PROLIFERATIVE ACTIVITY OF SAPONIN-REDUCING Carica papaya LEAVES EXTRACTS ON HUMAN LUNG FIBROBLAST CELL (IMR90)

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Abstract

Carica papaya belongs to Caricaceae family, which has been proven traditionally to treat dengue fever due to its pharmacological properties to increase platelet count. However, during the critical phase of dengue fever, the platelet count will decrease due to the blood vessel rupturing. Therefore, the main objectives of this study were to reduce the bitterness of Carica papaya extract by removing saponin and to study the effect of saponin-reducing extract on the proliferative activity of human lung fibroblast cell (IMR90). For preparative isolation of the saponin compound, peleg model was used to determine the maximum extract concentration, exhaustive time of extraction and total saponin content (TS) using different weights of dry Amberlite® IRA-67 resin. The remaining saponins in the extract were quantified by mean of RP-HPLC prior to material balance. Then, approximately 1.0 x 10⁴ cells of IMR90 were seeded onto 96-well plate and later treated with various concentrations of extracts for 3 days of incubation. The results showed that, the amount of saponin left in the extract was approximately the same as in the untreated extract (p<0.05). A short adsorption incubation time (2 hrs) was believed to affect the saponin adsorption efficiency. In fact, other bio-active constituents (e.g. polyphenolic compounds) might have been adsorbed as there was a significant depreciation of antioxidant properties on the treated extract (p<0.05). In conclusion, after three consecutively days of extracts-IMR90 cell incubation, the best EC₅₀ values of both untreated and saponin-reducing extracts were observed to be more than 24 hrs of exposure ranging from 104.08 ± 0.90 to 17040.47 ± 2.30 µg/ml. Meanwhile, saponin-reducing extract has been proven not to affect any normal cell growth but in fact it decreased 1.2-fold as compared to the extract containing saponin (control).

Keywords: Carica papaya, dengue fever, saponin, resin, adsorption, fibroblast, proliferation

Abstrak

Carica papaya tergolong dalam keluarga Caricaceae dan telah terbukti secara tradisional untuk merawat demam denggi kerana sifat farmakologinya yang boleh meningkatkan jumlah platelet. Walau bagaimanapun, kelata fasa kritikal demam denggi, pengiraan platelet akan berkurangan disebabkan oleh saluran darah yang pecah. Oleh itu, objektif utama kajian ini ialah untuk mengurangkan kepekatan fitokimia saponin untuk mengkaji kesan penjerapan saponin kepada aktiviti proliferasi sel fibroblas paru-paru (IMR90). Dengan persiapan pengasingan komponen saponin, model peleg telah digunakan untuk menentukan hasil maksimum kepekatan hasil ekstrak, masa pengekstrakan menyeluruh dan kandungan keseluruhan saponin dengan menggunakan berat resin...
1.0 INTRODUCTION

Dengue viruses, mosquito-borne members of the Flaviviridae family is the most important emerging viral disease of humans that in recent decades has become a major international public health concern [1]. In dengue virus-infected cell cultures, supernatant levels of secreted nonstructural protein NS1 (sNS1) correlate with infectious virus titers [2]. No vaccine is available for the prevention of dengue infection but immunogenic, safe tetravalent vaccines have been developed and still are undergoing clinical trials [3]. Acetaminophen may be used to treat patients with symptomatic fever [1]. There is a rapidly growing response to the use of medicinal plants by the Malaysian population [4]. Recently, the use of alternative medicine and the consumption of plant materials have increased in many countries in the world, mostly because plant-derived drugs and herbal formulation are commonly considered to be less toxic and side effects than synthetic ones [5]. A medicinal plant is any plant with one or more of its organ containing substances that can be used for therapeutic purposes or which are the precursor for the synthesis of useful drugs and produce definite physiological action on the human body [6].

