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In Vitro Evaluation of Cholesterol-Reducing Ability of Chitosan from Mangrove Crab (*Scylla serrata*) Shell Solid Dispersion using PVP K-30 as a Carrier

Uji In Vitro Penurunan Kadar Kolesterol Sistem Dispersi Padat Kitosan dari Cangkang Kepiting Bakau (Scylla serrata) Menggunakan PVP K-30 Sebagai Pembawa

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ABSTRACT

Background: Chitosan is a compound that can be synthesized from nature which can reduce the total serum cholesterol levels between 5.8−42.6% and decrease LDL (Low-Density Lipoprotein) between 15.1-35.1%. One of the natural resources containing chitosan derivative compounds is the shell of mud crab. Chitosan is insoluble in water but soluble in acidic solutions such as acetic acid. With such chitosan solubility, it is necessary to increase the solubility by making a solid dispersion system so that drug absorption can be faster. **Objectives:** The aims of this study is to determine the potential of chitosan solid dispersion system for reducing cholesterol. **Material and Methods:** The reduction of cholesterol levels was carried out by in vitro tests using UV-Vis spectrophotometer at a wavelength of 405 nm with Lieberman-Burchad reagent. The positive control used was simvastatin. There are 4 formulas, namely SD1, PM1, SD2, and PM2. This solid dispersion system uses polyvinyl pyrrolidone K-30 (PVP K-30) as carrier. **Results:** The characterization of chitosan has fulfilled all the characterization requirements that is organoleptic (shape and color) was creamy white, moisture content was 2.15%, ash content was 1.14%, ninhydrin test was positive purple, and deacetylation degree was 70.57%. The results of in vitro evaluation were obtained a dark green solution. The reducing percentage in cholesterol levels are SD1: 18.44%; PM1 : 18.11%; SD2 : 29.57%; and PM2 :12.01%. Simvastatin as a positive control has a percentage reduction in cholesterol levels of 30.07%. **Conclusion:** Chitosan has an activity as anticholesterol agent. SD2 (Solid Dispersion Chitosan: PVP K-30 = 1:2) has the higher percentage than other formulas for reducing cholesterol level comparable with the positive control.

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ABSTRAK

Latar Belakang: Kitosan merupakan senyawa yang dapat disintesis dari alam yang dapat menurunkan kadar kolesterol total serum antara 5,8−42,6% dan menurunkan LDL (*Low-Density Lipoprotein*) antara 15,1-35,1%. Salah satu sumber daya alam yang mengandung senyawa turunan kitosan adalah cangkang kepiting bakau. Kitosan sendiri tidak larut dalam air tetapi larut dalam larutan asam seperti asam asetat. Dengan kelarutan kitosan yang demikian maka perlu ditingkatkan salah satunya dengan membuat sistem dispersi padat agar penyerapan obat dapat lebih cepat. **Tujuan:** Penelitian ini bertujuan untuk mengetahui potensi sistem dispersi padat kitosan dalam menurunkan kolesterol. **Bahan dan Metode:** Penurunan kadar kolesterol pada penelitian ini dilakukan dengan uji *in-vitro* menggunakan spektrofotometer UV-Vis pada panjang gelombang 405 nm menggunakan pereaksi *Lieberman-Burchad*. Kontrol positif yang digunakan adalah simvastatin. Ada 4 formula yang dibuat yaitu SD1, PM1, SD2, dan PM2. Sistem dispersi padat ini menggunakan PVP K-30 sebagai pembawa. **Hasil:** Hasil karakterisasi kitosan telah memenuhi semua persyaratan yaitu organoleptik (bentuk dan warna) putih krem, kadar air: 2,15%, kadar abu: 1,14%, uji ninhidrin positif ungu, dan derajat deasetilasi : 70,57%. Hasil evaluasi in vitro diperoleh larutan berwarna hijau tua. Persentase penurunan kadar kolesterol adalah SD1: 18,44%; PM1: 18,11%; SD2: 29,57%; dan PM2: 12,01%. Simvastatin sebagai kontrol positif memiliki persentase penurunan kadar kolesterol sebesar 30,07%. **Kesimpulan:** Kitosan memiliki aktivitas sebagai antikolesterol. Formula SD2 (*Solid Dispersion* Kitosan: PVP K-30 = 1:2) memiliki presentase terbesar dari semua formula dalam menurunkan kadar kolesterol dan mendekati kontrol positif.

