

Research Article

FORMULATION OF NANOCURCUMIN USING LOW VISCOSITY CHITOSAN POLYMER AND ITS CELLULAR UPTAKE STUDY INTO T47D CELLS

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ABSTRACT

Using of curcumin as anti cancer agent is restricted by its low solubility, therefore it has low bioavailability. This obstacle can be solved by the development of curcumin nanoparticle. Nanoparticle technology has been started to be developed as an alternative solution to improve drug delivery profile, especially for the less bio-available chemical. This study was aimed to develop nanocurcumin formulation with low viscosity chitosan as the matrix and to study its ability to be taken into the cells *in vitro*. Method used in the formulation of nanocurcumin in this study is by ionic gelation followed by freeze drying. Entrapment Efficiency then assayed, and its stability was tested by incubating the formula into artificial intestinal fluid (AIF). Furthermore, its toxicity was evaluated, also its cellular uptake ability into T47D cell line. It was found that the Entrapment Efficiency in acetate buffer at pH 4 is higher than at pH 5. This formula also has a good stability in AIF. For the cellular uptake study through fluorescence microscope, it was found that the complex has an ability to penetrate cellular membrane into the cytosol. The cytotoxicity study tell us that the nanocurcumin is non-toxic to normal cell line. For the characterization of the nanoparticles, the average size of this particle is 269.8 nm, its zeta-potential is +18.63 mV, with spherical particle morphology. From the result of this study, it is concluded that formulation of nanocurcumin using low viscosity chitosan polymer as the matrix has a great potential as an alternative for anticancer therapy.

Key words: nanoparticle, curcumin, low viscosity chitosan, T47D cell line.

INTRODUCTION

Curcumin is a yellow polyphenol chemical contained in turmeric rhizome (*Curcuma longa*L.), it has various wide spectrum biological activities. Antioxidant activity of curcumin is related to its terminal aromatic hydroxyl group and β diketone. Double bond in its structure has been proved to take a main role in the anticancer and antimutagenic activities (Majeed *et al.*, 1995). The wide use of curcumin for the therapy is limited because of its low solubility in water so it has a low systemic bioavailability (Bisht *et al.*, 2007). Curcumin is also easily degraded in neutral to basic pH, and easily photo-degraded as well. (Strimpakos and Sharma; Goel *et al.*, (2008)).

Nanoparticle is a colloidal structure with the size ranged within 10-1000 nm (Rawat *et al.*, 2006). Nanoparticle can deliver chemicals better onto the small units in the body; overcome the resistance problem caused by body's physiological barriers related to pore-size factor; and its possible to be targeted, so it can reduce drug's toxicity and improve drug distribution efficiency (Rawat *et al.*, 2006). Chitosan is a natural biopolymer obtained by alkaline deacetylation of chitin (Hejazi and Amiji, 2003). Chitosan has various excellent properties as biocompatible, bio-degradable, low toxic and not immunogenic.

Chitosan is widely used together with TPP polyanion in various nanoparticle formulation study by ionic gelation method. Ionic gelation method is engaged to the forming of the complex by the two oppositely charged structures which then form nanoparticle gel. Ionic gelation is a very simple and easy preparation method (Racovita *et al.*, 2008).

Based on its linear polymer structure, chitosan is available in various molecular weight: short, middle, and long chain chitosan. This molecular weight takes influence in its solubility and viscosity. Short chain chitosan is dissolved easily in acidic organic solvents such as acetic acid, citric acid, and tartaric acid (Mao, *et al.*, 2009).

Based on the explanation above, a study of curcumin nanoparticle formulation has been done using low viscosity chitosan for solving curcumin restrictiveness' by formulating curcumin nanoparticle using polymer a role as a carrier to improving solubility and stability of curcumin. The particle formed is then characterized and evaluated for its stability, safety and the ability to penetrate cell membrane into the cytosol or also known as transfection study into breast cancer cell line T47D in vitro.

METHODOLOGY

Nanoparticle formulation

Selection and optimization

In this preliminary experiment, formulation optimization of the curcumin chitosan nanoparticle was run by mixing chitosan solution with a concentration of 0.02, 0.04, 0.06, 0.08, and 0.1% and curcumin solution with a concentration of 0.01, 0.02, 0.03, and 0.04%. Curcumin solution with a volume of 0.05 mL was put in eppendorf tube then 0.5 chitosan solution was added into acetate buffer solution at pH 4.0 and 5.0.

