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Effects of Curcumin and Pentagamavunon-0 Against Dengue-2 Virus Infection In Vero Cells; an In Vitro Study

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Abstract

The research aims was to determine the cytotoxic effects and the highest safe concentration of curcumin and PGV-0 on vero cells. This research also compare the effect of curcumin and PGV-0 against vero cells infected by Dengue virus (in vitro) in one and three days incubation period. The highest safe concentrations against vero cells from curcumin was 6.25 ppm and 1.5625 ppm for PGV-0. Immunocytochemistry test of curcumin and PGV-0 in both incubation period (one and three days) shows no significant difference (significant value 0.925), but RT-PCR result indicates that PGV - 0 have potential antiviral better than curcumin.

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Keywords: Dengue, curcumin, PGV-0, immunocytochemistry, RT-PCR

1. Introduction

Dengue Haemorrhagic Fever (DHF) is an infectious disease caused by dengue virus. Dengue is still a significant health problem in tropical and sub tropical countries, because the course of this disease was very fast, so if not addressed promptly, it can be fatal. The global prevalence of dengue infection has increased dramatically in recent decades. Dengue infection has now become an endemic in more than 112 countries in Africa, America, Eastern Mediterranean, Southeast Asia and Western Pacific. The World Health Organization (WHO) estimates that

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40% of the world's population or 2.5 billion people live in tropical and subtropical areas at risk for contracting the Dengue infection [1, 2].

Dengue virus is an RNA virus which is included in Flaviviridae family [3], infecting 50 to 100 million people per year. However, a vaccine which is commercially available has not been found as it is still in the development stage; specific therapy or effective antiviral drugs to treat dengue virus infection has also not been found yet. As a result, the development of antiviral drugs licensed for the treatment of patients remains an urgent need to prevent Dengue deaths.

Indonesia has many traditional medicinal plants that have been reported to have potent antiviral activity and a few have been used to treat people infected with Dengue. Examples of these are *Curcuma longa* plant which has curcumin content, that is, a yellow chemical compound especially on the roots of plants. Some studies suggest curcumin has preventive activity against viral agents, such as vasicular stomatitis (VSV), HSV 1 and 2, parainfluenza-3, reovirus-1, feline corona virus, feline herpes virus and other viruses with EC_{50} 0.019 to 0.105 μM [4]. Curcumin is also known to perform the inhibition of ubiquitin-proteasome system that causes a decrease in the production of one type of flavivirus which is *Japanese encephalitis* from neuroblastoma cells which are previously infected [5].

Curcumin has anticancer, antimutagenic, anticoagulant, antifertility, antidiabetic, antibacterial, antifungal, antiprotozoa, antivirus and antifibrosis activities [6]. Based on the analysis of the structure of curcumin, it is known that the pharmacological activities of curcumin are related to functional groups such as double bonds in the middle of the chain, β-diketon group, and fenolik hydroxy groups [7]. A study on relationship of structure to curcumin activity has been done by Sardjiman (1997) on the cell lines [8]. One of curcumin analogue compounds that have received patents as an anti-inflammation and introduced as a National Molecule (Molnas) is pentagamavunon-0 (PGV-0). PGV-0 compound [2,5-bis-(4' - hydroxy-3'- metosksibenzilidin)-cyclopentanone] is known have antioxidant and anti-inflammatory potential which is more powerful and less toxic than other curcumin analogue compounds [9] and has never been tested in vitro activity as antiviral. Accordingly, this study was conducted to determine the effects of curcumin and pentagamavunon-0 against Dengue-2 virus Infection.

The method used for the detection of Dengue virus in this study was RT-PCR and immunocytochemistry. Both examination techniques are known to have a high sensitivity; thus, it can detect the antigens with low levels. RT-PCR technique is known rapid and sensitive for the detection of viral RNA, but this method is expensive and requires certain tools and skills, whereas immunocytochemistry has the advantage of requiring no special equipment and can be done only by using a light microscope widely available in laboratories and requiring no certain skills

Nomenclature

DHF	Dengue Haemorrhagic Fever
WHO	World Health Organization
RNA	Ribonucleic Acid
VSV	Vasicular Stomatitis
PGV-0	Pentagamavunon-0

2. Method

This was a laboratory experimental study. Curcumin (1,7-bis (4'hidroksi - 3 methoxyphenyl)-1,6 heptadien, 3,5-dione) used Sigma brand, whereas curcumin analogue compound (PGV-0 or 2,5-bis- (4-hydroxy-3-metoksibenzilidin) cyclopentanone) was obtained from synthesis results in the pharmaceutical laboratory of UGM. Five mg of each compound was dissolved in 100 µl dimethyl sulfoxide (DMSO) as stock solution.

