



Original article

In vitro and *in vivo* studies of nanoparticles of chitosan-*Pandanus tectorius* fruit extract as new alternative treatment for hypercholesterolemia via Scavenger Receptor Class B type 1 pathway

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ABSTRACT

Pandanus tectorius fruit, a natural product rich in tangeretin and ethyl caffeate, has been reported to have potential as anti-hypercholesterolemia agent via Scavenger Receptor Class B type 1 (SR-B1) pathway. However, due to its semi-polar properties, *P. tectorius* extract exhibits poor solubility when used as a medical remedy. The extract's solubility can potentially be improved through a synthesis of nanoparticles of chitosan-*P. tectorius* fruit extract. This can also increase the extract's SR-B1 gene expression activity. To date, no studies of nanoparticles of chitosan-*P. tectorius* fruit extract and its pathway via SR-B1 have been published anywhere. In this study, cytotoxicity properties against HepG2 were explored by MTT. Then luciferase assay was used to detect their effectiveness in increasing SR-B1 activity. An *in vivo* study using *Sprague dawley* was carried out to observe the extract nanoparticles' effectiveness in reducing the cholesterol levels and the toxicity property in rat's liver. As the results showed, the extract nanoparticles had no cytotoxic activity against HepG2 cells and exhibited higher SR-B1 gene expression activity than the non-nanoparticle form. As the *in vivo* study proved, nanoparticle treatment can reduce the levels of TC (197%), LDL (360%), and TG (109%), as well as increase the level of HDL cholesterol by 150%, in comparison to those for the untreated high-cholesterol diet group. From the toxicity study, it was found that there was non-toxicity in the liver. It can be concluded that nanoparticles of chitosan-*P. tectorius* fruit extract successfully increased *P. tectorius* fruit extract's effectiveness in reducing hypercholesterolemia via SR-B1 pathway. Hence, it can be suggested that nanoparticles of chitosan-*P. tectorius* fruit extract is safe and suitable as an alternative treatment for controlling hypercholesterolemia via SR-B1 pathway.

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1. Introduction

Hypercholesterolemia is one of the significant risk factors for morbidity and mortality in the world. Hypercholesterolemia refers to a condition where the level of cholesterol in the blood is more than the normal limit of 200 mg/mL. The condition is marked by

increasing total cholesterol (TC) and low-density lipoprotein (LDL) levels, and decreasing high-density lipoprotein (HDL) level (Harada-Shiba, et al., 2012). Increased LDL level in the blood vessel can be a factor for the development and progression of atherosclerosis (Deepa and Varalakshmi, 2005). Atherosclerosis is a condition that occurs when the accumulation of lipids in the blood vessel leads to its blockage or narrowing, affecting blood circulation (Lewis et al., 2004). Atherosclerosis is the main cause of cardiovascular diseases, including stroke and heart attack (Deepa and Varalakshmi, 2005).

Hypercholesterolemia treatment drugs are available in the markets; however, these drugs are not suitable for extended consumption because they can develop side effects (Alsheikh-Ali et al., 2004). These include effects on the gastrointestinal tract (Geng et al., 2014) and liver damage (Mach et al., 2018). The most severe

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adverse reaction from long-term use of these drugs is rhabdomyolysis (Catapano and Alberico, 2012). These diseases will be the main causes of over 24 million estimated deaths in 2030 (WHO, 2017).

Hypercholesterolemia medicine has been produced using natural products such as plants. Some of these plants include Sweet star fruit (*Averrhoa carambola* L) (Pham et al., 2017), Tamarind (*Tamarindus indica* L) (Kuru, 2014), Kemuning (*Murraya paniculata* L jack) (Zhang et al., 2018), and Mahkota Dewa (*Phaleria. Macrocarpa* (Scheff.) Boelr), (Andriani, et al., 2015b). These plants can decrease the cholesterol levels in the blood as they contain high antioxidant and secondary metabolite compounds, such as phenol, flavonoids, tannins, and alkaloids (Baba et al., 2007, Maron et al., 2003).

Another relevant plant is *Pandanus tectorius*, one of the plants belonging to the mangrove group. The plant has been reported to have some beneficial uses, including as hypolipidemic agent (Zhang et al., 2013a; Liu et al., 2013), anti-inflammatory and antiviral agents (Adkar and Bhaskar, 2014), anti-oxidant agent, and antibacterial agent (Andriani et al., 2015a; 2017). The fruit of *P. tectorius* yields high total phenolic content and chemical constituents such as phenolic, flavonoid, steroid, triterpenoid, saponin, and glycosides (Andriani et al., 2015a). Phenolic and flavonoid are believed to facilitate the reduction of the cholesterol levels (Baba et al., 2007; Maron et al., 2003). The fruit consists of fifteen secondary metabolite compounds, ten phenolics and five semipolar, such as vanillin, *trans*-ethyl caffeate, tangeretin, chrysin, naringenin (Zhang et al., 2013b), and caffeoylquinic acid (Zhang et al., 2013a). The fruit and its compounds have been reported to have potential as anti-hypercholesterolemia agent (Andriani et al., 2019; Pangestika et al., 2020). However, the semipolar properties of *P. tectorius* extract cause it to exhibit poor solubility if it is used as a medical remedy.

One of the ways to potentially improve the extract's solubility is through a synthesis of nanoparticles of chitosan-*P. tectorius* fruit extract. This can also increase Scavenger Receptor Class B type 1 (SR-B1) gene expression activity. The SR-B1 is known as an HDL receptor that is present on the liver surface. This receptor is responsible for the disposal of lipid particles (cholesterol esters), mediating the uptake of cholesterol esters from HDL into the liver (Savel et al., 2012). The SR-B1 provides supports for removing free cholesterol.

Nowadays, the technology of nanoparticles in pharmaceutical fields is quickly developing. Materials that have nanoparticles size offer the most promising potency for the mucosal delivery of drugs and antigens (Rampino et al., 2013). One of the natural materials employed to make drug-containing nanocarriers is chitosan. Chitosan is a suitable raw material for nanoparticle synthesis because it is nontoxic and biocompatible (Hejazi and Amiji, 2003; Dutta et al., 2009; Agnihotri et al., 2004). Chitosan can be integrated with some functional substances, such as vitamins, minerals, proteins, and other active natural products, and these modifications can increase the ability of chitosan in the pharmaceutical field (Lopez-Caballero et al., 2005; Siripatrawan and Harte, 2010; Ojagh et al., 2010). Synthesis of chitosan nanoparticles is carried out using a method called ionic interaction or ionic gelation, where positively charged chitosan is set to react with negatively charged matrix. Additionally, chitosan has been formulated into tablets, matrix, and microparticles (Dash et al., 2011). Chitosan can prevent of absorption of fat or lipid in the gut or intestine, through electrolyte reaction. A positive charge present in the chitosan can bind negatively charged cholesterol, resulting in decreased cholesterol total in the blood plasma. It contains β -1 $\rightarrow$ 4 linked 2-deoxy-D-glucosamine (GlcN) and 2-deoxy-N-acetyl-D-glucosamine (GlcNAc) units (Rinaudo, 2006). Chitosan is derived from the deacetylation of chitin, which is isolated from crustaceans' exoskeleton, such as shrimps and crabs (Woo and Park, 2003, Dhillon et al., 2012, Kaur and Dhillon, 2013). Chitosan can dissolve

in pH below 6.0 (Kumbar et al., 2014). It is solubilised using acetic acid, which can protonate chitosan to become a positive charge ($-\text{NH}_3^+$). As a positive charge, chitosan can crosslink with a negatively charged matrix to become polyelectrolyte interaction or ionic gelation.

To date, no studies of nanoparticles of chitosan-*P. tectorius* fruit extract and its pathway via SR-B1 have been published anywhere. Thus, our current study aimed to verify the hypothesis that nanoparticles of chitosan-*P. tectorius* can be used as an alternative treatment for reducing the cholesterol levels and can increase SR-B1 gene expression.

2. Material and methods

The organic solvents and other reagents were of analytical grade and purchased from Sigma aldrich. The experimental animals were male *Sprague dawley* rats and purchased from Takrif Bistari Enterprise, Kuala Lumpur, Malaysia.

