INTERACTION OF CARICA PAPAYA LEAVES OPTIMUM EXTRACT ON VIRUS DENGUE INFECTED CELLS

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ABSTRACT: *Dengue fever is the most prevalent mosquito-borne disease in the tropics and the subtropics and becomes morbidity and mortality. Recently, there are no effective drugs or vaccine accepted against dengue. Nowadays, the development of plant-based medicine becomes important since plant has active biological compounds. The objective of this study were to investigate the cytotoxicity and in vitro antiviral activity of extracts on DENV-2 in Vero cells using Carica papaya dried leaves (CPD) extract with optimize extraction parameter. The extraction parameter involved were solid material to solvent ratio, time of extraction and amplitude of sonication which were analysed using Central composite design and the relationship between parameters were analyzed using ANOVA. Results showed that extraction parameters significantly (P<0.05) affect the yield of extraction while the F-value of 6.43 implies the ANOVA model is fit for purposes. The IC50 value for Carica papaya was 137.6 µgmL-1 and its selective index value was 75.85. Extract of Carica papaya reduced in foci formation via inhibitory effects on dengue infected cells, therefore promotes anti-viral activity.*

Keywords: Carica papaya, dengue, optimization, anti-viral activity, Vero cells

1. INTRODUCTION

Natural medicines have been demand than synthetic drugs since people are now beginning to be aware of its side effects and start looking for a natural remedy because they believe it is safe and non-toxic [1]. Medicinal plants contain active biological compounds that respond to prevent or cure diseases or helps promote the health of the body. Since time immemorial, many infectious diseases have been treated with the use of materials particularly natural herbs and medicinal plants. Furthermore, it is estimated that 80% of developing countries still rely on traditional medicines and make the plant-based materials as a source in the development of pharmaceutical instead of chemical drugs. Malaysia is an immense depository of medicinal plants that are used in traditional treatment. There is rapidly growing demand for natural medicines among Malaysian population.

Carica papaya, commonly known as "papaya or pawpaw" belongs to the family Caricaceae, have been used as medicine to treat various disease [2-3]. It was widely cultivated in tropical countries such as India, Bangladesh, Indonesia, the Philippines, Sri Lanka and the West Indies including Malaysia [4]. Many previous studies investigate the biological activities in papaya plant parts including leaves, shoots, fruits, flowers, seeds, bark, roots and latex. Papaya leaves have been widely used as folk medicine for centuries. It has been revealed to be potentially for treatment of cancer since it reported to exhibits the of anti-tumour activity and immunomodulatory effects [5]. Recent studies showed its biological importance as an antioxidant [6], wound healing [7]. Moreover, the aqueous extract of papaya leaves taken by dengue-infected patients has been increased the platelet count [4], white blood cells and neutrophils [6-7]. Although papaya leaves known to be non-toxic and safe for oral consumption [8-9], there is still no information on their cytotoxicity.

Dengue is an infectious disease caused by dengue virus (DENV), transmitted to humans by *Aedes aegypti* and *Aedes albopictus* mosquito bites [8]. DENV belongs to the genus name *Flavivirus*, family Flaviviridae, that cause human disease and parts of human pathogens [10-11]. It has four antigenically closely related serotypes of DENV-1, -2, -3 and -4 which caused a wide range of illness from mild dengue fever (DF) to severe dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS) [8, 12-13]. Until today, there is no specific drug or vaccine for dengue fever [1]. Vaccine development becomes difficult since not all four serotypes provide cross-protection immunity against each other. One key concern surrounding the utility of antiviral treatment for dengue is the rapid increased in platelet count and decline in viremia during the viral infection. Studies report that viremia levels were decreased 94-98 per cent as infections progressed from serum samples collected 0-2 days after fever onset to 3-5 days after fever onset [14]. The study was conducted to investigate the inhibitory potential *Carica papaya* extracts to inhibit DENV-2 replication on Vero cells as confirmed by cytotoxicity and antiviral assay.