Cytotoxic test were carried out using the MTT [3 - (4,5-dimetiltiazol-2-i1) -2,5 difeniltetrazolium bromide]. Cells at a density of 10^4 vero cells / wells distributed in wells (well plate) and incubated for 24 hours. Followed by the addition of curcumin and PGV-0 with 7 (seven) concentration series of 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 ppm respectively - each of 4 replications. Solvent used to dissolve the test compound is dimethylsulfoxide (DMSO). The cytotoxicity test results read by Elisa reader with a wavelength of 595 nm.

Immunocytochemistry test SBPC.

Vero cell culture at a density of 5 x 10^5 cells per well were grown in a well plate . Each given a deck glass that has been coated with poly elysin as the attachment of cells . Well plate is divided into two (2) incubation of the incubation groups (1) and three (3) days . Each group was divided into groups of Dengue - 2 virus infected 1 day incubation and were given curcumin, the positive control (cells infected by dengue 2 days of incubation 1) and cells infected by Dengue - 2 incubation 1 day but not given immunocytochemistry staining of primary antibody DSSE10 as immunocytochemistry control, and negative control (uninfected cells 1 day incubation). The division of the group on the infected cell for three (3) days is similar to that of Dengue - 2 infected incubation one (1) day.

Immunocytochemistry examination SBPC [10] .

Sample to be tested were fixed with cold methanol and washed with PBS. The slide then immersed in peroxidase blocking solution at room temperature for 10 minutes, to remove endogenous peroxidase activity, then washed with distilled water. Sample incubated in the background sniper (protein blocking solution) for 10 min at room temperature. Primary antibodies (monoclonal antibodies DSSE10 1:10) was added 100 mL per preparation (adjusted until all parts were flooded) and then incubated in a moist tray overnight. Preparations then washed with PBS for 2 x 2 minutes. Trekkie universal link (secondary antibody) was added to 30 mL per preparations and incubated at room temperature (25^0 C) for 15 minutes and washed with PBS for 2 x 2 minutes. Trekavidin - HRP reagent was added and incubated for 10 minutes, then washed with PBS for 2 x 2 minutes .

Substrate preparation is done with DAB chromogen : DAB chromogen betazoid 1 mL diluted with 100 mL betazoid susbtrate DAB buffer immediately before use. Sample incubated in DAB chromogen substrate above 30 mL per sample for 10 minutes, then washed with tap water. Cat Mayer hematoxylin (counterstain) was added to the preparations, and then incubated for 1-3 minutes, then washed under tap water and dried. Furthermore, dipped in alcohol, dried and cleaned and then covered with spilled entellan cover glass. Ready to be examined under a microscope at a magnification of 40x , 100x , 400x and 1000x. Cell preparations showed a brown color at the cytoplasm or contained brown granules around the cell means Dengue - 2 antigen positive, whereas cells showed blue or pale and there are no brown granules around the cell as a negative control. Results of Immunocytochemistry examination positive rate result was analyzed by ANOVA with Confidence Interval (CI) 95 % .

RT – PCR (Polymerase Chain Reaction)

RT-PCR examination begins with the extraction of RNA / RNA isolation of dengue virus with the manual High Pure Viral Nucleic Acid Kit (Roche Diagnostics GmbH, Mannheim Germany, cat no.11 858 874 001). The PCR process using RT-PCR (Superscript ™ Kit, III One Step RT-PCR System with Platinum, Invitrogen 125 74-026). Serotype-specific primers used were:

Table 1. Primer for One-Step RT-PCR (Yong *et al*., 2007) [11]

Virus Serotype	Primer	Primer Sekuens	Primer Position	Band Target
	Dcon	5'- AGT TGTTAGTCTACGTGGACCGACA-3'	1-25	
DEN 2	D2	5'- CGCCACAAGGGCCATGAACAG-3'	231-251	251 bp

PCR mix was made in tubes of 1.5 ml nuclease - free and done in the ice with: 2x reaction mix 12.5 mL, SuperscriptTMIII RT / Platinum ® Taq Mix as much as 0.5 mL, MgSO4 as much as 0-2 mL, primary Dcon (forward) in 1 mL, D2 primer (reverse) by 1 mL, RNase- free water as much as 0-3 mL and RNA as much as 5-10 mL, total volume of about 25 mL. The components were confirmed to the bottom of the tube by means of centrifugation. PCR mix put in a thermal cycler, then its run according to the program : (i) cDNA synthesis 1 cycle : 60^0C for 45 min; (ii) predenaturasi 1 cycle : 94^0C for 2 min; (iii) 30-35 amplification cycles: 94^0C for 30 sec (denaturation), 60^0C for 30 sec (annealing), 68^0C for 1 min (extension); (iv) final extension of 1 cycle : 68^0C for 5 minutes . RT - PCR products generated electrophoresed on 1.5% agarose gel and the 100 bp ladder used as a marker to analyze the products of PCR. The expected size band is 251 bp.

