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CHARACTERIZATION, ANTIOXIDANT ACTIVITY, AND IN SILICO MOLECULAR DOCKING OF CHITOSAN FROM SNAIL SHELL WASTE BY ULTRASONIC TECHNIQUE

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Abstract. Snails are often found in Indonesia, especially at Kediri, but the snail shell has no commercial value. This research report describes the characterization and antioxidant activity of chitosan from snail shell waste (chitosan-SSW) by ultrasonic technique and analyzes the potential of chitosan as an inhibitor of receptors of free radicals using an *in silico* molecular docking method. Characterization of chitosan-SSW was performed to analyze the content of water, protein, and functional groups as well as molecular weight, particle size, morphology, antioxidant activity, and *in silico* molecular docking. We found that chitosan-SSW with ultrasonic treatment had a high degree of deacetylation (DD) and high molecular weight (MW). The characteristic of chitosan-SSW was found to be as follows: water content of 0.43 %, protein content of 1.59 %, molecular weight of 2.198 kDa, and deacetylation degree value of 79.50 %. Importantly, chitosan-SSW had high antioxidant activity to potentially reduce free radical of DPPH with IC_{50} value of 2.44 μg/mL. Chitosan is predicted to have the potential as an inhibitor of lipoxygenase, CYP2C9, and NADPH-oxidase.

Keywords: chitosan-SSW, characterization, ultrasound, antioxidant, *in silico* molecular docking.

1. Introduction

Nowadays, waste has become a concern of world scientists. Snail waste has not been handled optimally, particularly in Indonesia. This biodegradable waste can be disposed of into the environment. However, there is no further use, which impacts the environmental damage.[1] Interestingly, the snail shell contains a high calcium carbonate content of 95-99 %,[2] which is potentially employed as a source of chitosan production. Several studies have reported degree of acetylation (DA) values of snail shell chitin of 120 % and 126.42 %. The degree of deacetylation is a crucial component, because it impacts the physical characteristics of chitin.[3-4] Chitosan is a cationic polymer derived from N-deacetylated chitin which can be used as a dye adsorbent,[5] a matrix for drug delivery,[6-7] a therapy for mending wounds,[8] and an antibacterial agent against *S. flava* and *B. cereus*.[9] Chitosan units with a chemical formula 2-amino-2-deoxy-glucose and 2-acetamino-dedoxy-D-glucose are connected through glycosidic bond (1 → 4).[6] The previous works investigate the modification of chitosan from snail shells by the traditional method with a hot plate magnetic stirrer for antioxidant and hypercholesterolemia applications. This isolation approach changes the heat, temperature, and solvent during extraction. The isolated chitosan performs antioxidant[10] and hypercholesterolemia function.[11] Antioxidants play an important role in helping protect body from free radicals to prevent adverse effects by neutralizing them. The role of antioxidants with a single pathway or group of antioxidants is to neutralize free radicals (radical scavengers), to reduce singlet oxygen by complexing pro-oxidants, which catalyze radical forms, and inhibit pro-oxidant enzymes that cause radicals such as lipoxygenase, xanthine oxidase, and NADPH oxidase will occur.[12-13]

On the other hand, chitosan has the potential to be developed as a drug candidate because of no side effects.[14] Snail shell waste can produce high-quality by-products and reduce environmental damage through extraction techniques with no change in its functional groups.[14] The ultrasonic method is a more effective extraction technique for antioxidant materials when compared to the thermal process.[15] The ultrasonic extraction method can accelerate the solvent and solute diffusion as well as heat transfer to increase the degree of deacetylation (DD) and molecular weight (MW). Also, it highly improves the extraction of

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chitin, increases the reaction rate while using solvents and a low energy input and enabling an easy adjust of the extraction temperature and time.[16] The findings of this study prove that the development of the ultrasonic treatment technique results in chitosan from snail shell waste (chitosan-SSW) with high DD values and molecular weights which exhibits high antioxidant activity.