Papaya (Carica papaya) belongs to the family Caricaceae and is known by different names across various continents [3]. Numerous scientific studies have been conducted to assess the biological activity on the part of Carica papaya including fruit, shoots, leaves, seeds, roots and latex [7]. Traditionally leaves have been used for treatment of a wide range of ailments, like in treatment of malaria, dengue, jaundice, immunomodulatory and antiviral activity [8]. The fresh leaves and extract of the plant Carica papaya are a traditional herbal treatment in developing countries for burns, soft tissue wounds and skin infection [9]. The leaf extract of Carica papaya evidenced significant antioxidant and free radical scavenging potential [10]. The leaf of Carica papaya is categorized as non-toxic because its LD50 > 15 g per kg body weight [5]. A recent study found that papaya leaf extract could prevent growth of cancer cells, including pancreatic cancer [11]. For the preparation of plant extracts, like those papaya, water is undoubtedly the safest and the most environmentally friendly and accessible solvents [12]. Various studies have been reported in the literature documenting the phytochemical content papaya leaves [1]. Phytochemical analysis of Carica papaya leaf extracts revealed the presence of alkaloids, glycosides, flavonoids, saponins, tannins, phenols and steroids [10] as shown in Table 1. Previous study revealed that the papaya leaf not only contained phenolic compound but it also had a substantial content of saponins, which was significantly higher than the level of phenolic compounds [11]. Saponins also give the leaves the bitter taste [13]. In the present study, in order to create the therapeutic product with less bitter taste, saponin adsorption was conducted.

Saponins are structurally amphiphilic, containing hydrophilic (carbohydrate) and hydrophobic (steroid or triterpene) moieties [11]. Saponin occur constitutively in a great many plant species, in both wild plants and cultivated crops [14]. In cultivated crops, the triterpenoid saponins are generally predominant, while steroid saponins are common in plants used as herbs or for their health-promoting properties [14]. The structural diversity and resulting wide range of polarities makes determination of individual saponins very difficult [15]. Chromatographic determination of saponins in plant material is still a challenge to the phytochemist [15]. The task of isolating saponins from plant material is complicated also by the occurrence of many closely related substances in plant tissues, and by the fact that most of the saponins lack a
chromophore [14]. Saponins are natural glycosides which possess a wide range of pharmacological properties including cytotoxic activity [16]. In fact, saponin-based adjuvants have the unique ability to stimulate the cell-mediated immune system, as well as to enhance different aspects related to saponins, including biological and pharmacological activities of saponins [16]. Most studies were performed on a relatively narrow range of cell lines [16]. The most notable exceptions in this respect are the reports, in which isolated compounds were assayed by National Cancer Institute (NCI) in anticancer drug discovery screen [16]. The pursuit of natural substances capable of controlling malignancies has led to considerable research on this property of saponins [14]. Generally, the choice of cell lines seem to be random, most studies with a wider range of lines use leukemia and solid tumors cells [7]. Very few studies compared cells from the normal cells or proliferation of normal cells. Therefore, this study revealed the effect of removing saponin in Carica papaya extract towards the proliferation of Human Lung Fibroblast Cell (IMR90). The main objectives of this study were to reduce the bitterness of Carica papaya extract by removing saponin and to study the effect of saponin-reducing extract on the proliferative activity of human lung fibroblast cell (IMR90).

### Table 1 Quantitative phytochemical screening of Carica papaya aqueous extracts [6]

<table>
<thead>
<tr>
<th>Phytochemical Components Present</th>
<th>Percentage Composition*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Leaves</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>0.019 ± 0.10</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>0.013 ± 0.01</td>
</tr>
<tr>
<td>Tannins</td>
<td>0.001 ± 0.10</td>
</tr>
<tr>
<td>Saponins</td>
<td>0.022 ± 0.10</td>
</tr>
<tr>
<td>Phenolics</td>
<td>0.011 ± 0.01</td>
</tr>
<tr>
<td>Steroids</td>
<td>0.004 ± 0.01</td>
</tr>
<tr>
<td></td>
<td>Roots</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>0.021 ± 0.01</td>
</tr>
<tr>
<td>Flavonoids</td>
<td>0.014 ± 0.10</td>
</tr>
<tr>
<td>Tannins</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Saponins</td>
<td>0.026 ± 0.01</td>
</tr>
<tr>
<td>Phenolics</td>
<td>0.011 ± 0.01</td>
</tr>
<tr>
<td>Steroids</td>
<td>0.006 ± 0.10</td>
</tr>
</tbody>
</table>

*Values are mean of three replications ± S.D.

