7. antiviral action

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RESEARCH ARTICLE

Antiviral Action of Curcumin Encapsulated in Nanoemulsion against Four Serotypes of Dengue Virus

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Abstract: ***Background*:** Curcumin has been used as a traditional medicine showing anti-inflammatory, antimicrobial, and antiviral properties. Despite the promising potentials, curcumin-based drug development is hindered due to its poor solubility and cell uptake.

***Objective*:** This study aims to produce curcumin nanoemulsion (nanocurcumin) and evaluate its physical characteristics and *in vitro* cell cytotoxicity and antiviral activity against dengue virus (DENV).

***Methods*:** Nanocurcumin was generated by self-nanoemulsion technique. Cytotoxicity was determined using MTT assay in A549 cell line. Anti-DENV properties were determined by calculation of inhibitory concentration 50 (IC_{50}) and plaque assay.

***Results*:** The resulting nanoemulsion showed uniform droplet size distribution with the average droplet size of 40.85 ± 0.919 nm. Nanocurcumin exhibited higher cell cytotoxicity compared to curcumin solution and may be explained by better cell uptake. Nanocurcumin treatment suppressed DENV growth, although no significant difference observed compared to the curcumin solution counterpart. Greater virus reduction was observed for DENV-1 and DENV-2.

***Conclusion*:** The synthesis of nanocurcumin improved curcumin physicochemical properties with potential as antiviral against DENV.

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1. INTRODUCTION

Dengue virus (DENV) is a positive-sense single-stranded RNA virus, a member of the *Flaviviridae* family, the causative agent of dengue disease. The virus is transmitted between human individuals through *Aedes* mosquitoes' bites. DENV can be differentiated into four different serotypes (DENV-1, DENV-2, DENV-3, and DENV-4) [1]. The global burden of dengue is estimated to reach up to 390 million infections occurring annually with Asian countries suffering

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from most of the burden [2]. As a dengue endemic country, Indonesia displays an increasing trend of dengue incidence rate from 1968 to 2013 [3]. DENV infection can manifest into various clinical symptoms, ranging from the classical dengue fever (DF) to the more severe forms of dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) [1, 4].

Although DENV infection often causes severe disease and fatality, currently there is no antiviral available for this disease. Efforts to discover drugs for this disease are sought, including the use of natural products as the potential source of antivirals [5]. Curcumin [1,7-bis (4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is one of the main substances found in the rhizome of *Curcuma longa* (L) and other *Curcuma* spp. [6]. This substance has been commonly used as a traditional medicine worldwide, including Indonesia. Curcumin has been reported to have strong antimicrobial, antiviral, and anti-inflammatory properties [7]. Several studies have reported the antiviral property of curcumin to Hepatitis C Virus (HCV) [8], Vesicular Stomatitis Virus (VSV), Herpes Simplex Virus (HSV) 1 and 2 [9], para-influenza 3, and Respiratory Enteric Orphan Virus (REO) 1 [10].

Antiviral activities of curcumin against DENV-2 has also been reported [11, 12]. The action against DENV or enveloped virus is believed through the inhibition of the Ubiquitin Proteasome System (UPS) [11, 13], which plays a role in viral replication [14]. Nevertheless, the development of curcumin as an antiviral drug is still hindered by its poor solubility and rapid hydrolysis in aqueous media, which leads to low bioavailability in the serum and tissue after administration [15]. Numerous approaches have been undertaken to solve, including the formation of nanoemulsion or nanocurcumin. It has been demonstrated that curcumin encapsulated in the nanocarrier system exhibited an increase in physicochemical stability and therefore its shelf-life [16]. In this report, we describe the potential antiviral activity of curcumin nanoemulsion against four Indonesian-derived DENV serotypes.