After the adding of chitosan solution, the mixture was homogenized using vortex for 20 seconds. Into the homogenous mixture, TPP solution with a final concentration of 0.03% was added and immediately homogenized again using vortex for 20 seconds. The dispersion of nanoparticles was observed to count the amount of undissolved curcumin for 7x24 hours to determine the optimum mix to be used for further processing.

Scale up and Freeze Drying

The optimum mixture of curcumin-chitosan obtained from the optimization process is 200 mL. Subsequently the solution was evaporated to remove the content of ethanol and was made into powder using freeze drying technique at - 40°C.

Nanoparticle characterization

Determining Nanoparticle size distribution

The size and the distribution of nanoparticle was measured using particle size analyzer (PSA). 5 mL aquadest then added to two drops of curcumin-chitosan nanoparticle at pH 4. This mixture mixed by tossing and turning the solution. 3 mL of the mixture was taken and put in a cuvette for analysis.

Observation of the nanoparticle morphology

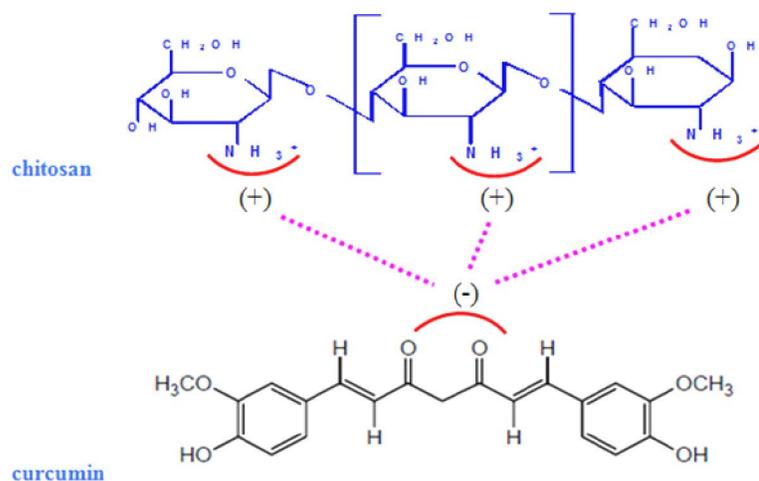
Observing the morphology of nanoparticles was done using transmission electron microscopy (TEM). Samples of nanoparticles were spilled over carbon coated copper grid and then by means of Auto Carbon Coated (JOEL JEC-560, Japan) for 5 seconds then dried at room temperature for 24 hours. Once the nanoparticle samples were dried, they coated again with carbon as mentioned above then copper grid was entered into the holder and samples were ready to be analysed by the voltage acceleration on 120 kV and 60,000 magnification.

Determining Entrapment Efficiency

10mg of curcumin-chitosan nano particles were weighed then dissolved in 10 mL of distilled water. 10 mL of ethyl acetate then added. The mixture was shaken and they ethyl-acetate phase being separated. Curcumin dissolved in ethyl acetate was then measured for the absorbance at a wavelength of 420 nm and the entrapment efficiency was the calculated.

Stability Test of the Nanoparticle

The nanoparticle powder were incorporated into erlenmeyer and were added with artificial intestinal fluid (AIF) of 100 mL. The solution was shaken using Orbital Incubator Shaker (Environ Shake) at 39°C and 50 rpm. Samples were taken in hour 0, 1, 2, 3, and 4 with replication for each samples. The samples were introduced into a separating funnel and were added with 5 mL of ethyl acetate. The mixture were shaken and the ethyl acetate phase was separated.



Gambar 1. Ionic interaction between chitosan and curcumin low pH.

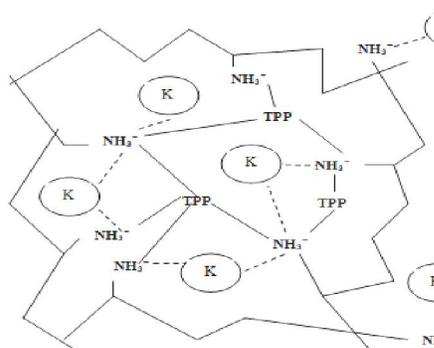


Figure 2. Chitosan-curcumin complex nanoparticle formation

The concentration of curcumin which was not encapsulated into nanoparticle and included in ethyl acetate was measured for its absorbance at wavelength of 419 nm.

