Novel Antiviral Investigation of Annona squamosa Leaf Extract against the Dengue Virus Type-2: In Vitro Study


ABSTRACT

Introduction: Dengue virus (DENV) infection is general mosquito-transmitted viral taint. It can lead to the dengue hemorrhagic fever (DHF) or dengue shock syndrome (DSS). Dengue is a solemn illness with no endowed antiviral medication or recognized vaccine. Therefore, we aimed to investigate the activity of Annona squamosa leaf extract (ASLE) against dengue virus type-2 (DENV-2) isolated from Surabaya, Indonesia in 2013 (NCBI accession number: KT012509). Methods: In this study, the antiviral activity of ASLE was evaluated against DENV-2 in Vero cells using Viral ToxGlo™ Assay. In addition, we used CellTiter-Glo® Luminescent Cell Viability Assay to set the amount of viable cells in culture based on quantitation of the ATP. Results: DENV-2 replication inhibited by ASLE in Vero cells with IC50 = 73.78 μg/mL and SI = 4.49 when cells were treated two days after virus infection, whereas its CC50 for cytotoxicity to Vero cells was 331.54 μg/mL. Interestingly, this is the first report on the investigation of ASLE against DENV-2. Conclusion: In summary, ASLE demonstrated the antiviral activity against DENV-2 with less toxicity, and high possibility as a drug candidate. Therefore, it might be suggested for in vivo assessment in the progress of a potent antiviral against DENV-2.

Key words: Annona squamosa, Antiviral activity, DENV-2, Vero cells.

INTRODUCTION

Dengue disease is a crucial mosquito-borne viral infectious disease in sub tropical and tropical regions. Approximately, it reported about 350 million cases in global and more than 2.5 billion people are at high risk. Indonesia is a tropical country and it is common home of mosquito vector species of dengue virus (DENV), Aedes aegypti, and Aedes albopictus. The infection is caused by four DENV serotypes (DENV-1 to DENV-4) belonging to the Flaviviridae family. Up to now, there is no effective an antiviral available for this dengue disease. However, the vaccination now been trialed in several endemic countries, including Indonesia.

Indonesia is a huge country in Southeast Asia with high of flora diversity in the world. There are more than 5,000 medicinal plants that available all around us. Consequently, medicinal plants are utilized by people for curing various diseases, including for viral related diseases. Various medicinal plants were found as an antiviral compounds, such as Amaryllidaceae, Annonaceae, Euphorbiaceae, and many others. Annona squamosa leaves contain of several active compounds, such as phytosterols, flavonoids, alkaloids, saponins, glycoside, phenolic compounds, and tannins. Those compounds demonstrated the various therapeutic properties, such as an antitumor, antioxidant, antidiabetic, antimicrobial, antiviral, anti-inflammatory, and antimalogenic activities. However, there are no reports regarding Annona squamosa leaf extract (ASLE) activity as an antiviral effect against DENV. Therefore, we aimed to investigate the activity of ASLE against DENV-2 isolated from Surabaya, Indonesia in 2013.

MATERIALS AND METHODS

Chemical reagents

The chemical reagents used in this study were the Minimum Essential Medium Eagle (MEM) (Sigma-Aldrich, USA), ethanol (≥99.8%, Sigma-Aldrich, USA), trypsin-EDTA (Sigma-Aldrich, USA), dimethyl sulfoxide (DMSO) (Merck, Germany), penicillin-streptomycin (Gibco, USA), CellTiter-Glo® Luminescent Cell Viability Assay (Promega, USA), fetal bovine serum (FBS) (Sigma-Aldrich, USA), and Viral ToxGlo™ Assay reagents (Promega, USA).

