CHOLESTEROL LOWERING EFFECT OF CHITOSAN NANOPARTICLES USING PARIJOTO FRUITS EXTRACT

Fania Putri Luhurningtyas*, Rissa Laila Vifta, Nur Syarohmawati, Mahardika Adhi Candra

Program Studi S1 Farmasi Universitas Ngudi Waluyo, Jl. Diponegoro No. 186, Ungaran Timur, Kabupaten Semarang, Jawa Tengah, 50512, Indonesia

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ABSTRACT

Parijoto (Medinilla speciosa Reinw. ex Blum) fruit is known to have pharmacological activity as cholesterol lowering levels. Its activity needs to be increased with nanoparticle system so that the active substance can bind 100% to the action target. This study aims to determine the formation of nanoparticles from parijoto fruit (NEBP) and activity test as a decrease in cholesterol levels. The formation of nanoparticles used variations of concentration and volume of chitosan and NaTPP. Anti-cholesterol testing is based on the amount of free cholesterol in the sample that reacted with Lieberman-Burchard into complex green compounds. The best formation of NEBP was 0.2% chitosan, 0.1% NaTPP and volume ratio 5:1. The particle size showed an average size of 269.3 nm (10-1000 nm). The result of the percent transmittance and polydispersity index were 99.379 (close to 100%) and 0.378 (PDI <0.5). The functional group-specific of NEBP was –OH, N-H, PO3. The morphology was round and non-uniform particles. NEBP can decrease 50% cholesterol levels with a smaller EC50 value was 89.08 compared to the extract (EC50 259.98 ppm). Nanoparticles of parijoto fruit is a potential candidate for anti-cholesterol drug.

Keywords: anti-cholesterol; fruit; Medinilla speciosa Reinw. ex Blum; nanoparticles.

INTRODUCTION

The consumption level of fat intake in Indonesian society is increasing. The increase of the level of fat intake is related to the incidence of cardiovascular disease. In 2030, there will be an estimated 23.6 million people who die of cardiovascular disease (Bastien et al., 2014; Kemenkes RI, 2018). Parijoto fruit is one of the plants preferred by researchers related to its health benefits, especially in degenerative diseases. Parijoto fruit extract is proven to have anti-diabetic, anti-oxidant, anti-bacterial and anti-cholesterol activities (Wachidah, 2013; Sa’adah et al., 2017; Sugiarti et al., 2017; Vifta et al., 2018).

Bioactive compounds contained in parijoto fruit have low weaknesses. That compounds are very sensitive to processing factors, thus increasing the amount of absorbed active substances. The activity of parijoto fruit metabolites needs to be improved by the application of nanotechnology so that the economic value of Parijoto fruit can be increased (Fathinatullabibat et al., 2014; Irawati et al., 2018).

Nanotechnology can be the main solution for active substances with low bioavailability. Nano allocation particles have a very large surface area that makes it easier to use more effectively and easily in passing through the intestinal wall (Elgadir et al., 2015). The nano method that is easy to implement uses polymeric ionic gelation. The basic principle for this method is the presence of electrostatic attraction between oppositely charged molecules. This force can occur between bioactive components and nanocapsule material (Rizvi et al., 2018).

Chitosan or α (1-4) 2-amino 2-deoxy a β-D-glucane is a form of deacetylation of chitin,
a biopolymer contained in the exoskeleton of crustaceans and insects. Chitosan has a sensitivity to pH because it is easily soluble at acidic pH (pH<6.5). Chitosan does not dissolve at a higher pH range. The advantages of chitosan as an encapsulate ingredient that chitosan can prolong the duration of drug activity, improve therapeutic efficiency and reduce side effects (Kleine-brueggeney et al., 2015).

Based on the description above, it is necessary to research the increasing pari joto fruit extract activity through the encapsulation process used chitosan and evaluate its activities as anti-cholesterol. This research is important to provide further results regarding herbal plants in the field of phytopharmaceutical, specifically in herbal plants as anti-cholesterol candidates.

METHODS

Materials

Dried pari joto fruit, chemicals used include ethanol 96%, ethanol pa, glacial acetic acid pa, Liebermann Burchard reagent, concentrated sulfuric acid, anhydrous acetic acid (Merck), chitosan powder (92% acetylation degree) from Zhejiang Golden-Shell Pharmaceuticals, NaTPP (Brataco) powder, distilled and redistilled water (Ikapharmindo Putra Mas), chloroform pa, cholesterol standard (Sigma).