Cytotoxicity Study

Cytotoxicity testing was performed by the MTT method using T47D cell line as a model. Confluent and an exact amount T47D cell lines were distributed into the chambers on 96 well microplate with the number of $5 \times 10^3 / 100 \mu\text{L} / \text{well}$. Cells were then incubated in the CO_2 incubator overnight for the adaptation until they were being ready for treatment. Chitosan curcumin nanoparticles

with predetermined concentrations incorporated into the wells/chambers *triplo* of $100 \mu\text{L}$ after washed once with phosphate buffer saline (PBS), then incubated into CO_2 incubator overnight. After incubation, the test solution was removed and then added 10 mL of 0.5% MTT solution in sterile PBS to each wells. Then the sample incubated in CO_2 incubator for 3 hours. The enzyme possessed by living cells would react to the MTT to form purple. The reaction was stopped by the addition of stopper reagent 3 hours after the adding of MTT, then incubated in room temperature and protected

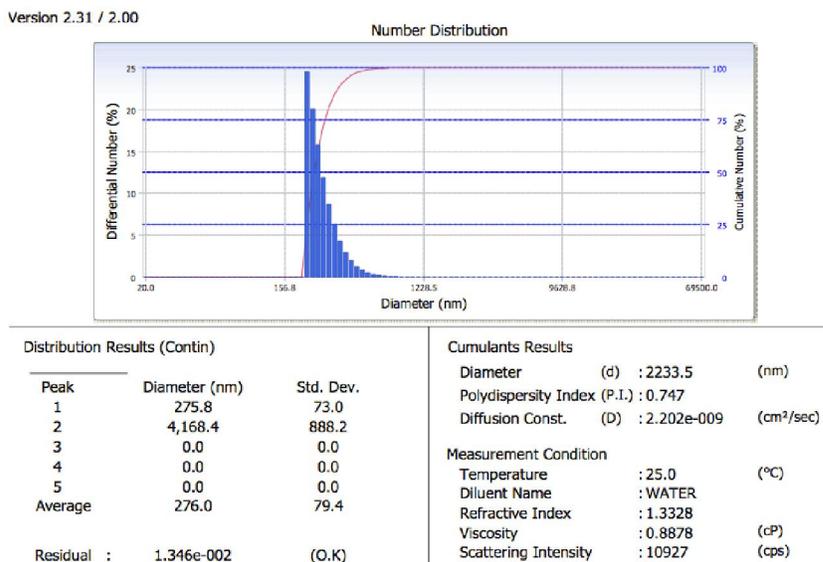


Figure 3. Size diameter and distribution of chitosan-curcumin nanoparticle by *Particle Size Analyzer*.

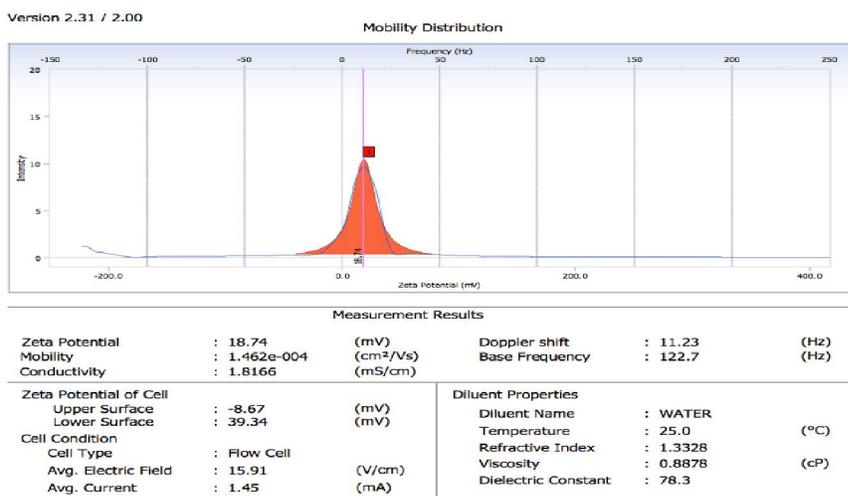


Figure 4. Zeta potential measurement of chitosan-curcumin nanoparticle.

from the light overnight. After the incubation, the purple crystal would be dissolved, then the absorbance could be read using ELISA reader at 595 nm wavelength.

