# *IN VITRO* AND *IN SILICO* STUDIES ON SELECTED MALAYSIA CULINARY PLANTS AGAINST DENGUE NS2B-NS3 PROTEASE

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# *IN VITRO* AND *IN SILICO* STUDIES ON SELECTED MALAYSIA CULINARY PLANTS AGAINST DENGUE NS2B-NS3 PROTEASE

**by**

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During the Master research project, bridging theory on books and practical in lab is tough for me as a fresh degree graduate at the initial stage. Therefore, I learned to seek technical advice from experts and set achievable objectives for completing the research work. Hereby, I claim all the work in this thesis are done by me and willing to take the responsibility for it.

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| | |
|---|---|
| Ac | Acetone |
| AMC | 7-Amino-4-methyl-coumarin |
| Arg | Arginine |
| Asn | Asparagine |
| Asp | Aspartic acid |
| Boc | *tert*-Butyloxycarbonyl |
| br | Broad |
| BuOH | n-Butanol |
| $CDCl_3$ | Chloroform-D |
| $CHCl_3$ | Chloroform |
| Conc. | Concentration |
| COSY | Correlation Spectroscopy |
| d | Doublet |
| DENV2 | Dengue Virus Type 2 |
| DMSO | Dimethyl sulfoxide |
| ESI | ElectroSpray Ionisation |
| EtOAc | Ethyl acetate |
| FEB | Free energy of binding |
| RFU | Relative fluorescence unit |
| g | Gram |
| Gly | Glycine |
| Hex | n-Hexane |
| His | Histidine |
| HMBC | Heteronuclear Multiple Bond Correlation |
| HPLC | High Performance Liquid Chromatography |
| HSQC | Heteronuclear Single Quantum Correlation |
| Hz | Hertz |
| $H_2O$ | Water |
| $IC_{50}$ | Half maximal inhibitory concentration |
| IR | Infrared |
| *J* | Coupling constant |
| kcal/mol | Kilocalories per mol |
| L | Litre |
| Lys | Lysine |
| m | Multiplet |
| MCA | Methyl Cumaryl Amide |
| MeOD | Methanol-D |
| MeOH | Methanol |
| Met | Methionine |
| MHz | Mega Hertz |
| mL | Milli litre |
| MS | Mass Spectrometry |
| *m/z* | Mass to charge ratio |
| nm | Nanometer |
| NPCC | Normal Phase Column Chromatography |
| Phe | Phenylalanine |
| PLC | Preparative Thin Layer Chromatography |
| ppm | Part per million |

| | |
|---|---|
| Pro | Proline |
| Q-Tof | Quadrupole time-of-flight |
| $R_f$ | Retention factor |
| RP-TLC | Reverse Phase Thin Layer Chromatography |
| RMSD | Root mean square deviation |
| $r^2$ | Correlation coefficient |
| s | Singlet |
| Ser | Serine |
| $SO_4$ | Sulfate |
| t | Triplet |
| Thr | Threonine |
| TLC | Thin Layer Chromatography |
| Tyr | Tyrosine |
| UV | Ultraviolet |
| Val | Valine |
| $^1$H-NMR | Proton Nuclear Magnetic Resonance |
| $^{13}$C-NMR | Carbon Nuclear Magnetic Resonance |
| δ | Chemical Shift |
| 2D | Two dimension |
| % | Percentage |
| μM | Micro Molar |
| Å | Angstrom |

**KAJIAN *IN VITRO* DAN *IN SILICO* KEATAS TUMBUHAN MASAKAN MALAYSIA TERPILIH TERHADAP PROTEASE DENGGI NS2B-NS3**

## ABSTRAK

Di Malaysia, statistik jangkitan denggi setakat tahun 2015 sudah melebihi 100,000 kes. Walaubagaimanapun sehingga kini tidak ada calon vaksin dan ubat untuk denggi yang boleh didapati di pasaran. Oleh itu, penemuan ubat untuk denggi adalah penting untuk mencari calon ubat yang sesuai. Kajian ini melibatkan pencirian kimia juzuk, eksperimen *in vitro* dan simulasi *in silico* untuk tumbuh-tumbuhan yang digunakan dalam masakan harian di Malaysia. Dalam kajian ini, kunyit, serai wangi, daun kari, daun selasih, daun pandan, daun kesum dan halia dipilih dalam saringan bioaktiviti terhadap NS2B-NS3 protease. Tumbuh-tumbuhan tempatan ini dipilih sebab mereka memaparkan keputusan yang menarik dalam penyaringan maya pada kajian yang lepas. Antara tujuh tumbuhan, daun kesum dan daun kari didapati sangat aktif dalam merencat denggi NS2B-NS3 protease dengan 93% dan 88% perencatan masing-masing. F2_1 ($IC_{50}$ 17.4 μM) diasingkan dari daun kesum menyerupai Vanicoside A mempunyai perencatan yang lebih baik daripada rujukan Panduratin A ($IC_{50}$ 94.60 μM) manakala Bismahanine ($IC_{50}$ 93.1 μM) diasingkan dari daun kari mempunyai perencatan yang serupa dengan Panduratin A. Dari hasil pendokan, sebatian F2_1 mempunyai interaksi polar dengan amino asid pada struktur krystal 3U1I manakala Bismahanine mempunyai interaksi ikatan hydrogen dengan triad pemangkin (His51, Asp75, Ser135) yang lebih baik daripada enzim protease. Walau bagaimanapun, F2_1 dan Bismahanine cenderung untuk beragregat pada kepekatan mikro molar dan $IC_{50}$ masing-masing 319.4 μM dan 327.3 μM dalam asai berasaskan detergen. Kajian masa depan boleh dilakukan pada pengubahsuaian kimia pada struktur Bismahanine bagi merekabentuk perencat denggi NS2B-NS3 yang lebih baik.

