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Original paper

Study the Interaction of Curcumin Nanoemulsion on 3T3/NIH and MCF-7 Cell Line Using Confocal Microscope

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Abstract

Background and aim: Curcumin is a phenolic compound that can be found naturally in the rhizome of Zingiberaceae plants and it has wide therapeutic effects, ranging from antioxidant activity, anti-inflammatory, hepatoprotector, anti-diabetic, anti-fibrosis, and anti-cancer. The limitations for the development of curcumin as therapeutic agent are due to its poor solubility in water and its low bioavailability.

Materials and methods: In this study, a formulation was fabricated to overcome those limitations into a nanoemulsion dosage form comprising castor oil as the oil phase. As surfactant and co-surfactant, cremophor RH40 and PEG 400 were used respectively. Several physical characterizations were performed to ensure the successful repetition of the formula. In addition, in vitro study was performed to find IC_{50} values using MTS assay in 3T3/NIH and MCF-7 cells, and the interaction between curcumin nanoemulsion with MCF-7 cell using confocal microscope.

Results: Curcumin nanoemulsion has a significantly lower IC_{50} compared to curcumin solution on 3T3/NIH cells indicating a better cytotoxic effects than curcumin solution. There was an indication of the ability of curcumin nanoemulsion and curcumin solution to penetrate the cell membrane. The curcumin nanoemulsion formula from previous study was successfully fabricated with satisfying results.

Conclusion: The presence of green fluorescent at around the cells nucleus from the observation using confocal microscope gave the initial conclusion of curcumin in the form of nanoemulsion and in the solution of having the ability to interact with the MCF-7 cell.

Keywords: *Curcumin, nanoemulsion, Anticancer, IC_{50}, 3T3/NIH, MCF-7.*

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Introduction

Curcumin or in chemical structure known as (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6 heptadiene-3,5-dione), is a phenolic compound that can be found naturally in the rhizome of Zingiberaceae plants, such as turmeric (Curcuma longa L.). Turmeric contains not only curcumin, but also demethoxycurcumin and bisdemethoxycurcumin [1]. Curcumin is the most abundant component of turmeric and it has wide therapeutic effects, ranging from antibacterial, antiviral, antifungal, antimalarial [2], antioxidant, and also as a chemopreventive agent for colon, breast, prostate, esophagus, lung, and oral cancer [1]. Curcumin is poorly soluble in water, so the oral bioavailability is very low due to curcumin is also slightly absorbed in the gastrointestinal tract [3]. Some approaches have been investigated to increase the bioavailability of curcumin include loading curcumin into liposomes or nanoparticles, forming curcumin-phospholipid complex, and also making a chemical modifying by synthesizing structural analogues of curcumin [3].

Cancer is one of the highest prevalence life-threatening disease in the world. Cancer is indicated by the abnormal proliferation of the cells [4]. Cancer treatment is usually not only toxic to its cells, but also to normal cells [5]. Curcumin as an anticancer is modulates the growth of cancer cells through regulation of multiple cell signaling pathway, including cell proliferation pathway (cyclin D1, c-myc), cell survival pathway (Bcl-2, Bcl-xL, cFLIP, XIAP, c-IAPI), caspase activation pathway (caspase-8, 3, 9), tumor suppressor pathway (p53, p21), death receptor pathway (DR4, DR5), mitochondrial pathways, and protein kinase pathway (JNK, Akt, AMPK) [5]–[7]. Curcumin also has an effect as an anti-proliferative agent by the inhibition of oxidative stress and angiogenesis. This process due to curcumin can induce the apoptosis of cancer cells [8].

Nanoemulsions are dispersion system of two immiscible phase and stabilized using an appropriate surfactant, and sometimes also used a co-surfactant to enhance the stability. Visually, nanoemulsions has a clear appearance and look like a homogeneous system. Nanoemulsion system can solubilize a very hydrophobic substances, so it can increase the dissolution rate of the hydrophobic drugs, then expected to enhance systemic bioavailability of the drug [9], [10]. Castor oil based nanoemulsion containing curcumin can enhance the physicochemical stability, permeability and storage time of curcumin, based on our previous study [11].

In this report, we characterized the curcumin nanoemulsion physically by determining the particle size, polydispersity index, zeta potential, and its morphological behavior. We also determined the cytotoxicity of curcumin nanoemulsion in 3T3/NIH and MCF7 cell lines using MTS assay for 24h incubation, and the interaction between castor oil based nanoemulsion containing curcumin and cancer cells (MCF-7) and also normal cell lines (3T3/NIH) was observed using confocal microscope for 3 and 6h incubation.

