Antiviral activities of curcumin and 6-gingerol against infection of four dengue virus serotypes in A549 human cell line *in vitro*

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ABSTRACT Dengue virus (DENV) is the most geographically widespread arbovirus causing dengue disease epidemics in tropical and subtropical regions. Nature provides abundant plants as a source for lead molecules against various diseases including DENV infection. We investigated the antiviral effect of curcumin and 6-gingerol, the major active constituent of turmeric (*Curcuma longa* Linn.) and ginger (*Zingiber officinale* Roscoe), respectively, against all four serotypes of DENV infecting human lung epithelial carcinoma (A549) cell line *in vitro*. Both compounds generated cell cytotoxicity to A549 cells at CC50 values of 108 µM for curcumin and 210 µM for 6-gingerol. The compound curcumin showed antiviral properties as described by IC$_{50}$ of 20.60, 13.95, 25.54, and 12.35 µM, while 6-gingerol of 14.70, 14.17, 78.76, and 112.84 µM for DENV-1, -2, -3, and -4, respectively. Different levels of antiviral properties were observed between DENV serotypes. Our findings suggest that the antiviral assay of compounds against DENV should be performed to all four serotypes and not limited to a particular serotype. In conclusion, curcumin and 6-gingerol exhibit antiviral properties against DENV infection and could provide a new therapeutic approach for dengue disease treatment strategies.

KEYWORDS 6-gingerol; A549; antivirus; curcumin; dengue virus; natural product

1. Introduction

Dengue virus (DENV) is considered the most geographically widespread arthropod-borne virus with an increased frequency of epidemics and severe disease manifestation in hyperendemic countries (Guzman and Harris 2015). It is estimated that 390 million cases of DENV infections occur worldwide annually (Bhatt et al. 2013; Guzman and Harris 2015). Over the past 50 years, the world has experienced a dramatic increase in dengue incidence to almost 30-folds (Guo et al. 2017), facilitated by virus transmission predominantly by *Aedes aegypti* and to a lesser extent *Aedes albopticus* mosquito vectors (Guzman et al. 2016). The four DENV serotypes (DENV-1, -2, -3, and -4) can cause a wide range of disease in human, from asymptomatic, classical fever Dengue Fever, to the life-threatening conditions Dengue Hemorrhagic Fever and Dengue Shock Syndrome (Martina et al. 2009).

The current disease management for DENV infection is limited to early detection, fluid replacement, and symptomatic therapy, although research has proven the association of viral load with the severity of the disease in the patients (Wang et al. 2003). The first-ever dengue vaccine Dengvaxia (CYD-TDV) still has limited use in the target population below nine years old (Dighe et al. 2019) and the development of anti-DENV drugs has been in a slow process. There is no licensed antiviral therapy available to date and only four small molecule anti-DENV drugs have entered phase I and phase II clinical trials. Although these drugs meet the required safety profile, they could not reduce viral load as expected (Tian et al. 2018). With the current unavailability of antiviral therapy for DENV infection, a search for compounds having antiviral effect needs to be established. One of the possible sources of a compound with anti-DENV activities will be from natural sources.

Indonesia has been known to have an abundant source of medicinal plants. Turmeric (*Curcuma longa* Linn.) and ginger (*Zingiber officinale* Roscoe), both from the family *Zingiberaceae*, have been used by generations of people around the world as a dietary spice, food preservative, and traditional herbal medicine (Soleimani et al. 2018). Turmeric contains a major active constituent called curcumin (Soleimani et al. 2018), while ginger contains 6-

gingerol (Kubra and Rao 2012; Wang et al. 2014). Researchers have shown the antioxidant, anti-inflammatory, anti-neoplastic, anti-microbial, and antiviral abilities of curcumin (Gupta et al. 2013; Kocaadam and Şanlier 2017) and 6-gingerol (Chrubasik et al. 2005). Curcumin, which is considered a non-mutagenic and non-genotoxic agent (Soleimani et al. 2018), has shown its antiviral properties against *Flaviviridae* viruses such as Zika Virus (Mounce et al. 2017), Hepatitis C Virus (HCV) (Anggakusuma et al. 2014), Japanese Encephalitis Virus (Dutta et al. 2009), and DENV-2 (Padilla-S et al. 2014). Fresh ginger has shown its inhibitory effect against human respiratory syncytial virus (HRSV) (Chang et al. 2013) and HCV (El-adawi et al. 2011).

