>
<th>Rt</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot plate magnetic stirrer</td>
<td>97.896</td>
<td>81.538</td>
<td>249</td>
<td>0</td>
<td>249</td>
</tr>
</tbody>
</table>

Surface plot analysis can be seen in Fig.-2 and Table-2, showing the 3D topography visualization in black and white (B and W) and colored spectrum. The table above also reveals the statistical analysis data of the surface roughness. In this case, Rp is the highest peak value from the average line.\(^{17}\) Apart from the Rp
value, the Rv value was calculated. Rv indicates the lowest valley value from the average line. The Rp and Rv values in this method indicate the range of the pixel. Meanwhile, Ra is the arithmetical mean deviation. The Ra value signifies the mean range of the valleys and the peaks. A higher Ra value means rougher the sample surface. To measure the deviation value of the Ra, the Rq value was also considered. Rq (root mean square deviation) value was obtained from squared Ra.

**Antioxidant Activity Analysis**

The results of IC$_{50}$ values chitosan from *Lissahatina Fulica* shell analysis are shown in Fig.-3 and Table-3.

![Graph showing IC$_{50}$ values for chitosan and quercetin](image)

(a) LogIC$_{50} = 0.3713 \ \mu g$

(b) LogIC$_{50} = 0.2457 \ \mu g$

IC$_{50}$ value of chitosan from snail shell waste was 2.35 g/mL. The IC$_{50}$ value of quercetin was lower than that of snail shell chitosan. In addition, chitosan with a high MW value has a higher ability to scavenge free radicals. A high DD and MW value also affects its antioxidant properties. The DD value of chitosan 94.3-95.1% the lower the IC$_{50}$ value, the higher the antioxidant activity and high MW can increase intermolecular bonds. MW value of 318 kDa has lower antioxidant activity than MW 12 kDa. The higher the DD value of chitosan, the more H, OH- and NH$_2$+ groups and NH$_3$+ which react with unstable free radicals to become stable. The mechanism of chitosan as an antioxidant is that chitosan has nitrogen from the amine group having a lone pair of electrons that can bind to a proton that forms an ammonium group NH$_3$+ so that free radicals can react with hydrogen ions NH$_3$+ to form stable molecules.

**Acute Toxicity Analysis**

The toxicology test was performed on white male rats in this study, as in Table-4:

![Table showing acute toxicity test results](image)

Table-4 shows that the control group given distilled water had no death, and white male rats were well active. At a dose of 3000 mg/kg BW, the treatment group did not die for 14 days of observation, but lethargy occurred on average for 180 minutes. Moreover, glycated chitosan did not show a toxic effect on cell culture and female Wistar Sprague Dawley mice.

**CONCLUSION**

The chitosan isolation method with a hot plate magnetic stirrer method of chitosan isolation that is cheap, clean, and simple. Thus, a hot plate magnetic stirrer can be a solution for the extraction stage of chitosan from the chitin stage to the chitosan of snail shell waste. This study uncovered that snail shell waste is
plentiful and has a high quality of chitosan. The researchers also found that chitosan with a hot plate magnetic stirrer affected water content, protein content, high molecular weight, small particle size, good morphology, very strong antioxidant value, and non-toxic in vivo tests of Wistar *Rattus norvegicus* rats.

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