Butyrylcholinesterase Inhibitory Activity and GC-MS Analysis of Carica papaya Leaves

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Abstract – Carica papaya is a medicinal and fruit plant owing biological activities including antioxidant, antiviral, antibacterial and anticancer. The present study aims to investigate the acetyl (AChE) and butyryl (BChE) cholinesterase inhibitory potentials of C. papaya extracts as well as their chemical compositions. The chemical composition of the active extract was identified using a gas chromatography-mass spectrometry (GC-MS). Ellman enzyme inhibition assay showed that the alkaloid-enriched leaf extract of C. papaya possessed significant anti-BChE activity with an enzyme inhibition of 75.9%. GC-MS analysis showed that the alkaloid extract composed mainly the carpaine (64.9%) – a major papaya alkaloid, and some minor constituents such as aliphatic hydrocarbons, terpenes and phenolics. Molecular docking of carpaine revealed that this molecule formed hydrogen bond and hydrophobic interactions with choline binding site and acyl pocket. This study provides some preliminary findings on the potential use of C. papaya leaf as an herbal supplement for the prevention and treatment of Alzheimer’s disease.

Keywords – Carica papaya, Cholinesterase inhibitor, GC-MS, Alkaloid, Carpaine, Alzheimer’s disease

Introduction

Alzheimer’s disease (AD) is a progressive neurodegenerative disorder associated with memory impairment and cognitive deficit and deaths related to AD has been reported to increase by 89% between 2000 and 2014. It is estimated that 10% of the world’s population over the age 65, are affected by this disease. At present about 18 million of people around the world have this disease and the number is expected to increase approximately up to 34 million by 2020.¹,²

Acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) enzymes catalyse the hydrolysis of the neurotransmitter acetylcholine (ACh) which plays an essential role in memory and learning. According to the cholinergic hypothesis, inhibition of AChE increases the levels of acetylcholine in the brain.² Likewise, current clinical approved drugs used for the management of mild to moderate AD patients rely on AChE inhibitors, which promotes an increase in the concentration and prolong the duration of action of synaptic Ach.³ A recent systematic review and meta-analysis of 43 randomized placebo-controlled clinical trials showed that anticholinesterases improved cognitive function, global symptomatology, and functional capacity, further decreasing patients’ mortality.⁴ BChE, which primarily associated with glial cells cleaves Ach in a similar manner. The importance of BChE can be postulated in advanced stage AD patients. The AChE activity reduced up to 85% in brain of AD patients, while the activity of BChE increases substantially.⁵,⁶ Therefore, restoring the Ach level via inhibiting BChE could be the propitious target for the management of the progression of AD.

While drugs widely used for the treatment of AD is the acetylcholinesterase inhibitors (AChEi) such as donepezil,
rivastigmine, galantamine—and the glutamate antagonist memantine, all of which have limited effectiveness and has been reported to exert certain level of side effect. Therefore, a reliance in natural product was sorted in addressing a potential treatment option that offers a significantly reduced side effects. Over the years, studies have been carried out in identifying and isolating natural molecules for design and development of new anti-AD drugs. However, the gap in addressing a potent natural product with substantial outcome remains vague leading to continuous efforts devoted toward discovery of potent natural compounds for the management of AD.

_Carica papaya_ L., or commonly known as papaya, is one of the major tropical fruit consumed worldwide. Malaysia, which is blessed with 12,000 species of flowering plants of which 1300 with medicinal properties, is the top five papaya exporter. _C. papaya_ leaves have been used in folk medicine for centuries and studies have shown its beneficial effect as an antiinflammatory agent, anticancer, wound healing properties, antitumour and immune-modulatory effects and an antioxidant. A study by Halim and colleagues investigating toxicity of _C. papaya_ leaves extract on Sprague Dawley rats revealed that it was safe for oral consumption. Furthermore, it has been reported that extracts and pure compounds derived from _C. papaya_ to possess a wide variety of pharmacological activities including antioxidant, antimicrobial, antihypertensive, antiplasmodial, antifungal and anti-inflammatory.