Kata kunci: Cangkang kepiting bakau; Kitosan; Penurunan kolesterol; Dispersi padat; Uji *in vitro.*

INTRODUCTION

Along with the modernization of life, humans are fast-paced in their activities. This causes some people tend to consume fast food (fast food) which contains a lot of fat so that it can increase cholesterol levels in the blood or hypercholesterolemia (Puspitasari, 2014).

Chitosan is a compound that can be synthesized from nature which can reduce total serum cholesterol levels between 5.8−42.6% and decrease LDL (Low-Density Lipoprotein) between 15.1-35.1% (Ylitalo et al., 2002). There are two basic mechanisms of chitosan in binding fat, which involve attraction such as magnetic poles which have two opposite charges and a charge neutralization mechanism, namely, chitosan covers the active site of fat and protects it from attack and decomposition of lipid enzymes (Pagala, 2011).

One of the natural resources containing chitosan derivative compounds is crab. Indonesia is the largest crab exporting country for the international market with a fairly fantastic value so that it produces a lot of crab shell waste. Crab shells contain chitin (18.70% - 32.20%) which can be converted into chitosan (Maidin Nasir Alfian, 2017). The synthesis of chitosan from mangrove crab *(Scylla serrata)* shells was carried out in three stages, namely deproteination, demineralization, and deacetylation. The characterization of chitosan carried out included tests of water content, ash content, degree of deacetylation, yield, ninhydrin, and organoleptic (Anas, Ajwar, 2017; Rasio et al., 2017).

Chitosan is insoluble in the air but soluble in acidic solutions such as acetic acid (Taufan et al., 2010). With such chitosan solubility, it is necessary to increase it by making a solid dispersion system so that drug absorption can be faster. The use of a solid dispersion system also has many advantages including,

reduced particle size (reduced), increasing the wettability of the drug so that it is more easily dissolved (Trianggani & Sulistiyaningsih, 2018). Solid dispersion is a mixture of one or more active substances which are dispersed into an inert carrier in the solid-state. The method of making solid dispersions in this study is solvent evaporation because it has the best advantage, namely, it can prevent the decomposition of drug ingredients (Trianggani & Sulistiyaningsih, 2018).

The selection and comparison of the carrier material also determine the success in increasing the dissolution rate of the drug. According to research, it has been proven that the manufacture of solid dispersions with polyvinyl pyrrolidone K-30 (PVP K-30) as a carrier can increase the dissolution rate of the drug (Umar et al., 2014). The ratio of active ingredients: carrier used in this study was chitosan: PVP K-30 = 1:1 and 1:2 (11). The solid dispersion will be compared with a physical mixture with the same ratio.

The reduction of cholesterol levels in this study was carried out by in vitro tests. In vitro test was carried out by observing the decrease in cholesterol levels when added to the amount of chitosan which was seen using a UV-Vis spectrophotometer at a wavelength of 200-800 nm (Adu et al., 2019).

This study aims to see the ability of solid dispersion of crab shell chitosan in lowering cholesterol levels. This needs to be done for the development of chitosan, which is the second most abundant product that can be synthesized from natural ingredients whose utilization is still not too widespread as an active pharmaceutical ingredient, especially anticholesterol.

MATERIAL AND METHODS

Materials

The material used in this research are cholesterol powder (analytical grade) that purchased from Sigma Aldrich, chloroform (technical grade) that purchased from Merck, Acetic anhydride (analytical grade) that purchased from Merck, H_2SO_4 (analytical grade) that purchased from Merck, chitosan from mangrove crab *(Scylla serrata)* shells, PVP K-30 (technical grade), and pristine simvastatin that given by Hexpharm Jaya company.

Methods

Chitosan Characterization

Organoleptic test

Organoleptic test of chitosan was included evaluation of the shape and color of the chitosan. Typically, chitosan shows flaky powders and is light brown to white (Nasional, 2013)**.**

Moisture content test

The sample of chitosan was weighed as much as 0.5 grams. Then heated using an oven at 105ºC for 1 hour. After that, it was cooled using a desiccator for 3 hours. Weighed the results of chitosan that has been in the oven. It was alculated using the formula (Zahiruddin et al., 2008):

$$\%\ moisture\ content = \frac{(a-b)}{c} \times 100\%$$

a= weight of container + wet sample (grams)

b= weight of container + dry sample (grams)

c= weight of wet sample

Ash content test

Carefully weigh 2-3 g of the sample into a porcelain (or platinum) dish of known weight, for the liquid sample, evaporate over a water bath to dry. Charcoal over a burner flame, then ash in an electric furnace at a maximum temperature of 550°C until complete ashing (occasionally opening the furnace door slightly to allow oxygen to enter). Cool in a desiccator, then weigh it until obtained the constant weight (SNI 01-2891-1992).

