# ANTI-DENGUE VIRAL ACTIVITY OF *Carica papaya* LEAVES EXTRACT

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## ABSTRACT

Dengue fever is an infectious tropical disease, which is considered a worldwide public health problem. However, until today no licensed vaccine is approved for dengue virus. The effort to develop dengue vaccine is made complicated by the four closely related dengue virus serotypes. Nowadays, the search for anti-dengue viral activities in natural plant products has been increasing. Thus, the development of plant-based antiviral agent will have the potential to fight against dengue fever. This study aimed to investigate the anti-dengue activity of *Carica papaya* leaves extracts and its fraction as well as determination of possible active compound and mechanism involved. The analyze of polysaccharide, glycosaponin and total protein were carried out by standard methods. Viability of Vero cells treated with aqueous extract of *Carica papaya* leaves was estimated by neutral red uptake assay. The microscopic observation on cytopathic effect was observed to determine the highest tolerable dose of *Carica papaya* leaves extract in Vero cells. The cytotoxicity of *Carica papaya* extracts on Vero cells was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method. Antiviral assays of aqueous extract of *Carica papaya* leaves were performed in three different stages, pre-, post- and continuous treatment on Vero cells against DENV-2 by foci forming unit reduction assay. Further antiviral assays of *Carica papaya* leaves crude extract and its fraction was investigated. Foci reduction on DENV-2 infected cells treated with the extracts for post- and continuous treatment were determined. Number of RNA reduction of DENV-2 was investigated by SYBR® green quantitative reverse transcription polymerase chain reaction method. Finally, the possible active compounds in the extracts was carried out using high performance liquid chromatography (HPLC) analysis. In this study, the results showed that the methanol matured leaves contained the highest concentration of total polysaccharide than aqueous matured leaves with 0.23 $mgmL^{-1}$ and 0.22 $mgmL^{-1}$ respectively. In addition, glycosaponin content in methanol matured leaves was the highest followed by methanol young leaves with 82% and 75% respectively, as well as in total protein content of 35.9% and 29.6% respectively. The extract did not reveal cytotoxic effects on Vero and C6/36 cells at all tested concentrations as well as its fractions. However, the methanol and ethanol extracts were found to be more toxic than the water extract. Anti-adsorption effect was shown at $IC_{50}$ = 435.3 $\mu gmL^{-1}$ with SI = 7.6. The extract also inhibited DENV-2 replication in Vero cells with $IC_{50}$ = 1413 $\mu gmL^{-1}$ and SI = 2.3 when added after adsorption to the cells. The $IC_{50}$ against DENV-2 was 137.6 $\mu gmL^{-1}$ and SI = 23.9 when cells were treated 5 hours before virus infection and continuously up to four-day post-infection. From the HPLC analyze, the possible active compound of catechin, quercetin and cinnamic acid were found in the water extract of *Carica papaya* leaves. In conclusion, the *Carica papaya* leaves extract showed slight inhibition of DENV-2 replication by decreasing foci number and size. The aqueous extract of *Carica papaya* leaves possesses the ability of inhibiting the activity of DENV-2. Thus, this extract is worth to be further investigated and might be advantageous as a primary source in the treatment of dengue and as a potential element for drugs formulation.