Preparation of Annona squamosa leaf extract (ASLE)

Annona squamosa leaves were collected from Lumajang, East Java, Indonesia. Taxonomic identification of Annona squamosa was carried out by Purwodadi Botanical Garden, Indonesian Institute of Sciences, Purwodadi, Indonesia (approved with the reference number: 003/IPH.06.HM/VII/2017). The preparation of ASLE conducted according to Fadholly et al. (2020).
Preparation of Vero cells and DENV-2 isolate

The Vero cells was purchased from ATCC: The Global Bioresource Center, USA which cultured in medium containing 2% penicillin-streptomycin and 10% FBS (Gibco, USA) and incubated at 37 °C with 5% CO₂. A confluent monolayer of Vero cells was detached with trypsin-EDTA and incubated at 37 °C for 5 minutes. Furthermore, medium was added, pipet gently, and counted by using a hemocytometer (Paul Marienfeld, Germany). Cells were cultured into 96-well plate with density 1×10⁶ cells/10 mL and incubated at 37 °C with 5% CO₂. A confluent monolayer of Vero cells was detached with trypsin-EDTA and incubated at 37 °C for 5 minutes. Furthermore, medium was added, pipet gently, and counted by using a hemocytometer (Paul Marienfeld, Germany). Cells were cultured into 96-well plate with density 1×10⁶ cells/10 mL and incubated at 37 °C with 5% CO₂.

In this study, we used DENV-2 which originally isolated from Surabaya, Indonesia. We used a clinical DENV-2 isolate identified by the Dengue Study Group, Institute of Tropical Diseases, Universitas Airlangga (NCBI accession number: KT012513). Previous study revealed that all DENV-2 strains isolated in Surabaya were classified into Cosmopolitan genotype. The clinical isolates propagated and maintained as previously described by Kotaki et al. (2016). After titration of the virus isolate, the stocks were stored at -80 °C for further experiments.

Cytotoxicity assay

The confluent monolayers of Vero cells were prepared on a 96-well plate (1×10⁶ cells/mL) as previously described by Untoro et al. (2019). We used CellTitre-Glo® Luminescent Cell Viability Assay (Promega, USA) in method to set the number of cells culture based on the ATP quantitation. Furthermore, CellTitre-Glo® Luminescent Cell Viability Assay was addressed for cytotoxicity test by following the manufacturer’s guidelines.

Antiviral activity assay

The confluent monolayers of Vero cells were prepared on a 96-well plate (1×10⁶ cells/mL), and the titer of DENV-2 was added in 2×10⁴ FFU/well. In this study, we used Viral ToxGlo® Assay (Promega, USA) as a method intended to identify the cytopathic effect induced by a viral infection. In addition, we used Viral ToxGlo® Assay reagents following the manufacturer’s guidelines. Furthermore, the calculation of the selectivity index (SI) was defined as the comparison with ratio of 50% cytototoxic concentration (CC₅₀) and 50% antiviral concentration (IC₅₀) as previously stated by Zandi et al. (2012).

RESULTS AND DISCUSSION

Dengue is a mosquito-borne disease whose broad incidence has upward dramatically in the last ten years. Indonesia is one of the greatest dengue-endemic nations in the worldwide which approximately 100,000 incidences of dengue are informed per annum. The East Java, West Java, and Central Java are considered for being the most prevalent province for dengue.

Interestingly, in this study, we used DENV-2 isolate (NCBI accession number: KT012513) from the previous study conducted by Kotaki et al. (2016). It had a higher ratio of illness severity as compared to the other serotypes, such as DENV-1, DENV-3, and DENV-4 in Brazil. Recently, secondary infection with DENV-2, DENV-3, and DENV-4 increased the risk of severe dengue infection within Southeast Asia. Furthermore, DENV-2 in vitro proliferation was already established and standardized. In addition, we rendered molecular phylogenetic modeling and tree visualization by applying Molecular Evolutionary Genetics Analysis X (MEGA X) software with the maximum likelihood method based on the envelope glycoprotein gene from Indonesia and other countries (Figure 1). The phylogenetic tree was validated by performing the analysis on 1000 bootstrapped input datasets and cross-referencing with the Tamura-Nei substitution model.

Nowadays, the use of traditional herbal medicines to manage various illness is accretion globally. Medicinal plants are a potential source for the development of new antiviral drugs. The plants contained of various chemical composition with the potency to prevent viral replication and control the viral infection. Plants have been considered to have an antiviral action and some have been accustomed to manage viral taints in humans and animals. In brief, the member of Annonaceae, Zingiberaceae, Cucurbitaceae, Fabaceae, Myrtaceae, Caricaceae, Meliaceae, Poaceae, Acanthaceae, Euphorbiaceae, Halymeniaceae, Piperaceae, and many other families have been reported as an anti-DENV. However, up to now, there is no effective antiviral or vaccine available for the dengue disease.