Observation of the Cellular Uptake

T47D cells were seeded in six-well plates with coverslip coated at the bottom of the well. The cells incubated in CO₂ incubator until

starvation. Cell media was replaced with a new media. 10µg/100µL of curcumin nanoparticle was added afterwards, then incubated. After 4 hours of incubation, methanol was added for 30 seconds then discarded immediately. Coverslip containing the cells were attached on the object glass to be observed with a fluorescent microscope (Zeiss).

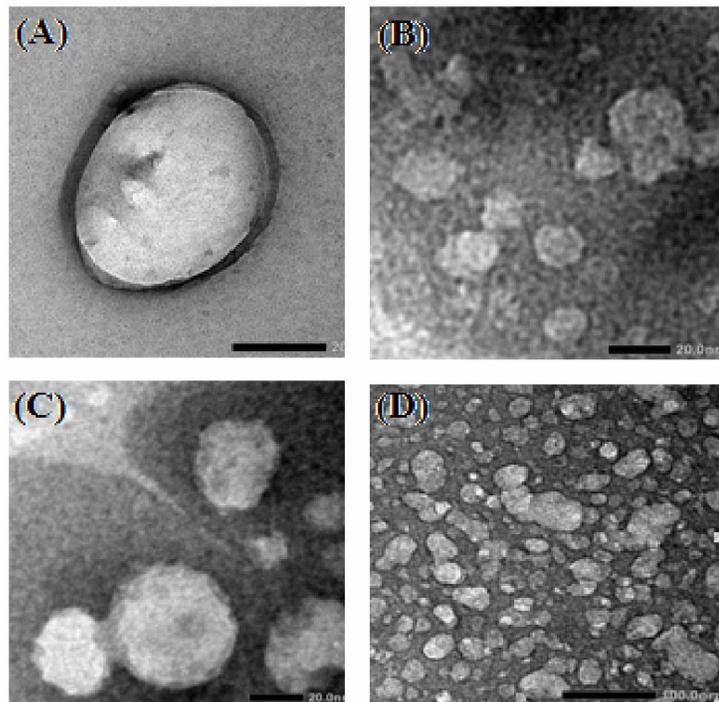


Figure 5. Transmission Electron Microscopy (TEM) picture of chitosan-curcumin nanoparticle (pH 4.0 formulation), 120 kV voltage, 200k magnification (A & B) and chitosan-curcumin nanoparticle (pH 5.0 formulation) 120 kV voltage, 100k magnification (C & D)

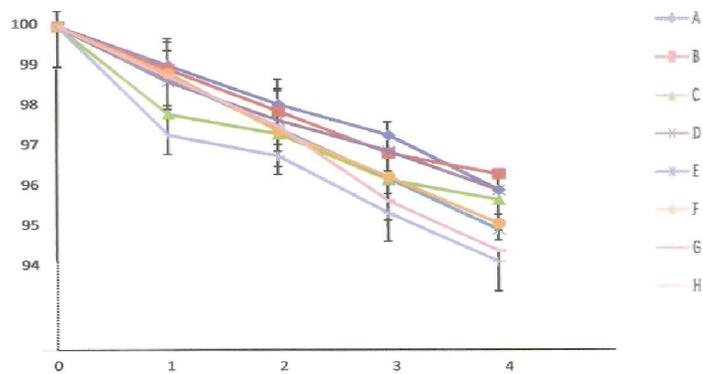


Figure 6. Nanoparticle stability in artificial intestinal fluid (AIF). Nanocurcumin formulation at pH 4.0 within concentration 0,01:0,02 (A), 0,01:0,04 (B), 0,02:0,02 (C), 0,02:0,04 (D) and formulation at pH 4.0 within concentration 0,01:0,02 (E), 0,01:0,04 (F), 0,02:0,02 (G), 0,02:0,04 (H).

