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- Immune Response against Hepatitis B Virus after Vaccination among Low Birth Weight and Preterm Newborns: A Retrospective Cohort Study in Magelang District Central Java
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The Effects of Curcumin Against Dengue-2 Virus Based on Immunocytochemistry Technique

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ABSTRACT

Introduction: Dengue is the most important mosquito-borne flavivirus disease. The number of Dengue cases in Indonesia in 2010 range from 150,000 cases with the deaths of around 1,317 people. Huge number of cases have made Indonesia was the first ranked as the state with the highest Dengue cases in the ASEAN region and the world's second ranking after Brazil. The drugs or antibiotics that can be administered effectively to cure this disease has not been found yet. Many study have been done and some that have been reported include viral RNA synthesis inhibitors, protein inhibitors of NS3 helicase and protease and inhibitors that inhibit Dengue virus maturation. Curcumin have preventive activity against several viruses: *vasicular stomatis* (VSV), HSV 1 and 2, *parainfluenza* - 3, *reovirus* - 1, *feline corona virus*, *feline herpes virus* . Curcumin also known have ubiquitin proteasome inhibition system was able to decrease the production of *Japanese ensefalitis* virus.
Objectives: This study aims to determine safe concentrations of curcumin against vero cells (cytotoxic test results) and know the Dengue-2 antiviral potency of curcumin.
Methods: Including quasi-experimental study. The anti viral potency of curcumin seen from the result of immunocytochemistry Streptavidin Biotin Peroxidase Complex (SBPC). Data were analyzed by ANOVA.
Results: The results showed that secure concentrations from cytotoxic of curcumin against vero cells is 6.25 ppm. The calculation of positive rate from immunocytochemistry in vero cells infected by Dengue - 2 incubation 1 and 3 days were the result is significantly different than the control.
Conclusion: The secure concentration of curcumin against vero cells was 6.25 ppm and curcumin was able to lower the positive rate due to Dengue-2 infection.

Key Words : Dengue virus, Curcumin, Immunocytochemistry.

INTISARI

Pendahuluan: Dengue adalah penyakit flavivirus paling penting yang ditularkan oleh nyamuk. Jumlah kasus Demam Berdarah Dengue (DBD) di Indonesia pada tahun 2010 berkisar 150.000 kasus dengan kematian sekitar 1.317 orang. Jumlah kasus yang besar ini membuat Indonesia menjadi peringkat pertama sebagai negara dengan kasus DBD tertinggi di kawasan ASEAN dan peringkat kedua di dunia setelah Brazil. Sampai saat ini belum ditemukan obat – obatan ataupun antibiotik yang efektif yang dapat diberikan untuk menyembuhkan penyakit ini. Berbagai senyawa dilaporkan mencakup penghambat sintesis RNA virus, inhibitor NS3 protein helikase dan protease serta inhibitor

yang menghambat pematangan virus Dengue. Kurkumin terbukti memiliki aktivitas preventif terhadap beberapa virus, antara lain: *vasicular stomatis* (VSV), HSV 1 dan 2, *parainfluenza*-3, *reovirus*-1, *feline corona virus*, *feline herpes virus*. Kurkumin juga diketahui mampu melakukan penghambatan sistem *ubiquitin-proteasome* yang menyebabkan penurunan produksi virus *Japanese ensefalitis.*
Tujuan: Penelitian ini bertujuan untuk mengetahui konsentrasi yang aman dari kurkumin terhadap sel vero (hasil uji sitotoksik) dan mengetahui potensi antiviral kurkumin terhadap Dengue-2.
Metode: Termasuk penelitian kuasi eksperimental. Potensi anti viral kurkumin dilihat dari hasil uji imunositokimia *Streptavidin Biotin Peroxidase Complex* (SBPC). Data dianalisis dengan ANOVA.
Hasil: Hasil uji sitotoksik menunjukkan konsentrasi kurkumin yang aman terhadap sel vero adalah 6,25 ppm. Hasil perhitungan nilai *positive rate* dari uji imunositokimia pada sel vero yang diinfeksi Dengue-2 inkubasi 1 hari dan 3 hari dengan perlakuan kurkumin dibandingkan kontrol adalah berbeda nyata.
Simpulan: Konsentrasi kurkumin yang aman terhadap sel vero adalah 6,25 ppm dan diketahui kurkumin mampu menurunkan nilai *positive rate* akibat infeksi Dengue-2.