2.1. Preparation of *P. tectorius* fruit extract

Fresh fruits of *P. tectorius* collected from Pantai Teluk Ketapang were sliced thinly into small pieces, dried in a freeze dryer for 5 days, and ground into fine powder using sample blender. The fine powder was weighed using digital balance, then extracted. The extraction was carried out using successive extraction method by Andriani et al., (2011) that was slightly modified. In this study the powder (500 g) was dissolved with several solvents having different polarity, such as hexane, ethyl acetate, and methanol, starting with a solvent with the lowest polarity, and then continuing successively with solvents having higher polarity. The extraction was conducted until the solvent turned colourless. Subsequently, the extract was filtrated and evaporated using a rotary evaporator to yield thick or viscous samples named hexena extract (PHE), ethyl acetate extract (PEE), and methanol extract (PME). The extracts were weighed to calculate the w/w ratio between them and the dried fine powder. Further, the extracts were used to synthesize with the nanoparticles, and the nanoparticles were assayed using *in vitro* and *in vivo* studies.

2.2. Synthesis of nanoparticles of chitosan-*P. tectorius* fruit extract

Nanoparticles of chitosan-*P. tectorius* fruit extract was synthesized using ionic gelation method, following the protocol by Mohammadpourounighi et al. (2010), with some modification made to the concentration of acetic acid and ratio between chitosan and sodium tripolyphosphate (NaTPP). About 1 mg/mL chitosan solution was prepared by dissolving chitosan powder in 0.1% (v/v) acetic acid solution and stirring for 24 h, subsequently adding NaTPP 1% and 1 mL Tween 80 0.1% under stirring for 60 min. Subsequently, 1 mL of the *P. tectorius* fruit extract was added and stirred for 15 min, then put in ultrasonicator for 5 min. The supernatant and nanoparticle pellet were centrifuged for 15 min at 1120g to separate them. Finally, the pellet was freeze-dried to yield a dry powder. The dry powder and the solution of nanoparticles were characterized by fourier-transform infrared spectroscopy (FT-IR), particle size analyzer (PSA) using Malvern Instruments, Worcestershire, UK, and scanning electron microscopy (SEM) using Philips XL30 SEM, Eindhoven, Netherlands.

2.2.1. Particle size analyzer (PSA)

Particle size analysis was carried out to characterize the nanoparticle size and particle distribution. The analysis was done using Malvern Zetasizer Nano ZS particle size analyzer (Malvern

Instruments, Worcestershire, UK), an instrument for quickly measuring and determining particle size and particle distribution. The nanoparticle samples were put into 1 mL cuvettes and placed inside the particle size analyzer to be characterized. The method used to measure the particle size was laser diffraction, where particles that passed through the visible ray scattered the light at certain angles based on the particle sizes. The size distribution was recorded by the computer system.

2.2.2. Fourier transform infrared (FTIR)

Analysis of FTIR was used to investigate the functional group to analyze the structure of formed nanoparticles. This analysis can be also used to check the success of formed nanoparticles as a way to compare pure chitosan peak and chitosan nanoparticles peak shown in the histogram. The equipment used was spectrophotometer of Fourier transform infrared (IRTreccer-100, Shimadzu). Less than 0.1 g dry chitosan nanoparticles and the chitosan - *P. tectorius* fruit extract NPs powder was placed in the KBr plate, and the spectrum was recorded in the transmittance mode. The range of IR spectrum recorded was between 4000 and 600 cm^{-1} . The spectrum was recorded using Attenuated Total Reflectance (ATR) technique.

2.2.3. Scanning electron microscopy (SEM)

SEM analysis was used to analyse the morphology of nanoparticle surface area. The instrument used was Philips XL30 SEM, Eindhoven, Netherlands. The dried sample was put on cylinder metal plate for the next coating with gold (Au). The plate was placed inside a magnetron sputtering coater equipped with a vacuum pump. The sample coated with gold was put on the sample plate. The sample was scanned by an electron beam and magnified images of varying scales of the sample's surface area were displayed on the computer screen.

2.3. In vitro study

2.3.1. Cytotoxicity assay

Cytotoxicity assay was conducted utilizing 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT) (modified from Lee et al., 2004) to determine the cell viability. The concentration of the HepG2 cell line seeded was 2.5×10^5 cells/well. About 50 μL of cells and 50 μL sample of nanoparticles of chitosan-*P. tectorius* fruit extract were inserted into the 96-well plate, and each sample was measured in triplicate, while untreated cells were designated as the control. Untreated cells were HepG2 cell line to which only complete media was added. The concentration of samples used was 100 $\mu\text{g}/\text{mL}$ and two-fold dilution to 1.5625 $\mu\text{g}/\text{mL}$. Then, 100 μL of complete media was added into each well, and the plate was incubated at 37 $^{\circ}\text{C}$, 5% CO_2 for 72 h. After incubation, 20 μL of MTT solvent was added to each well, and it was covered using aluminum foil. The medium or supernatant was discarded carefully from each well and was replaced with 100 μL dimethyl sulfoxide (DMSO). The well was thoroughly mixed by the pipette to require the MTT formazan to dissolve. The 96-well plate was covered with aluminium foil and incubated for 10 min. Finally, the optical density (OD) was measured by a Varioskan reader at 570 nm.

2.3.2. SR-B1 gene expression assay

Concentration samples of nanoparticle of chitosan-*P. tectorius* fruit extract used 50 $\mu\text{g}/\text{mL}$ and 2-fold dilution to 3.125 $\mu\text{g}/\text{mL}$. The trichostatin A (TSA) at the final concentration of 0.5 $\mu\text{g}/\text{mL}$ was used as the positive control and untreated cells as the negative control. This experiment used the ONE-Glo luciferase assay reagent (Promega), and the cell titer-fluor cell viability assay reagent (Promega) in luciferase assay. In a well of 96-well plate, the 95 μL medium seeded SR-B1 stable line of HepG2 cells (3×10^4 cells/well)

was added 5 μL of various concentrations of samples. Each concentration of samples and control were analyzed with three replications. About 20 μL cell-Titer flour reagent was added into the well and it was shaken for 30 s. Then 90 μL ONE-Glo luciferase reagent was added into the well. Finally, the plate was incubated at room temperature for at least 3 min. The luminescence activity was recorded using a 96-well plate luminescence reader. Relative luciferase activity was obtained by normalizing the firefly luciferase activity against internal control (renilla luciferase).

2.4. In vivo study

A letter of approval for ethical animal testing (UMT/JKEPHT/2018/26) was obtained from Universiti Malaysia Terengganu (UMT). The experiment was conducted at the animal laboratory, Institute of Marine Biotechnology (IMB), UMT, and the experiment guidelines set by the university were followed. As many as 32 male *Sprague dawley* rats were used for the animal testing. They were randomly divided into 4 groups (group A, B, C, and D). Group A was the normal group, group B was the high cholesterol group, fed with high cholesterol diet food (HF), group C was the drug control group, fed with 10 mg/kg body weight (BW) simvastatin, and group D was the nanoparticle group, fed with 500 mg/kgBW nanoparticles of chitosan-*P. tectorius* fruit extract. HF was made from a mixture of standard food, 1% cholesterol, 0.1% cholic acid, 8.5% oil vegetable, and water.

The rats were housed in a temperature-controlled room with a 12 h dark-light cycle and given free access to water. The experiment was completed in 7 weeks, which included 1 week of adaptation period for all groups, 2 weeks of HF feeding to groups B, C, and D to increase the cholesterol levels, 2 weeks of cholesterol treatment to groups C and D, and 2 weeks of recovery for all groups.

2.4.1. Analysis of blood serum: measuring total cholesterol (TC) levels, high-density lipoprotein levels (HDL), low-density lipoprotein (LDL), and triglyceride (TG) levels

Every week blood was withdrawn from the rats' tail veins using a sterile needed and collected into 1.5 mL centrifuge tube. It was then centrifuged to separate the serum (colorless) from blood. About 1 mL of the blood yielded 0.5 mL of serum. Further, the serum was analysed to measure TC, HDL, LDL, and TG levels. The total cholesterol levels in the blood serum was quantified using the enzymatic-colorimetric method. This assay was conducted using an enzymatic method.

2.4.2. Histological change of rats' livers

The rats' livers were collected and immersed in 10% buffered formalin overnight. After the liver tissues were fixed, they were processed using a tissue processor machine (Leica, Nussloch, Germany) for ± 26 h. The processes in the tissue processor machine involved dehydration process (utilizing 50% alcohol for 1.5 h, 70% alcohol for 1.5 h, 80% alcohol for 1.5 h, 90% alcohol for 1.5 h, and 100% alcohol for 1.5 h 3 times), clearing process (using mixture of alcohol + toluene (50:50) for a 0.5 h and only toluene overnight), and impregnation process (utilizing paraffin wax for 2 h 3 times).