2. MATERIALS AND METHODS

2.1. Materials

The human alveolar epithelial cell line A549 (CCL-185) and baby hamster kidney BHK-21 (CCL-10) cells were from the culture collection at Eijkman Institute for Molecular Biology and maintained in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1% of Antibiotic-Antimycotic (all from Gibco-Thermo Scientific) in a humidified 37°C incubator with 5% CO_2 supplementation. Four DENV strains representing four DENV serotypes from Indonesia were isolated from clinical isolates and propagated in Vero (CCL-81) cells and maintained in low passage number to minimize mutation accumulation. The DENV-1 JMB-034 was isolated from a dengue patient in Jambi [17] while the other three strains, *i.e* DENV-2 SUB-011, DENV-3 SUB-006, and DENV-4 SUB-007 were isolated from patients in Surabaya, East Java [18, 19]. Curcumin powder (98.2% purity) obtained from Combiphar, Indonesia. Castor oil, Cremophor RH 40, and PEG 400 were obtained from idCHEM, Korea. Dimethyl sulfoxide (DMSO) was obtained from Applichem, Indonesia.

2.2. Curcumin-loaded Nanoemulsion

Curcumin was loaded in the nanoemulsion system including castor oil, Cremophor RH 40, and PEG 400, as previously described [16]. Briefly, castor oil (oil phase), Cremophor RH40 (surfactant), and PEG 400 (co-surfactant) were mixed with the ratio of 1:8:1 using a magnetic stirrer at 100 *rpm* for 2 hours to form the homogenous oil phase. Further, the oil phase was placed in a sonicator bath (Branson 5510) for 1 hour at 25°C to complete the SNE process. Subsequently, the product was added to deionized water with a concentration of 2 mg/mL and stirred for 15 minutes at 100 *rpm* until a clear and homogenous system was formed. As a comparison, a curcumin solution was prepared in DMSO.

2.3. Evaluation and Characterization of Curcumin-loaded Nanoemulsion

The particle size as well as the size distribution of the nanoemulsion were characterized using DelsaNano C Particle Analyzer (Beckman Coulter)

which was measured based on the photon correlation spectroscopy. Cryo-transmission electron microscopy (cryo-TEM) was employed to analyse the morphology of nanocurcumin. Briefly, 10 µL of nanocurcumin was dropped on a 400 mesh cryo-TEM grid and allowed to dry for 1 minute before being stained with 10 µl of uranyl acetate. The grid was allowed to dry and placeinto the JEOL 1010 cryo-TEM instrument at Eijkman Institute for Molecular Biology to inspect the nanocurcumin morphology under 80 kV at 20,000 × magnification.

Encapsulation efficiency was determined by calculating the ratio between the amount of curcumin entrapped and the initial amount of curcumin in nanoemulsion, and drug loading were determined by calculating the ratio between amount of curcumin entrapped and volume of nanoemulsion. 1 mL of the curcumin nanoemulsion was centrifuged at 14000 rpm for 15 minutes and the supernatant was diluted in DMSO. The percentage of curcumin encapsulated was measured using UV/Vis spectrophotometer (Beckman DU 7500i) at the wavelength of 430 nm based on the standard calibration of curcumin in DMSO provided.

$$\% \text{ Encapsulation Efficiency} = \frac{\text{Amount of curcumin entrapped}}{\text{Initial amount of curcumin in nanoemulsion}} \times 100\%$$

$$\text{Drug Loading} = \frac{\text{Amount of curcumin entrapped}}{\text{Volume of nanoemulsion}}$$

2.4. Cell Viability Assay

The cytotoxicity of nanocurcumin was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) in Vybrant MTT Cell Proliferation kit (Thermo-Scientific). Briefly, A549 cells were seeded into wells of 96-well plate (Corning) with a density of 10^5 cells/well. Confluent cells were treated with nanocurcumin or curcumin solution using a 2-fold serial dilution, ranging from 1 to 100 µg/mL, performed in triplicates. Vehicle controls were prepared as 1% v/v of nanoemulsion vehicle or DMSO in a medium. Further, the cells were incubated at 37°C incubator, 5% CO_2 for 48 hours. Following the incubation period, the supernatant was removed from each well and replenished with 100 µL of fresh medium followed by the addition of 10 µL of 12 mM MTT solution. The formation of formazan was allowed for 2 hours at 37°C, 5% CO_2 where blue colour was developed. The reaction was continued with the addition of 100 µL of sodium dodecyl sulphate-hydrochloric acid (SDS-HCl) and further incubation for 18 hours. Subsequently, the sample absorbance was determined by UV-Vis spectrophotometry at 570 nm. A dose-response curve was obtained using non-linear regression (curve fit) and the cytotoxic concentration was calculated to determine the concentration required to reduce the cell viability by 50% (CC_{50}).