Kata kunci: Virus Dengue, kurkumin, imunositokimia.

INTRODUCTION

Dengue is the most important mosquito-borne flavivirus disease. People living in the tropical and subtropical areas are at risk of Dengue virus infection. More than Dengue infected cases occur worldwide each year. The number of Dengue cases in Indonesia in 2010 range from 150,000 cases with the deaths of around 1,317 people. Huge number of cases have made Indonesia was the first ranked as the state with the highest Dengue cases in the ASEAN region and the world's second ranking after Brazil[1].

Dengue infection is caused by Dengue virus transmitted to humans by the mosquito. These viruses belongs to the Flaviviridae family of RNA viruses. Dengue virus has four serotypes of Dengue-1, Dengue-2, Dengue-3 and Dengue -4. Clinical syndroms in humans caused by Dengue virus ranging from an acute self-limited febrile illness (Dengue fever, DF) to Dengue Haemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS)[2].

The drugs or antibiotics that can be administered effectively to cure this disease has not been found yet. Handling Dengue cases was usually done only to relieve symptoms with intravenous fluids.[3] Many have been studied extensively and some that have been reported to act as inhibitors of RNA synthesis, protein inhibitors of NS3, protease inhibitors that inhibit Dengue virus maturation and polianion monoclonal antibody that prevents binding to host cell receptors[4, 5].

Curcumin is known have the preventive activity against several viruses, such as vasicular stomatis (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[6]. Curcumin also known have ubiquitin proteasome inhibition system was able to decrease the production of *Japanese ensefalitis* virus[7]. Curcumin, a hydrophobic polyphenol compound derived from the rhizome of the herb *Curcuma longa* an Indonesian traditional medicinal plants. Some of this considerations underlying the use of curcumin was tested in Dengue virus.

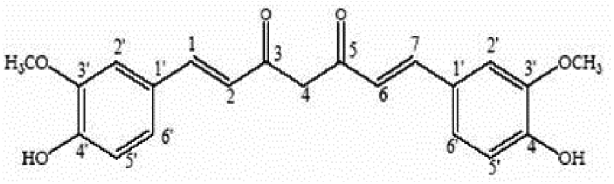

Figure 1. Structure of [1,7-bis-(4'-hidroksi-3'-metoksifenil)hepta-1,6-diena-3,5-dion] (curcumin)[10]

Curcumin has a wide spectrum of biological and pharmacological activities, such as anticancer, antimutagenic, anticoagulant, antifertilitas, antidiabetic, antibacterial, antifungal, antiprotozoa, antiviral and antifibrosis[8]. The pharmacological activities of curcumin associated with as double bonds in the curcumin's central chain, β-diketone group, and a hydroxy phenolic group[9]. This study was conducted to determine the cytotoxic effects of curcumin and to test the activity of curcumin against Dengue-2 in vitro. The Antiviral potency of curcumin known by Streptavidin Biotin Peroxidase Complex (SBPC) immunocytochemistry. Here is a chemical structure of curcumin.

MATERIAL AND METHODS

This study was an experimental study. Five mg of Curcumin (1,7-bis (4'hidroksi - 3 methoxyphenyl)-1,6 heptadien, 3,5-dione) was dissolved in 100 µl dimethyl sulfoxide (DMSO) as stock solution.