The livers were sectioned with a thickness of no more than 4 μm using a rotary microtome machine (Leica, Nussloch, Germany). The liver sections were immersed into a water bath at 38 $^{\circ}\text{C}$ and left to float for 3 min. The floating organs were fished out and placed on glass slides coated with albumin, which acted as a tissue adhesive. Subsequently, the slides were dried in the oven at 38 $^{\circ}\text{C}$ for 24 h.

The specimen slides were subjected to a staining process following the Hematoxylin and Eosin (H&E) protocol to clarify the structure and to make it easier for evaluation. The slides were sub-

merged in xylene I (2 times) to remove wax clinging to the tissues, followed by 100%, 100%, 90%, 80%, 70% and 50% ethanol for 3 min for each concentration, then followed by hematoxylin for 15 min. Subsequently, the slides were washed under running tap water for 10 min, submerged in 1% acid alcohol for 3–5 dips, and washed under running tap water for 3–5 min. Furthermore, the slides were submerged in eosin for 3 dips, then in 95%, 95%, 100%, and 100% ethanol for 3 min for each concentration, and lastly in xylene for 5 min (2 times). The stained slides were dried in the oven at 40 °C for 10–15 min and dipped in xylene for 1 min.

Finally, the slides were mounted with DPX mounting, covered with a coverslip, and left to dry overnight. Analysis of cell degeneration of rat's livers was conducted under a light microscope, and photomicrograph was taken using an image analyzer microscope (Leica, Nussloch, Germany) at 20x magnifications.

2.5. Statistical analysis

All data analyses were performed in triplicate. All data were expressed as means $\pm$ standard deviations (S.D). Data were normalized using Kolmogorov-Smirnov. Differences between groups were analyzed using one-way analysis of variance (ANOVA) (SPSS version statistic 20), and *post hoc* test was performed using Duncan test, in which *p*-value < 0.05 was considered significant.

3. Results and discussion

3.1. *P. tectorius* fruit extract

As many as 30 fresh fruits of *P. tectorius* yielded 4.5 kg of dried fruits. Successive extraction method yielded three extracts, namely PHE (15 g), PEE (10 g) and PME (520 g). PME had the highest percentage of extract obtained at 11%, followed by PHE and PEE at 0.33% and 0.22%, respectively.

3.2. Synthesis and characterization of nanoparticles of chitosan-*P. tectorius* fruit extract

Chitosan nanoparticles can be formed at certain chitosan concentrations. In addition, NaTPP concentration must also be controlled to prevent the formation of broad particles. The addition of NaTPP in the production of chitosan nanoparticles is as a cross-linked that allows the structure of chitosan to become tight and rigid, making the size of chitosan smaller. NaTPP was chosen as a crosslinker because it has more negative charges compared to other polyanions, such as sulfate and citrate; thus, NaTPP has a stronger interaction with the amine group in chitosan (Zeng, et al. 2010). NaTPP has quite low toxicity, is non-mutagenic and non-carcinogenic, and is thus suitable for biomedical applications. Qualitatively, the nanoparticles of chitosan-*P. tectorius* fruit extracts could be seen from the increasing changes in turbidity or cloudiness of the solution. The changes indicated the occurrence of electrostatic interaction. Thenanoparticles formed were characterized using FTIR, PSA and SEM.

The size analysis of the nanoparticles of chitosan-*P. tectorius* fruit extracts utilized the zetasizer instrument. To obtain the best particle size distribution, the nanoparticles of chitosan-*P. tectorius* fruit extracts were synthesized using 18 design formulations (Table 1). Smaller size distribution of sample is indicated by smaller polydispersity index (PDI) value. Based on the experiment, particle size and PDI value were smaller when the method of sonication used was probe sonication compared to bath sonication. This is because probe sonication has a higher force than bath sonication and breaks the particles directly.

A significantly different effect on the formed particle size was observed when different concentrations of acetic acid were added to the nanoparticle synthesis (Fig. 1). Acetic acid can protonate chitosan to become a positive charge ($-\text{NH}_3^+$). Higher concentration of acetic acid changes NH_2 to NH_3^+ , and due to this, the probability polyelectrolyte interaction will increase. In this experiment, the smallest particle size was gained by adding 2% acetic acid solution.

Furthermore, as shown in Table 1, the smallest particle size was present in sample 2c. It can be seen from Fig. 2 that the peak of sample 1a was sharper than the peak of sample 2c. This means that sample 1a had a more homogenous size than sample 2c. Among the 18 design formulations, the best formulation for creating chitosan-*P. tectorius* nanoparticles was formulation 1a. This is because formulation 1a had the smallest PDI, which indicated that it was the most stable among all samples. Danaei et al. (2018) reported the correlation of particle size and PDI value of the samples with their quality as nano-carrier system. This means that particle size and PDI value have important influence as nano-carrier system for drugs. Value of PDI between 0.01 and 0.5 correlates with nanoparticles that have good homogeneous degree. The sample having the smallest PDI value indicated that the sample was the most stable among all samples, while the sample having high PDI value meant large particles can easily be made to become the stable particle.

Each FTIR spectrum of the main components was compared to see peak changes between the main components, to investigate the functional group in the samples, and to examine if ionic interaction successfully occurred in the nanoparticle formation. Fig. 3 shows FTIR spectra of nanoparticle components such as chitosan, NaTPP, and *P. tectorius* fruit extract. A special spectrum of chitosan was present from the broad peak at 3277 cm^{-1} that corresponded to the OH stretching, which overlapped the NH stretching in the same region, the peak at 1639 cm^{-1} that corresponded to CONH_2 , and the peak at 1570 cm^{-1} that corresponded to the bending vibrations of $\gamma\text{ NH}_2$. The $\gamma\text{ NH}_2$ peak was sharper than the CONH_2 peak that showed high levels of chitosan deacetylation (shown in Fig. 3). Deacetylation is the process of removing acetyl groups. Chitosan is produced from chitin through deacetylation by removing the acetyl group (HN-COCH_3) to be replaced by NH_2 .

There was a new peak in the FTIR spectrum of nanoparticles of chitosan-*P. tectorius* fruit extract compared to the FTIR spectrum of chitosan nanoparticles. Peak shifts, changes in peak characteristics, and appearance of new peaks were caused by strong electrostatic interactions between polycation (in chitosan) and polyanion (from NaTPP solution and *P. tectorius* fruit extracts (Li et al., 2010). As shown in Fig. 3, the peak shift occurred at wavenumber from 3277 to 3255 cm^{-1} that resulted in an overlap of N–H and O–H stretching vibration, from 1639 to 1633 cm^{-1} and from 1570 to 1533 cm^{-1} that released C–O stretching. The new peak appeared at 1151 , 1060 , and 891 cm^{-1} , which resulted in P=O and P–O stretching (Rodrigues et al., 2012). These results indicated that an interaction occurred among amino groups of chitosan and negatively charged TPP and crude extract of *P. tectorius*. The change of peak absorbance of yielded nanoparticles indicated the formation of inter and intramolecular reactions in the yielded nanoparticles (Dounighi et al., 2012; Neves et al., 2014).

The morphology of the surface area of pure chitosan, chitosan nanoparticles, and nanoparticles of chitosan-*P. tectorius* fruit extract are shown in Fig. 4. The shapes of pure chitosan, chitosan nanoparticles, and nanoparticles of chitosan-*P. tectorius* fruit extract were respectively irregular, similar to fiber, semi-squared, and spherical nanostructures. There were significant differences before (Fig. 4b) and after (Fig. 4c) adding *P. tectorius* fruit extract. The extract was expected to influence the characteristic of nanoparticles of chitosan-*P. tectorius* fruit extract, which can make the interaction more solid.

Table 1

Particle size and particle distribution of 18 formulations designs with the variation of acetic acid concentrations, the ratio of volume/volume (v/v) between chitosan and NaTPP solution, and variation of type of sonicator.