$$\%\ ash\ content = \frac{w1 - w2}{w\ (g)} X100\%$$

w = Sample weight (g)

w1 = Weight of sample + cup after ashing (g)

w2 = Weight of empty cup (g)

Ninhydrin test

The ninhydrin test was used to show the presence or absence of an amine group in chitosan. The presence of amine groups in the chitosan sample was indicated by color change into purple (Ylitalo et al., 2002)

Deacetylation degree test

The degree of deacetylation of chitosan was determined by several factors, namely the concentration of NaOH used, the temperature and the duration of the deacetylation process. The standard percent degree of deacetylation is 70% (Dompeipen et al., 2016). The degree of deacetylation of chitosan was determined by FTIR spectroscopy and its spectrum was evaluated in the frequency range of 1000-4000 cm-1. The DD of chitosan will be calculated using the following equation (Heidari et al., 2018):

$$Deacetylation\ Degree\ (DD) = 97{,}67 - (26{,}486 \times (\frac{A_{1655}}{A_{3450}}))$$

Solid Dispersion Preparation Method

The Solvent Evaporation method is carried out by dissolving the drug substance, namely chitosan with a carrier material into an organic solvent that dissolves it which is then evaporated by drying using water bath and oven (Trianggani & Sulistiyaningsih, 2018). In this study, chitosan was dissolved in 2% acetic acid in a ratio of 1:50 then PVP K-30 dissolved with 96% ethanol in a ratio of 1:5. Afterwards, the chitosan solution was mixed with PVP K-30 solution following the formula (Table 1) and evaporated in

water bath at 50-60ºC until a precipitate was formed. The resulting precipitates was then dried in the oven at 50ºC for 2 h (Imtihani et al., 2021).

Physical Mixture Preparation Method

The physical mixture was made by mixing the drug substance, namely chitosan, with the carrier material, namely PVP K-30 in a ratio of 1:1 and 1:2 in a tumbler for 5 minutes.

Table 1. The Formula of Solid Dispersions (SD) and Physical Mixtures (PM).

Formula	Chitosan (55mg)	PVP K-30
SD1	1	1
PM1	1	1
SD2	1	2
PM2	1	2

Preparation of Stock Solution (Cholesterol 1000 ppm)

Cholesterol powder as much as 100 mg was dissolved in chloroform to obtain a volume of 100 ml solution (concentration 1000 g/ml), and stored in the refrigerator at a temperature of 2 - 8ºC (Adu et al., 2019).

Determination of Maximum Wavelength

The 1000 ppm cholesterol stock solution that has been made was taken 0.5 ml and added chloroform ad 5 ml to obtain a 300 ppm cholesterol solution. 1 ml of acetic anhydride was added, then vortexed for 30 seconds and soaked in cold water and keep in the dark for 30 minutes. 0.1 ml of concentrated H_2SO_4 was added and homogenized with a vortex for 30 seconds and then soaked in cold water and keep in the dark for 90 minutes. Measurements were made using a UV-Vis spectrophotometer at a wavelength of 200-800 nm (Adu et al., 2019; Kenny, 1952).

Preparation of Standart Solution

Cholesterol mother liquor with a concentration of 1000 ppm was made in 5 concentration series, namely 0.5; 0.75; 1; 1.25; and 1.5 ml of chloroform ad 5 ml were added, so that each solution was produced with concentrations of 100, 150, 200, 250, and 300 ppm. Each solution was added 1 mL of acetic anhydride solution then vortexed for 30 seconds and soaked in cold water and keep in the dark for 30 minutes. Then 0.1 mL of concentrated H_2SO_4 was added to each solution and the solution mixture was homogenized using a vortex for 30 seconds, then soaked in cold water and keep in the dark for 90 minutes and the absorbance was measured at a maximum wavelength of 405 nm according to the previous measurement results (Adu et al., 2019; Maidin et al., 2017).