EXPERIMENTAL DETAILS

Plant materials and extraction

Fresh leaves of *Carica papaya* collected locally in Taman Universiti, Skudai Johor were washed and separated from the midrib and extracted using a blender. A ratio of 3:5 was made by homogenized *C. papaya* leaves with distilled water. The resulting juice was then subjected to lyophilisation to produce powder forms of the extracts and stored in airtight bottle at 4ºC.

Extraction of *Carica papaya* leaves using Ultrasound Assisted Extraction Method

The extraction method of *Carica papaya* dried leaves (CPD) was referred to Vuong *et al.* (2014) [15] with some enhancement by using ultrasonic assisted extraction. The experiment was carried out based on one factor at a time (OFAT) method to determine points for a ratio of solid material to solvent, time of extraction and amplitude of sonication which yield the optimal *C. papaya* leaves extract in term of percentage yield. The point for each parameter was

selected to be used further in the optimization process as the central point.

For the extraction process of CPD, the range for solid to solvent ratio, the amplitude of sonication and time of extraction were chosen from the result of the preliminary study. The factor level of each processing parameters is shown in Table 1 as follows:

Table 1.0 Factor level of each processing parameters

Factor Name	Factor Levels
Solvent to raw material ratio (g/g)	1:10 - 1:20
Amplitude of Sonication	20 - 40
Time of Extraction (min)	2 - 4

The experiment was run according to Central Composite Design (CCD) under Response Surface Methodology (RSM) scope that was developed by Design Expert (Version 7.0.0) with 20 experimental runs and using three factorial variables to optimize the extraction of CPD. The weighed samples were soaked in water according to the ratio for each run. Next, the samples were sonicated using ultrasonic probe sonicator (Fisher Scientific, United States) in a closed system by covering the flask with aluminium foil to prevent from loss of active compounds and maintaining the solvent to solid ratio towards the experiment as suggested by Wahid *et al.,* (2010) [16]. The CPD aqueous samples were filtered and further lyophilized using freeze dryer. Table 1.1 shows the non-coded and coded levels of independent variables involved in this study.

Table 1.1 Non-coded and coded levels of independent variables of experimental design

Coded Variables Level (Z_i)	Non-coded Variables Level		
	Ratio of solid to solvent (g/g) X_1	Amplitude of Sonication X_2	Time of Extraction X_3
+1	1:10	20	2
0	1:15	30	3
-1	1:20	40	4

Determination of Percentage Yield *C. papaya* leaves Extract

The yield of freeze-dried for CPJ was calculated based on the dry weight basis using the following equation 1:

$$\text{Yield (\% w/w)} = \frac{Mass\ of\ freeze{-}dried\ extract\ (g)}{mass\ of\ dried\ leaves\ (g)} \times 100$$

Preparation of extracts

For the cytotoxicity and antiviral assays, a stock solution was prepared by dissolving 2.0 g of extract in 100 mL of cell medium. The stock solution was filtered and sterilised (0.20 µm pore, Minis- art) then further diluted with culture medium to the desired concentration for the assays.

Preparation of medium

Powdered Eagle's minimum essential medium (EMEM: Sigma) was used in this study. A total of 3.7 g of sodium bicarbonate was added, dissolved with 1 L of ultrapure water and the pH of the medium was adjusted to 7.0. The medium was then filter sterilized using 0.22 µm PES membrane filter (TPP, Switzer- land) under vacuum condition.

Cells and virus

Vero cell from the African green monkey kidney cells (ATCC No. CCL-81) was used for the MTT and foci forming unit reduction (FFURA) assay were propagated and maintained in the growth media containing 1× Eagle's minimum essential medium (EMEM: Sigma) supplemented with 10% fetal bovine serum (FBS: Gibco, NY, USA) and incubated at 37ºC with 5% CO_2 [17]. For virus concentration, 2 mL of Dengue virus type-2 (DENV-2) New Guinea C strain (NGC) working solution was prepared by diluted 20 µL virus stock with 1960 µL of 1× EMEM with 2% FBS prior to antiviral assay.