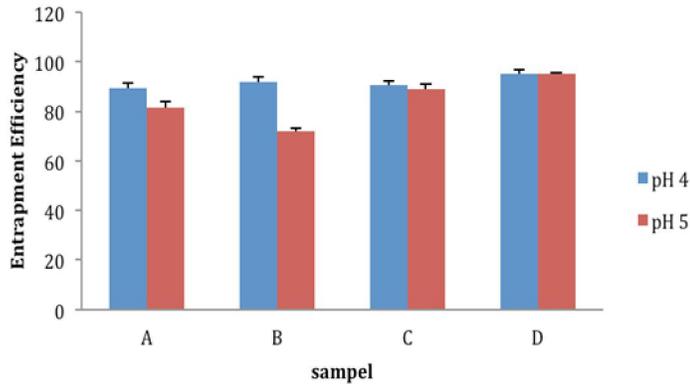


Figure 7. Entrapment efficiency of nanocurcumin with 0,01:0,02 (A), 0,01:0,04 (B), 0,02:0,02 (C) and 0,02:0,04 (D) concentration of chitosan at pH 4.0 and pH 5.0.

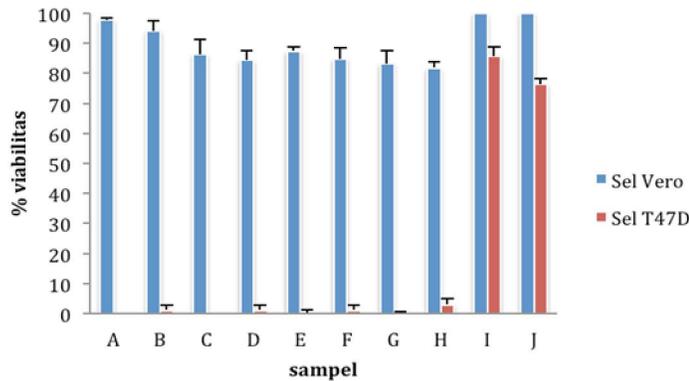


Figure 8. Citotoxicity of curcumin-chitosan low viscous toward Vero and T47D cell culture. Nanocurcumin formulation at pH 4.0 within concentration 0,01:0,02 (A), 0,01:0,04 (B), 0,02:0,02 (C), 0,02:0,04 (D) and at pH 5.0 within concentration 0,01:0,02 (E), 0,01:0,04 (F), 0,02:0,02 (G), 0,02:0,04 (H), chitosan pH 5.0 within concentration 0,02 (I) dan curcumin within 0,02 (J).

RESULT AND DISCUSSION
Nanocurcumin formulation

The optimal composition of curcumin-chitosan in acetate buffer pH 4.0 was 0,01:0,02, 0,01:0,04 (B), 0,02:0,02 (C), and 0,02:0,04 (D); and in acetate buffer pH 5.0 was 0,01:0,02 (E), 0,01:0,04 (F), 0,02: 0,02 (G), and 0,02:0,04 . These formulations were scaled-up into 200 mL and were evaporated to remove the content of ethanol in the solution. This process was followed by freeze drying to dries the solution into powder. The colour of nanocurcumin powder obtained from freeze drying was range from yellow to orange.

The formulation of chitosan-curcumin nanoparticles based on ionic gelation method. Chitosan in the low pH condition have positive charge because its amine group (NH₂) will be protonated as NH₃⁺. The NH₃⁺ will be bind to the negative charge of PGV-0 form an ionic interaction between chitosan and PGV-0 (Figure 1). TPP added to the formula acted as stabilizer. TPP (highly negative charge compound) will bind to the chitosan (positive charge polymer) for a cross-linked nanoparticle (Figure 2).