Samples	[Acetic Acid]	[Cs]	Vcs:V NaTPP	Sonicator Bath		Sonicator Probe		Information
				Size (nm)	PDI	Size (nm)	PDI	
1 a	0.1	1	2:1	404.2	0.362	160.4	0.279	The smallest PDI
2 a			3:1	157.6	0.449	128.9	0.365	
3 a			4:1	181.8	0.459	140.4	0.408	
1b	0.5	1	2:1	217.4	0.597	134.5	0.394	
2b			3:1	355.4	0.455	149.1	0.433	
3b			4:1	336.7	0.454	181.3	0.523	
1c	2	1	2:1	212.6	0.593	142.4	0.505	The smallest Size
2c			3:1	159.5	0.582	110.5	0.456	
3c			4:1	150.2	0.8	137.2	0.519	

Notes: Cs = chitosan, NaTPP = sodium tripolyphosphate, PDI = polydispersity index, V = volume.

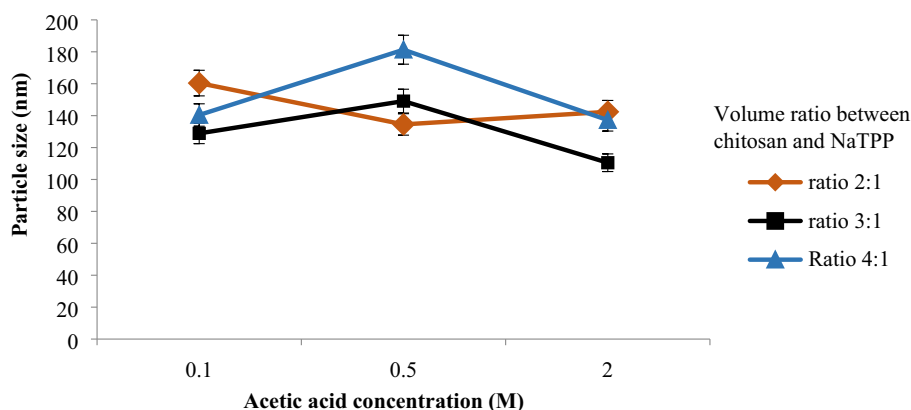


Fig. 1. Effect of different concentration of acetic acid toward the particle size using probe sonication.

3.3. In vitro study

3.3.1. Cytotoxicity properties

The cytotoxicity activities of all nanoparticle samples were categorized as non-cytotoxic since the IC_{50} values obtained was $>30 \mu\text{g/mL}$ (Fig. 5). The results are in line with those reported by Andriani et al. (2020),

The highest concentration of nanoparticles ($100 \mu\text{g/mL}$) did not kill 50% of HepG2 cell line population. The sample with IC_{50} value less than $30 \mu\text{g/mL}$ exhibited significant cytotoxicity activity. This indicated that the sample has potential as an anti-cancer agent (Moshi et al., 2004; Saetung et al., 2005). The samples with IC_{50} value more than $30 \mu\text{g/mL}$ were considered as having noncytotoxic properties, meaning that the samples can be used as anti-diabetic, anti-cholesterol, and anti-inflammatory agents (Andriani et al., 2011). Cao et al. (1998) reported that a sample with IC_{50} value less than $5 \mu\text{g/mL}$ is strongly toxic, $5\text{--}10 \mu\text{g/mL}$ toxic, $11\text{--}30 \mu\text{g/mL}$ mildly toxic and more than $30 \mu\text{g/mL}$ non-cytotoxic. The results revealed that nanoparticles of chitosan-*P. tectorius* fruit extract can be further assayed for use as anti-cholesterol, anti-inflammatory, and anti-diabetic agents plus others.

3.3.2. The effect of *P. tectorius* fruit extracts and nanoparticles of chitosan-*P. tectorius* fruit extracts on transcriptional activity of SR-B1 promoter

The SR-B1 is a promoter that has an essential role in reverse cholesterol transfer (RCT) mechanism (Grundy, 2016). As a promoter, SR-B1 can facilitate the uptake of selective cholesterol that brings the HDL, which can decrease the total cholesterol levels in the blood vessels (Mathews et al., 2013). In this study, the luciferase activity controlled by PPRE plasmid was expressed as the num-

ber of fold change in relative to the negative control. Higher number of fold change compared to the negative control (>1) indicates a positive result. This means that the sample has higher SR-B1 gene expression activity compared to the negative control. The nanoparticles of chitosan-PHE, nanoparticles of chitosan-PEE, and nanoparticles of chitosan-PME showed the fold change number more than 1 (Fig. 6), and thus the nanoparticles were considered as active in increasing SR-B1 promoter. From the observation, the highest transcriptional activity of SR-B1 promoter was the nanoparticles of chitosan-PEE with fold change value of 5.8-fold at the effective concentration of $12.5 \mu\text{g/mL}$, followed by the nanoparticles of chitosan-PME with fold change value of 5.2-fold at the effective concentrations of 6.25 and $12.5 \mu\text{g/mL}$, and the nanoparticles of chitosan-PHE with fold change value of 2.7-fold at the effective concentration of $6.25 \mu\text{g/mL}$, compared to the negative control (Fig. 6).

This experiment compared the activities of *P. tectorius* fruit extract (PME) and nanoparticles of chitosan-*P. tectorius* fruit extract (chitosan-PME), as shown in Fig. 7. Methanol extract was chosen in further test because the extract is more reactive and had more stock. The results showed that the nanoparticles of chitosan-PME had higher SR-B1 promoter activity than the PME. The highest activity of nanoparticles of chitosan-PME was at effective concentrations of $6.25 \mu\text{g/mL}$ and $12.5 \mu\text{g/mL}$. At the same concentration ($6.25 \mu\text{g/mL}$), the nanoparticles of chitosan-PME had higher SR-B1 promoter activity than PME. The activity of nanoparticles of chitosan-PME was 1.2 times higher than pure PME. This high activity may be caused by the size of nanoparticles, where small particle size can increase the surface area that in turn can increase the probability of its interaction and reaction, (Duceppe and Tabrizian, 2010; Nagpal et al., 2010) and finally increase the SR-B1 promoter activity of the nanoparticles. Thus, the small size

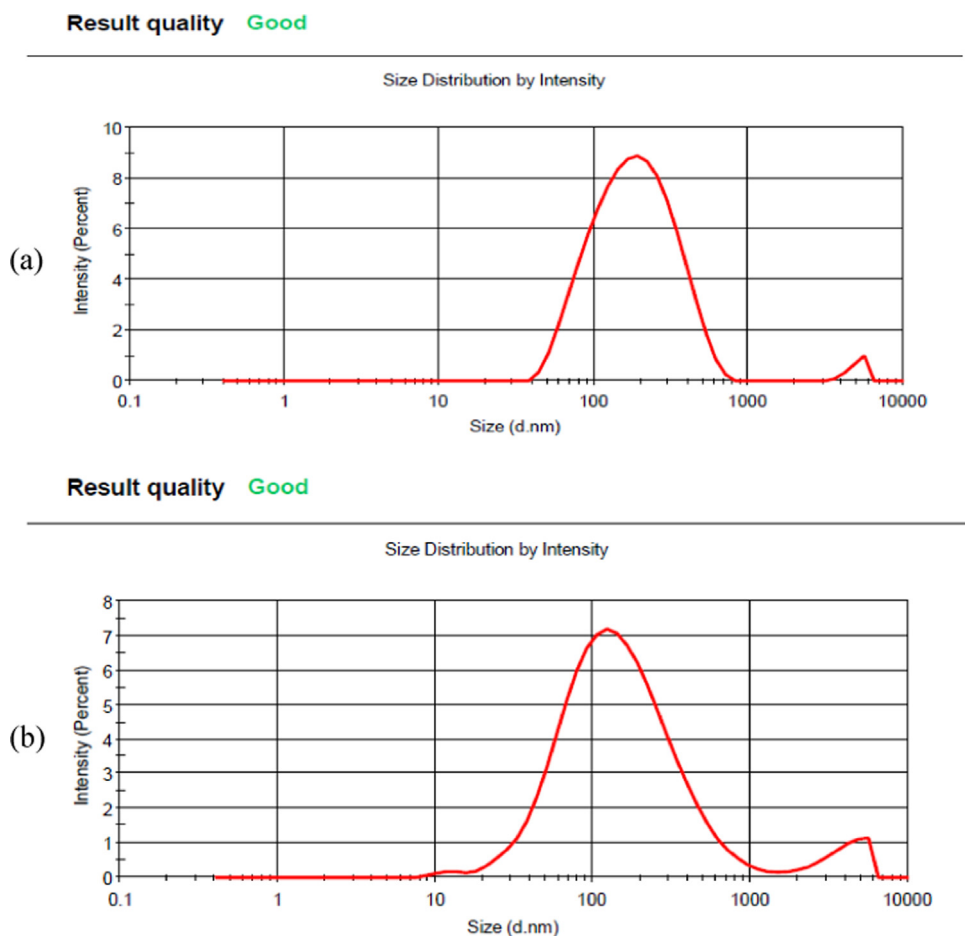


Fig. 2. Size distribution by intensity of nanoparticles of chitosan-*P. tectorius* fruit extracts with formulation 1a (a) and 2c (b).