Cytotoxic test were carried out using the MTT [3 - (4,5-dimetiiazol-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 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 that used to dissolve the test compound was dimethylsulfoxide (DMSO). The cytotoxicity test results read by Elisa reader with a wavelength of 595 nm, the absorbance data obtained converted into living cells or the percentage of cell viability that can be calculated with the following formula.

$$\text{Percent of living cells} = \frac{\text{Absorbance cell treatment - Absorbance media}}{\text{Absorbance of control cells - Absorbance media}} \times 100\%$$

Immunocytochemistry SBPC

Vero cell at a density of 5 x 10^5 cells per well were grown in a well plate. Each given a deck plate 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 of incubation 1 days) and cells infected by Dengue - 2 incubation 1 day but not given DSSE10 as primary antibody of immunocytochemistry staining 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 one (1) day incubation.

Immunocytochemistry SBPC[11]

Sample to be tested were fixed with cold methanol and washed with PBS. The sample

then inundated 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 minutes at room temperature. Primary antibodies (DSSE10 monoclonal antibodies 1:10) was added 100 mL per sample (adjusted until all parts were flooded) and then incubated in a moist tray overnight. Sample then washed with PBS for 2 x 2 minutes. Trekkie universal link (secondary antibody) was added to 30 mL per sample 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.

One (1) mL DAB chromogen betazoid diluted with 100 mL betazoid susbtrate DAB buffer immediately before used. Sample incubated in DAB chromogen substrate above 30 mL per sample for 10 minutes, then washed with tap water. Mayer hematoxylin (counterstain) was added to the sample then incubated for 1-3 minutes, washed under tap water and dried. Furthermore, dipped in alcohol, dried and cleaned and then covered with entellan and 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. Positive rate result from Immunocytochemistry examination was analyzed by ANOVA with 95 % Confidence Interval (CI).

RESULT AND DISCUSSION

Cytotoxic test of curcumin

Test is intended to determine the cytotoxic of curcumin. Test performed by the MTT method. 3 - (4,5-dimetiiazol-2-i1) -2,5 difeniltetrazolium bromide (MTT) is absorbed into the cells and reduced the mitochondria-dependent reaction into formazan crystals that can be measured using ELISA reader spectophotometric at λ 595 nm. Dimethyl-sulfoxide (DMSO) which is used as a solvent of curcumin known to have no effect on vero cell death. Research using DMSO as a solvent in the cytotoxicity test against HSC-4 did not affect the cell growth[12,13].

Percentage living of vero cells after administration by curcumin can be viewed as the following graph.

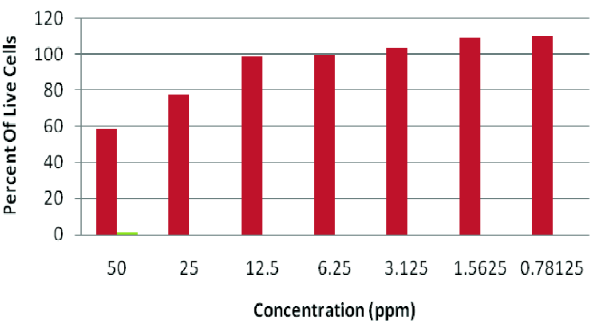

Figure 2. Percentage living vero cells after administration of curcumin

Figure 2. Showed the result of curcumin cytotoxic test against vero cells. The decrease of the concentration of curcumin was positively correlated with the percentage of living cells. The results showed cytotoxic concentrations of curcumin that safe to vero cells was 6.26 ppm is due to the results of the calculation of percent concentration of living cells has reached 100%.

In vero cell cultures treated with curcumin at rise concentrations was appears the vero cell

morphological changes. Vero cell death mechanisms at the molecular level as a result of administration of curcumin can't be known by the cytotoxicity assay. Reactivity curcumin was capable of interacting with cellular components such as DNA, membrane lipids and other cellular proteins that will affect biological processes in the cell such as cell cycle, metabolism and apoptosis[14]. Curcumin is known have the ability to stimulate apoptosis in various cancer cell culture[15]. Viewed lipophilic nature, curcumin easily related to the cell and modulate nuclear transcription factors or protein kinases, leading to caspase-activated DNAse enters the nucleus and degrade DNA[12]. Depth studies related to the apoptotic process can be performed to determine this.