### 2.0 EXPERIMENTAL

#### 2.1 Preparation of Carica papaya Leaves

Carica papaya leaves were obtained from consisten sources in Banting, Selangor, Malaysia. Papaya leaves were identified by comparing the authentic sample and confirmed with the experts. However, there is no official voucher specimen deposited in Herbarium in this research. The mature papaya leaves were stored at 20°C prior to processing to minimize deterioration of the polyphenolic compounds. The leaves were then cut into small pieces and freeze-dried. Papaya leaves were ground by using Kenwood grinder before sieved to ≤1 mm particle size using a 1 mm stainless steel mesh sieve [11]. The powder was stored at 4 °C prior to extraction process.

#### 2.2 Extraction of Carica papaya Leaves Juice Extract

Papaya leaves juice extract was prepared in 3 replications (n = 3) and was shaken using Environment Shaker using 1:20 ratio where the polar solvent used was distilled water. The temperature of the shaker was kept at 25 °C and the weight of 1 ml papaya leaves juice extract was weighed using analytical balance (Shimadzu, Japan) to get the concentration of extract (mg/ml) [17]. The extraction process was carried out for 48 hrs to determine the optimum yield and exhaustive extraction time via Peleg model.

A Peleg mathematical model was chosen for this study because the data were graphed yield (g) to the contrary extraction time (min) showed characteristic curve of sorption processes [18]. Two constant K in this model show that K₁ is value for γ-intercept and K₂ is the gradient value through kinetics curves and plateau to the linear graph of time (t)/concentration (ρ) versus time (t) [17]. Peleg model is shown as:

\[
\rho (t) = \rho_0 ± t/\left(K_1 + K_2 \cdot t\right)
\]

Where \( \rho \) is concentration extract (g/ml) at t (min), \( \rho_0 \) is concentration at t = 0 (g/ml), K₁ is constant for Peleg 1 (min,g/ml) and K₂ is constant for Peleg 2 (ml/g). Concentration (\( \rho \)) at t = 0 assume as 0, so linear Equation (2) derived

\[
\frac{1}{\rho (t) - \rho_0} = K_1 + K_2 \cdot t
\]

Peleg 1 constant (K₁) is relate to rate of concentration (B₀) at specific time process (t = t₀)

In Equation (3)

\[
B_0 = 1/K_1
\]

The Peleg capacity constant K₂ relates to maximum extraction. When t \( \to \infty \) (extraction reached between the dissolved substance in a sample of bulk volume of the extract). Equation (4) gives relation between the equilibrium of extract concentration and constant of K₂

\[
\rho \to \infty = 1/K_2
\]

#### 2.3 High Performance-Liquid Chromatography (HPLC)

Reversed phase HPLC was applied for chromatographic analysis. The Waters HPLC System consisted of a high pressure constant flow pump (Model 510), auto injector (WISP 712), a UV spectrophotometric detector and a data station (840 with a Digital 350 computer). Chromatographic separation was performed by using a column of µBondapak, C₁₈, with particle size 10 µm (internal diameter 3.9 mm x length 120 mm) and with a water-
acetonitrile mobile phase, as well as UV detection at 203 nm. A 20 µl volume of sample was injected onto the column.

2.4 Adsorption of Saponin (Batch Mode) by Amberlite® IRA-67

A total of 0.1 g of Amberlite® IRA-67 resin (St. Louis, MO USA) which had been conditioned reacted with 20 ml of papaya leaves extract of in 250 ml sterile Erlenmeyer flask without any adjustment of pH value at with 3 replications (n = 3) based on the previous study [19]. 250 ml Erlenmeyer flask containing a mixture of resin and papaya leaves juice extract is then shaken with Environmental Shaker at a speed of 120 rpm and a temperature of 30 °C for 2 hrs. Determination of total saponin content adsorbed determined by using colorimetric method wherein the ratio of total saponin content is done through statistical analysis and data presentation charts. The same procedure is used for 0.5 g Amberlite® IRA-67 resin.