2.5. Antiviral Activity Value Determination

The anti-DENV activity of nanocurcumin against DENV-1 to -4 was assessed by measuring the virus titre after exposure to different concentrations of nanocurcumin or curcumin solution, using standard plaque assay method [20, 21]. The A549 cells were seeded at 10^5 cells/well in 96-well plate and subjected to infection with DENV-1, DENV-2, DENV-3, or DENV-4 with multiplicity of infection (m.o.i) value of 1 (hypothetically one virus particle per cell). Treatment of cells with calculated concentrations of nanocurcumin or curcumin solution was done according to the three treatment methods. In the pre-entry treatment, known titre of DENV were mixed with known concentrations of nanocurcumin or curcumin solution followed by inoculation of the mixture into A549 cell monolayers for 1 hour at 37°C, 5% CO_2. Following the incubation period, inoculant was aspirated, and cells were washed with complete RPMI-10% FBS medium to remove unbound viruses before replenishment with fresh complete medium and incubation for 48 hours at 37°C, 5% CO_2. On the other hand, in the after-entry treatment, cells were initially inoculated with known titre of DENV and incubated for 1 hour at 37°C to facilitate virus-cell binding and adsorption. The cells were then washed with complete medium and replenished with medium containing known concentrations of nanocurcumin or curcumin solution and incubated for 48 hours. at 37°C, 5% CO_2. Meanwhile, in the full-treatment method, the mixture of DENV and nanocurcumin or curcumin solution was added to cell monolayer and without removal and wash steps, allowed to react at 37°C, 5% CO_2 for 48 hours. After incubation, the supernatant was trans-

ferred into microtubes for the virus titration using plaque assay in BHK21 cells. To assess the cell viability upon virus infection and curcumin treatment, MTT assay was performed as described above.

2.6. Statistical Analysis

Statistical analysis was performed using IBM SPSS software v.21. The parametric student's t-Test was used to compare the means between two independent groups. The *p*-value of < 0.05 was considered statistically significant.

3. RESULTS AND DISCUSSION

Curcumin is not yet claimed as a drug due to low soluble in water, light sensitive, and low bioavailability upon oral as well as vascular routes. The low bioavailability of curcumin is a combination of its low solubility, dissolution and absorption rates, and high hepatic metabolism [8]. Various methods have been carried out to improve the lack of curcumin as a drug, including the analogue synthesis, chemical pro-drug formation, combination with food components, and many others. Nanotechnology was known to provide a novel approach for pharmaceutic-associated problem drugs including curcumin [22, 23].

We successfully synthesized nearly monodisperse negatively charge curcumin nanoemulsion with the average droplet size of 40.85 ± 0.919 nm, 0.366 ± 0.165 for polidispersity index value and zeta potential of -7.039 ± 0.532 mV. The TEM analysis confirms the spherical morphology of nanoemulsion with uniform size (Fig. **1**). The negative charge of the nanoemulsion was caused by the use of Cremophor RH 40, a dissociated fatty acid ester with low toxicity and high biological compatibility [24], which can form negatively charged free fatty acids. A high amount of surfactant in the formula contributed to the droplet stabilization through droplet surface coverage. The % of encapsulation efficiency was 99.40% and the value of drug loading was 1.988 mg curcumin/mL nanoemulsion.