## ABSTRAK

Demam denggi adalah penyakit tropika berjangkit, yang dianggap sebagai masalah kesihatan umum di seluruh dunia. Walaubagaimanapun, sehingga hari ini tiada vaksin berlesen diluluskan untuk virus denggi. Usaha untuk membangunkan vaksin denggi adalah rumit disebabkan oleh empat serotip virus denggi yang berkait rapat. Pada masa kini, pencarian aktiviti anti-denggi dalam produk tumbuhan semulajadi semakin meningkat. Oleh itu, pembangunan agen antiviral berasaskan tumbuhan akan mempunyai potensi untuk melawan demam denggi. Kajian ini bertujuan untuk mengkaji aktiviti anti-denggi dari ekstrak daun betik (*Carica papaya*) dan pecahannya serta penentuan kemungkinan sebatian dan mekanisme aktif yang terlibat. Analisis polisakarida, glikosaponin dan jumlah protein dilakukan dengan kaedah piawai. Daya tahan sel Vero yang dirawat dengan ekstrak air daun *Carica papaya* dianggarkan dengan esei pengambilan neutral merah. Pemerhatian mikroskopik pada kesan sitopatik diperhatikan untuk menentukan dos tertinggi daun *Carica papaya* yang boleh ditoleransi dalam sel Vero. Sitotoksisiti ekstrak *Carica papaya* pada sel Vero ditentukan oleh kaedah 3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolium bromida. Pemeriksaan antiviral ekstrak air daun *Carica papaya* telah dilakukan dalam tiga peringkat rawatan berlainan, sebelum, selepas dan berterusan pada sel Vero terhadap virus DENV-2 melalui esei pengurangan unit pembentukan foci. Pemeriksaan lanjut antiviral ekstrak mentah *Carica papaya* dan pecahannya disiasat. Pengurangan foci terhadap sel-sel yang dijangkiti DENV-2 yang dirawat dengan ekstrak untuk rawatan selepas dan berterusan ditentukan. Bilangan pengurangan RNA DENV-2 disiasat oleh kaedah SYBR® hijau kuantitatif transkripsi terbalik tindak balas rantai polimer. Akhirnya, kemungkinan sebatian aktif dalam ekstrak dilakukan dengan menggunakan analisis kromatografi cecair berprestasi tinggi (HPLC). Dalam kajian ini, keputusan menunjukkan bahawa daun matang metanol mengandungi kepekatan tertinggi polisakarida daripada daun matang akues masing-masing dengan 0.23 $mgmL^{-1}$ dan 0.22 $mgmL^{-1}$. Di samping itu, kandungan glikosaponin dalam daun matang metanol adalah yang tertinggi diikuti oleh daun muda metanol masing-masing dengan 82% dan 75%, serta jumlah protein masing-masing sebanyak 35.9% and 29.6%. Ekstrak itu tidak mendedahkan kesan sitotoksik pada sel Vero dan C6/36 pada semua kepekatan yang diuji serta pecahannya. Walaubagaimanapun, ekstrak metanol dan etanol didapati lebih toksik daripada ekstrak air. Kesan anti-penjerapan ditunjukkan pada $IC_{50}$ = 435.3 $\mu gmL^{-1}$ dengan SI = 7.6. Ekstrak ini juga menghalang replikasi virus DENV-2 dalam sel Vero dengan $IC_{50}$ = 1413 $\mu gmL^{-1}$ dan SI = 2.3 apabila ditambahkan selepas penjerapan ke sel. $IC_{50}$ terhadap DENV-2 adalah 137.6 $\mu gmL^{-1}$ dan SI = 23.9 apabila sel dirawat selama 5 jam sebelum jangkitan virus dan berterusan sehingga empat-hari selepas jangkitan. Dari analisis HPLC, sebatian aktif katekin, kuersetin dan asid sinamik yang terdapat dalam ekstrak air daun *Carica papaya*. Sebagai kesimpulan, ekstrak daun *Carica papaya* menunjukkan sedikit perencatan replikasi DENV-2 dengan mengurangkan bilangan dan saiz foci. Ekstrak air daun *Carica papaya* mempunyai keupayaan merencat aktiviti DENV-2. Oleh itu, ekstrak ini adalah bernilai untuk disiasat lebih lanjut dan mungkin berfaedah sebagai sumber utama dalam rawatan denggi dan sebagai unsur berpotensi dalam formulasi ubat.

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| | | |
|---|---|---|
| Ab | - | Antibody |
| ADE | - | Antibody Dependent Enhancement |
| AML | - | Aqueous Mature Leaves |
| AYL | - | Aqueous Young Leaves |
| CMC | - | Carboxymethyl Cellulose |
| CPE | - | Cytopathic Effect |
| DAB | - | 3'-diamiobenzidine |
| DENV | - | Dengue Virus |
| DHF | - | Dengue Hemorrhagic Fever |
| DMSO | - | Dimethyl Sulfoxide |
| DPPH | - | 2,2-diphenyl-1-picrylhydrazyl |
| DSS | - | Dengue Shock Syndrome |
| FBS | - | Fetal Bovine Serum |
| FFURA | - | Foci Forming Unit Reduction Assay |
| HPLC | - | High Performance Liquid Chromatography |
| HTD | - | Highest Tolerated Dose |
| MML | - | Methanol Mature Leaves |
| MNTD | - | Maximum Non-Toxic Dose |
| MYL | - | Methanol Young Leaves |
| m.o.i | - | multiplicity of infection |
| MTT | - | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NRU | - | Neutral Red Uptake |
| O.D | - | Optical Density |
| PBS | - | Phosphate-Buffered Saline |
| PR | - | Protease |
| P/S | - | Penicillin/Streptomycin |
| RNA | - | Ribonucleic Acid |
| RT-PCR | - | Reverse Transcription Polymerase Chain Reaction |
| SPE | - | Solid Phase Extraction |
| TPB | - | Tryptose Phosphate Broth |

# CHAPTER 1

# INTRODUCTION

## 1.1 Dengue Research Background

In recent decades, dengue fever has emerged as the most important viral disease of human and become major international public health concern. More than 100 countries worldwide have been affected by dengue fever (DF) and dengue hemorrhagic fever (DHF) (Sanyaolu *et al.*, 2017). Every year, up to 400 million people are infected mainly in tropical and sub-tropical regions around the world (Dos Santos *et al.*, 2019) including Southeast Asia. America and Asia have been expected with the highest risk zones of dengue transmission due to densely populated humans and vectors and surrounding climate suitability, with Asia bears up to 70% of this burden (Bhatt *et al.*, 2013). Until date, there is no specific anti-dengue therapeutic, but self-supportive care and specialized medical care for serious illness is very effective.