Instrumentations

Maceration tools, filter paper, laboratory glassware, rotary evaporator (RE 100-Pro), water bath (Memmert), analytic balance (OHAUS), magnetic stirrer (Thermo Scientif Cimarec), a set of centrifugation devices (PLC Series), UV-Vis spectrophotometer (Shimadzu UV Mini 1240), Particle Size Analyzer (Malvern), Fourier Transform Infrared/FTIR spectrophotometer (Perkin Elmer Spectrum 100) and Scanning Electron Microscopy (Phenom Pro-X).

Determination of plants

Parijoto fruit was obtained from Colo Village, Kudus Regency, Central Java at the beginning of March 2019 with the specification of purplish-pink fruit with a sour taste. The sample was determined at the UPT. Herbal Materia Medica Laboratory, Batu, Malang Health Office to ensure the authenticity of plants used and avoided mistakes in plant selection.

Extraction

Parijoto was macerated by soaking 200 grams of pari joto dried powder which had been mashed with 2 L ethanol 96% (1:10). The maceration was carried out for 2 days (48 hours) and followed by re-maceration. Macerate was evaporated by a rotary evaporator at 80°C and it was concentrated using water bath at 80°C. The concentrated extract was calculated as percent yield and percent moisture content.

Nano extract procedure

NEBP making procedure consists of 2 steps, namely (1) optimization of the concentration of chitosan:NaTPP, and (2) optimization of the volume of chitosan : NaTPP. The extract was weighed 100 mg and dissolved in 100 mL of ethanol, mixed with 15 mL of redistilled water. The liquid extract was taken by 10 mL then 50 mL of chitosan solution was added with various concentrations (0.1%; 0.2%; 0.3% w/v). In the next step, the solution was stirred at 400 rpm for 20 minutes. Then, gradually added 10 mL NaTPP with varying concentrations (0.1%; 0.2% w/v). After that, it was homogenized using a magnetic stirrer to form nanoparticles at 400 rpm for 20 minutes and the mixture entered centrifugation process at 3000 rpm for 15 minutes. The obtained supertanant was characterized by particle size, percent transmittance, specific functional groups, and shape morphology.
Test cholesterol reduction activity and data analysis

The in vitro assay of anti-cholesterol activity was based on research procedures from Anggraini et al. (2018). The nano extract and crude extract (25, 50, 75, 100, 125 and 150 ppm) were taken 4 mL, transferred to a test tube with a lid, then added with 1 mL of 1000 ppm cholesterol stock solution. They were mixed until homogeneous then added 2 mL of anhydrous acetic acid and 0.1 mL concentrated H$_2$SO$_4$. The solution was incubated for 15 minutes in a dark place (the container was covered with aluminum foil) until the color changed to green. The study was replicated 3 times and the color results obtained were read with a UV-Vis spectrophotometer at a maximum wavelength of 623.20 nm. The blank reagent used was 5 mL chloroform plus 2 mL anhydrous acetic acid and 0.1 mL concentrated H$_2$SO$_4$. The negative control used was 1 mL of 1000 ppm cholesterol solution in 5 mL of chloroform, plus 2 mL of anhydrous acetic acid and 0.1 mL concentrated H$_2$SO$_4$.

The parameter for decreasing cholesterol levels is a decrease in color intensity (green). The green color is a reaction between the Liebermann-Burchard reagent and ergocalciferol (cholesterol); a decrease in color intensity is due to the reaction between cholesterol and secondary metabolites (flavonoids) in the parijoto fruit nano-extract. Percent reduction in cholesterol levels used absorbance value data obtained from measurements of NEBP and parijoto fruit extract compared to standard cholesterol solutions.

The percentage formula for decreasing cholesterol levels = (Cholesterol Solution Absorbance - Sample Absorbance) / (Cholesterol Solution Absorbance) x 100%. The statistical test used data of the percentage reduction in cholesterol levels from each concentration treated. The application used SPSS version 24 with the One-Way ANOVA post hoc Tukey HSD to determine the differences in each sample treatment. The EC$_{50}$ value was obtained based on a linear regression calculation of the reduction in cholesterol levels from each concentration. The EC$_{50}$ value was the concentration of the sample which can inhibit cholesterol levels by 50%. The purpose of determining the EC$_{50}$ is to determine the concentration of the dosage which is expected to produce an effect of reducing cholesterol levels by 50%. The result of the equation $y = a + bx$ can be calculated EC$_{50}$ using the formula:

$Y = a + bx$

$50 = a + bx$

$(x) EC_{50} = (50-a) / b$

RESULTS AND DISCUSSION

Determination

Plant determination was carried out at the UPT. Materia Medica Batu, Batu City, Malang, East Java. Plant identification key of parijoto is 1b-2b-3b-4b-12b-13a-14bb-17b-18b-19b-20b-21b-22b-23b-24b-25b-26b-27a-28b-29b-30b-31a-32b-33a-34a-36b-1b-4b-6b-9b-10b-14b-15b-16a-17b-18b-20b-23b-24b-25b-27b-1b-3a-4a.

Parijoto is a shrub (1-2 meters high) typical of the Muria Mountains, Kudus. The fruit is similar to kersen, purplish red, rounded, and has a distinctive hemispherical section attached to the petals, 5-8 mm in diameter. The seeds are round, larges, mall, and white (Figure 1).

Figure 1. Parijoto fruit
Characterization of Parijoto Fruit Extract

The yield of extract was 10.48% w/v. The percentage of the extract’s water content was 4.07%. The identification of moisture content in the extract was supposed to know the minimum limit or range of the amount of water content in the material (extract). The higher the water content is, the easier it is to grow fungi and molds, so that they can reduce the biological activity of extracts in the retention period (Mohammed et al., 2017).

Characterization of nano parijoto fruit extract (NEBP)

The nanoparticles were made broadly into 2, i.e. top-down and bottom-up. Nano extract in this study used the bottom-up method. It arranges atoms or molecules and combines them through chemical reactions to form nanostructures. Nano parijoto fruit extract (NEBP) is made by the ionic gelation method, which was based on the principle of crosslinking between chitosan cation groups and polyanion in NaTPP.

The NEBP formulation used variations in the concentration of chitosan and NaTPP. The concentration of chitosan used in the formula was obtained by conducting a literature study. The concentration of chitosan was 0.1%, 0.2% and 0.3% w/v, the NaTPP concentration was 0.1% and 0.2% w/v (Sulistyawati et al., 2017). The results of the formation of nanoparticles based on variations in the concentration of chitosan: NaTPP are presented in Table 1.

| Chitosan:NaTPP | Particle size (nm) | PDI | %T   |
|---------------|-------------------|-----|------|
| 0.1:0.1       | 292.4             | 0.426 | 99,269 |
| 0.2:0.1       | 269.3             | 0.372 | 99,379 |
| 0.3:0.1       | 1097              | 0.549 | 96,618 |
| 0.1:0.2       | 315               | 0.332 | 99,109 |
| 0.2:0.2       | 777.9             | 0.513 | 98,274 |
| 0.3:0.2       | 1171              | 0.476 | 98,025 |

PDI: Polydispersity index
%T: Percent transmittance

The formation of NEBP 0.2% w/v chitosan and 0.1% w/v NaTPP produced the best nanoparticle characteristics. These results follow the study about the synthesis of red mangosteen peel extract which the concentration of 0.2% chitosan and 0.1% NaTPP gave the best physical and functional properties with a particle size of 214.4 nm in Garcinia forbesii extract nanoparticles and 285.2 nm in Garcinia extract mangosteen (Mardliyanti et al., 2012).

Prevention of particle formation at a micro size, chitosan must use concentrations below 0.3%. If the concentration of chitosan used is too small, it will produce small and easily aggregated particle sizes, and it causes a larger particle size. Increasing the concentration of NaTPP with the same concentration of chitosan will also cause a larger particle size. This is because the high concentration of NaTPP results in an increase in the availability of amine groups to combine small particles into larger particles (Ningsih et al., 2017). The small size of nano extract can increase the absorption by the mechanism of diffusion through the intestinal wall and reaches the blood compared to large particles (Shah et al., 2016).

Results of variations in chitosan volume:NaTPP based on particle size, polydispersity index, and transmittance value showed that the best formula obtained was chitosan 0.2% w/v and NaTPP 0.1% w/v with a volume ratio of 5:1 (Table 2). The volume
ratio greatly influences particle size, polydispersity index, and percent transmittance. Nano parijoto fruit extract made with a volume ratio of 5:1 has the smallest particle size and polydispersity index of 269.3 nm and 0.372. Increasing the ratio of chitosan and NaTPP will produce nanoparticles with smaller particle size, smaller polydispersity index, and larger percent transmittance.