2. Experimental

2.1. Materials

Snail shells were obtained from Kediri, East Java, Indonesia. The materials used to isolate chitosan from shells were NaOH 4 %, Merck. P.A., HCl 6 % Merck. P.A, NaOCl 0.315 % Merck. P.A, NaOH 70 % Merck. P.A., absolute methanol (Merck, Germany), 1,1-Diphenyl-2-picryl-hydrazyl (DPPH), absolute acetic acid (Sigma Aldrich, Steinheim, Germany), and distilled water.

2.2. Isolation of Chitosan from Snail Shell Waste with Ultrasonic Technique

The snail shell powder was isolated to get chitosan by using four stages, *i.e.*, demineralization, deproteination, depigmentation, and deacetylation by ultrasonic technique. At the demineralization stage, to snail shell powder, it was added 6 % HCl at a ratio of 10:1 (v/w) using ultrasonic-assisted for 3 h with the frequency of 50 kHz. Afterwards, the mixture was filtered; the solid form was washed with aquadest, and dried at 373 K for 5 h until a constant weight. Then, 4 % NaOH was added to the demineralized powder at a ratio of 10:1 (v/w) for 3 h at a frequency of 50 kHz. The outcome was then strained and washed with aquadest. The solid form was hold in the oven at 373 K for 5 h until a constant weight was achieved. At the depigmentation stage, to the snail shell powder from the deproteination process, 0.315 % NaOCl was added at a ratio of 10:1 (v/w) for 3 h during ultrasonification. The solid form was strained and washed with aquadest. The solid form was then heated in and oven at 368 K for 2 h until a constant weight was obtained. At the deacetylation stage, 70 % NaOH was added to the snail shell powder at a ratio of 10:1 (v/w) using ultrasound for 6 h. The ultrasonifier probe used 50 kHz, and the solid form was strained and washed with aquadest until pH 7. The obtained solid form was then heated in an oven at 368 K for 24 h until a constant weight was obtained.

2.3. Ash and Protein Content of Chitosan from Snail Shells (Chitosan-SSW)

The ash and protein content was determined according to AOAC standards.[17]

2.4. Deacetylation Degree (DD) Test

The chitosan was analyzed by using the FTIR method to determine the Degree of Deacetylation (DD).[18]

2.5. Scanning Electron Microscopy (SEM)

SEM image analysis at 150X magnification with the image J software and region of interest was selected and then proceed by using OriginPro software.

2.6. Particle Size Analyzer (PSA)

Chitosan from snail shell waste was analyzed with a Dynamic Light Scattering (DLS) method.

2.7. Chitosan Molecular Weight

GPC analysis was carried out using 0.3M aqueous AcOH and 0.2M aqueous NaOAc as eluents on an Agilent PL-GPC 50 integrated GPC/SEC system with a refractive index detector, PSS NOVEMA MAX columns set (1 guard column, 10 μm, 2 analytical columns 1000 Å, 10 μm, and 1 analytical column 30 Å, 10 μm). The pullulan used to calibrate the system ranged from 180 to 1,220,000 KDa. In 1.5 mL of mobile phase, the chitosan-SSW samples of 2 mg were dissolved overnight. The solutions were injected (100 μL) using a sterile 0.22 μm PTFE filter. At 313 K, the analytical flow rate was 1 mL/min. The number average molecular weight (M_n), the weight average of the molecular weight (M_w), the molecular weight of the highest peak (M_p), and the polydispersity (PD) index were used to express the results of the GPC study.[19]

2.8. DPPH Antioxidant Activity Test of Chitosan from Snail Shells

The antioxidant activity test was carried out by the modified 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging method.[20]