Curcumin is known to inhibit A549 cell proliferation by inducing apoptosis and there is a linear relationship between inhibitory effect and curcumin concentration [25, 26]. In this study, we compared the cytotoxicity and viral inhibitory characteristics of nanocurcumin to the DMSO-based curcumin solution counterpart. The CC_{50} value represents the concentration of an active substance that can induce a 50% reduction of the cell population. Treatment of different concentrations of nanocurcumin to the A549 human cell line generated a CC_{50} value of 52.97 µg/mL (Table **1**). On the other hand, treatment with DMSO-based curcumin solution generated a higher CC_{50} value (61.51 µg/mL). A significant decrease in cell viability was observed after treated with both nanocurcumin and curcumin solution at a concentration of 100 µg/mL (Fig. **2**). No significant impact was observed on cell viability treated with vehicle controls. A typical lower cell viability was observed in nanocurcumin-treated cells than in curcumin solution. Cell viability of higher than 80% was observed at treatments using concentrations below 12.5 µg/mL. Based on this observation, the concentration tested for an antiviral activity was 1, 5, and 10 µg/mL. The selection of A549 cells as an infected host was due to its suitability for DENV studies [20]. There was no significant change in viability when A549 cells were treated with both compounds up to 50 µg/mL (Fig. **2**). Higher toxicity of nanocurcumin on A549 cells compared to curcumin solution was possibly caused by the differences in the cellular uptake; nanoemulsion system presumably was more readily taken up to the cells due to the composition.

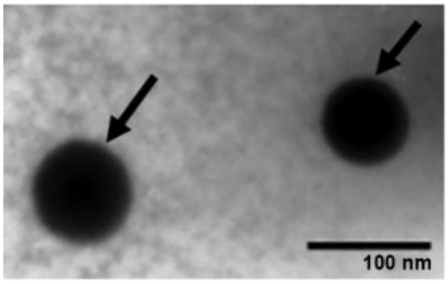

Fig. (1). The TEM analysis of curcumin nanoemulsion. Magnification of 20,000 ×. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

The inhibitory effect of nanocurcumin against DENV was measured as a percent of viable virus titre in the supernatant of treated cells to the viral titre in control medium. From three different viral infection approaches (pre-entry, after-entry, and

Table 1. The *in vitro* characteristics of nanocurcumin and curcumin solution in A549 cell system as determined by cell cytotoxicity 50 (CC_{50}) and inhibitory concentration 50 (IC_{50}) values.

Parameters	Nanocurcumin	Curcumin Solution
Cell cytotoxicity/CC_{50} (µg/mL)	52.97	61.51
Inhibitory concentration/IC_{50} of challenge virus (µg/mL)	-	-
DENV-1	0.96	1.12
DENV-2	2.61	4.03
DENV-3	22.62	35.9
DENV-4	15.13	17.24

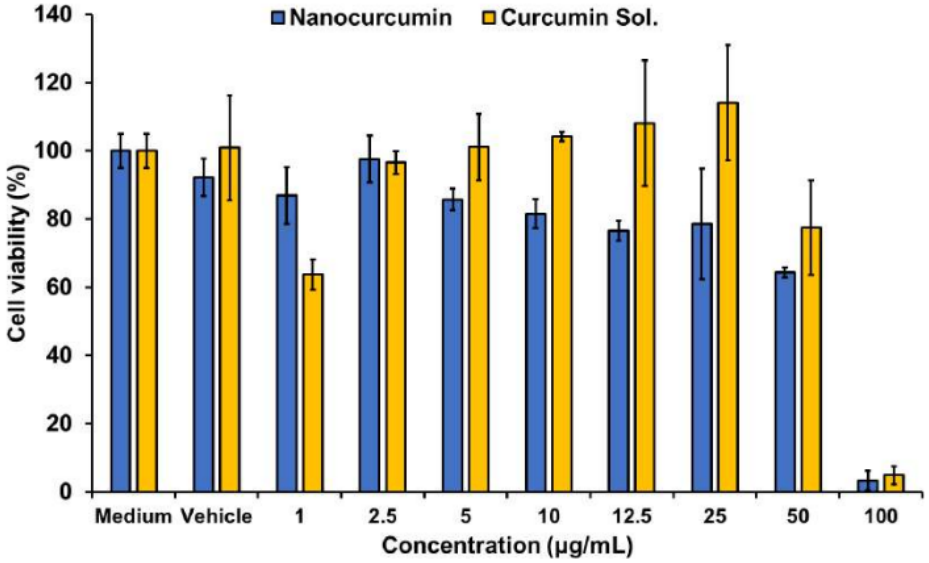

Fig. (2). A549 cell viability treated with different concentrations of nanocurcumin or curcumin solution. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