Identification of Cholesterol

Each sample and simvastatin (positive control) 10 mg was added to 5 ml of 300 ppm cholesterol solution, the mixture was homogenized with a vortex for 30 seconds and incubated for 60 minutes at 37ºC. Then centrifuged for 5 minutes at 4000 rpm. The supernatant was taken and transferred in a closed test tube

then it was added with 1 ml of acetic anhydride then vortexed for 30 seconds and soaked in cold water and keep in the dark for 30 minutes, then 0.1 ml of concentrated H_2SO_4 was added and homogenized with a vortex and soaked in cold water and keep in the dark for 90 minutes. The absorbance was measured at a maximum wavelength of 405 nm according to the previous measurement results (Adu et al., 2019; Maidin et al., 2017). After that, the absorbance of the test was measured by the final cholesterol level compared with the standard curve with a simple linear regression formula (Lindawati & Ningsih, 2020):

$$Y = bx+a$$

y = Dependent variable (Absorbance)

x = Independent variable

a = Constant

b = Regression coefficient

then calculate the percent reduction in cholesterol levels with the formula:

$$A= \frac{C - B}{C} * 100$$

A: % decrease in cholesterol levels
B: Final cholesterol level
C: Initial cholesterol level

RESULTS AND DISCUSSION

The characterization of chitosan carried out included tests of organoleptic, moisture content, ash content, ninhydrin, and degree of deacetylation. The results of the evaluation are presented in table 2.

Table 2. Result of Chitosan Characterization from Mangrove Crab (*Scylla serrata*) Shell

Parameter	Chitosan Characterization	Result	Interpretation
Shape	Flakes to powder	Powder	Good
Color	Light Brown to white	Creamy White	Good
Moisture Content	2-10%	2.15%	Good
Ash Content	≤2%	1.14%	Good
Ninhydrin	(+) Changes to purple	(+) Changes to purple	Good
Deacetylation Degree	>70%	70.57%	Good

From the characterization of chitosan, it can be stated that chitosan has fulfilled all the characterization requirements that exist from organoleptic (shape and color) was creamy white (Yanti et al., 2018), moisture content was 2.15% (2-10%) (Pratiwi, 2014), ash content was 1.14% (≤2%) (Pratiwi, 2014), ninhydrin test was positive purple(Ylitalo et al., 2002), and deacetylation degree was 70.57% (>70%)(Dompeipen et al., 2016).

This research was preceded by measuring the maximum wavelength with a concentration of 300 ppm in the range of 200 - 600 nm. The maximun wavelength was obtained at 405 nm that can be seen in table 3. The peak on scanning using a UV-Vis spectrophotometer for all samples and a positive control showed a peak at a wavelength of around 300 nm and 400 nm that showed in figure 1. However, according to the reference, the maximum wavelength of cholesterol is at a 410 nm (Adu et al., 2019; Burke et al., 1974). So the maximum wavelength was chosen around 400 nm, namely 405 nm which shows the highest absorbance.

Table 3. The Scanning Result of Maximun Wavelenght.

Wavelength	Absorbance
402	1.968
403	1.969
405	1.970
406	1.967
407	1.963

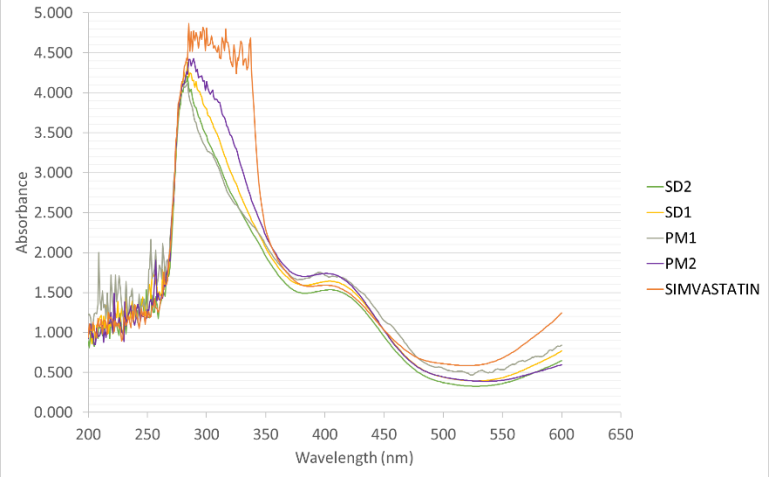

Figure 1. Cholesterol level reduction by the sample and positive control

The process is continued by making a standard cholesterol solution curve with five variations of cholesterol solution levels (Table 4). With the standard curve, it can be used to find a linear regression equation so that it can be used in the search for a level whose absorbance has been measured.