Human alveolar epithelial A549 cells have been proven suitable for use as a DENV infection model *in vitro* (Yohan et al. 2014). The use of A549 cell line in DENV research was based on the evidence of viral antigen detection in macrophages and vascular endothelial cells of the lung (Jessie et al. 2004). The antiviral effect of curcumin and 6-gingerol to DENV has been reported although limited to a particular serotype (Padilla-S et al. 2014; Sharma et al. 2015). The differing genetic of DENV serotypes may cause differences in their virulence (Holmes and Burch 2000). Hence, an antiviral study against all four DENV serotypes in human cells will give more insights into dengue pathogenesis. In this study, we investigated the antiviral effect of curcumin and 6-gingerol on the growth of all four DENV serotypes in human A549 cell line. Findings from this study may be beneficial for the development of anti-DENV drug therapy from natural resources as alternative therapeutic approach for dengue disease.

2. Materials and Methods

2.1. DENV serotypes

Four DENV serotypes of Indonesian isolates were used. All virus stocks were generated from a low-passage number (maximum of five passages) of the virus in Vero cells. The DENV-1 strain JMB-034 was isolated from a dengue patient in Jambi (Haryanto et al. 2016). The other serotypes DENV-2 strain SUB-011, DENV-3 strain SUB-006, and DENV-4 strain SUB-007 were isolated from patients in Surabaya, East Java (Aryati et al. 2013; Wardhani et al. 2017). The harvested culture supernatants containing viable DENV were stored at -80 °C until use and their titers were measured using standard plaque assay method in BHK21 cells, as described previously (Yohan et al. 2014; Sasmono et al. 2015).

2.2. Cell cultivation and maintenance

Cell lines were obtained from ATCC. The human (*Homo sapiens*) alveolar epithelial carcinoma A549 (ATCC CCL-185) and Syrian golden hamster (*Mesocricetus auratus*) fibroblast BHK-21 (ATCC CCL-10) cells were grown and maintained in RPMI 1640 Medium (Gibco-Thermo Fisher

Scientific, CA, USA). The African green monkey (*Cercopithecus aethiops*) Vero (ATCC CCL-81) cells were maintained in Minimum Essential Medium (Gibco). The media were supplemented with 10% fetal bovine serum (FBS), 2 mM of L-glutamine, 100 U/mL of penicillin, and 100 µg/mL of streptomycin (all from Gibco). Cells were maintained in a humidified 37 °C incubator with 5% CO_2 supplementation (Yohan et al. 2014).

2.3. A549 cell viability test

Curcumin ($\geq$ 65%, HPLC) and 6-gingerol ($\geq$ 98%, HPLC) were purchased from Sigma-Aldrich in powder and lyophilized forms, respectively. Both compounds were solubilized using dimethyl sulfoxide (DMSO) (Sigma-Aldrich, USA) as a vehicle into stock solutions of known molarity. The stock solutions were then diluted into working solutions using RPMI medium supplemented with 10% FBS. The cytotoxicity of the compounds was tested in A549 cells, seeded 1×10^5 cells/well of 96-well plate and grown overnight at 37 °C, 5% CO_2. Serially diluted compounds were added into the cells monolayer in triplicate and tested with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) using the MTT Cell Proliferation Assay Kit (Trevigen, Gaithersburg, MD, USA) after 48 h of incubation at 37 °C, 5% CO_2, according to the protocol described by the manufacturer. Following the formation of formazan complexes, the plate was read at 570 nm using a microplate reader. The percentage of cell viability was measured as a comparison to the medium-only controls. A dose-response curve was obtained using non-linear regression (curve fit) and the cytotoxic concentration was calculated as median cell cytotoxicity (CC_{50}) in which the concentration of compound that reduced the cell's viability by half.