3. Results and Discussion

3.1. Cytotoxicity test of curcumin and PGV-0

The cytotoxic test of curcumin and PGV-0 used seven concentrations; 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.78125 ppm. Solvent used to dissolve the test compound was dimethylsulfoxide (DMSO). Observation of living cells was performed with MTT method. MTT was used to evaluate cytotoxic changes of a compound based on tetrazolium salt into a colored product (formazan) by the dehydrogenase succinate mitochondrial enzyme with the help of cellular NADH. Living vero cell percentages were calculated based on a comparison between the treatment absorbance of the test compound and the vero cell controls. The percentage of living vero cells after administration of curcumin and PGV-0 can be viewed in the following graph:

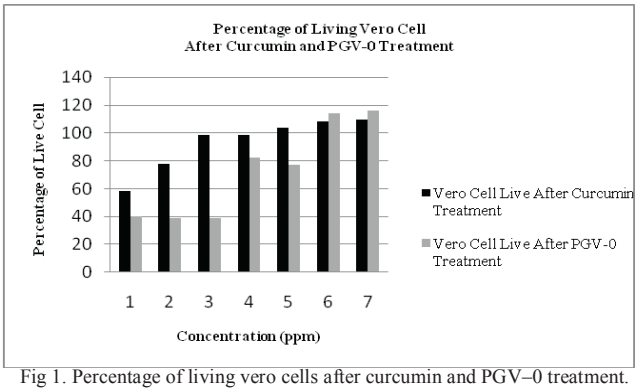

Fig 1. Percentage of living vero cells after curcumin and PGV–0 treatment.

The result of the cytotoxic test for curcumin was 6.25 ppm and PGV-0 was 1.5625 ppm. Both concentrations were obtained from the calculation of the percent of living cells over 100%. Cytotoxicity potential of PGV-0 was higher than curcumin. It's seen from a safe concentration value of vero cells for PGV-0 was smaller than curcumin. Increased cytotoxicity potential of PGV - 0 was likely due to changes in β - diketon groups on curcumin into cyclopentanone group, leading to increased cytotoxicity activity against vero cells.

3.2. Immunocytochemistry to Determine Antiviral Ability of curcumin and PGV-0

The result of immunocytochemistry test on vero cells infected with Dengue-2 virus on first day incubation and treated using curcumin and PGV-0 is as follows:

Table 2 . Positive rate of immunocytochemistry test on vero cells infected by Dengue-2 virus on first day incubation.

		Compound		
		Curcumin	PGV-0	Positive Control
Repeat 1	MP %	2 ± 1.25	3.3 ± 3.23	18.6 ± 11.2
	P %	2.6	3.43	20.4
	N %	97.4	96.57	79.6
Repeat 2	MP %	6.6 ± 3.27	4.2 ± 4.75	10.5 ± 3.3
	P %	7.6	4.23	14.48
	N %	92.4	95.77	85.52
Repeat 3	MP %	2 ± 1.56	3.9 ± 3.69	
	P %	2.11	4.51	
	N %	97.88	95.49	
Average	**MP %**	**3.53 ± 2.03**	**3.8 ± 3.89**	**14.55 ± 7.25**
	P %	**4.1**	**4.06**	**17.44**
	N%	**95.89**	**95.94**	**82.56**

Description: MP=mean positive, P=positive, N=negative

Immunocytochemistry test results on third day incubation shown in the following table:

Table 3. Positive rate of immunocytochemistry test on vero cells infected by Dengue-2 virus on third day incubation

		Compound		
		Curcumin	PGV-0	Positive control
Repeat 1	MP %	9.1 ± 5.19	5.6 ± 8	13.7 ± 8.14
	P %	9.3	7.7	20.3
	N %	90.7	92.3	79.7
Repeat 2	MP %	16.7 ± 10	18.5 ± 14.79	29.3 ± 18.37
	P %	5.73	18.05	28.2
	N %	84.27	81.95	71.8
Repeat 3	MP %	15.1 ± 10.33	11.1 ± 5.74	
	P %	15.24	12.8	
	N %	84.76	87.2	
Average	**MP %**	**13.63 ± 8.506**	**11.73 ± 9.48**	**21.5 ± 13.25**
	P %	**10.09**	**12.85**	**24.25**
	N %	**86.57**	**87.15**	**75.75**

Description: MP=mean positive, P=positive, N=negative

Anova test results on a comparison of positive rate of curcumin and PGV-0 both on first and third day incubation obtained significance value (0.925). Thus, it's known that there was no significant difference between curcumin and PGV-0 treatment from first and third day incubation (the ability of curcumin and PGV-0 in lowering the positive rate of Dengue virus infection was the same).