Materials and Methods

Curcumin powder (98,3% purity) from PT Combiphar, Bandung, Indonesia; Castor oil, cremophor RH40, PEG 400 all from idCHEM, Korea; Dimethylsulfoxide (DMSO) from Merck, Darmstadt, Germany were used as a solvent for curcumin and thus as vehicle controls in the experiments; 3T3/NIH and MCF-7 cell lines from ATCC, Manassas, USA; DMEM medium, MEM medium, Fetal Bovine Serum (FBS), 1% Antibiotic-Antimycotic, Trypsin EDTA 0.25%, Dulbecco's Phosphate Buffer Saline (PBS) all from Gibco, Thermo Fisher Scientific, Waltham, USA; MTS reagent; Tryptan blue; DAPI; Paraformaldehyde; Uranyl acetate.

Preparation of curcumin nanoemulsion

Curcumin nanoemulsion was prepared using self-nanoemulsification (SNE) technique as previously described [12]. Briefly, castor oil as the oil phase, cremophor RH40 as a surfactant, and PEG 400 as a co-surfactant, were mixed with the ratio of 1:8:1 using a magnetic stirrer for 2 hours to form the oil phase of the nanoemulsion. The oil phase was then placed in a sonicator bath for 1 hour at 25°C. The SNE formed was then added to deionized water with the ratio 1:5 with the oil phase and stirred for 15 minutes until a clear and homogeneous system was formed.

Characterization studies of curcumin nanoemulsion

The curcumin nanoemulsion was characterized with a DelsaTMNano C Particle Analyzer (Beckman Coulter) to determine the particle size, polydispersity index, and zeta potential. Cryo-transmission electron microscopy (cryo-TEM) was employed to analyze the morphology of the curcumin nanoemulsion. Briefly, the curcumin nanoemulsion was dropped on a 400 mesh cryo-TEM grid and was allowed to dry before being stained with uranyl acetate. The grid was allowed to dry and placed into the cryo-TEM instrument at Eijkman Institute of Molecular Biology, Jakarta, Indonesia to inspect the nanoemulsion morphology under 80 kV, 20,000× magnification.

Cell viability assay to measure curcumin nanoemulsion cytotoxicity

The cytotoxicity of curcumin nanoemulsion was assessed using MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay. Briefly, 3T3/NIH and MCF-7 cell lines were seeded into wells of 96-well plate with a density of 5000 cells/well. Cells were then incubated for 24 hours. Confluent cells were treated with filtered curcumin solution or curcumin nanoemulsion and then incubated at 37°C and 5% CO_2 for 24 hours. Following the incubation period, MTS reagent solution was added to each well, the cells were then incubated for 3 hours. The sample absorbance was then determined by microplate reader at 490 nm. A dose-response curve was obtained using a non-linear regression (curve fit) and the cytotoxic concentration was calculated to determine the concentration required to reduce the cell viability by 50% (IC_{50}). Statistical analysis was determined using independent-t-test.

Interaction determination of curcumin nanoemulsion using confocal microscope

The interaction of curcumin nanoemulsion was assessed using confocal microscope. Briefly, MCF-7 cell line was seeded into the bottom of confocal dish with a density of 100,000 cells/well. Cells were then incubated at 37°C and 5% CO_2 for 24 hours. Confluent cells were treated with filtrated 12.5 µg/ml curcumin-only solution or curcumin nanoemulsion and then incubated at 37°C and 5% CO_2 for 3 and 6 hours. Supernatant was removed and cells were rinsed using DPBS. Following the procedure, cells were fixed using paraformaldehyde and stained by DAPI. The interaction study was observed by confocal microscope.

Results

Characterization of curcumin nanoemulsion

Curcumin nanoemulsion was successfully formed, with the average droplet size 36.83 ± 9.64 nm and polydispersity index was 0.278 ± 0.113, indicated that the globules in the formulation were uniform in size and no aggregation occurs [15]. These results were confirmed by TEM observation in figure 1, showing that the spherical nanoemulsion droplets were uniform in size. Zeta potential value of curcumin nanoemulsion were -0.92 mV.