Antiviral Test with SBPC immunocyto-chemistry

Antiviral test of curcumin is known through immunocytochemistry test. This immunocyto-chemistry using biotin labeled secondary antibody that can recognize the primary antibodies and using the enzyme labeled streptavidin conjugated with horseradish peroxidase and chromogen substrate mixture to detect antigens on the cell or tissue with a high sensitivity, so that the low level of antigen can be detected. Straptavidin Biotin Peroxidase Complex (SBPC) base reaction was a very strong bond between streptavidin and biotin.[16]

Test results showed SBPC immunocyto-chemistry with DSSE10 primary antibodies able to detect Dengue-2 virus infection at 1 day incubation that indicated by the light brown color wass formed and the 3 day incubation formed brown look older. Positive control, negative control were used to control the work. The specificity of immunocyto-chemistry should be validated with the negative control and positive control showed the antibody binding.[17] The immunocytochemistry assay results can be seen in figure 3 below.

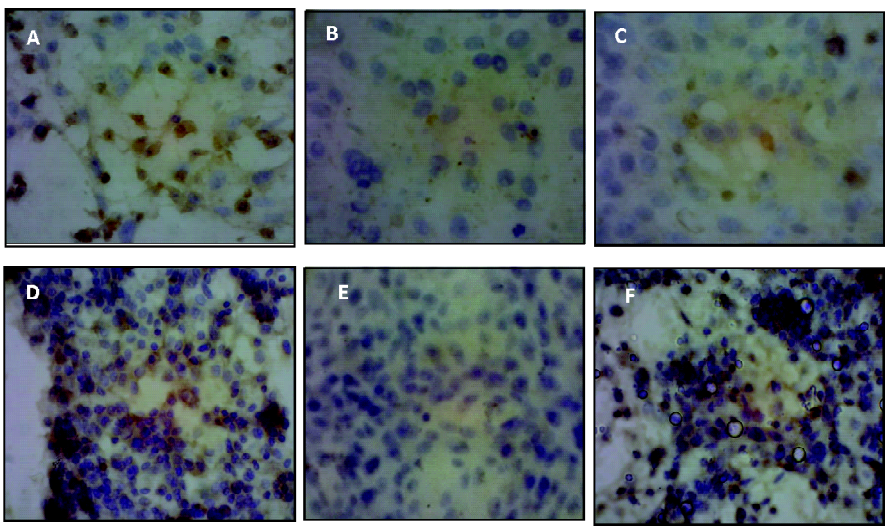

Figure 3. Microscopic photograph at a 40 x 10 magnification of vero cells infected by Dengue-2 virus were incubated 1 and 3 days and treated with curcumin examination by SBPC immunocyto-chemistry. One (1) days positive control (A), one (1) days negative control (B), curcumin treatment with 1 days incubation of Dengue-2 infection (C), 3 days positive control (D), 3 days negative control (E), curcumin treatment three 3 days incubation of Dengue-2 infection (F).

Figure 3. showed the effect of curcumin on vero cells infected Dengue - 2 incubation 1 and 3 days. At vero cell were incubated 1 day infection (both positive control (A) or treated with curcumin (C)) are visible positive reaction. A positive reaction seen by the presence of light brown colour on vero cell cytoplasm. Negative control 1 (one) day (B) and negative controls 3 (three) days (E) appears blue colour on cytoplasm. Sample which incubated 3 days of infection looks more positive reaction when compared with 1 day incubation infection. On microscopic observation brown color intensity in vero cells were incubated 3 days of infection seen dark brown colour on cytoplasm.