However, for dengue hemorrhagic fever, if not treated properly, it has a mortality rate as high as 10-20% (Zakaria *et al.*, 2019). A single dengue vaccine, Dengvaxia®, are first licensed vaccine for the prevention of dengue disease. However, due to its dengue-specific complexities, their long-term effectiveness against all four dengue virus serotypes still have an adverse effect on production (Godói *et al.*, 2017). The clinical development pathway brings a diverse of risk to all vaccine development efforts especially which were dengue-specific (Thomas and Yoon, 2019). Furthermore, this vaccine is no longer available due to the delay in the use of Dengvaxia® after some findings on the side effects of this vaccine in humans (Zakaria *et al.*, 2019).

Dengue fever, the tropical infectious disease has been rapidly expanding over the world. Dengue cases in Malaysia has becomes seriously increasing since the first reported case in 1902. The epidemic is not limited only in urban areas, but also involve rural areas in Malaysia. The awareness of dengue fever irrespective of age, led to the search for the best way to fight this virus. The current prevents of dengue infections are only in general based on public awareness such as vector control and surveillance alert. However, periodic vector control has not been able to stop its rapid emergence and global spread. To keep the infection rates low and to prevent outbreaks, some medical treatments have been practiced such as liquid injection for reducing body temperature and to avoid prolonged fever.

There is strong need of effort towards the development of safe and effective treatment to cure dengue. However, efforts to develop antiviral agents for dengue have encountered some difficulties. It is currently unclear mechanism since the four closely related, but antigenically distinct serotypes of dengue virus often undergo mutations. Therefore, antiviral would have to be effective against all the serotypes. Until now, there is no licensed, commercially available vaccines or specific therapeutics for dengue virus. Although numerous groups have already made vaccine formulation, they are still experiencing long-term problems and are still undergoing clinical trials to develop a safe, affordable and effective vaccine against all serotypes. In addition, any other possible treatments including traditional medicines should be investigated to test their effectiveness in controlling this problem.

Since ancient time, human has used plant for medicinal properties. Indian integrative medicine such as Rigveda, Atharvaveda and Ayurveda have categorized plants for drugs, essence, food, poisons and agricultural purpose (Jain *et al.*, 2008). Research efforts to explore the potential of new agents for antivirus increased in recent years because of low toxicity and high selective antiviral substances from products of plant origin (Newman and Cragg, 2016).

Medicinal plants are widely used to prevent infectious diseases and inflammation and has been supported by a lot of clinical evidence (Borchers *et al.*, 1997). Evidence linking plants to have antiviral properties are like *Parietaria diffusa* and *Urtica dioica* against Feline immunodeficiency virus (Uncini Manganelli *et al.*, 2005) and olive leaf extract inhibit spread of HIV-1 (Lee Huang *et al.*, 2003; Bao *et al.*, 2007).

In many part of the world, the leaves, fruits and latex are widely used traditionally to treat various types of ailments like asthma, rheumatism, fever, diarrhea, boils and hypertension (Zakaria *et al.*, 2006). Based on previous studies, this species have antibacterial, antifungal, anthelminthic and antimalarial properties (Melariri *et al.*, 2011) which these properties show that the activity of *Carica papaya* leaves extract against dengue infection.

Furthermore, a human study conducted by Ahmad *et al.* (2011) and Kala (2012) showed that juice of *Carica papaya* leaves can cure dengue fever. However, the interactions mechanism of *Carica papaya* leaves extract on dengue-infected cells are still remain unknown. Therefore, this study hypothesized that *Carica papaya* leaves extract can inhibit dengue by decrease the viability and change the morphological of dengue virus by certain active compound in the extracts. To prove that the cell culture study will be conducted and the interactions of *Carica papaya* leaves extract with dengue-infected cells will be investigated.

## 1.2 Problem Statement

Dengue fever remains a significant public health concern in Malaysia and was established in Malaysia ever since the first reported case of dengue in 1902 (Ahmad *et al.*, 2018). Rapid urbanization, climate changes and abandoned areas results in vector breading causes rise in dengue outbreaks. Despite reactive efforts by the government in Malaysia, dengue cases continue to increase from years to years. Unfortunately, there are no specific therapeutics agents, and no vaccine commercially against dengue.

New anti-dengue drug leads are therefore urgently needed. Traditional healers have long used plants to prevent or cure infections. It is important that anti-dengue drug development has to be pursued further, with the highly active products, to preclinical and clinical testing.