2.5 Total Saponin Content (TSC)

The total saponin content was determined by using the colorimetric method [20]. An aliquot of 200 µl filtered papaya leaves juice extract was dissolved in 200 µl 8% (w/v) vanillin reagent that was earlier dissolved in ethanol, followed by 2 ml 72% (v/v) sulphuric acid. The mixture was stirred gently using rod glass before being soaked in water bath (Memmert) at 60 °C for 4 mins and the absorbance was measured at 544 nm. Aescin was used as the external calibration curve standard.

2.6 EC50 Values of Carica papaya Leaves Extract Cell Culture

Human Lung Fibroblast Cell (IMR90) were plated in 96-well tissue culture, treated white plates with clear bottoms at a density of 40,000 cells/well. Cells were allowed to grow to confluence. Approximately 1.0 x 10⁴ cells of IMR90 (cell seeding) were seeded on 96-well plate and then incubated for 24 hrs in an incubator (37 °C, 5% CO₂). After overnight incubation, serial of Carica papaya leaves extract concentrations were treated onto the IMR90 that had been seeded. Then, the incubation period was continued for 24, 48 and 72 hrs to observe the effect of the extract on cell growth. At each interval days of incubation, 10 µl of PrestoBlue® solution was added into the well plates, and then incubated at 37 °C for 3 hrs prior to absorbance analysis using a micro-ELISA reader at 570 nm. The absorbance of the treated wells in the visible region correlates with the number of viable cells as follows:

\[ \text{Viable cells (%) } = \frac{(A_c - A_e)}{(A_c - A_b)} \times 100 \]  \hspace{1cm} (5)

Where \( A_c \) is the absorbance of control, \( A_t \) is the absorbance of treated samples, and \( B \) is the absorbance of the blank [21]. EC50 values were determined based on the log concentration and normalized cell growth (%) by referring to the probit values.

2.7 Statistical Analysis

All data was analysed using the Statistical Package for the Social Sciences (SPSS) version 16.0. The one-way ANOVA, Pearson’s correlation and statistical comparisons using t-test were used. For the determination of significant differences between the samples, the probability of significant levels determined in 95% (p<0.05). Data was presented as means ± standard deviation (SD) of mean values. A p<0.05 was considered significant.

3.0 RESULTS AND DISCUSSION

3.1 Extraction of Papaya Leaves Juice Extract

Peleg model was chosen since there are similarities between the extraction process and sorption kinetics mechanism [22]. For that reason, weight of extraction of the extract (mg) against time (minutes) are identical in form with a graph of moisture sorption against time. Therefore, the K1 and K2 value were obtained from the equation \( y = 0.1043\ln(x) + 0.2609 \) where K1 and K2 are 12.011 (min.ml/g) and 0.9862 g/ml respectively (Figure 1). The initial rate extraction and the exhaustive extraction concentration yield are 0.084 min.ml/g and 1.02 g/ml respectively. Therefore, based on those predicted values, the exhaustive time of extraction was predicted at 23 ± 1.2 hrs. The extraction was repeated (n = 3) and the exhaustive extract concentration was obtained approximately with the predicted value. The extract was later used as an initial concentration \( (C_0) \) of EC50 analysis.

![Figure 1 Relationship between Time (min) against the Concentration (mg/ml) to get initial rate extraction and the exhaustive concentration yield](image-url)
3.2 Saponin-Resin Adsorption Studies

3.2.1 Total Saponin Content (TSC)

The exhaustive time to achieve the highest TSC was determined using the peleg model. The extract containing high amount of saponin was subjected to saponin-resin adsorption studies to determine the efficacy of the resin in adsorbing saponin. Finally, the TSC was correlated with extractive concentrative to determine the amount of TSC at exhaustive time of extraction in Figure 1.