2.4. DENV antiviral activity testing

The A549 cells were seeded at 1×10^5 cells/well in 96-well plate and subjected to infection with DENV-1, DENV-2, DENV-3, or DENV-4 with multiplicity of infection (MOI) value of 1, described hypothetically as one virus particle per cell. Compounds treatment was done using 10, 25, and 50 µM of curcumin and 50, 100, and 200 µM of 6-gingerol, selected as sub-cytotoxic concentrations following the CC_{50} values determined from A549 cell viability test. Treatment of cells with calculated concentrations of compounds was done using co-treatment method, where the mixture of DENV and compounds was added to cell monolayer and allowed to react at 37 °C, 5% CO_2 for 48 h, without removal of inoculant or wash steps, as described previously (Chen et al. 2013). Following the incubation period, the supernatant was transferred into microtubes for the virus titration using plaque assay in BHK21 cells. The calculated virus titer was normalized to the medium only control (without compound or 0 µM) and presented as a percent of inhibition. The median inhibitory concentration (IC_{50}) was measured as the concentration of compound that can inhibit 50% of virus titer. A vehicle control is used to monitor the effect of DMSO diluent in the system.
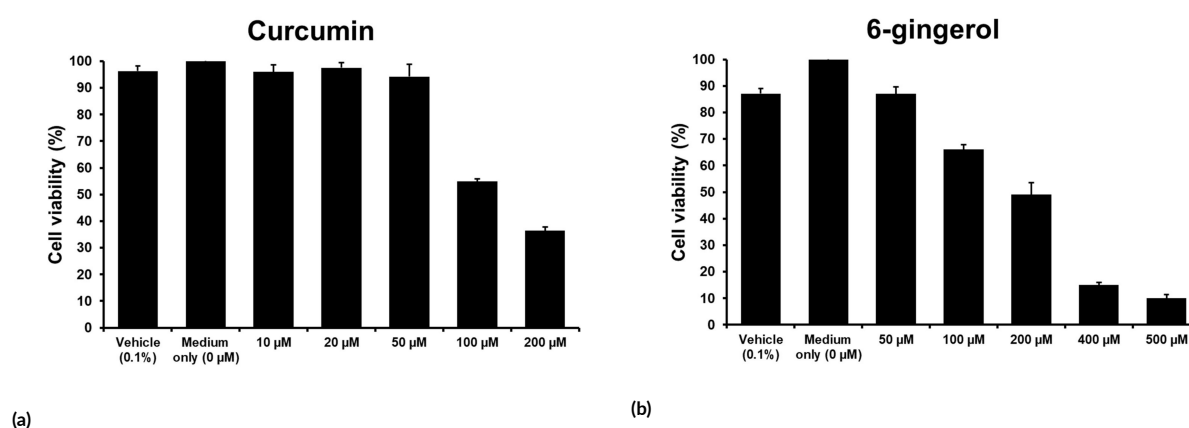
FIGURE 1 . Cell cytotoxicity of different concentrations of curcumin (a) and 6-gingerol (b) to A549 cells, as determined by CC_{50}. Both compounds showed significant dose-dependent cell cytotoxicity. Treatment of cells with 0.1% of DMSO was used as vehicle control.

The vehicle control was prepared as 0.1% v/v of DMSO, a concentration that was higher than the highest concentration of compounds in the testing.

2.5. *Statistical analysis, CC_{50} and IC_{50} calculations*

Statistical analyses were conducted using SPSS Statistics software version 17.0 (SPSS Inc., USA). Mean and standard error of mean (SEM) from three independent experiments were calculated. The comparison between groups in experimental results was analyzed using one-way ANOVA, followed by Tukey's post hoc test. A *p*-value less than 0.05 was considered as statistically significant. The CC_{50} and IC_{50} values were calculated using AAT Bioquest Quest Graph IC_{50} calculator (available online at https://www.aatbio.com/tools/ic50-calculator) based on the four-parameters logistic equation to generate a sigmoid function.

3. Results and Discussion

3.1. *Cytotoxicity of curcumin and 6-Gingerol to A549 cells*

The cells' cytotoxicity properties of curcumin and 6-gingerol were measured using standard MTT assay for measurement of cells' viability. Both curcumin and 6-gingerol reduced the viability of A549 cells in a dose-dependent manner. The median cell cytotoxicity CC_{50} of curcumin and 6-gingerol were measured at 108 µM and 210 µM, respectively (Figure 1). Both curcumin and 6-

TABLE 1 The antiviral activities of curcumin and 6-gingerol against four DENV serotypes as determined by median inhibitory concentration (IC_{50}).

Compound	IC_{50} of compound to DENV challenge (µM)			
	DENV-1	DENV-2	DENV-3	DENV-4
Curcumin	20.60	13.95	25.54	12.35
6-gingerol	14.70	14.17	78.76	112.84

gingerol significantly reduced the A549 cell viability in a dose-dependent manner (R^2=0.9132, p = 0.0029 and R^2= 0.9149, p = 0.0001, for curcumin and 6-gingerol, respectively).

3.2. *Antiviral activities of curcumin and 6-gingerol against DENV*

Following the CC_{50} data, the antiviral testing of curcumin and 6-gingerol was set at sub-cytotoxic concentrations. Curcumin and 6-gingerol significantly inhibited the growth of all DENV serotypes (Figure 2 and 3, respectively). For curcumin, all concentrations tested significantly inhibited the growth of all DENV serotypes compared to the medium-only group (p < 0.05), except in two experimental conditions at 10 µM and 25 µM in DENV-3 (Figure 2). On the other hand, for 6-gingerol, all concentrations tested show a significant reduction in the growth of all DENV serotypes (p < 0.05) except at 50 µM in DENV-3 (Figure 3).