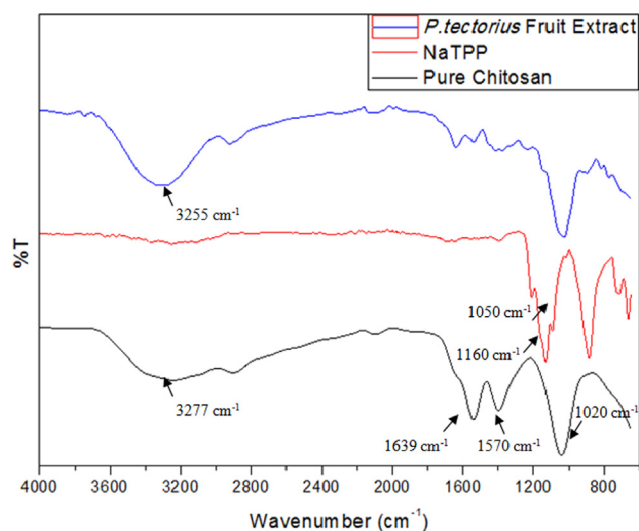


Fig. 3. Infrared spectra of chitosan (red line) and nanoparticles of chitosan-*P. tectorius* fruit extract (blue line).

of nanoparticles highly influences the increase in the transcriptional activity of SR-B1 promoter.

Duceppe and Tabrizian (2010), Nagpal et al. (2010), and Wang et al. (2011) reported that the nanoparticle form of natural product samples will have a positive impact on their bioactivity compared to their initial form (non-nanoparticle form). In this

experiment, the nanoparticles of chitosan-PME and chitosan-PEE exhibited higher activity of SR-B1 promoter gene expression. As an HDL receptor present on the liver surface, SR-B1 plays an important role in lipid metabolism. It can mediate the selective uptake of cholesterol bound to HDL to be inserted into the liver to be metabolized and finally removed as bile acid or impurities from the body. Thus the higher the SR-B1 gene expression, the higher the absorption of cholesterol, and the lower the cholesterol level in the blood, which will suppress the buildup of plaque in the bloodstream and reduce the development of atherosclerosis (Andriani et al., 2015b).

3.4. In vivo study using male Sprague dawley rats

3.4.1. Relationship between body weight and food consumption during 42-day treatment

Generally, there was a positive correlation between the body weight (BW) of rats and their consumption of food (Figs. 8 and 9). The changes in the body weight and food intake during 7 weeks were categorized into a baseline phase (week 1), high cholesterol phase (week 2–3), treatment phase (week 4–5), and recovery phase (week 6–7) as shown in Fig. 8 and in Fig. 9 respectively. Before the experiment, standard diet was given to all the rats for 7 days. To induce hypercholesterolemic condition in rats, high-cholesterol diet food (HF) was given to them. Feeding HF for 2 weeks to group B, C, and D increased the body weight, but the increment was not significant compared to group A (Fig. 8). Increase in body weight was also observed by Zhong et al. (2011), which used rabbits to examine the effect of probucol in

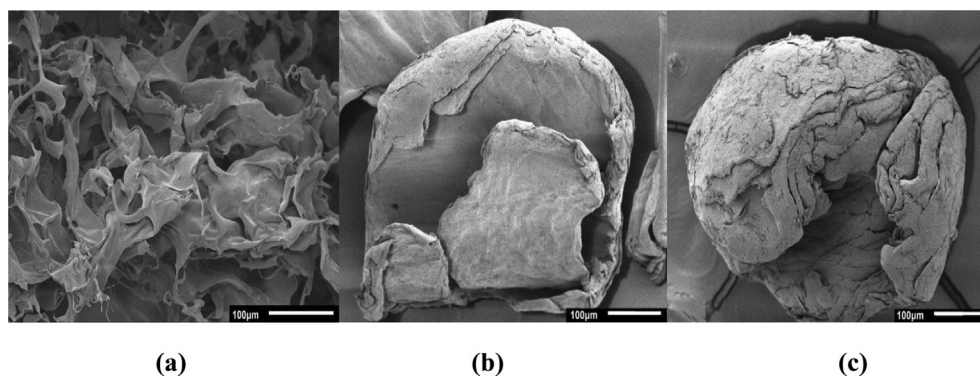


Fig. 4. Surface morphology of pure chitosan (a), chitosan nanoparticles (b) and nanoparticles of chitosan of *P. tectorius* fruit extract (c) using SEM.

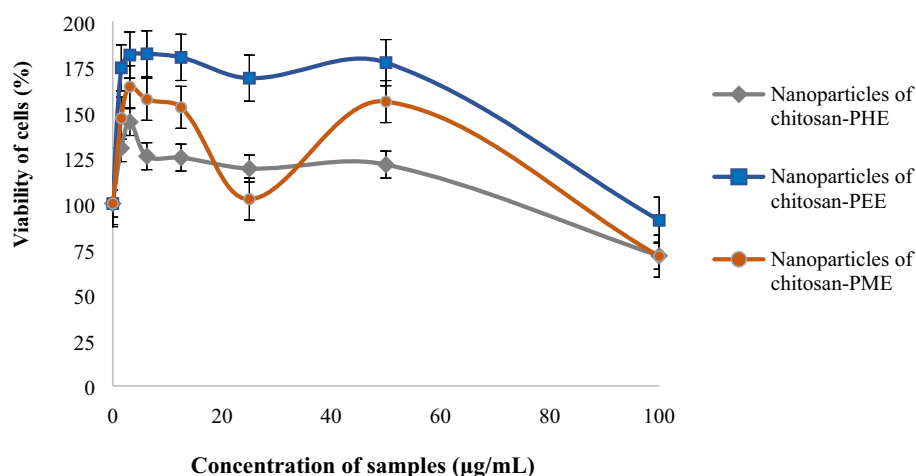


Fig. 5. Cytotoxicity property of nanoparticles chitosan loaded by PHE, PEE, and PME against HepG2 Cell Line. The values are presented as mean $\pm$ SD.

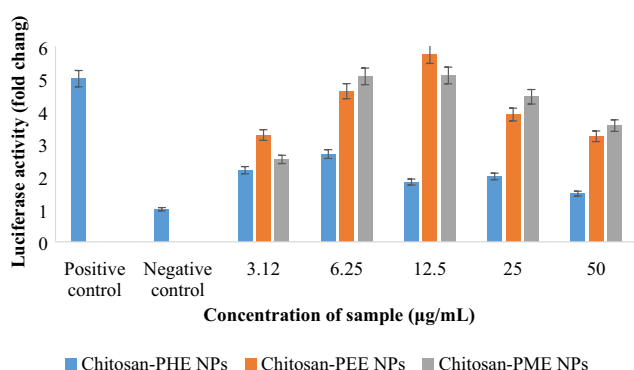


Fig. 6. Luciferase activities among nanoparticles of chitosan-PHE, PEE, and PME in increasing SR-B1 expression. Data are presented as mean $\pm$ SD ($n = 3$). PHE = hexane extracts of *P. tectorius* fruit, PEE = ethyl acetate extracts of *P. tectorius* fruit, PME = methanol extracts of *P. tectorius* fruit, NPs = nanoparticles.

alleviating atherosclerosis. The HF fed to experimental rats will increase their body fat deposits and stimulate an increase in adipocyte size and count (Kwak et al., 2012). Consequently, the accumulation of fatty acid in hepatocyte causes an increase in relative body weight. However, there was a study that showed a decrease in body weight. Peng et al. (2013) observed the influence of meal on non-alcoholic fatty liver and obesity, where there was a reduction in body weight as a consequence of a decrease in body fat deposition. A decrease in body weight can also be due to reduced weight of other vital organs (Al-Jarallah et al., 2013).

In week 4 (beginning of treatment phase), an increase in body weight was observed in all the groups, but the increase for groups B, C, and D was not significant compared to group A. The increase in body weight could be caused by lack of activity. Fewer activities mean that calorie intake needs to be reduced to have a good balance (Boyle and Sara, 2010). If the number of calorie intake is greater than the number of calories burnt, a buildup of energy in the body (in the form of fat) can cause the body weight to increase.