Based on Figure 2, the exhaustive extraction time to produce maximum TSC (48.32 ± 2.8 mg Aes/g) at temperature of 25°C was 8294.44 mins (5.74 ± 0.2 days). The results were by far too long as compared with the concentration-based extract peleg model which was 23 ± 1.2 hrs (Figure 1) (p<0.05). For that reason, the amount of saponin available in the extract at 23 ± 1.2 hrs was predicted approximately 25 ± 2.7 mg.

The extract was treated with the Amberlite® IRA-67 for the saponin adsorption. However, there was no reduction of TSC as compared with the control (p<0.05). However there were some significant changes occurred to the antioxidant properties on the treated extract (p<0.05) (data not shown) which could possibly affect the cell growth. Therefore, a thorough saponin-resin kinetic adsorption studies has to be carried out to determine the maximum TSC that could be extracted from the extract prior to cell culture work.

Figure 2 Logarithm relationship between Total Saponin Content (TSC) against extraction time

3.2.2 Adsorbent Capability of Amberlite® IRA-67

The efficacy of saponin-Amberlite® IRA-67 resin adsorption study was carried out on a different weight of dry resin (0.1 g and 0.5 g). The result in Figure 3 revealed that different weight of resin did not affect significantly on the adsorption of saponins (p<0.05). However, other bio-constituents might have been adsorbed from the extract due to the fact that there was colour changes on the beads (Figure 4). Moreover, 2 hrs of incubation time might not be enough to adsorb saponin adequately as adsorption capacities is prominently depending on the adsorption time [23].

Figure 3 Total saponin content (TSC) removed from the extract by means of different weight of Amberlite® IRA-67 resin. Similar superscripta represents insignificant different (p>0.05)

Figure 4 Total phenolic content (TPC). Different superscripta represents significant different (p<0.05)

3.2.3 Resin Beads Colour Changes

Changes in colour was observed after of saponin-resin adsorption process. Originally, the resin Amberlite® IRA-67 was a semi-translucent white and it changed to a dark-grey beads (Figure 4). This phenomenon was possibly due to the polymer chains expansion which makes hydrophilic ionic resin remain hydrated thus causing changes in colour of the beads [24] via ion exchange mechanism and other interactions such as Van der Waals forces and hydrogen bonds [25].
Figure 5 (a) Beads of resin Amberlite® IRA-67 visually prior to treatment; b) Beads of resin Amberlite® IRA-67 after treatment - changes in colour observed

3.3 EC₅₀ Value Determination

Based on the peleg prediction model, the exhaustive time of extraction to obtain the highest amount of extract was at 23 ± 1.2 hrs. The highest extract concentration was used as an initial concentration (C₀) of EC₅₀ analysis. Serial dilution of both sample extract were prepared ranging from 15.625 to 1000 µl/ml from the initial extract concentration of 1.10 ± 0.12 g/ml. Figure 4 shows the trend of normalized cell growth (%) treated with Carica papaya leaves with saponin (control) and saponin-reducing extract. Both extracts exhibit different growth profile as time of exposure increase (p<0.05). The doubling effect were seems to be mostly affected to the treated extract. For that reason, the effective concentration (EC₅₀) analysis was carried out to determine the lowest concentration to sufficiently obtain 50% of a maximum effect estimate in 100% with respect to the best treatment exposure time [26]. Probit analysis was used for calculating confidence interval and plotting the dose-response curve as shown in Figure 5 [27].

Based on Table 2, the extracts with saponin at 72 hrs (104.08 ± 0.90 µg/ml) and saponin-reducing at 48 hrs (17040.47 ± 2.30 µg/ml) were considered the best EC₅₀ for further cell work study. Those extracts were the lowest concentration to achieve 50% cell growth at
an exposure time of greater than 24 hrs. However, the saponin-reducing extract needs approximately 164-fold higher concentration than the extract containing saponin. This phenomenon could possibly due to the reduction of polyphenolic compounds properties (Figure 4) which has affected cell population. A short adsorption incubation time (2 hrs) due to time constraints was believed to affect the saponin adsorption efficiency thus affected the proliferation of cell. Moreover, cell proliferative and adaptive mechanism and extract concentration were conceivably contributed to the cell behaviors (e.g. cytotoxicity) against different exposure duration [28].