2.9. *In Silico* Molecular Docking

Protein structure xanthine oxidoreductase (PDB ID 3NRZ), Lipoxygenase (PDB ID 1N8Q), CYP2C9 (PDB ID 1OG5), and NADPH-oxidase (PDB ID 2CDU) from PDB was prepared using Autodock Tools in which the protein was cleaned from water molecule bond, metal atom and lignan which were removed from the protein structure, then added by polar hydrogen atom and gesteiger content was saved in pdb format. The structure of chitosan lignan molecule ($C_{56}H_{103}N_9O_{39}$) (CID ID: 71853) from pubChem was changed into 3D format with Open Babel PyRx and the parameter optimization of the structure geometry and chemical force field was carried out. Docking was conducted by using HEX 8.0 software with the Vina Autodock evaluation function. The obtained results were analyzed to get the energy and amino acid residual rate by using Accelerys Discovery Studio 4.1 (Dassault Systems Biovia).

3. Results and Discussion

The snail shell used in this study is a typical that of an Asian species of *Lissachatina fulica*. The chitin isolation process begins with demineralization followed by deproteination and depigmentation. Demineralization aims to remove the mineral content in the snail shell. In the demineralization process with the addition of HCl, it is necessary to convert the mineral calcium present in the surface into $CaCl_2$ (white residue) and release CO_2 and H_2O.[21] During the deproteination process, a viscous liquid is formed, which tends to precipitate. In contrast, the depigmentation process changes the color of the brown snail shell powder to a pale white color. The three processes described above produce chitin, a pale white powder with a distinctive snail smell. The chitin preparation is followed by the deacetylation process. During the deacetylation process, a viscous brown liquid is formed. In this process, the acetyl group is released from the chitin polymer. The chitosan obtained from this reaction is a pale white powder with a distinctive snail smell and the average yield as shown in Table 1.

According to Table 1, it can be seen that the high yield of chitosan-SSW was achieved using the isolation process with the ultrasonic technique. This process requires an additional energy input at high level, namely 120–1200 W with a frequency of 20–80 Hz. These findings are in a good agreement with the results of Albu *et al.* where it was shown that ultrasonic techniques lead to high yields.[15]

The isolated chitosan-SSW was analyzed for proximate (moisture content and protein content) and characterized in terms of the degree of deacetylation (DD), molecular weight (MW), particle size, and morphology. The physical characteristics of the chitosan-SSW obtained by using the ultrasonic technique are summarized Table 2

Table 1. Yield of isolated chitosan-SSW using ultrasonic technique

Process	Yield of chitosan-SSW
Demineralization	31.13%±0.62
Deproteination	95.28%±0.88
Depigmentation	98.48%±0.15
Deacetylation	81.85%±0.33

Table 2. Characteristics of chitosan-SSW using ultrasonic technique

Parameters	chitosan-SSW
Water content	0.43%
Protein level	1.595%
Degree of deacetylation (*DD*)	79.50%
Molecular weight (M_w)	2,198 kDa
Particle size	59.820 µm

The results of the physical characterization of chitosan-SSW revealed a water content of 0.43 %. The water content in this study met the quality requirements of Chitosan Lab Protan Japan > 10 %. The water content is known to affect the quality of chitosan. The water content value in this study is lower than the water content of chitosan-SSW by Oyekunle and Omoleye (1.73 %).[22] Protein content in chitosan-SSW prepared by using the ultrasonic technique is low too. This method also minimizes the protein content in the isolated chitosan (1.59 %) which meets the European Food Safety Authority (EFSA) chitosan quality standard.[23] The protein content in this study is much lower when compared to the results of Ningrum *et al.* (4.8%).[24]

The FTIR spectrum of the isolated chitosan-SSW is presented in Fig. 1. Chitosan isolated by the ultrasonic method has the NH functional group as evidence by the absorption band at 753 cm^{-1}. Likewise, the presense of absorption bands at 895 cm^{-1} (ring CO stretch) and 1029 cm^{-1} (outer ring CO stretch) is a characteristic of the C-O bonds present in the chitosan macromolecule. Another absorption band in the chitosan-SSW spectrum at 1416 cm^{-1} implies the presence of CH_2 and CH_3 groups. Absorption bands at 1565 cm^{-1} and 1662 cm^{-1} indicate amide II and primary amide band 1. An absorption band at 2930 cm^{-1} corresponds to a symmetric stretching vibration of the CH_3 bond, and an absorption band at 3265 cm^{-1} corresponds to a stretching vibration of the NH bond. An absorption band at 895 cm^{-1} belongs to a stretching vibration and proves the presence of β-1,4-glycosidic bonds in the chitosan macromolecules.