The inhibition profiles were increased along with the increasing concentration of compounds compared to the medium only control. Vehicle control resulted in not significant reduction of virus titers compared to control (data not shown). We observed a variable median inhibitory concentration IC_{50} results for each DENV serotype (Table 1).

The compound curcumin has IC_{50} of 20.60, 13.95, 25.54, and 12.35 µM against DENV-1, -2, -3, and -4, respectively (Table 1). Curcumin showed the highest antiviral activities to DENV-4 compared to other DENV serotypes. Nevertheless, IC_{50} values for all serotypes were quite similar. For 6-gingerol, the compound was inhibiting the growth of DENV with IC_{50} values of 14.70, 14.17, 78.76, and 112.84 µM for DENV-1, -2, -3, and -4, respectively (Table 1). While highest antiviral activity was observed for DENV-2, the compound 6-gingerol showed higher antiviral potency for DENV-1 and DENV-2 compared to DENV-3 and DENV-4.
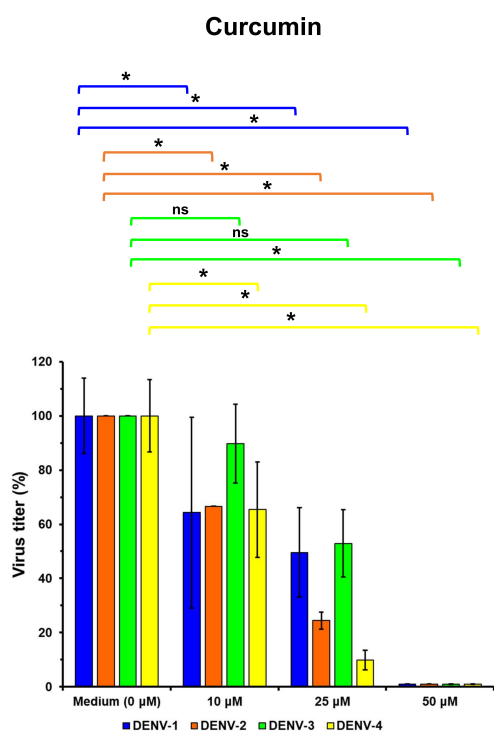
Curcumin

6-gingerol

FIGURE 2 Antiviral activity profiles of curcumin against all four DENV serotypes, measured as percentage of viable virus titer in treatment groups to the medium only control after 48 h of assay in A549 cells. ns, not significant; *, $p < 0.05$.

FIGURE 3 Antiviral activity profiles of 6-gingerol against all four DENV serotypes, measured as percentage of viable virus titer in treatment groups to the medium only control after 48 h of assay in A549 cells. ns, not significant; *, $p < 0.05$.

3.3. Discussion

The significant increase in dengue research during the past decades covers topics on dengue virology, pathogenesis, and immunology and progress in developing antivirals, vaccines, and new vector-control strategies that are important for dengue control and prevention (Guzman and Harris 2015). We tested the antiviral properties of curcumin and 6-gingerol against all four serotypes of wild strain DENV on A549 cell lines. The A549 cell line was used for its susceptibility to DENV infection and showed superior suitability compared to other human cell lines (Yohan et al. 2014).

As an active compound from nature, curcumin and 6-gingerol can also be toxic and induce apoptosis to cells (Kaushik et al. 2012; Impheng et al. 2015). We found that curcumin has cytotoxic property although it did not significantly affect A549 cells' viability in concentrations up to 50 μM with CC_{50} of 108 μM after 48 h of cell treatment (Figure 1a), a result that is comparable to a previous study by Kaushik et al. (2012). In the other hand, 6-gingerol showed cytotoxicity to A549 cells with CC_{50} of 210 μM (Figure 1b), comparable to other result observed elsewhere (Kim et al. 2008).