The polydispersity index value is used to estimate the range of particle size distributions that exist in a sample and finds out whether there is aggregation (Bunglavan et al., 2014). The smaller the polydispersity index value is the more homogeneous the particle size. The polydispersity index value (Table 1 and 2) ranges from 0.3 to 0.7 showed wide particle size distribution. Non-uniform particle size was caused by the tendency of particles to agglomerate to form larger particle aggregates. Factors that can influence particle size and polydispersion index are the concentration of chitosan and crosslinker, the ratio of volume and mass between chitosan solution and crosslinker, stirring speed and stirring time (10). Percent transmittance (% T) is used to measure the clarity of a solution or dispersion system. The percent transmittance value ranges from 98.025-99.379%. Transmittance values close to 100% show clear and transparent dispersion.

**Characterization of functional groups nano parijoto fruit extract**

The interaction between parijoto fruit extract and chitosan-NaTPP is needed to determine the ability of coating. One method that can be used to determine the presence of extract is Fourier Transform Infra-Red (FTIR). FTIR in this study used 4000-400 cm\(^{-1}\) wavenumbers for extract samples, NaTPP, and chitosan. Parijoto fruit extract used wavenumbers of 4000-600 cm\(^{-1}\). FTIR results can be seen in figure 2.

**Table 2.** The results of NEBP formulation based on volume variation of Chitosan: NaTPP

| Volume Chitosan: NaTPP | Particle size (nm) | PDI  | %T       |
|------------------------|--------------------|------|----------|
| 2:1                    | 2.168 x 10^4       | 0.441| 98.643   |
| 5:1                    | 269.3              | 0.372| 99.379   |
| 8:1                    | 315.7              | 0.493| 99.127   |
| 10:1                   | 346.2              | 0.612| 98.997   |

PDI: Polydispersity index, %T: Percent transmittance

![Figure 2](image-url)
The transmittance graph of FTIR results on nano parijoto fruit extract (*Medinilla speciosa* Reinw. Ex Blume) shows that there was an interaction between chitosan, NaTPP, and nano parijoto fruit extract which was indicated by the shift of wavenumber in the group (-OH) from 3370 cm\(^{-1}\) (chitosan), 3387 cm\(^{-1}\) (NaTPP) and 3365 cm\(^{-1}\) (parijoto ethanol extract) to 3371 at nano parijoto fruit extract (Napsah *et al.*, 2014).

At N-H uptake also experienced a shift in wavenumber from twin uptake 1654 and 1598 cm\(^{-1}\) (chitosan) to 1645 cm\(^{-1}\) at nano parijoto fruit extract. This change showed the deformation of the N-H group to be one peak because of the cross-linking process (Dewandari *et al.*, 2014). The stretching of the PO\(_3\) group at wavenumber of 1045 cm\(^{-1}\) showed the formation of cross bonds between amino groups from chitosan and anionic groups in NaTPP (Lusiana *et al.*, 2018).

**Nano morphology of parijoto fruit extract**

Physical particle characterization was carried out by Scanning Electron Microscopy (SEM) to observe the morphology and determine the particle size. This method is an efficient way to obtain images of the specimen’s surface. Data obtained from SEM is a two-dimensional photo that displays the specimen's surface and particle size. The morphological results of the NEBP were round and non-uniform with particle sizes ranging from 100-1000 nm (Figure 3). This is shown by the graph of particle size ranges obtained from measurements using a particle size analyzer.

Morphology of NEBP based on SEM photos had a round shape and was not uniform. Measurement of particles applied SEM to obtain particles with sizes ranging from 100-1000 nm. The SEM measurement results have followed the graph of particle size ranges obtained from measurements using particle size analyzers in the range of 100-1000 nm.

![Figure 3. Results of scanning electron microscopy nano Parijoto fruit extract in 4000x zoom](image)
Determination of maximum waves and operating time

Determination of maximum wavelength and operating time using the 100 ppm cholesterol which was reacted with anhydrous acetic acid and concentrated H_2SO_4 produced the following results: the maximum wavelength of 623.20 and 15th-minute operating time. Determination of the value of the operating time is used to determine the timing of the reaction perfectly and stably.