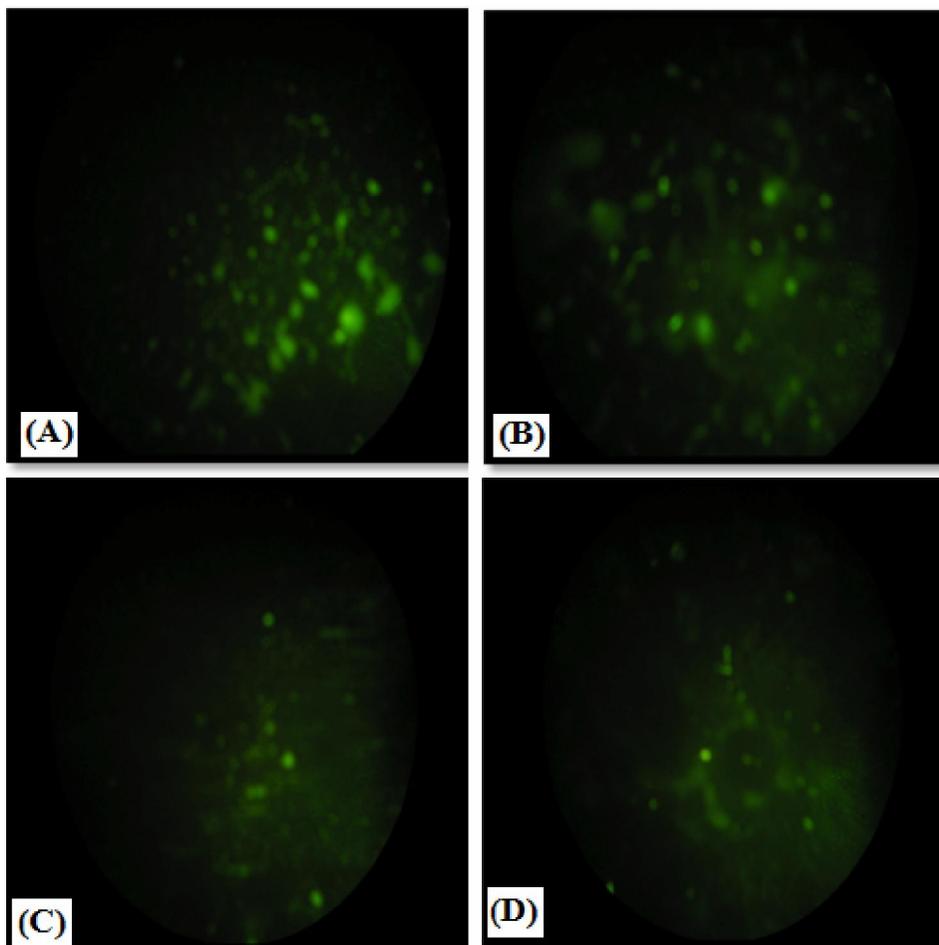


Figure 9. Cellular uptake studies of chitosan-curcumin nanoparticle using fluorescence microscope. pH 4.0 formulation within concentration 0,01:0,02 (A,B), and pH 5.0 formulation within concentration pH 5 0,02:0,04 (C,D).

Characterization of Curcumin Nanoparticle formulation using Particle Size Analyser

Particle size was obtained using *Particle Size Analyser* at Balai Inkubasi Teknologi BPPT PUSPIPTEK Serpong. The observation was done at 25°C temperature with water as the solvent. From the result of the observation, the formulation of nanocurcumin has average particle size of 269.8 nm (Figure 3) and 18.74 mV of zeta potential (Figure 4). Obtained particles have size range in nanometer, as aimed. Nanoparticle colloid has many beneficial properties namely nano-size makes it much more stable sedimentate slowly. Particles with

the size less than 400 nm has a good properties in drug delivery (Rao *et al.*, 2010). Polydispersibility index obtained from the measurement of particle size was 0,656. Polydispersibility index describes the distribution of particle sizes present in the preparation of nanoparticles, the smaller the number of polydispersibility index, the more uniform the sizes of the particles, if there is a significant difference of size between the larger particles and the smaller ones, it will affect the particles' characteristic. The larger the size of the particles, the particle will settle more easily (Manmode, 2009).

Characterization of Curcumin nanoparticle formulation using (TEM)

Characterization using *Transmission Electron Microscopy* (TEM) was performed at the laboratory of chemistry, Faculty of Mathematics and Natural Science, Gadjah Mada University. The results (figure 5) showed that the nanoparticles character of chitosan-nanocurcumin was average shaped spheres. It also seemed that some nanoparticles bind together, forming larger size. Curcumin-chitosan interactions occurred by electrostatic interactions between cationic chitosan's amine groups with the carbonic groups of curcumin. The strength of the interactions was influenced by the pH of the media, the higher the pH, the weaker the strength of the chitosan to bind curcumin will be. This was indicated on the nanoparticles that bind together because they do not perfectly encapsulated.