The research revealed that ASLE inhibited the DENV-2 in Vero cells with IC₅₀: 73.78 μg/mL, CC₅₀: 331.54 c, and SI: 4.49 (Figure 2). Furthermore, we tested the ASLE activity in various concentrations and observed that ASLE exhibited low cytotoxicity effect within all evaluated concentrations (viability >50%) (Figure 3). In detail, we used various extract concentrations, such as 200 μg/mL, 100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, and 6.25 μg/mL. The treatment of ASLE showed that the reduction of DENV-2 replication is demonstrated by the lowest concentration of extract (6.25 μg/mL). Our findings showed that ASLE exhibited the consequential antiviral action against DENV-2 in Vero cells. Furthermore, this study also suggested the leading antiviral activity of ASLE is possible in consequence of its action towards the stages of intracellular replication of the virus in place of the early stages of its replication sequence such as virus entry. Nevertheless, the completed frameworks of the activity required to be discovered for an anti-DENV medication. Further investigation might be applied by using the suitable model, for example cultured human cells.

Annona is one of 129 genera of the Annonaceae family which contained of 119 species. Annona squamosa is commonly called by people
by interfering the attachment and reducing the viral replication. The inhibition mechanism of the extract was performed that the most effective concentration of ASLE showed the availability of acetogenins, alkaloids, flavonoids, as sri kaya (Indonesia) or sugar apple (English). It is also known for its delicious sweet taste. The Province of East Java, Indonesia is considered as the endemic of Annona squamosa even though it is widely distributed to all the regions. The fruit is marketed as family fruit for daily consumption or as a source of the other food products in the home industry. On the other hand, phytochemical screening of ASLE showed the availability of acetogenins, alkaloids, flavonoids, glycosides, phenolic, saponins, steroids, tannins, terpenoids, and many other compounds. Therefore, Annona squamosa considered as the medicinal plant for many diseases, such as cancer, cardiac disease, diabetes, mucous diarrhea, and many other diseases.

According to the ASLE, previous research revealed that the cytotoxic effect of chitosan-based nanoparticles of ASLE on cervical cancer cells (HeLa) had an IC₅₀ value with 344.48 µg/mL in concentration. Even though, many studies on the biological effects of Annona squamosa were conducted, there are only two reports have shown Annona squamosa as an antiviral against HIV and avian influenza virus. Furthermore, Lisina and Piramanayagam (2014) also demonstrated its binding properties by the in silico study between the HIV protease and ASLE: Annona squamosa leaf extract; ATP: Adenosine triphosphate; CC₅₀: 50% cytotoxic concentration; CO₂: Carbon dioxide; DENG: Dengue virus; DENV-2: Dengue virus type-2; DMSO: Dimethyl sulfoxide; EDTA: Ethylenediaminetetraacetic acid; FBS: Fetal bovine serum (FBS); HIV: Human immunodeficiency virus; IC₅₀: 50% antiviral concentration; MEGA X: Molecular Evolutionary Genetics Analysis X; MEM: Minimum Essential Medium Eagle; SI: Selectivity index.

Figure 3: Evaluation of the anti-DENV-2 activity of ASLE in Vero cells. A: 6.25 µg/mL of ASLE; B: 12.5 µg/mL of ASLE; C: 25 µg/mL of ASLE; D: 50 µg/mL of ASLE; E: 100 µg/mL of ASLE; F: 200 µg/mL of ASLE; G: Control.

CONCLUSION

In summary, ASLE demonstrated the antiviral activity against the DENV-2 with less toxicity, and high possibility to be considered as a drug candidate. Therefore, in vitro assessment might need to be conducted for further DENV-2 antiviral identification.

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DISCLOSURE STATEMENT

The authors have no conflicts of interest to declare.

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17. Ansori ANM, et al.: Novel Antiviral Investigation of Annona squamosa Leaf Extract against the Dengue Virus Type-2: In Vitro Study


GRAPHICAL ABSTRACT

Dengue Virus Type-2 (DENV-2)

Annona squamosa Leaf Extract (ASLE)

Cytotoxicity and Antiviral Activity

> Viral ToxGlo Assay
> CellTiter-Glo Luminescent Cell Viability Assay

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