full-treatment), we observed that the full-treatment method generated the most consistent viral inhibitory profile (data not shown). The full-treatment of nanocurcumin and curcumin solution to DENV-infected A549 cells showed a decrease in DENV titre after 48 hours of incubation along with the increase in compound concentration (Figs. **3-6**). The decrease in viral titre was observed in all four DENV serotypes with greater inhibitory profiles detected for DENV-1 (Fig. **3**) and DENV-2 (Fig. **4**). The MTT assay performed on infected A549 cells revealed cell viability of about 80%, although slightly lower cell viability was observed in DENV-4-infected cell system (Fig. **6**). There were no statistically significant differences between DENV titre treated with either nanocurcumin or curcumin solution.

We analysed cell viability from the challenge tests to ensure that cell death was related to the inhibitory effect of curcumin on the dengue virus and that the decrease of viral titre was not due to cell death. Overall, cell viability slightly dropped to not less than 80% after exposure to all dengue serotypes, with the exception in DENV-4 challenge test, which displays cell viability around 70% (Fig. **6**). The decrease in viral titres due to cell death can be regarded as inconsequential to the experiment.

The viral titre values after incubation with nanocurcumin and curcumin solution were calculated from plaque assay test results. The viral titre of all DENV serotypes decreased after treated with either nanocurcumin or corresponding solution

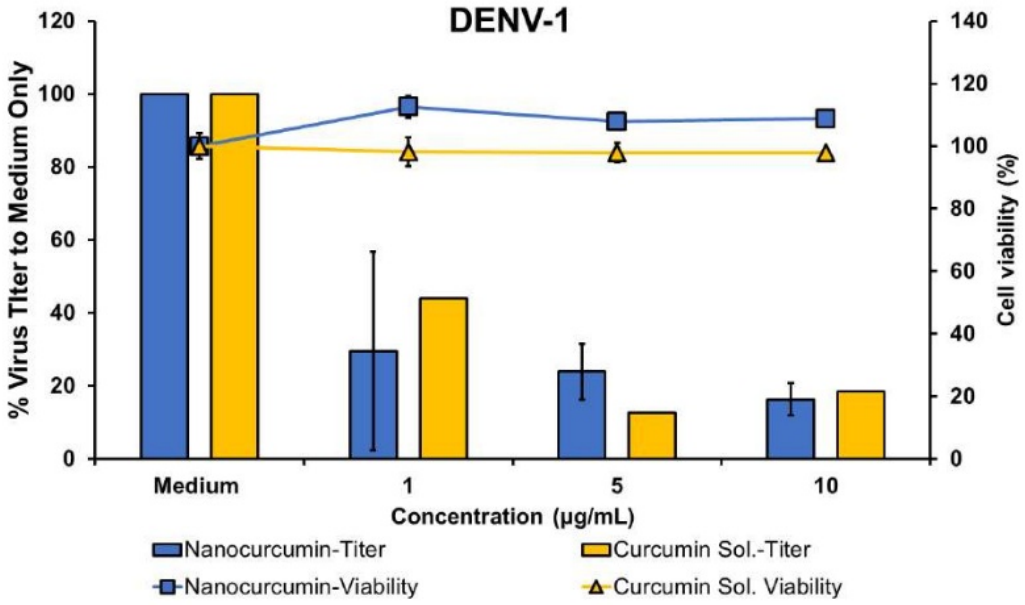

Fig. (3). Inhibitory effect of nanocurcumin and DMSO-based curcumin solution to the replication of DENV-1 (bars) and the corresponding A549 cell viability (lines) during challenge assay in a full-treatment approach. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