In week 7 (after the recovery phase), the body weight increased for group A, C, and D but decreased for group B. It is believed that the loss of body weight for the latter group was due to the stress experienced by the animals when they were subjected to intervention procedures, particularly during withdrawing of the blood. The stress led to loss of appetite, resulting in decreased body weight (Jeong et al. 2013). However, as reported by Guillén et al. (2008), no significant change in body weight was observed during their 10-week experiment on the squalene effect towards reducing incidence of atherosclerosis.

The food consumption of rats during the 7-week treatment is shown in Fig. 9. Standard diet or HF was given to the rats as much as 50 g/rat/day. However, HF eaten by each rat was different, where some rats ate only a small amount of HF. The different amount of HF intake had an impact on the rats' cholesterol levels. The lesser the amount of HF consumed, the lower the cholesterol levels in the blood. The decrease in food intake after 14 days of HF feeding and treatments of simvastatin and nanoparticles might have been caused by loss of appetite due to the intervention done to the rats. They were subjected to daily restraint stress for specific

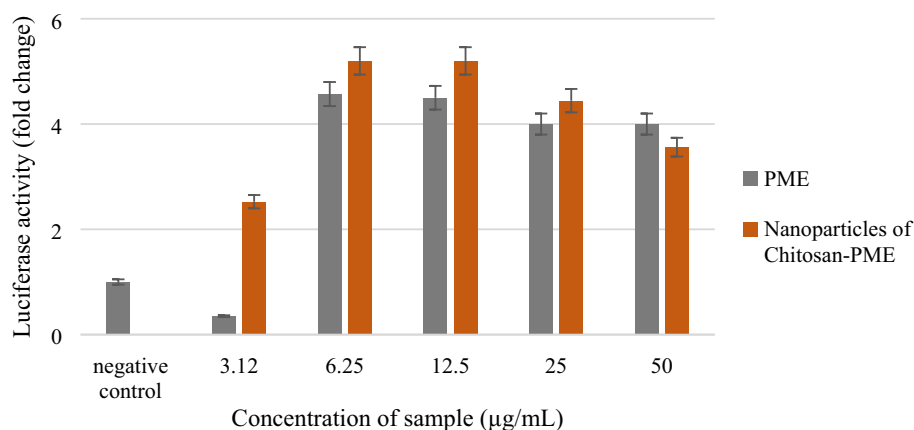


Fig. 7. Comparison of luciferase activity value of SR-B1 promoter given by nanoparticles of chitosan-PME and pure PME. The values of data are presented as mean $\pm$ SD. Note: PME = methanol extracts of *P. tectorius* fruit.

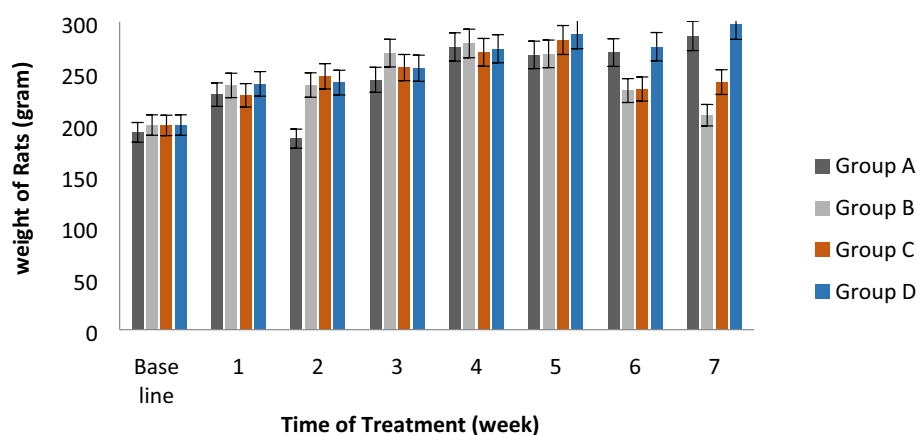


Fig. 8. Body weight during 42-day treatment of normal group (A), cholesterol (B), simvastatin drug (10 mg/kgBW) (C) and nanoparticles of chitosan-*P. tectorius* fruit extract (500 mg/kgBW) (D).

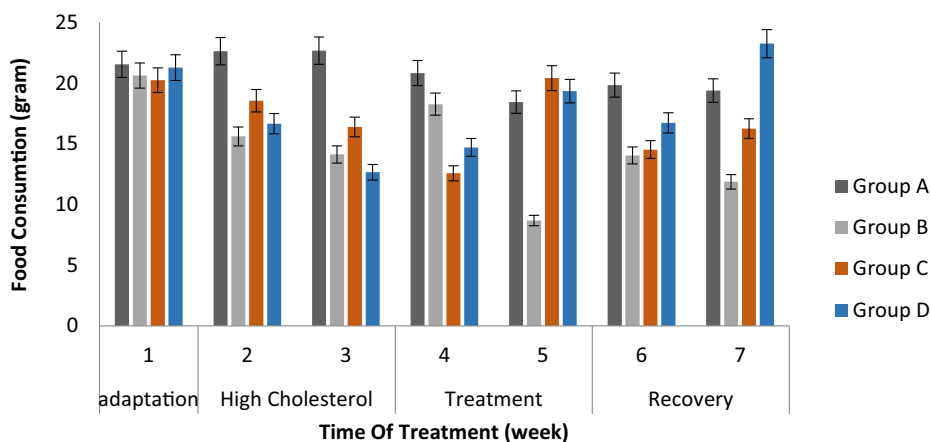


Fig. 9. Food consumption during 42-day treatment by normal group (A), cholesterol (B), simvastatin drug (10 mg/kgBW) (C), and nanoparticles of chitosan-*P. tectorius* fruit extract (500 mg/kgBW) (D).

purposes, such as withdrawing their blood. This is an unavoidable stress situation that causes automatic shifts in body temperature, mean arterial pressure, heart rate increase, and behavioural changes in rats. During this phase, the rats were stressed, and thus their desire to eat decreased, and in turn their food intake and body weight decreased (Jeong et al., 2013).

3.4.2. The effect of nanoparticles of chitosan-*P. tectorius* fruit extract toward total lipid profile: total cholesterol (TC) levels, high density lipoprotein (HDL) levels, low density lipoprotein (LDL) levels and triglyceride (TG) levels

Influences of HF toward the total lipid profile (TC levels, TG levels, HDL levels, and LDL levels) in the rats are shown in

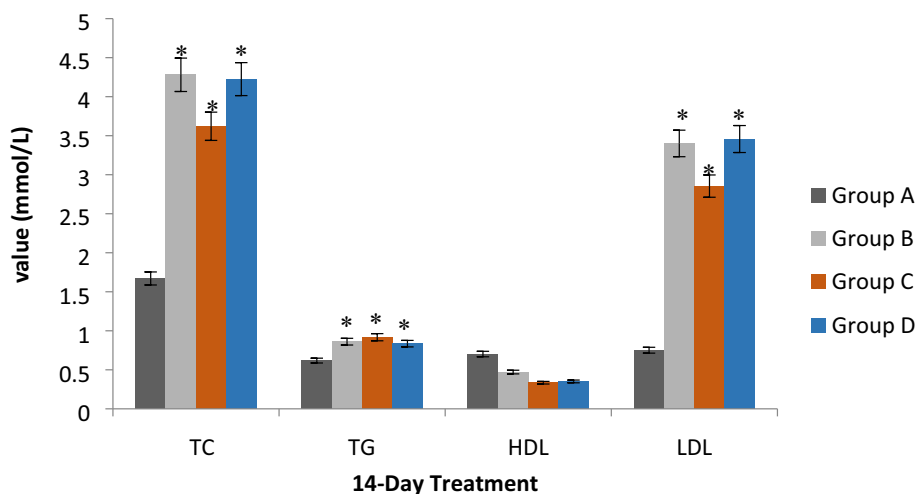


Fig. 10. Effect on total cholesterol (TC), triglyceride (TG), high density Lipoprotein (HDL) and low density lipoprotein (LDL) levels for normal group (A), cholesterol (B), simvastatin (10 mg/kgBW) (C), and nanoparticles of chitosan-*P. tectorius* fruit extract (500 mg/kgBW) (D) at day 14 of treatment. Data are presented as mean $\pm$ SD, with the number of rats per group $n = 8$. * $p < 0.05$ compared to normal group (A) using the Duncan test.