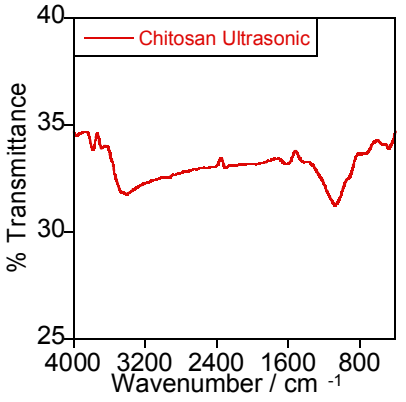


Fig. 1. FTIR spectrum of the chitosan-SSW prepared by the ultrasonic technique

The success of the deacetylation process is determined by the percentage of *DD*. That is, the higher the *DD* value, the higher the purity of the chitosan produced as shown by Yuan *et al*.[25] In this study, the highest DD value was 79.50 % due to the deacetylation process using ultrasonic techniques. This *DD* value is higher when compared to chitosan, which was produces by using the magnetic-reflux method by Kusumaningsih (74.78–77.99 %)[26] or the hot plate method by Waryani *et al*. (75.13 %).[26] It should be noted that the *DD* value of snail shell chitosan without ultrasonic treatment was 52.56 %.[27] The isolation method, temperature, and sufficient reaction time resulted in the high degree of deacetylation of the chitosan obtained in this study. The results of this study are in a good agreement with the data of the researches conducted by Hossain and Iqbal[28] and Srinivasan *et al*.[29] Chitosan-SSW used in this study had a molecular weight of 2,198 kDa. Based on the molecular weight of this polymer, it can be concluded that the resulting polymer should have a good stability, according to the results of research conducted by Xuan Du and Xuan Voung.[30] Chitosan-SSW is categorized as chitosan having a high molecular weight. Medium and high a molecular weight chitosan (M_w in the range from 60 kDa to 150 kDa) are known to have a superior stability.[31]

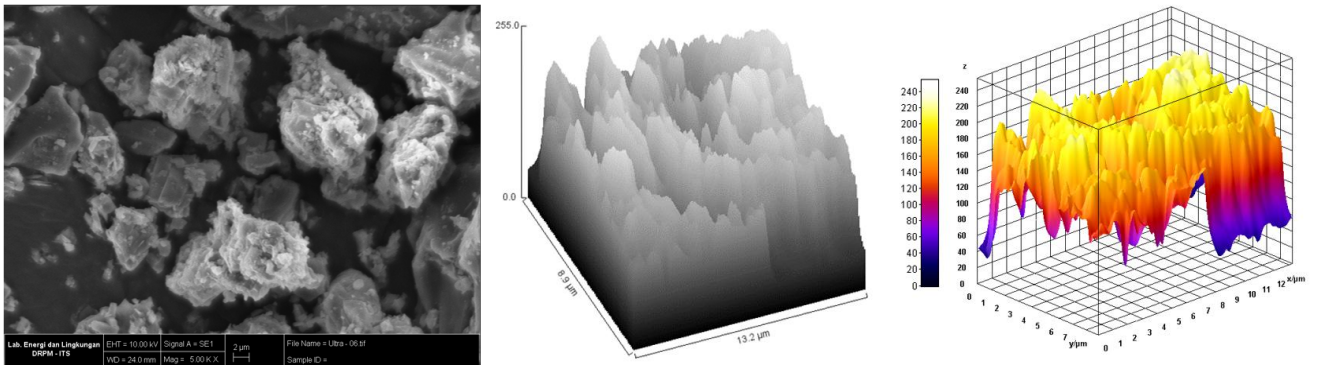
Another important physical characteristic of chitosan is its particle size value. As measured by the dynamic light scattering technique, the average particle size of chitosan-SSW is 59.820 µm. In terms of morphology, the prepared snail shell chitosan was a white powder with a distinctive snail smell.