Results of statistical test on positive rate calculation showed that the ability of curcumin and PGV - 0 in lowering the positive rate of Dengue virus infection was the same. However, when compared with the positive rate of positive control (cells infected with Dengue-2 virus but not treated with the test compound), it is known both curcumin and PGV-0 could reduce the positive rate due to Dengue virus infection.

Research of Nurminha (2011), immunocytochemistry test with WDSSB5 antibodies obtained positive rate 100 % on C6/36 cells infected by Dengue-3 virus [12] . In cells infected with positive rate of 100 %, strong positive reaction of brown color was very clear in the cytoplasm of cells, many giant cells and irregular cell shape.

3.3. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

The electrophoresis results of RT-PCR products in this study can be seen in the following figure:

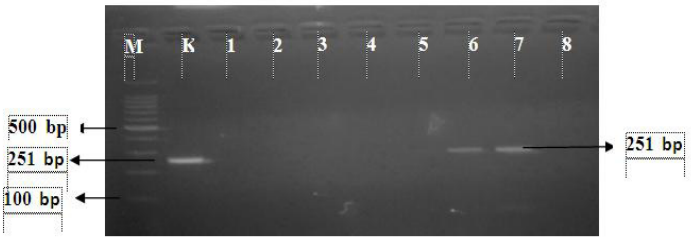

Fig 2. Photo of electrophoresis results of RT-PCR products from vero cells infected with Dengue-2 virus and treated with curcumin and PGV-0. M : 100 bp ladder marker, K : positive control of Dengue-2 virus, Lane 1: negative control of first day incubation (without infection), Lane 2: positive control of Dengue-2 virus on first day incubation, Lane 3 : first day incubation treated with curcumin, Lane 4: first day incubation treated with PGV-0, Lane 5: negative control of third day incubation, Lane 6 : positive control of Dengue-2 virus on third day incubation, Lane 7: third day incubation treated with curcumin, Lane 8: third day incubation treated with PGV-0.

RT-PCR examination could detect Dengue-2 virus in vero cells infected in the incubation period of day 3 (viral positive control) and a treatment sample on day three of viral infections treated with curcumin. In day 1 infection, both viral control and treatment with curcumin and PGV-0 had not seen any positive antigen of dengue-2 by RT-PCR examination.

Comparison of the results of RT-PCR and immunocytochemistry showed that immunocytochemistry test could detect Dengue-2 antigen in the first incubation period, while the results of first day incubation using RT-PCR was negative. Research by Harris [13] suggested that the detection of RNA Dengue could be performed on *Ae. aegypti* individually using One Step RT-PCR from the first day after viral inoculation. At the study, *Ae.aegypti* was infected parenterally with Dengue-2 virus with a titer of 10^3 PFU. According to Jittmittraphap [14] , PCR was able to detect the RNA virus in the mosquito's body with an incubation period of 7 days with a titer of 1-10 PFU. Research of Widiastuti [15] stated that RNA from Dengue-3 virus in infectious mosquito on 1- 4 day incubation could not be detected using RT-PCR, although the concentration of RNA was performed; Dengue-3 virus RNA could be detected after 5 day incubation. Yasmon [16] stated that a false negative on RT-PCR examination could be caused by the number of viral particles in the sample which was too low.

Positive results of Dengue-2 antigens shown in the third day incubation of curcumin treatment. Mean while incubation of Dengue-2 virus infection treated with PGV-0 in third days was negative. This suggested that the PGV-0 have a Dengue-2 antiviral better than curcumin. This was possible because there was a major difference between curcumin and PGV-0 on the central cluster chemical structure. Removal of the active methylene group and

the carbonyl group at the center of the structure of curcumin led to be non-polar, so it became more soluble in lipid and was more easily interact with the walls of bacteria and viruses. So that from this research the PGV-0 is more potent than curcumin as Dengue antivirus.

4. Conclusions

PGV-0 was more toxic than curcumin to the vero cell and the comparison effect of curcumin and PGV-0 on vero cells in first and third day incubation by Dengue-2 infection showed that the result of positive rate was not significantly different (from immunocytochemistry test). The results of RT-PCR showing positive Dengue-2 antigen only on day 3 of curcumin treatment, which indicated that PGV-0 had better antiviral activity against Dengue-2 compared to curcumin.

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