Fig. 10. At D-14, the TC levels, TG levels, HDL levels, and LDL levels of groups B, C, and D were significantly different compared to group A ($p < 0.05$). Increase in the TC levels for groups B, C, and D was caused by HF given daily for 14 days. HF that consists of 1% cholesterol and 0.1% cholic acid can stimulate the increase in TC levels (Wang et al., 2010).

At D-28 of treatment, serum lipid profiles of group D (oral administration of nanoparticles of chitosan-*P. tectorius* fruit extract) were significantly different compared to group B ($p < 0.05$) (Fig. 11). This meant that nanoparticles of chitosan-*P. tectorius* fruit extract were able to decrease TC levels, TG levels, LDL levels, and increase HDL levels in male *Sprague dawley* rats. Compared to group B, the TC levels decreased by 197%, TG levels by 104%, and LDL levels by 360%. HDL levels increased by 150%.

TC is made up of mostly LDL (60%). LDL is also referred to as “bad cholesterol”. It is one of the lead factors for atherosclerosis. The higher the LDL level, the more cholesterol will be transported from the liver to peripheral tissues. This increases the accumulation of cholesterol in the blood vessel, which causes atherosclerosis diseases (Mathews et al., 2013). According to Akpanabiatu et al.,

(2005), a 2 mg/dL decrease in LDL can lead to a decrease of 1% of cardiovascular diseases.

HDL on the other hand is “good cholesterol”. HDL has a crucial role in reverse cholesterol transport (RCT) mechanism. It can clean excessive cholesterol present in peripheral tissues by bringing it to the liver to be metabolised (Trapani et al., 2012; Mathews et al., 2013). Song et al., (2014) reported that the HDL receptor can reduce atherosclerosis risk.

As the results showed, nanoparticles of chitosan-*P. tectorius* fruit extract have anti-hypercholesterolemic potency. The small size of the nanoparticles is very beneficial in the delivery of the drugs. The nanoparticles can enter the smallest capillary artery, and they can also protect the bioactive compounds from *P. tectorius* fruit present in them from adverse environmental conditions, such as extreme pH, oxygen and other digestible enzymes. Therefore, they can increase drug delivery until target (McClements, 2015; Chu et al., 2008).

Chitosan has the ability to reduce total cholesterol levels (Aranaz et al., 2014; Xia et al., 2011). It is a natural copolymer with many beneficial properties, such as non-toxic, biocompatible, and

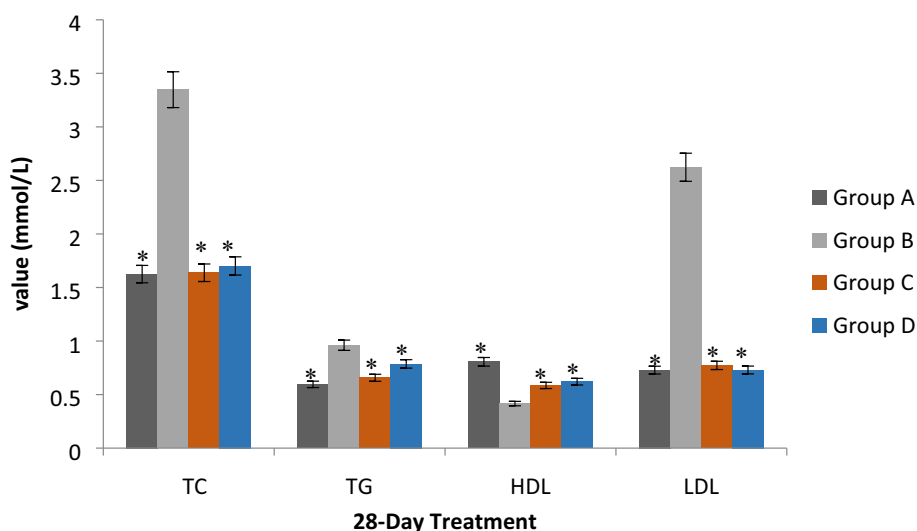


Fig. 11. Effect of total cholesterol (TC), triglyceride (TG), high density Lipoprotein (HDL) and low density lipoprotein (LDL) levels for normal group (A), cholesterol (B), simvastatin (10 mg/kgBW) (C), and nanoparticles of chitosan-*P. tectorius* fruit extract (500 mg/kgBW) (D) at D-28 of treatment toward the administration of nanoparticles of chitosan-*P. tectorius* fruit extracts. Data are presented as mean $\pm$ SD, with the number of rats per group $n = 8$. * $p < 0.05$ compared to normal group (A) using the Duncan test.

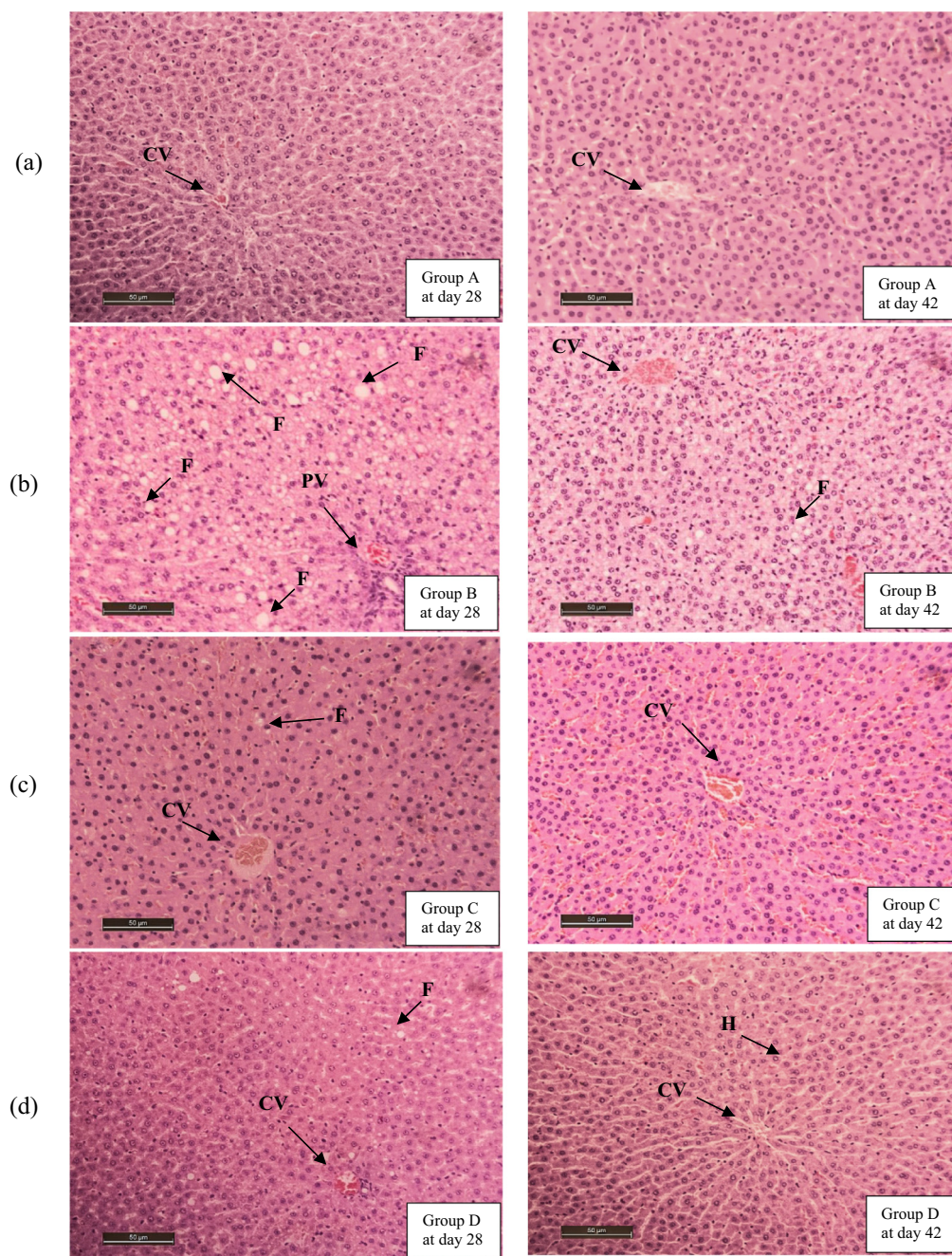


Fig. 12. Histology of liver tissue of group A, B, C, and D at day 28 and day 42 of treatment with magnification 20x. CV = Central Vein, PV = Portal vein, F = fatty liver degeneration.

biodegradable (Hejazi and Amiji, 2003; Dutta et al., 2009; Agnihotri et al., 2004). Chitosan is a fibrous substance that can inhibit fat transfer by preventing absorption of fat or lipid in the gut or intestine through electrolyte reaction. A positive charge present in chitosan can bind negatively charged cholesterol, resulting in decreased cholesterol total in the blood plasma. Lattimer and Haub (2010) reported that dietary fibres, such as cellulose and chitosan, have specific mechanism for controlling blood sugar levels and healthy weight, and lowering the cholesterol levels.