The characterization of chitosan with SEM (Fig. 2) showed the morphology and topography with clearly visible surface pores. Furthermore, the results of processing using the origin image 2D and 3D programs on the morphology of chitosan are given in Fig 2. The variables that explain the surface roughness of chitosan from the 2D images are presented in Table 3.

Table 3. Calculated surface roughness results

	Rq	*Ra*	*Rsk*	*Rku*	*Rp*	*Rv*	*Rt*
chitosan-SSW by the ultrasonic technique	100.382	86.867	1.404	2.295	255	0	255

Notes. Root mean square deviation (*Rq*), Arithmetical mean deviation (*Ra*), Skewness of the assessed profile (*Rsk*), Kurtosis of the assessed profile (*Rku*), Highest peak (*Rp*), Lowest valley (*Rv*), the total height of the profile (*Rt*).



a b c

Fig 2. SEM image of chitosan-SSW (a), 2D (b) and 3D (c) visualization with ImageJ

Based on the data in Table 3, all parameters concerning the surface roughness of chitosan-SSW have an *Rku* value less than 3. This means that the chitosan produced in this study is categorized as platykurtoic (a uniform distribution of the chitosan roughness). Chitosan-SSW is one of the bioactive compounds used in healthcare and a natural compound for medicine development. In this study, the antioxidant activity test for chitosan-SSW was carried out through two approaches, namely *in silico* and antioxidant activity with DPPH.

Testing of antioxidant activity has been carried out using a free radical compound 1,1-diphenyl-2-picrylhydrazyl (DPPH). This test using the DPPH method is often used in research because it has the appropriate level of stability.[32-33] The DPPH method was chosen because it provides inexpensive, easy, fast, and reasonably accurate results. In addition, this method can be used for both a liquid and solid samples. The method is not specific for certain components of antioxidant compounds.[34] The antioxidant activity of chitosan-SSW prepared by the ultrasonic method was analyzed based on the 50 % inhibition value (IC_{50}). The results of antioxidant activity are following. The IC_{50} value calculation shows that the chitosan snail shell has high antioxidant activity, indicated by the IC_{50} value or inhibitory concentration of 2.44 µg/mL. This value is higher than the IC_{50} of vitamin C (0.99 µg/mL). The lower the IC_{50} value, the higher the antioxidant activity because less concentration is needed to inhibit DPPH by 50 %. The antioxidant activity data are represented in Table 4.

Table 4. Antioxidant activity

Parameter	chitosan-SSW	L-asorbic acid
DPPH, IC_{50} (µg/mL)	2.44	0.99

Chitosan-SSW can act as a natural antioxidant because its molecules donate hydrogen atoms or electrons to DPPH. DPPH is oxidized or turned into a reduced form, and a residue is formed in a DPPH-H group. The decrease in the DPPH concentration is proportional to the number of hydroxyl groups as contributors of hydrogen atoms. This study found that chitosan-SSW has a potential as an antioxidant through its ability to scavenge free radicals such as cellular/mitochondrial ROS. The chitosan polymer molecule has many hydroxyl groups (C6) and amine groups (C2). Hydroxyl radicals play an essential role in oxidative agents, reacting with chitosan chains *via* hydrogen atoms linked to carbon atoms to form carbohydrate radicals. The hydroxyl and amine groups in the chitosan macromolecule play an essential role in free radical scavenging.[35-36] This study is similar to Jafari *et al*. in which it was shown that a higher molecular weight leads to a lower antioxidant activity.[35]