Due to these extraordinary therapeutic properties, this study aims to study the anticholinesterase activities of _C. papaya_ leaves in understanding its therapeutic strategies towards AD.

**Experimental**

**Chemicals and reagents** – Solvents (n-hexane, chloroform and ethanol) used for extraction were of analytical grade (Mercks, Germany). Reagents and chemicals used for Elman assay: acetylthiocholine iodide and acetylcholinesterase from electric eel, bovine serum albumin, 5,5′-dithiobis-2-nitrobenzoic acid, and butyrylcholinesterase from equine serum, and S-butyrylthiocholine chloride and physostigmine were purchased from Sigma-Grade (Mercks, Germany). Reagents and chemicals used for extraction were of analytical form and ethanol) used for extraction were of analytical natural compounds for the management of AD.

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**Plant extraction of _C. papaya_ leaves** – The dried leaves of _C. papaya_ L. (Caricaceae) were purchased from a local herbal and nutraceutical company – Herbagus Sdn. Bhd (Malaysia). The dried leaves were ground into fine powder using a mill grinder. The powdered leaves (200 g) were first extracted with n-hexane (1: 10 w/v), then followed by 95% ethanol for 48 hours, respectively. The supernatant of each extract was filtered and then concentrated under reduced pressure to yield the dried extract. The resulted hexane (44.20 g) and ethanolic (27.2 g) extracts were kept in -20 °C.

**Alkaloid extraction of _C. papaya_ leaves** – Approximately 20 g of the ethanolic extract was dissolved in 10% (v/v) acetic acid and stirred for 24 hours. After that, the acidified suspension was filtered to give a clear supernatant. The supernatant was then adjusted to pH 9 with ammonia solution to liberate the alkaloid constituents. The basified solution was extracted with chloroform for several time until colourless. The chloroform fraction was combined and evaporated under reduced pressure to yield the alkaloid-enriched extract (0.23 g). Similar to crude extracts, the sample was kept in -20 °C prior to biological activities evaluation.

**Cholinesterase inhibitory assays** – Cholinesterase inhibitory activity of the test samples was determined by Ellman’s microplate assay with modification. For AChE/BChE inhibitory assay, AChE/BChE enzyme, acetylcholine iodide, S-butyrylthiocholine chloride, known inhibitors, physostigmine was used as the reference standards. Absorbencies of the test samples were corrected by subtracting the absorbance of their respective blank (test samples in methanol with acetylthiocholine iodide, S-butyrylthiocholine chloride and 5,5′-dithiobis-2-nitrobenzoic acid). A set of five concentrations was used to estimate the 50% inhibitory concentration (IC_{50}). Each test was conducted in triplicate.

**GC-MS analysis** – Phytochemical analysis of _C. papaya_ active extract was carried out on a hyphenated Agilent 6890N Network GC system coupled to an Agilent 5973i mass selective detector (Agilent Technologies, Germany) according to the method previously described. Separation was performed on a HP-5MS column (30 m x 0.25 mm, 0.25 μm film thickness; Agilent Technologies, Germany) with helium as the carrier gas at a constant flow rate of 1.2 mL/min. The injection volume was 1 μL with a splitless mode. The initial column temperature was held at 70 °C for 2 minutes and then increased to 280 °C at a rate of 20 °C/min. The final column temperature was maintained at 280 °C for another 20 minutes. The temperatures of the injector and the detector were 250 °C and 280 °C, respectively. The interface temperature was set to 300 °C. Mass acquisition was performed in the range of 40-550 m/z using electron impact ionisation at 70 eV. The components detected in the sample were identified by
performing spectral database matching against the National Institute of Standards and Technology database (Gaithersburg, MD, USA). The identity of the detected compounds was evaluated by comparing the mass of their molecular ions, base ions, fragment ions, as well as their peak intensities with those reference standards in the database. The detected compounds with >90% spectral matching quality were considered acceptable.