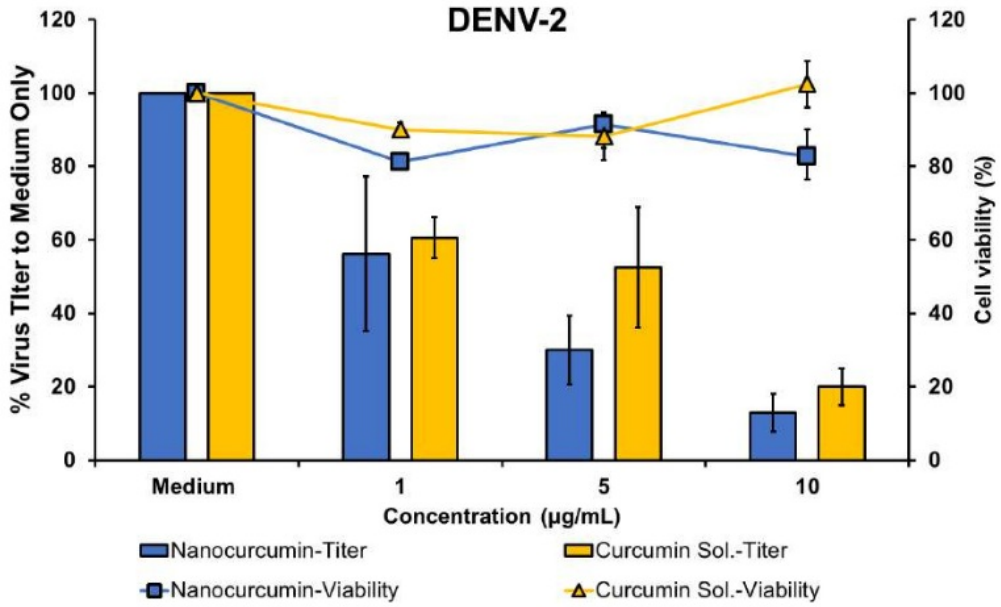

Fig. (4). Inhibitory effect of nanocurcumin and DMSO-based curcumin solution to the replication of DENV-2 (bars) and the corresponding A549 cell viability (lines) during challenge assay in a full-treatment approach. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

(Figs. **3-6**). However, DENV-1 (Fig. **3**) and DENV-2 (Fig. **4**) gave a better response compared to both DENV-3 (Fig. **5**) and DENV-4 (Fig. **6**). These findings urge that antiviral activity testing against DENV should consider the use of all four serotypes and not only a representative serotype. Moreover, we used DENV strains isolated from patients that may be considered as wildtype viruses rather than the highly adapted prototype or laboratory strains of DENV that may have accumulated mutations through the time. Based on the data above, the curcumin-loaded in nanoemulsion

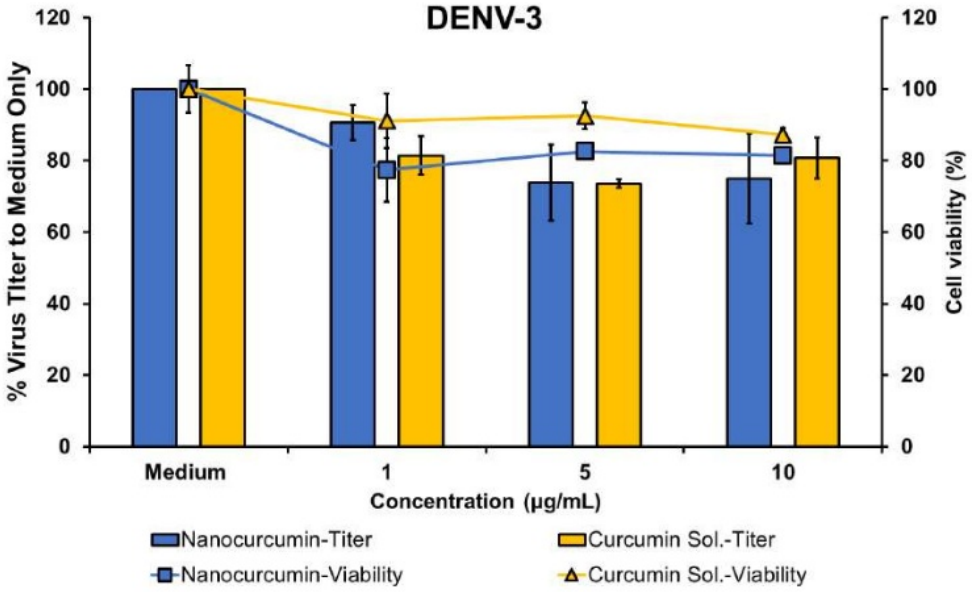

Fig. (5). Inhibitory effect of nanocurcumin and DMSO-based curcumin solution to the replication of DENV-3 (bars) and the corresponding A549 cell viability (lines) during challenge assay in a full-treatment approach. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