In Malaysia, *Carica papaya* leaves juice have been used traditionally in folk medicine to cure dengue fever. This is possibly due to the presence of a wide variety compounds in juice of *Carica papaya* leaves known for their antioxidant and anti-inflammatory effect (Sudhakar and Theivanai, 2014; Gupta *et al.*, 2017). However, there is limited scientific evidence for its anti-dengue activity *in-vitro*.

The underlying mechanism and responsible compounds that act as potential anti-dengue agents remains unknown. Moreover, it is not known whether each fraction of *Carica papaya* leaves extract is able to give a similar positive effect to inhibit dengue virus and which compound of *Carica papaya* leaves fractions having a high anti-dengue inhibition. Therefore, this project was carried out in order to yield a novel insight and better understanding on how *Carica papaya* leaves extract and its fraction can inhibit dengue virus and likely lead to the development of anti-dengue supplement.

## 1.3 Objectives of the Study

The objectives of study were:

1. To evaluate the *in vitro* inhibitory potential of *Carica papaya* leaves extract and its fraction towards dengue virus infection type-2 (DENV-2).
2. To determine the mechanism of dengue inhibitory activities of *Carica papaya* leaves extract and its fraction.
3. To determine the possible active compounds which responsible for anti-dengue properties.

## 1.4 Scope of Study

The scope of this research are as listed below:

1. Extraction of *Carica papaya* leaves extract using Soxhlet extraction method. Water, methanol and ethanol were used as a solvent.
2. Phytochemical screening of primary metabolites (polysaccharides and glycosaponins) in *Carica papaya* leaves extract by standard methods of Malaysian Standard MS 2409:2011.
3. Fractionation of *Carica papaya* leaves extract using solid phase extraction (SPE).
4. Evaluation of cytotoxicity profile of *Carica papaya* leaves extract on different cell lines (Vero and C6/36 cells) by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (MTT).
5. Determination of antiviral activities of *Carica papaya* leaves extract against dengue-infected Vero and C6/36 cell line by foci forming unit reduction assay (FFURA).
6. Detection and quantification of viral RNA in the plasma by viral RNA quantification using quantitative transcription polymerase chain reaction analysis (qRT-PCR)
7. Determination of possible anti dengue active compound in *Carica papaya* leaves extract and its fraction using HPLC analysis.

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## LIST OF PUBLICATIONS

### Journal with Impact Factor

1. **Abd Kadir, S. L.**, Ya'akob, H. & Mohamed Zulkefli, R. (2013). Potential anti-dengue medicinal plants: A review. *Journal of Natural Medicines, 67* (4), pp. 677-689. doi: 10.1007/s11418-013-0767-y **(IF: 1.966)**

### Non-Indexed Conference Proceedings

1. **Abd Kadir, S. L.**, Ya'akob, H., Mohamed Zulkefli, R. and Moghaddam, E. (2014) Screening of antiviral activity in *Carica papaya* aqueous extract on dengue virus type-2. *5th International Conference on Biotechnology for the Wellness Industry*. Kuala Lumpur.

2. **Abd Kadir, S. L.**, Ya'akob, H., Mohamed Zulkefli, R. and Moghaddam, E. (2014) The effects of *Carica papaya* leaves extract against dengue virus infected Vero cells. *2014 Asian Conference on the Life Sciences and Sustainability.* Hiroshima, Japan.

3. Ya'akob, H., Mohamed Zulkefli, R., Abd Rashid, S. N. A., Zainol, N. A., Sadek, N. A., Aman Nor, N. F., **Abd Kadir, S. L.,** Shaiful Amri, A. S., and Nik Mat Daud, N. N. N. (2015) Anti-Dengue: A novel Polyherbal anti-dengue capsule. *17th Industrial Art and Technology Exhibition (INATEX) 2015.* Universiti Teknologi Malaysia, Johor.

4. Shaiful Amri, A. S., Ya'akob H., **Abd Kadir, S. L**., and Husin, F. (2016) Acute oral toxicity study and anti-viral properties of Carica papaya leaves extract against DENV. *6th International Conference on Biotechnology for the Wellness Industry*. Melaka.

5. **Abd Kadir, S. L.**, Ya'akob, H., Shueb, R. H., Shaiful Amri, A. S. and Husin, F. (2016) Cytotoxic effects of *Carica papaya* leaves aqueous extract on C6/36 and Vero 76 cell line. *Asia Dengue Conference.* Kuala Lumpur.

6. Shaiful Amri, A. S., Ya'akob H., **Abd Kadir, S. L.**, and Husin, F. (2016) Prototype development of anti-dengue supplement from Carica papaya leaves, polyherbal extract and virgin coconut oil. *Asia Dengue Conference.* Kuala Lumpur.