Reduction of the cholesterol level in the Liebermann-Burchard method

The Lieberman-Burchard method was used to determine the amount of free cholesterol found in samples that reacted to green compounds that could be measured using UV-Vis spectrophotometers. The more concentrated form of green from the solution shows the high free cholesterol contained in the sample solution. The Liebermann-Burchard reagent consists of a mixture of anhydrous acetic acid with a small amount of concentrated sulfuric acid. Concentrated sulfuric acid was useful for cutting the hydroxyl group on cholesterol and then oxidized to 3.5 kolesterol (3.5 cholestadienes), resulting in a green color. Anhydrous acetic acid to form acetyl was derived from steroids.

The reaction carried out in this method must be free of water, because the reaction was very sensitive and unstable to water. The presence of water can affect the reaction process and make the compounds formed become unstable. The addition of anhydrous acetic acid is useful for removing water content and ensuring the system to be free of water to form derivative products of acetyl from steroids. The removal of the water content by anhydrous acetic acid is carried out by binding to the OH and H. Water groups makes the anhydrous acetic acid turn into acetic acid which will not react with concentrated cholesterol and sulfuric acid (Suptijah et al., 2011).

The percentage of cholesterol decrease levels in the concentration of NEBP was greater than the extract (Table 3). The result of EC_{50} values was inversely proportional to the level of activity of compounds in the sample in reducing cholesterol levels. The smaller the EC_{50} value is, the stronger the activity of decreasing cholesterol levels.

| Concentration (ppm) | Percentage of Inhibitory Cholesterol Levels (%) | NEBP   | Extract |
|---------------------|-----------------------------------------------|--------|---------|
| 25                  | 14.61                                         | 6.82   |
| 50                  | 25.22                                         | 14.29  |
| 75                  | 41.88                                         | 20.78  |
| 100                 | 62.98                                         | 24.35  |
| 125                 | 71.75                                         | 27.60  |
| 150                 | 78.38                                         | 28.25  |
| EC_{50}             | 89.08                                         | 259.98 |

The ability of NEBP to reduce cholesterol levels by as much as 50% only requires a small concentration of 89.08 ppm. NEBP can increase the effectiveness of parijoto fruit extract in reducing cholesterol levels which is equal to 3 times greater than the extract. Statistical analysis using Tukey HSD as well as variations in the concentration of nano extract (150-75 ppm) resulted in a reduction in cholesterol levels which differed significantly from the activity of decreasing cholesterol extract (p-value 0.000). Modification of nanoparticles will help increase the absorption of compounds contained in the extract by increasing the surface area so that the amount of the absorbed substance will increase or, in other words, also increase the effectiveness of the extract (Ferreira et al., 2018). Anti-cholesterol activity in the NEBP concentration of 25 ppm was comparable with the extract concentration of 50 ppm (p-value 1.000). The smallest concentration of nano extract can reduce the dose as much as 2 times compared to the extract, although, based on statistical results, showed no significant difference between the two treatments.

The content of the active ingredient which is considered to have cholesterol-lowering activity in parijoto fruit is flavonoids. Flavonoids are biologically polar and soluble in water, so they are absorbed poorly due to...
the large particle size. The particle size of these metabolites makes it difficult to absorb through the mechanism of passive diffusion since the lipid solubility is not good so that the ability of compounds to penetrate or penetration to lipid membranes is limited (Anggraini et al., 2017). Nanotechnology is recommended because there are some side effects on the formula that is already available in the market; one of which is influenced by the factor of non-compliant patient because the formulations use large doses and are less effective, there is no clear target specificity. Nanotechnology can affect the bioavailability and the increase of absorption of active ingredients due to an increase in particle surface area and solubility and has a longer residence time because it is trapped by intestinal mucosa. The increased surface area is due to the smaller particle size, so that the residence time in the intestine will be longer and ensnared by the intestinal mucosa (Napsah et al., 2014). Increasing the effectiveness of parijoto fruit extracts using the ionic gelation nanoparticle technology method can increase effectiveness by using lower doses.

CONCLUSION
Parijoto fruit extract nanoparticles were successfully synthesized with 0.2% chitosan and 0.1% NaTPP. The best volume ratio of chitosan:NaTPP was 5:1. Chitosan-encapsulated parijoto fruit extract based on the percentage reduction in levels and EC₅₀ values had a greater cholesterol-lowering activity compared to parijoto fruit extract (p<0.05). Nano parijoto fruit extract can be used as a potential candidate for anti-cholesterol drugs. It is necessary to find out the optimum dosage of nano extract in vivo test assay.

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