**Molecular docking** – Molecular docking of carpaine was performed according to the method described. Crystal structures of BChE from Homo sapiens were obtained from Protein Data Bank with PDB ID: 2WIJ. The molecular docking was carried out and the most populated cluster was chosen to further analysis. The analysis of the binding interactions was conducted using Accelrys Discovery Studio (Accelrys Inc., San Diego, CA, USA).

**Results and Discussion**

Cholinesterase enzymes are the promising target for Alzheimer disease (AD) drug discovery. To date, cholinesterase inhibitors are clinically preferred medications for the treatment of mild and moderate forms of AD due to its efficacy and less adverse effects. Cholinesterase inhibitors inhibit acetylcholine from further degradation which could aid in reducing memory and learning impairments. Therefore, the search for potential anticholinesterase inhibitor from natural products that contain various classes of phytochemicals is one of the global strategies for the prevention and treatment of AD.

*C. papaya* is a medicine and fruit plant enriched with different types of dietary phytochemicals such as fatty acids, terpenes, vitamin E, flavonoids, phenolic acids etc. These phytochemicals possess potential therapeutic properties such as antioxidant, antiviral, anticancer antiinflammation etc. In our search for the anti-neurodegenerative lead compounds from Malaysian flora, papaya leaves extracts were prepared from sequential solvent extraction (hexane and ethanol) and were screened for their potential cholinesterase inhibitory activities using Ellman method. The cholinesterase inhibitory activities of these extracts are summarized in Table 1.

For the screening purposes, cholinesterase enzymes were treated with 0.2 mg/mL of extract and fractions, and physostigmine was used as the standard for quality control. The result showed that ethanolic extract of *C. papaya* has mild inhibition on BChE with a percentage inhibition of 34.6% which was about 5 times greater than the hexane extract (6.9%). The active ethanolic extract was further fractionated by acid-base extraction to concentrate the alkaloid constituents. It is worthy to note that the alkaloid-enriched fraction showed more than two folds’ increment in BChE inhibition activity (75.0%) compared to the ethanolic extract at the same screening dose. Further evaluation showed that the BChE inhibition constant (IC$_{50}$) of alkaloid fraction was 175.0 ± 2.0 μg/mL. All *C. papaya* leaves extracts unable to inhibit AChE enzyme at the dose of 0.2 mg/mL indicating the extracts and the intrinsic phytochemicals might selective towards inhibiting BChE. BChE enzyme is localised in neurons, glial cells and in neuritic plaques and tangles of Alzheimer patients. There are great interests towards BChE inhibitor where several studies showed that the activities of BChE increased for the advanced Alzheimer cases. Therefore, chemical entities owing BChE inhibition and/or dual inhibitory potential can be used as the alternative for

<table>
<thead>
<tr>
<th>Sample</th>
<th>AChE (μg/mL)</th>
<th>BChE (μg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane extract</td>
<td>&gt;0.2</td>
<td>6.9 ± 2.1</td>
</tr>
<tr>
<td>Ethanol extract</td>
<td>&gt;0.2</td>
<td>34.6 ± 10.6</td>
</tr>
<tr>
<td>Alkaloid fraction</td>
<td>&gt;0.2</td>
<td>75.9 ± 9.7</td>
</tr>
<tr>
<td>Physostigmine</td>
<td>98.9 ± 4.7</td>
<td>96.7 ± 2.5</td>
</tr>
</tbody>
</table>

Data presented as Mean ± SD (n=3)

*Screening of extracts and fraction at 0.2 mg/mL
*Screening of physostigmine at 0.1 mg/mL