Determination of cytotoxicity

The extract was subjected to toxicity studies to find out the maximum non-toxic dose which could not toxic to the Vero cells. The assay was carried out by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT: Promega, WI, USA), the dye will be reducing to colour formazan by viable cells [18] . The desired concentration of *C. papaya* leaves aqueous extract were added to a monolayer of Vero cells in 96-wells cell culture microplate (Corning, NY) in triplicate. The treated cells were then incubated at 37ºC with 5% CO_2 for four days and MTT assay was then performed. The optical density of each well was measured at 570 nm using 96-well plate reader (TECAN, Switzerland).

Determination of antiviral activity

In vitro antiviral effects of extracts was evaluated in Vero cells by foci forming unit reduction assay (FFURA) [13]. To determine the effects of continuous treatment, different concentrations of each extract were added to the Vero cells, 5 h pre-infection and continuously for 4 days post-infection. After 5 h of pre-infection treatment, 200 FFU of DENV-2 was inoculated into a confluent monolayer of Vero cells and incubated at 37℃ for 1 h. After 1 h of viral adsorption, the cells were washed with PBS to eliminate the unabsorbed viruses and overlay medium of 3% of carboxymethyl cellulose (CMC: Sigma-Aldrich, USA) containing 2× EMEM with 4% FBS was added 500 µL of the desired final concentration of the extracts was added to each well. 500 µL of an equal volume of 2× EMEM, 4% FBS with 3% CMC was added into the control wells (-v). All of the treatment was conducted in duplicate. Plates were incubated at 37ºC with 5% CO_2 for four days to allow viral foci to form [17].

Foci Forming Unit Reduction Assay

After four days post-infection, viral foci were visualized using peroxidase-based foci staining assay [13]. Briefly, the cell culture medium was discarded and washed with PBS three times. 10% paraformaldehyde was added to fix the cells for 30 min. Cells were washed three times with PBS. 1% NP40 or IGEPAL (Sigma, St. Louis, USA) was added to permeabilize the cell for 10 min following by washing with PBS three times. Cells were blocked with 3% skim milk solution (MP Biomedicals, France) prepared in PBS for 2 h. All treatment was done at room temperature. After another three times washing with PBS, the cells were incubated with dengue hyperimmune serum (Merck, USA) diluted in 1:500

using 1% skim milk solution at 37 ºC for 1 h. Cells were then washed three times with PBS and incubated with goat anti-mouse IgG conjugated with peroxidase (Merck, USA) at a final concentration of 1:250 in 1% skim milk solution. Finally, 3'-diaminobenzidine peroxidase substrate (DAB: Thermo Scientific, USA) was added to each well to stain the virus foci in dark room for 20 min following three times washing with distilled water and drying. DENV-2 foci were counted under a SMZ 1000 stereomicroscope (Nikon, Japan) and the titre of virus was expressed as a foci-forming unit (FFU). The percentage of foci reduction (RF%) compared with negative control was calculated as:

RF (%) = (C-T) × 100/C

Where C is the mean of the number of foci for negative control wells and T is the mean of the number of foci in treated wells.

Statistical analysis

The 50% cytotoxic concentration (CC_{50}) and the 50% inhibitory concentration (IC_{50}) for *C. papaya* leaves extract value were calculated from dose-response curves after linear regression using GraphPad Prism for Windows, Version 5 (Software Inc., USA 2005). The selective index (SI) was determined as the ratio of CC_{50} to IC_{50} [17]

2. RESULTS AND DISCUSSION

Effect of ratio solid to solvent, amplitude and time of sonication on Percentage Yield

The effects of extraction temperature, extraction time and ratio solvent to raw material on percentage yield of CPD extract were discussed. The percentage yield of the water extract of CPD was found to be between the ranges of 9.42% to 16.93%.