Antiviral activities of curcumin against DENV-2 has been reported (Padilla-S et al. 2014; Balasubramanian et al. 2019). The activity against DENV or enveloped virus is believed through the inhibition of Ubiquitin Proteasome System (UPS) (Chen et al. 2013; Padilla-S et al.
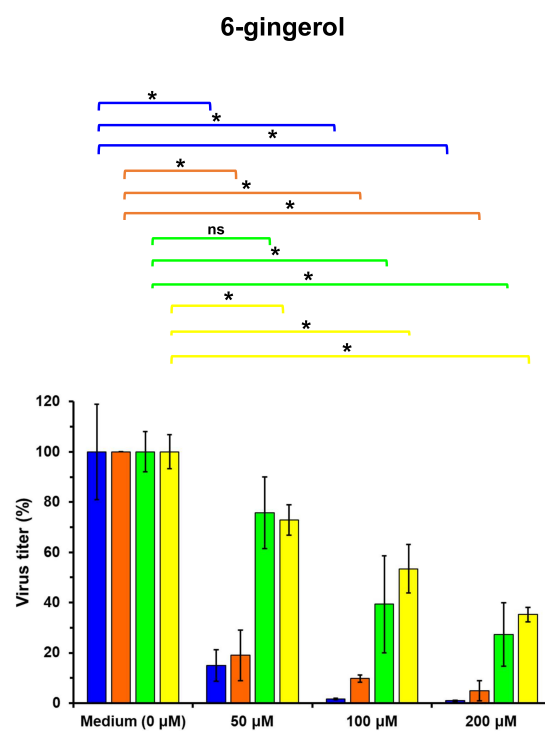
2014) which plays a role in viral replication (Glickman and Ciechanover 2002). In this study, we found that curcumin could inhibit all four DENV serotypes in the same manner (Figure 2), although different levels of IC_{50} was observed for each serotype (Table 1). Our result suggested similar antiviral potency of curcumin to all DENV serotypes. The effect of curcumin to DENV-2 was in accordance with previous result by Padilla-S et al. (2014). To the best of our knowledge, this is the first study to explore the antiviral ability of curcumin against all four serotypes of wild-type DENVs. The possible mechanisms of curcumin's antiviral properties include membrane-disturbing properties (Chen et al. 2013), altering membrane fluidity (Anggakusuma et al. 2014), and inhibiting cell binding (Mounce et al. 2017).

The compound 6-gingerol is known to exhibit a variety of biological activities including anticancer, anti-inflammation, and antioxidant (Wang et al. 2014). In this study, 6-gingerol, also revealed anti-DENV properties shown by the reduction of viral growth along with the increasing 6-gingerol concentration in the *in vitro* system (Figure 3). Previous study demonstrated the ability of fresh lipophilic juice of ginger to cause complete inhibition of HCV replication, a member of Flavivirus family like DENV (El-adawi et al. 2011). Fresh ginger is effective against HRSV-induced plaque formation on HepG2 and A549 cell lines by blocking viral attachment and internalization by interfering G protein and F protein (Chang et al.

2013). The administration of 6-gingerol was manifested as inhibition of fatty acid synthase (FASN) expression (Impheng et al. 2015), which is a biosynthetic pathway to establish DENV replication complexes (Heaton et al. 2010). The aqueous extract of ginger rhizome inhibited the activity of matrix metalloproteinase (MMP)-2 and MMP-9, while upregulating the expression of tissue inhibitor metalloproteinase (TIMP)-1 and TIMP-2 in DENV infected cells (Sharma et al. 2015). Interestingly, the antiviral activity of 6-gingerol was found to be higher in DENV-1 and -2 compared to DENV-3 and -4. The fact that 6-gingerol has different antiviral properties to four DENV serotypes is of merit for further confirmatory exploration whether different genetic of DENV serotypes attributed to these findings.

Our results add information on the antiviral properties of curcumin and 6-gingerol against all four serotypes of DENV. In addition, we promote that antiviral assay of compounds against DENV should be performed to all four serotypes and not limited to a particular serotype. Nevertheless, our study has limitations. The study design was aimed only to inspect the antiviral properties of curcumin and 6-gingerol in four DENV serotypes. The biological pathway mechanisms underlying the virus inhibition effect were not sought. More in-depth studies are needed to confirm our findings. In addition, the availability of different compound sources with different purities in the market may also give impact on the results generated. To the best of our knowledge, this is the first study describing the antiviral effects of curcumin and 6-gingerol to all four DENV serotypes.

4. Conclusions

In conclusion, curcumin, the major active compound of turmeric and 6-gingerol, the major active constituents of ginger, have shown antiviral activities against all four serotypes of DENV. Curcumin and 6-gingerol may have potentials in the development of anti-DENV drug therapy from natural resources and could provide an alternative therapeutic approach for dengue disease treatment strategies.

Acknowledgments

Authors' contributions

RTS, SH, ED, RH, BY designed the study. ED, RH, SH, RTS performed study supervision. JANH, SNH, DD, BY carried out the laboratory work. JANH, SNH, DD, BY analyzed the data. JANH, DD, BY, RTS wrote the manuscript. All authors read and approved the final version of the manuscript.

Competing interests

RTS serves as member of IJBiotech's editorial board. Other authors declare that they do not have competing interests.

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