Immunocytochemistry results are qualitative methods, but very sensitive, specific and valid for Dengue diagnostic[18]. This method allows researchers to determine the sub -cellular compartement containing antigen. Widiastuti research (2011)[19] showed that the SBPC immunocytochemistry with DSSE10 antibodies have a high sensitivity and specificity diagnostic (100 % and 91 %).

The calculation of the positive rate.

The calculation of the positive rate results from SBPC immunocytochemistry using DSSE10 monoclonal antibody on vero cell looks like in table 1 below .

Table 1. Positive value rate vero cells infected by Dengue-2 incubation 1 day and 3 days

Incubation	Curcumin (6.25 ppm)	Positive Control
The first day	3.53 ± 2.03	14.55 ± 7.25
The third day	13.63 ± 8.506	21.5 ± 13.25

Table 1 shows the value of a positive rate of vero cells infected by Dengue - 2 incubation 1 and 3 days to be curcumin treatment. When compared to the positive controls seem a significant difference, so it can be interpreted that curcumin can decrease the positive rate values due to Dengue - 2 infection.

Curcumin is known to suppress viral replication with proteasome inhibitors. The endocytosis process for virus penetration was regulated by the Ubiquitin Proteasome System (UPS) . Ubiquitin Proteasome System has a role in replication, maturation and assembly of viruses. Provision of proteasome inhibitors such as MG123 is known to inhibit the production of virus[20]. Study of Dutta et al., (2009)[7] state that curcumin can decreased the viral particles of *Japanese encephalitis* through ubiquitin proteasome mechanism. Effect of curcumin administration against *Japanese encephalitis* was expected gives similar results when given in Dengue virus, because both types of virus are in the same type of flavivirus, but further research is needed to know about it.

CONCLUSION

The results showed that secure concentrations from cytotoxic of curcumin against vero cells is 6.25 ppm. The calculation of positive rate from immunocytochemistry in vero cells infected by Dengue - 2 incubation 1 and 3 days were the result is significantly different than the control, it means curcumin can lower positive rate due to Dengue-2 infection.

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e. ***Materials and methods*****:** The materials and methods should be clear enough to allow experiments to be reproduced. Previously published research procedure should be cited, and important modifications of it should be mentioned briefly. If the conducted research involved the use of human subjects or animal laboratory, it should be stated that the clearance from the Research Ethics Committee was obtained. The Editor may request a copy of the clearance document or informed consent form for verification.

f. ***Results and Discussion*****:** The Results should be presented with clarity and precision and explained without referring to the literature. The original and important findings should be stated. The Results should be illustrated with figures or tables where necessary but these should be kept to the minimum. The Discussion should interpret the findings in view of the results obtained against the background of existing knowledge. The Discussion should highlight what is new in the paper. Any assumption on which conclusions are made must be stated clearly

g. ***Conclusions:*** State the Conclusions in a few sentences at the end of the paper.

h. ***Acknowledgments***: The Acknowledgments should be presented at the end of the text and before the references. Technical assistance, financial support and advice may be acknowledged.

i. ***Tables:*** The tables should be kept to a minimum and be designed to be as simple as possible. Each table should be numbered consecutively in Arabic numerals and supplied

with a heading and a legend. Tables should be self-explanatory without reference to the text.

j. ***Figure:*** The figures should be numbered consecutively with Arabic numerals. Graphics should be prepared using applications capable of generating high resolution GIF, TIFF, JPEG or Powerpoint before pasting in the Microsoft Word manuscript file. The figures should be constructed in such a manner that they can be understood without reading the text. Appropriate symbols should be used on graphs and explained in the legends. Graphs should not duplicate results presented in tables. Title and comments of the figures and photographs should be provied on separate page using MS Word.

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..... it has been reported[1] .....

..... according to Sardjito[2] .....

..... Winstein & Swartz[3] conducted .....

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Sample References

Scientific Journal

1. *Standard journal article*

   You CH, Lee KY, Chey RY, Menguy R. Electro-gastro-graphic study of patients with unexplained nausea, bloating and vomiting. Gastroenterology 1980; 79(2):311-14.

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