Based on the 3D graphs shown in Figure 1.1 until 1.3, solid to solvent ratio was the parameter that exerted the greatest effect on yield of CPD extract. Even though it was predicted that time of extraction and amplitude of sonication would increase the percentage yield of CPD extract somewhere near the central point based on the preliminary study, the experimental design is slightly diverted from the estimation.

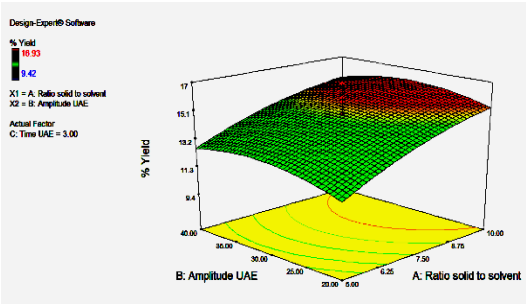

Figure1.1: Interaction between solid to solvent ratio and amplitude UAE on percentage yield

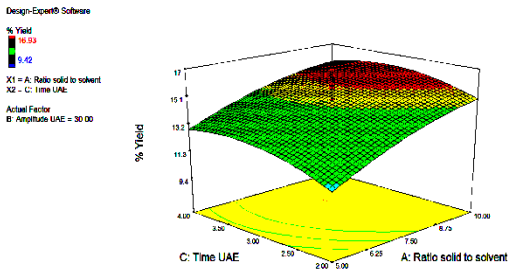

Figure 1.2: Interaction between solid to solvent ratio and extraction time on percentage yield

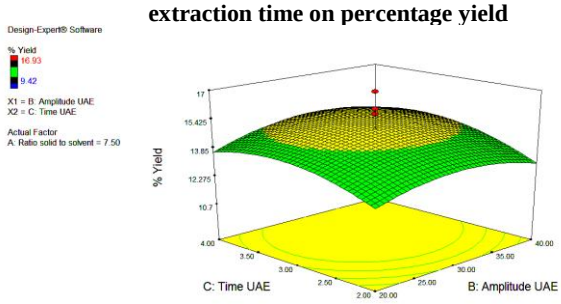

Figure 1.3: Interaction between the amplitude of UAE and time of extraction on percentage yield

Additionally, the significant impact of time of extraction and amplitude of UAE were thought to be overshadowed by the strong capability of vacuum pump in sublimating all the moisture content up to 98-99% in the extract during freeze-drying process causing loss of water-soluble compounds in the solvent system, consequently, resulting in less yield of CPD extract. Freeze drying is one of many processes that were used in food preservation by removing the moisture content that can cause product decomposition and growth of mould. Unlike most drying process that involves high temperature, freeze drying is suitable for *C. papaya* leaves extract that contains many heat-labile compounds such as enzyme papain.

Percentage yield of CPD extract was found to increase with high amplitude and longer extraction time. But, there was decrement could be observed when the amplitude exceeds 40 as in run 14 with amplitude was at the highest peak (46.82). Similar decrement trend was observed with prolonging of extraction time exceed three minutes of sonication. During the specific process time, percentage yield trend line will become plateau as it has reached maximum capability in leeching out compounds into the solvent system. Although high amplitude might enhance the plant extraction process, prolong exposure to it will cause phytocompounds degradation due to disruption cause by strong sound waves.

Cytotoxicity of extracts on Vero cells

MTT assay was used to determine cytotoxicity of each extracts on Vero cells and the CC_{50} value of each compound was calculated using Graph Pad Prism Version 5 (Graph Pad Software Inc., San Diego, CA.). Vero cells were treated by extracts for 4 days which was the same duration used for antiviral activity assay.