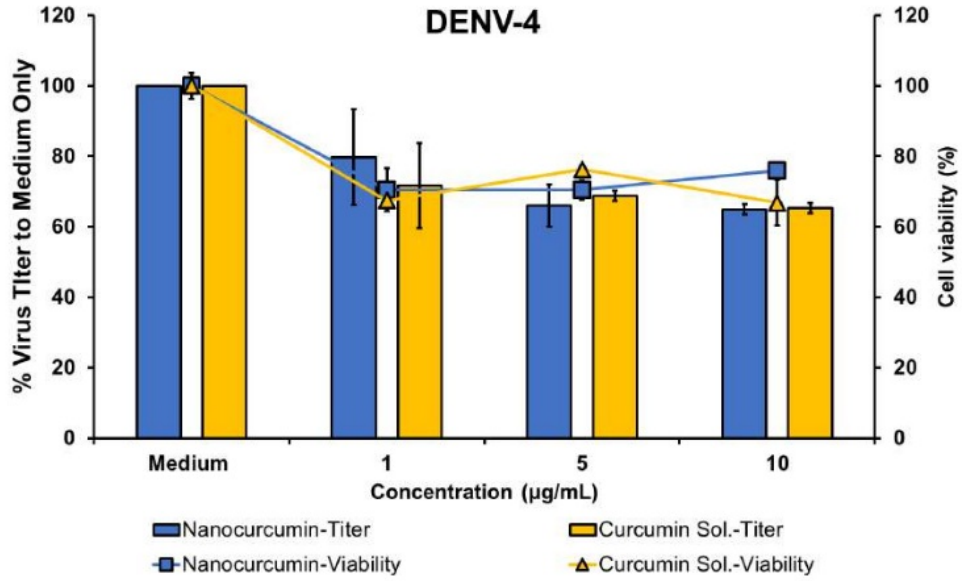

Fig. (6). Inhibitory effect of nanocurcumin and DMSO-based curcumin solution to the replication of DENV-4 (bars) and the corresponding A549 cell viability (lines) during challenge assay in a full-treatment approach. (*A higher resolution / colour version of this figure is available in the electronic copy of the article*).

improves the physicochemical properties of curcumin while keeping the potent inhibitory effect to four DENV serotypes. Curcumin in the nanoemulsion shows promising and safer formula as compared to DMSO-based curcumin solution hence replacing the formulation using DMSO, an uncommon solvent in the pharmaceutical products.

4. CURRENT & FUTURE DEVELOPMENTS

Curcumin has a promising potential as a therapeutic agent, especially as an antivirus. However, it is known that curcumin has poor cell uptake and also poor solubility in aqueous solution, therefore the bioavailability of curcumin is low. Based on several studies to solve the problem of curcumin,

the use of nanocarrier can exhibit an improvement in physicochemical stability. This result is also confirmed by our previous study. Our current study showed that curcumin nanoemulsion has a potential value as an antivirus against DENV. In the future, an accelerated stability test and antiviral test against DENV using a different method from Plaque Assay will be done to prove that curcumin nanoemulsion can improve the physicohemical properties and bioavailability of curcumin, and can be used as an anti-dengue drug for commercial purposes.

CONCLUSION

Curcumin loaded nanoemulsion consisted of castor oil, Cremophor RH40 and PEG 400, with the average droplet size of 40.85 ± 0.919, which increased the physical properties of curcumin while keeping the potent inhibitory effect to four DENV serotypes and hence replacing the solution formula, using a toxic organic solvent, which is DMSO.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

The human alveolar epithelial cell line A549 (CCL-185) and baby hamster kidney BHK-21 (CCL-10) cells were from the culture collection at Eijkman Institute for Molecular Biology, Jakarta, Indonesia.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

The data that support the findings of this study are available from the corresponding author [HR] upon request.

FUNDING

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CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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