3.4.3. Effect of nanoparticles of chitosan-Pandanus tectorius fruit extract on lipid accumulation in liver tissue

Microscopic liver tissues of experimental rats from groups A, B, C, and D at day 28 and 42 are shown in Fig. 12. Liver tissues of the rats from group A (normal) showed a normal cell architecture

(Fig. 12a). Liver tissues of the rats from group B (high-cholesterol diet) showed significant morphological changes in the form of fatty liver degeneration, indicated with a black arrow in Fig. 12b. Similar result was reported by Pangestika et al. (2020). Experimentally, fatty liver degeneration can occur due to feeding high-cholesterol diet (HF) for 14 days. HF containing cooking oil, cholesterol, and cholic acid can increase cell membranes' sensitivity against free radicals. Free radicals will form inside the cell from the oxidation reaction and will attack unsaturated fat in the liver cell or organelle membrane. This process causes peroxidation of lipids (Wadhwa et al., 2012). Lipid peroxide damages the reticulum endoplasm, thereby interrupting lipoprotein formation. This prevents the fat in the liver from being transported to peripheral tissues. Hence, there is an increased accumulation of fat in the liver, which leads to fatty degeneration in the liver cell.

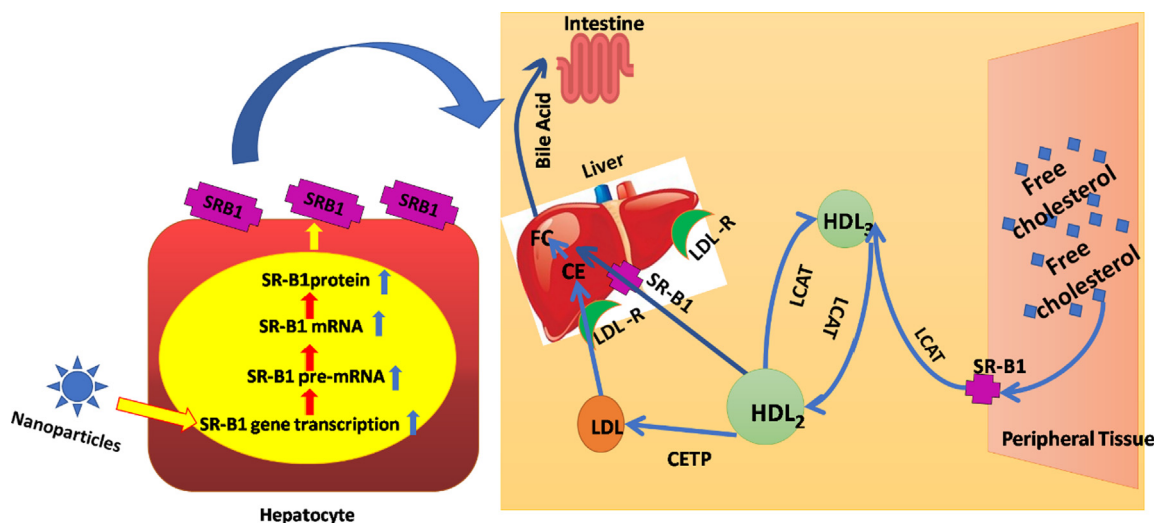


Fig. 13. Effect of nanoparticles of chitosan-*P. tectorius* fruit extract in reducing hypercholesterolemia through increasing of SR-B1 gene expression (Modified from Andriani et al., 2015b). HDL = High-density Lipoprotein, LDL = Low-density Lipoprotein, SR-B1 = Scavenger Receptor Class B, LCAT = Lecithin Cholesterol Acyltransferase.

The liver tissues of the rats from group C (simvastatin) showed accumulation of hepatic lipid droplet that was relatively lower than group B (Fig. 12c). The liver tissues of the rats from group D (nanoparticles of chitosan-*P. tectorius* fruit extract) showed result almost similar to group A, where only a little lipid droplet was present in the liver (Fig. 12d). As was demonstrated in this study, nanoparticles of chitosan-*P. tectorius* fruit extract could reduce the accumulation of lipid droplets in hepatic tissue cells of hypercholesterolemic rats. It was able to do so by suppressing biosynthesis of cholesterol. Therefore, it can be considered as safe an alternative oral treatment for hypercholesterolemia.

In the *in vivo* study, the nanoparticles of chitosan-*P. tectorius* fruit extracts were shown to have cholesterol-lowering effect. The effect may be due to the ability of nanoparticles of chitosan-*P. tectorius* fruit extracts to increase SR-B1 gene expression. SR-B1 gene plays a major part in mediating cholesterol transport in HDL and delivering cholesterol into the liver in the RCT pathway. It can reduce total cholesterol levels by binding the ester cholesterol (CE) brought by HDL, then metabolizing it in the liver to become bile acids, which is released together with feces (Rigotti et al., 2003; Trigatti et al., 2003). Hence, higher expression of SR-B1 gene in the liver leads to increased cholesterol-lowering effect. In brief, oral administration of *P. tectorius* fruit extract in the form of chitosan nanoparticles was proven to increase SR-B1 gene expression compared to non-nanoparticle form.

3.4.4. Purposed mechanism of nanoparticles of chitosan-*P. tectorius* fruit extracts through SR-B1 gene expression

From the *in vivo* study, the nanoparticles of chitosan-*P. tectorius* fruit extracts were found to have cholesterol-lowering effect. This effect could be attributed to the ability of nanoparticles of chitosan-*P. tectorius* fruit extracts to increase SR-B1 gene expression. SR-B1 gene plays a major part in mediating cholesterol transport in HDL and delivering cholesterol into the liver in the reverse cholesterol transport (RCT) pathway (Andriani et al., 2015b). The purposed mechanism of SR-B1 in decreasing cholesterol levels after treatment by nanoparticles of chitosan-*P. tectorius* fruit extracts is shown in Fig. 13. In hepatocyte cells, nanoparticles fed to rats stimulated the increase of SR-B1 gene expression in the liver surface. Nanoparticles of chitosan-*P. tectorius* fruit extracts could increase the uptake of HDL-cholesteryl ester (CE) by the hepato-

cytes through up regulation of SR-B1 expression. Increased synthesis of SR-B1 receptor in the liver resulted in more HDL-CE binding to the SR-B1, where CE was hydrolysed to free cholesterol. Free cholesterol in the hepatocyte is mostly metabolized to bile acids or is eliminated directly through the bile and feces. More free cholesterol (FC) released can reduce the cholesterol levels in the blood. Hence, the enhancement of SR-B1 gene expression has a linear correlation with the decreasing in total cholesterol levels. Successful reduction of total cholesterol levels by binding the ester cholesterol (CE) with HDL was also reported by Rigotti et al. (2003) and Trigatti et al. (2003).

4. Conclusion

Nanoparticles of chitosan-*P. tectorius* fruit extract were successfully obtained using the ionic gelation method. The *in vitro* study revealed that the nanoparticles were able to increase SR-B1 gene expression higher than extracts in non-nanoparticle form. Furthermore, from the *in vivo* study, it was found that the nanoparticles of chitosan-*P. tectorius* fruit extract were able to decrease not only the TC, LDL, and TG levels but also increase the HDL levels. The most important result was obtained from the toxicity study, which showed that the nanoparticles of chitosan-*P. tectorius* fruit extract had non-toxic effect. Thus, as shown in the current study, nanoparticles of chitosan-*P. tectorius* fruit extract have very good potential to be used as a new alternative treatment for hypercholesterolemia via SR-B1 pathway. Further studies on molecular levels are needed to support the potency of nanoparticles of chitosan-*P. tectorius* fruit extract in terms of being anti-hypercholesterolemia.

Declaration of Competing Interest

The authors declared that there is no conflict of interest.

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