Table 4. Results of Absorbance Measurement of Standard Cholesterol Solution

Consenstration (ppm)	Absorbance
100	0.705
150	1.004
200	1.290
250	1.594
300	1.970

Based on the results of the resulting standard cholesterol solution curve, it can be seen that the curve follows Lambert-Beer's law in the form of a straight line with a value of r^2 = 0.9971, a = 0.00624, b = 0.0646. So that the linear regression equation (y = a±bx) obtained is y = 0.00624.x + 0.0646. Figure 2 is shown the standard cholesterol solution curve.

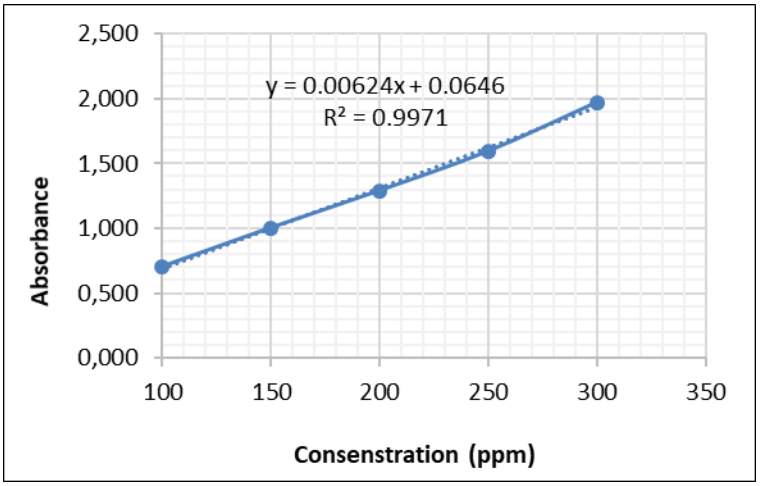

Figure 2. Standard cholesterol solution curve

The correlation coefficient value states a linear relationship between the concentration and the resulting absorption. Based on the results of r^2 obtained, it is stated that the correlation coefficient gives linear results because it meets the acceptable criteria, namely 0.99 or close to 1.00 (Miller et al., 2000).

Measurement of cholesterol-lowering activity using the Lieberman-Burchard method where each sample was replicated 3 times and produced a dark green color. The result is then calculated the final cholesterol level by comparing it with the standard curve that has been obtained and the average percentage reduction in cholesterol levels from each sample is obtained (Adu et al., 2019; Maidin et al., 2017). From the results of observations and calculations that have been carried out, the result of absorbance and percent reduction in cholesterol levels by chitosan are shown in table 5.

Table 5. The reducing percentage in cholesterol levels by the sample and positive control

Sample	Initial Cholesterol Concentration (ppm)	Final Cholesterol Concentration (ppm)	Reduction Percentage (%)
SD1	300	245.26	18.44
PM1		246.32	18.11
SD2		211.23	29.56
PM2		263.95	12.01
Simvastatin		209.78	30.07

The result of decreasing cholesterol content between solid dispersion and physical mixture showed that the solid dispersion sample had a greater reduction than the physical mixture. SD1 was better than PM1 and SD2 was better than PM2 with the same ratio of chitosan and PVP K-30. This indicates that there is a difference in the reduction in cholesterol levels between the solid dispersion and the physical

mixture. This is because solid dispersion is a product consisting of at least two different components, namely the hydrophobic active ingredient and the hydrophilic matrix of the active ingredient which will turn into crystalline, dissolved, or amorphous. This solid dispersion is used to accelerate the drug dissolution process (Chiou & Riegelman, 1971). The use of this solid dispersion can help increase the absorption of drugs that are poorly soluble in water. In addition, it can also increase the speed of dissolution by changing the drug form to amorphous (Trianggani & Sulistiyaningsih, 2018).

CONCLUSION

The solid dispersion system of mangrove crab (*Scylla serrata*) shell chitosan extract can reduce the cholesterol levels from the in vitro test. From the sample, the best results obtained for SD2 are 29.56% that is solid dispersion of chitosan: PVP K-30 =1:2. This value is comparable to the positive controlsimvastatin 10 mg.

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CONFLICT OF INTEREST

Authors declare that there is no conflict of interest

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