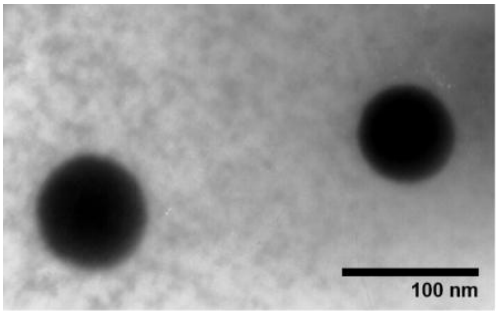

Fig. 1. Morphological analysis of curcumin nanoemulsion under cryo TEM (20,000x magnification).

IC_{50} determination of curcumin nanoemulsion using 3T3/NIH and MCF-7 cell lines

Cell viability in both 3T3/NIH and MCF-7 were decreased along with the increase of the sample concentration. From this result, MCF-7 showed better sensitivity when interact with curcumin, than in 3T3/NIH cell.

Table 1. IC_{50} value of curcumin nanoemulsion and curcumin solution

Cell Line	Curcumin Nanoemulsion		Curcumin Solution	
	IC_{50} (µg/mL)	Deviation Standard	IC_{50} (µg/mL)	Deviation Standard
3T3/NIH	68,568	± 2,811	76,981	± 3,457
MCF-7	15,625	± 2,401	17,175	± 0,517

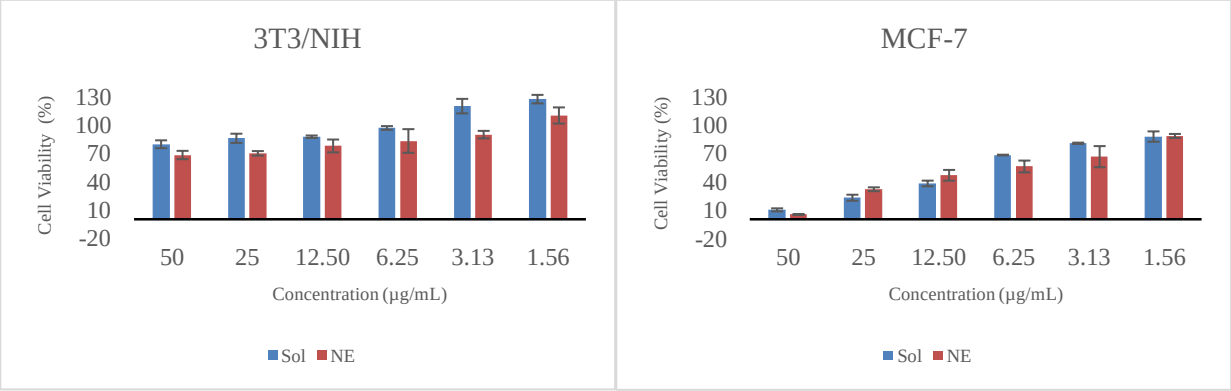

Fig. 2. Cell viability assay using different concentrations of curcumin nanoemulsion and curcumin solution on 3T3/NIH and MCF-7 cells (blue bar, Sol = curcumin solution; orange bar, NE = curcumin nanoemulsion).

Interaction determination of curcumin nanoemulsion using confocal microscope

The blue color in the figure 3 means DAPI stained the nucleus of the cells, and green color means curcumin as an autoflourescence agent. Result in figure 3 showed that curcumin can interact with MCF-7 cell.

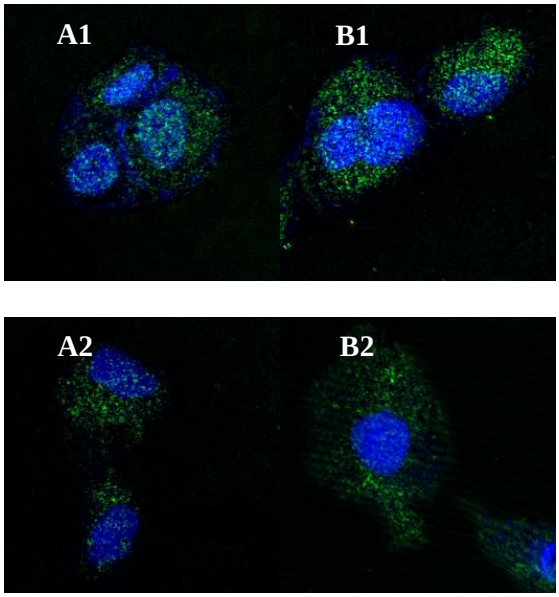

Fig. 3. Cell penetration study using confocal microscope on MCF-7 cells; (A1) Curcumin nanoemulsion (3 h incubation, (B1) curcumin solution (3h incubation), (A2) curcumin nanoemulsion (6 h incubation), (B2) curcumin solution (6 h incubation). (DAPI - blue light, curcumin - green light), with 60X magnification.