**Table 2. Identified phytochemicals in the alkaloid fraction of *C. papaya* leaves.**

<table>
<thead>
<tr>
<th>Peak label</th>
<th>Retention time (min)</th>
<th>Compound</th>
<th>Peak Area (%)</th>
<th>Chemical class</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>8.4</td>
<td>2,4 -Di-tert-butylphenol</td>
<td>3.4</td>
<td>Phenol</td>
</tr>
<tr>
<td>2</td>
<td>8.7</td>
<td>Dihydroactinidilide</td>
<td>1.2</td>
<td>Terpene</td>
</tr>
<tr>
<td>3</td>
<td>10.0</td>
<td>(-)-Loliolide</td>
<td>4.5</td>
<td>Terpene</td>
</tr>
<tr>
<td>4</td>
<td>14.5</td>
<td>Eicosane</td>
<td>2.1</td>
<td>Aliphatic hydrocarbon</td>
</tr>
<tr>
<td>5</td>
<td>16.2</td>
<td>Nonacosane</td>
<td>6.9</td>
<td>Aliphatic hydrocarbon</td>
</tr>
<tr>
<td>6</td>
<td>19.4</td>
<td>Vitamin E</td>
<td>1.4</td>
<td>Phenol</td>
</tr>
<tr>
<td>7</td>
<td>30.2</td>
<td>Carpaine</td>
<td>64.8</td>
<td>Alkaloid</td>
</tr>
</tbody>
</table>
Fig. 1. (a) A representative GC chromatogram of alkaloid fraction of *C. papaya* leaves. (b) Mass spectra of identified carpaine in the *C. papaya* leaf and the standard carpaine in NIST library.
AChE inhibitors for severe cases. A number of phytochemicals have been reported for their selective BChE inhibitory effect including lipophilic constituents and flavonoids from *Stenochlaena palustris*, garcinexanthone G from *Garcinia atroviridis*, and indole alkaloids from *Nauclea officinalis*. The apparent positive result of the alkaloid fraction initiate further analysis to reveal its chemical composition using gas chromatography-mass spectrometry (GC-MS) method.

The active alkaloid fraction of *C. papaya* leaves was analyzed for its phytochemicals using GC-MS at a fix concentration of 10 mg/mL. The identified compounds are summarized in Table 2. In total, seven lipophilic compounds, constituting 84.5% of the sample by percentage of peak area, were identified in the alkaloid fraction (Fig. 1). The fraction composed mainly carpaine (64.8%) – a major macrocyclic lactone alkaloid in the leaves of *C. papaya*, followed by a portion of non-alkaloid constituents such aliphatic hydrocarbons, volatile terpenes and phenolics. Aliphatic hydrocarbons - eicosane and nonacosane are the second and fifth major components comprising of 9.0% of the active fraction. Volatile terpenes were the third dominant chemical class, accounting for 5.7% of the active fraction, and this figure was contributed by loliolide (4.5%) and dihydroactinidiolide (1.2%). Lipophilic phenols such as 2,4 -di-tert-butylphenol (3.4%) and vitamin E (1.4%) were the trace components in the active fraction.

Molecular docking is used in the drug discovery to predict the molecular interactions of the compound and enzyme of interest. In the present study, the major compound of alkaloid fraction, carpaine was docked into the active site of BChE enzyme (PDB code: 2WIJ). Fig. 2 shows the interaction of carpaine with residues of BChE. Carpaine was well fitted well within the active site of BChE with the free energy of binding of -11.82 kcal/mol. Carpaine formed hydrogen bonding and hydrophobic interactions with the interface of choline binding site and acyl pocket domains (Table 2). Val 288 of the acyl pocket formed hydrogen bonding with carbonyl group of carpaine (3.3 and 4.6 Å) and Trp 82 formed hydrophobic interaction with lactone of carpaine (2.5 Å). A hydrogen interaction was observed with Pro 285 residue of the enzyme.

In conclusion, this study underscores the potential of *C. papaya* leaf alkaloid fraction and its major alkaloid compound as potential nutraceutical for memory enhancing agent. Alkaloid fraction showed promising BChE inhibition activity and GC-MS data revealed that the major compound was carpaine. Subsequently, molecular docking result showed that carpaine fitted well within active site of BChE enzyme. Future work will be conducted to access the blood brain barrier penetration potential of the fraction and in vivo study using suitable animal model for its therapeutic applications.

Fig. 2. Binding orientation of carpaine with butyrylcholinesterase enzyme residues at the active site of BChE.
Acknowledgments

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References

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