Table 2 EC\textsubscript{50} Values of Carica papaya leaves (with saponin) extract and Carica papaya leaves (saponin-reducing) for 24, 48 and 72 hrs

<table>
<thead>
<tr>
<th>Day/hour</th>
<th>Carica papaya leaves (with saponin) extract (µg/ml)</th>
<th>Carica papaya leaves (saponin-reducing) extract (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (24 hr)</td>
<td>3435.38 ± 1.20</td>
<td>44532.3 ± 12.80</td>
</tr>
<tr>
<td>2 (48 hrs)</td>
<td>721164.47 ± 15.30</td>
<td>17040.47 ± 2.30*</td>
</tr>
<tr>
<td>3 (72 hrs)</td>
<td>104.08 ± 0.90*</td>
<td>38888.2 ± 11.56</td>
</tr>
</tbody>
</table>

*Values are expressed as mean of three replications ± S.D. (n = 3).

The results clearly verified that low EC\textsubscript{50} from Carica papaya leaves extract required only small amount of dosage to stimulate cell growth. In fact, the longer the exposure time, the lower the EC\textsubscript{50} value were observed to increase cell proliferation (p<0.05). Moreover, previous study has stated that, saponin based adjuvants have the ability to modulate the cell mediated immune system as well as to enhance antibody production and have the advantage that only a low dose is needed for adjuvant activity [29]. Actually, papaya leaves used as a traditional herbal treatment in developing countries for burn, soft tissue wounds and skin infection [9]. This condition can be related to the proliferative effect of papaya leaves extract.

Furthermore, the cell morphology and its micro-environment in 96-well plate was visually observed under a reversible light microscope. After 48 hrs of treatment, it was observed that the extract with saponin proliferated effectively as compared to the saponin reducing extract. This can be proved by the formation of population doubling and the increasing IMR90’s network colonies as compared to the saponin-reducing extract. In fact, the cell’s shape started to increase its size and overlap to each other. However, both extracts show proliferative effect only after 24 hrs of treatment. This was possibly due to growth nature of fibroblast cells which requires growth factors and a long period of time as the cells began to adapt to their new chemically induced micro-environment [30]. Therefore, Carica papaya leaves which underwent saponin-resin adsorption exhibit lower cell yield. In fact, it requires higher dose of extract (EC\textsubscript{50}) to increase cell proliferation. Meanwhile, previous study by Vuong et al. [11] claimed that saponins in papaya leaf are bioactive compounds that exhibit potential in limiting cell proliferation of certain pancreatic cancer cell lines. However, no recent study shown that saponin has the antiproliferative effect towards normal cells. Thus, removing saponin could only affecting other essential bio-active constituents (e.g. polyphenolic content) in the extract which affects the ability of the extract to stimulate normal cell growth.

Figure 7 Cell morphology of IMR90 of Carica papaya leaves extract (with saponin) [Diagram a, c and e] and saponin-reducing Carica papaya leaves extract [Diagram b, d and f] at 24, 48 and 72 hrs. The presence of black particle were clarified as the dead cells and the debris in media.

4.0 CONCLUSION

In conclusion, it was observed that both with saponin and saponin reducing extracts influenced the proliferation of normal cell. The EC\textsubscript{50} of saponin reducing extract was shifted to a higher concentration as compared to the extract containing saponin by way of increasing cell growth (p<0.05). The insignificant depreciation of total saponin content...
(p>0.05) after treatment using resin Amberlite® IRA-67 was possibly due to the inefficient adsorption time where there were not much saponin compounds adsorbed in a short period of time. However, other bio-active constituents (e.g. phenolic) might have adsorbed onto resin which resulted in cell growth deficiency. Further study on the kinetic equilibrium of saponin-resin adsorption is required to determine the maximum absorbed TSC prior to cell culture work. Finally, this preliminary studies have proven that both extracts has an undoubtedly potential and reputation as an alternative remedy to cure severe dengue fever (dengue hemorrhagic fever (DHF)) by increasing the changes of repairing ruptured blood vessels.

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