Discussion

Curcumin is difficult to be claimed as a drug because its poorly soluble in water, degraded by light and has low bioavailability through both oral and vascular routes. The low bioavailability of curcumin is influenced by its solubility, low dissolution and absorption rates, and also high metabolism in the liver [13]. Various methods have been carried out to improve the weaknesses of curcumin as a drug, including making analog synthesis, chemical modification to be a pro-drug, combination with food components, and utilizing drug delivery systems on the nanoscale. Nanotechnology was known to provide a solution to the problem of the low bioavailability of curcumin [14]. The application of nanotechnology to curcumin was intended to increase solubility, dissolution rate and stability in vitro and in vivo. In addition, in the previous research, curcumin encapsulated in nanoemulsion system showed improvements in physicochemical stability, permeability, and storage time.

Particle sizes smaller than 200 nm are important for cellular delivery system, because smaller particle size can fastly distributed in the blood circulation and accumulate at the tumor site due to increase in permeability and retention effects [16]. Based on the result in the characterization of curcumin nanoemulsion, low zeta potential was caused by cremophor RH40 as a non-ionic surfactant. Cremophor RH 40 can dissociate fatty acid ester which can form negatively charged free fatty acids, causing the low zeta potential value. The large amount of surfactant used in the formula also coated the droplet surface, forming rather good distances between single nanoemulsion droplets.

MTS assay was employed for the IC_{50} determination of curcumin nanoemulsion and solution in 3T3/NIH and MCF-7 cell lines. The concentration of curcumin nanoemulsion and solution used were 50, 25, 12.5, 6.25, 3.13, and 1.56 µg/mL. IC_{50} value represents the concentration of active substance that can reduce 50% of the cell population. This value is used to determine the maximum possible concentration in inhibitory assay, which will not produce ambiguous result. We analyzed cell viability to ensure that cell death was related to the inhibitory effect of curcumin on the cell lines, and the decrease of cell population was not due to cell death.

The IC_{50} value in the table 1 shows that, IC_{50} of curcumin nanoemulsion in both 3T3/NIH and MCF-7 cell lines were lower than the curcumin solution. This data indicated that curcumin nanoemulsion has better cytotoxicity effect compared with curcumin solution. In the MCF-7, the IC_{50} value is significantly different compared with the IC_{50} in 3T3/NIH cell. This results indicated that curcumin has a potential effect for an anti-cancer agent. Based on statistical analysis, the IC_{50} value between curcumin emulsion and solution was significantly different for 3T3/NIH cells and not significant for MCF-7 cell.

MCF-7 used as the cell model for the interaction study of curcumin nanoemulsion and curcumin solution using confocal microscope. MCF-7 was used because based on IC_{50} determination data, MCF-7 had lower IC_{50} value compared with 3T3/NIH cell. Interaction study was evaluated in two different incubation time, that was 3 and 6 hours. The purpose of using two different incubation time was to understand the behavior of curcumin inside the cells and also to predict the optimum penetration time for curcumin. Curcumin solution showed better curcumin fluorescence intensity compared with curcumin nanoemulsion. This results indicated that the amount of curcumin that penetrated the cells in curcumin solution was higher than in curcumin nanoemulsion.

Curcumin solution used DMSO as the solvent of curcumin. DMSO can break the cell membrane and make the membrane permeability increase, so curcumin can penetrate the cells easily. The carrier of nanoemulsion was not toxic and not break the cells, so the amount of curcumin penetrated the cell was lower than in curcumin solution. There was no significant result between two different incubation time. This happened because curcumin needs more time to make the increasement of cellular uptake. Curcumin in solution intensity was lower in 6h incubation compared with 3h, because DMSO breaks the cell membrane more severe than in 3 h, so that curcumin was rinsed by the DPBS before fixed by paraformaldehyde.

Conclusion

Curcumin nanoemulsion in this study shows spherical in shape and uniform in size. Curcumin nanoemulsion demonstrated a lower IC_{50} value than curcumin solution in both 3T3/NIH and MCF-7 cells. The interaction and penetration study indicates that a number of curcumin can penetrate into the cell. To confirm this conclusion, various experiments in vitro and in vivo still need to be done on the relevant experimental models.

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Conflict of interests

The authors declare that there is no conflict of interests.

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