Stability of Short chain Chitosan-curcumin Nanoparticle

Stability of nanoparticle was tested by dissolving the nanoparticles into artificial intestinal fluid (AIF), then determining the amount of free curcumin of solution at hour-0 to hour-4, using UV-Vis Spectrophotometer at the maximum wavelength (λ) obtained from the scanning of the standard curve. In this experiment, obtained that the maximum wavelength is at 419 nm. The stability profiles of the nanocurcumin both in the two acetate buffer media at pH 4 and 5 in this experiment, showed a similar curve shape. The release of curcumin from nanoparticle formulation because of there is binding competition between curcumin and electrolyte with chitosan. Another factor affecting curcumin release is at pH 7, NH_3^+ in chitosan will transform into NH_3 . The stability of nanoparticle showed (Figure 6) that 90% of curcumin still in the nanoparticle state. The addition of TPP as stabilizer, give cross-linked effect between chitosan (positive charge) dan TPP (negative charge) by ionic interaction on the particle (Figure 2).

The curve at pH 4 acetate buffer media showed that the formula is more stable than at pH 5 acetate buffer media. At pH 4, chitosan have higher positive charge than pH 5. consequently, higher positive charge (chitosan)

give stronger ionic interaction with PGV-0 (negative charge).

Entrapment Efficiency

The entrapment efficiency was determined to find out the ratio between curcumin loading content that can be entrapped into nanoparticles versus total curcumin used in the formulation. The entrapment efficiency value of nanocurcumin in acetate buffer pH 4 and in acetate buffer pH 5 was about > 70 % (Figure 6). Thus, the formulation of nanocurcumin in acetate buffer pH 4 showed a greater entrapment efficiency value. At pH 4, the positive charge of the chitosan is higher than pH 5. At low pH (pH 4), amine group in the chitosan has been protonated, give the higher positive charge on the polymer. The higher positive charge, the higher negatively charge drug bind to the polymer to form nanoparticle complexes will be.

Cytotoxicity of Short chain Chitosan-curcumin Nanoparticle

Cytotoxic test was conducted to determine the level of nanocurcumin cytotoxicity against T47D cell line as the model of breast cancer cells, and Vero cell line as the model of normal cells. Nanocurcumin application against model cells should not affect the correlation between the percentage of the living cells by the concentration of nanocurcumin, with the method MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide using for evaluating living cells. The test method is based on the conversion of yellow tetrazolium salt by succinate hydrogenase in mitochondria into formazan crystals. The purple from formazan formed is proportional to the number of living cells that can be measured using ELISA reader.

The results (figure 8) show that nanocurcumin has toxicity effect on T47D cell line but not on normal cell (Vero cell line). Curcumin which has anti cancer activities give the toxic effect on the cancer cell line. T47D is a mammary carcinoma cell line, so it can be concluded that effect of curcumin only happened on that cell line not on Vero cell line. Nanoparticle formulation increase the uptake

of curcumin into the cell and increase the effect of anti-cancer to the cancer cell line (T47D).

Cellular Uptake of Short chain Chitosan-curcumin Nanoparticle

Cellular uptake was examined to see the ability of the nanoparticles to enter the cells. The observation was carried out using fluorescent microscopy (Figure 9).

Curcumin will give a green fluorescent due to the chromophore group in this molecule. The results proved that the formulated curcumin was well penetrating into the cells, as the cells became observed became fluorescent.

The complex coaservation method that was used to bind the positive charge of chitosan and the negative charge of curcumin possibles to the nanoparticles to bind with the negative charged plasma membrane, then undergo an endocytosis process, and finally the nanoparticles entered into the nucleus to express the protein. The observation using fluorescent microscopy showed that the nanocurcumin in acetate buffer pH 4.0 provided a higher intensity of the green fluorescent compared to the nanocurcumin in acetate buffer pH 4.0. Thus, nanocurcumin on acetate buffer pH 4.0 has a higher ability to enter the nucleus.

CONCLUSION

Nanocurcumin formulation with low viscosity chitosan can be developed with ionic gelation method which is producing nanocurcumin particle with average size as 269,8 nm, zeta potential (+) 18,63 mV and has a viscosity spherical shape.

Nanocurcumin using low viscous chitosan as the matrix has good stability in artificial in testinal fluid and still stable until

four hours, with stability value range between 94,04-96,27 %.

Nanocurcumin using low viscosity chitosan as the matrix is non-toxic against Vero normal cell line with the percentage of viable cells more than 80 %.

Nanocurcumin using low viscous chitosan as the matrix has an ability to penetrate into T47D cell *in vitro*.

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