Molecular docking is a tool to detect the molecular mechanism of pharmacologically bioactive compounds. Molecular docking interacts with chitosan polymers which are thought to have activity against the receptors, namely Protein xanthine oxidoreductase, lipoxygenase, CYP2C9, and NADPH oxidase. These receptors are free radicals produced from enzymatic reactions. The bioactive compounds used in this research support the test results of chitosan-SSW activity as an antioxidant. The bioactive compound used is chitosan with the formula ($C_{56}H_{103}N_9O_{39}$). The target protein correlates with antioxidant effects on hypercholesterolemia, NADPH oxidase (NOX) protein, cytochrome, lipoxygenase, and xanthine oxidoreductase. Molecular docking was used to study the potential of bioactive compounds that inhibit target proteins' inhibition of intracellular antioxidant mechanisms. The following molecular docking results are presented in Fig. 3.

Fig. 3 shows that the binding energy of chitosan with Lipoxygenase is -597.5 kcal/mol with six amino acid residues (ASN227, GLU682, ASP597, GLY619, ASP678, and ASP228). Chitosan with CYP2C9 is -292.0 kcal/mol with six amino acid residues (ARG307, GLU206, GLU300, THR304, GLN192, and PHE168). Chitosan with NADPH-oxidase is -148.2 kcal/mol with four amino acid residues (ASP141, LYS165, GLU137, and GLU309). Xanthine oxidase is 813.2 kcal/mol with four amino acid residues (ILE358, GLU373, ALA203, and GLU356). The results of the docking analysis showed negative binding affinity values for lipoxygenase, CYP2C9, and NADPH-oxidase, meaning that the more negative the free energy points, the higher the stability of a bond between the ligand and the stable receptor. The difference in the value of the affinity bond is strongly influenced by the type of interaction between the bioactive compound chitosan that binds to antioxidant receptors.

The presence of hydrogen bonds, conventional hydrogen bonds, and carbon-hydrogen bonds in the docking results has a vital role in determining the size of a substantial affinity value and interestingly, in CYP2C9, there are other types with π orbitals. Hydrogen bonds play an important role in binding bioactive compounds. Fig. 3 shows the amino acid residues visible in van der Waals force. The van der Waals force acts as a stabilizer for the complex. Multiple van der Waals forces will increase the solvent-accessible surface (SAS) value and stabilize the bond between the ligand and protein.[37]

This study shows that the bioactive compound chitosan is one of the most potent antioxidant products. The bioactive compound chitosan can be an inhibitor of the target protein of xanthine oxidoreductase, lipoxygenase, CYP2C9, and NADPH-oxidase. Inhibition of chitosan was found in six amino acid residues, mostly in lipoxygenase. Chitosan can inhibit lipoxygenase enzyme activity and can reduce cholesterol levels.