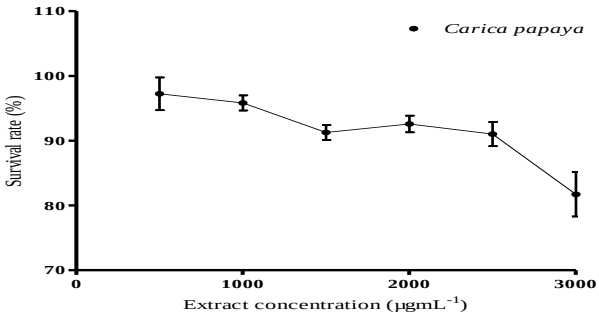

Figure 1.4: Cytotoxicity of C. papaya leaves aqueous extract on Vero cells.

The extracts showed significant reduction in foci formation as shown in Figure 1.4. The CC_{50} of extracts was 10437 (μgmL^{-1}). The IC_{50} value for Carica *papaya* of cells from 5 h before virus infection up to 4 days post-infection was 137.6 μgmL^{-1} and its selective index value was 75.85.

Continuous treatment of antiviral assay

In the continuous treatment of antiviral assay, Vero cells were pre-treated with extracts and infected with DENV-2 – incubated at 37°C, 1h for virus adsorption. Cells were washed and treated for a second time with extracts and incubated for four days.

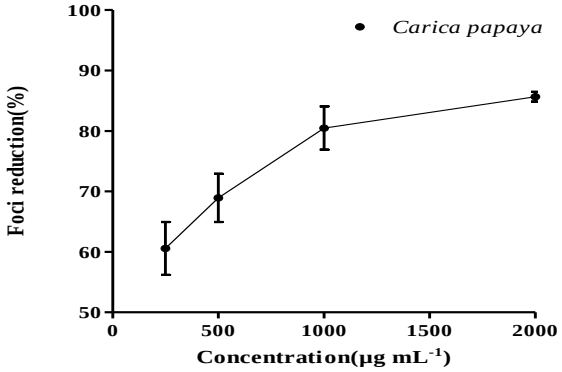

Figure 1.5: Anti-viral effects of continuous treatment with *Carica papaya* against DENV-2 replication. Cells were treated with the extracts at 5 h before infection and continuously treated with fresh extracts up to 4 days post-infection.

Early results from our study suggest that *Carica papaya* extract exhibits *in vitro* anti-dengue activity against intracellular replication of DENV-2. In the antiviral assay, the cells were pre-infected with DENV-2 for 1 hour at 37ºC and *C. papaya* leaves aqueous extract was added thereafter (Figure 1.5). This action gives enough time for the DENV-2 to penetrate the cells and starts the replication process. It was suggested that aqueous extract from plants used in traditional medicine practices are potential sources of antiviral agents [17]. Components in *C. papaya* leaves previously reported to potentially have anti-dengue activity. Extraction of *Carica papaya* leaves juice have been done elsewhere against dengue fever [7]. Thus, it may prove the inhibition activity of *Carica papaya* leaves aqueous extract towards DENV-2. The observed activity may be due to the high amount of phenolic compounds particularly flavonoid which is known to possess anti-dengue activities [18]. Quercetin was the only compound among all tested flavonoids that consistently showed significant antiviral activity against DENV-2 in Vero cells [11]

Although the best method to measure antiviral activity would be by plaque assay, the type of cell used in this study seems suitable and likewise rapid test with foci forming reduction assay. However, to confirm the activity and to achieve high reproducibility, RNA expression should be measured by qRT-PCR [13].

4. CONCLUSION

In conclusion, results from this study suggest that *Carica papaya* extracts show a promising anti-dengue activity via inhibitory effects on dengue-infected cells as depicted by decreasing number of the viral foci. The mechanism of how these extracts inhibited DENV-2 infection is not clear. However, the antiviral replication effects are most prominent when the infected cells were treated with the extracts after virus adsorption and attachment. This implies that all extracts act intracellularly on the infected cells. The bioactive compound in the extracts that are responsible for its anti-dengue activity should be further identified and characterized.

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