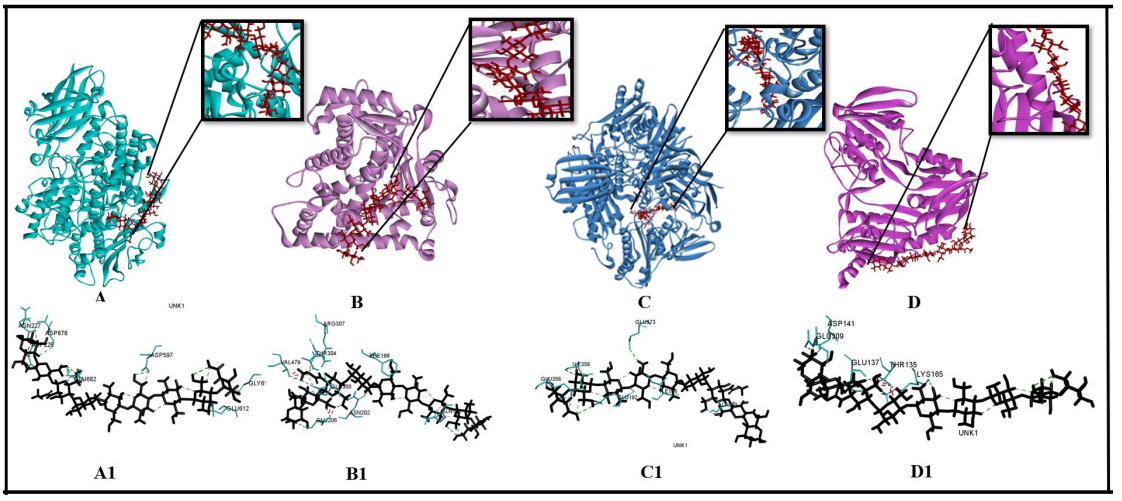


Fig. 3. Model of the Interaction of Chitosan Bioactive Compounds against Lipoxygenase, CYP2C9, NADPH-oxidase, and Xanthine oxidase: (A) Overview Chitosan–Lipoxygenase; (A1) the 3D Structure of the Chitosan–Lipoxygenase.; (B) Overview Chitosan–CYP2C9; (B1) the 3D Structure of the Chitosan–CYP2C9.; (C) Overview Chitosan–NADPH-oxidase; (C1) the 3D Structure of the Chitosan–NADPH-oxidase.; (D) Overview Chitosan–Xanthine oxidase; (D1) the 3D Structure of the Chitosan–Xanthine oxidase

4. Conclusions

To summarize, the characteristics of chitosan-SSW was found to be: (i) water content of 0.43 %, (ii) protein content of 1.59 %, (iii) molecular weight of 2.198 kDa, and (iv) deacetylation degree value of 79.50 %. Micro-structural characteristics of chitosan-SSW have revealed a surface roughness with a *Rku* value of 2.9. An antioxidant activity with an IC_{50} value of 2.44 µg/mL confirms its potential as an antioxidant.

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ДОСЛІДЖЕННЯ ВЛАСТИВОСТЕЙ, АНТИОКСИДАНТНА АКТИВНІСТЬ ТА *In Silico* МОЛЕКУЛЯРНИЙ ДОКІНГ ХІТОЗАНУ З ВІДХОДІВ ЧЕРЕПАШОК РАВЛИКА ЗА ДОПОМОГОЮ УЛЬТРАЗВУКОВОЇ МЕТОДИКИИ

Анотація. *Равлики поширені в Індонезії, особливо в Кедірі, але черепашки равлика не мають комерційної цінності. У цій роботі описано характеристику й in vitro оцінку біоактивності хітозану з відходів черепашок равликів (хітозан-SSW), отриманого за допомогою ультразвукової методики, та проаналізовано потенціал хітозану як інгібітора рецепторів вільних радикалів за допомогою методу молекулярного докінгу in silico. Мета дослідження властивостей хітозану-SSW – аналіз вмісту води, білка та функціональних груп, а також молекулярної маси, розміру частинок, морфології, оцінки антиоксидантної активності молекулярного докінгу in silico. Встановлено, що хітозан-SSW, отриманий за допомогою ультразвукової обробки, мав високий ступінь деацетилювання (DD) і високу молекулярну масу (MW). Встановлено характеристики хітозану-SSW: вміст води 0,43 %, вміст білка 1,59 %, молекулярна маса 2198 кДа, значення ступеня деацетилювання 79,50 %. Важливо, що хітозан-SSW мав високу антиоксидантну активність для потенційного зменшення вільних радикалів DPPH зі значенням IC_{50} 2,44мкг/мл. Передбачається, що хітозан має потенціал як інгібітор ліпоксигенази, CYP2C9 і NADPH-оксидази.*

Ключові слова: *хітозан-SSW, характеризація, ультразвук, антиоксидант, in silico молекулярний докінг.*