**_IN VITRO_ AND _IN SILICO_ STUDIES ON SELECTED MALAYSIA CULINARY PLANTS AGAINST DENGUE NS2B-NS3 PROTEASE**

## ABSTRACT

In Malaysia, dengue infection was reported more than 100,000 cases in year 2015. To date there is no marketed drug candidate available for Dengue disease. Therefore, drug discovery for dengue is important to find a suitable drug candidate. This study involves characterization of chemical constituent, *in vitro* experiment and *in silico* simulation for anti-dengue from culinary plants in Malaysia. In this study, turmeric, lemon grass, curry leaves, sweet basil, screw pine, Vietnamese mint and ginger were screened for bioactivity against dengue NS2B-NS3 protease. The rational of choosing these native culinary plants because they show interesting result in virtual screening in previous study. Out of seven plants, Vietnamese mint and curry leaves were found highly active in inhibiting dengue NS2B-NS3 protease with 93% and 88% inhibition, respectively. Compound F2_1 ($IC_{50}$ of 17.4 μM) isolated from Vietnamese mint which is similar to be Vanicoside A showed better inhibition than reference Panduratin A ($IC_{50}$ of 94.60μM) while Bismahanine ($IC_{50}$ of 93.1 μM) isolated form curry leaves has similar inhibition activity with Panduratin A. From docking result, compound F2_1 formed polar interaction with amino acid residues of protease crystal structure, 3U1I while Bismahanine has hydrogen bond interactions with the catalytic triad (His51, Asp75, Ser135) of protease which is more preferable. However, F2_1 and Bismahanine tend to aggregate at micromolar concentration and the $IC_{50}$ increased to 319.4 μM and 327.3 μM respectively in the detergent-based assay. Future studies especially on the chemical modification of the structure of Bismahanine could be carried out in order to discover more potent dengue protease inhibitors.

# CHAPTER ONE

# INTRODUCTION

## 1.1 Statement of Problem

Dengue disease, an arthropod-borne viral infection, has affected more than 100 countries worldwide. Every year, nearly 390 million cases of this infection are reported and over 3.9 billion people are at risk (Bhatt *et al.*, 2013). There is an increasing trend of dengue cases due to climate change, flourishing of tourism industry (Freedman *et al.*, 2006), unorganized urbanization and poor water management system. In Malaysia, it is an endemic and epidemic disease which increases yearly. In the year 2013, 43,346 cases reported and this number increased drastically to 103,610 cases in 2014 (WHO, 2014) and 107,079 cases in the year 2015 (WHO, 2015).

To date, there is no efficient marketed drug to treat dengue. Lack of suitable animal model (Zompi & Harris, 2012) for *in vivo* study slow down the dengue drug discovery compounded with the fact that part of the mechanisms of dengue virus infection is still unclear (Thullier *et al.*, 2001).

Only few peptides (Yin *et al.*, 2006), non-peptide (Ganesh *et al.*, 2005), small molecules (Deng *et al.*, 2012) and natural compounds (de Sousa *et al.*, 2015) have been reported to have inhibition activity towards dengue NS2B-NS3 protease. For instance, aprotinin inhibits dengue virus activity at submicromolar concentrations. However it cannot be developed as drug due to its large unstable peptide structure that would envelops enzyme and blocks substrate from approaching the active site and form non-competitive inhibition (Leung *et al.*, 2001). Ribavirin, a RNA virus inhibitor, suppresses virus replication *in vitro* but shows protective effect in animal study (Leyssen *et al.*, 2008). Kalata B1 cyclotide, one type of plant protein, which is designed and synthesized from natural cyclotide has potent inhibition from dengue NS2B-NS3 protease (Gao *et al.*, 2010). The findings of cyclohexenyl

chalcone derivatives isolated from *Bosenbergia rotunda* as competitive inhibitors to DEN2 NS2B-NS3 protease (Kiat *et al.*, 2006) have encouraged researchers to find more potential drugs from natural product.

Natural product has been established as excellent lead for therapeutic field, for example, taxol from *Taxus brevifolia* that treats cancer and artemisinin from *Artemisia annua* is widely used in treating malaria (Phillipson, 2001). Vinblastine from *Catharanthus roseus (L.)* is a vinca alkaloid and chemical analogue of vincristine. Both compounds are chemotherapy medication used to treat acute lymphocytic leukemia, acute myeloid leukemia, Hodgkin's disease, neuroblastoma, and small cell lung cancer (Lahlou, 2013).

Interestingly, the endemic cases of dengue usually occur in developing or underdeveloped countries with inadequate facilities where most patients could not afford expensive treatment. Drug discovery and development of dengue disease should not contribute to high-priced medicines that burden the patients. Therefore exploration of plant compounds is suitable as they are natural and cheap sources for remedy. Malaysia is rich in flora and fauna with over 16,000 flowering plants (Napis *et al.*, 2001) and 3000 species of medicinal plants, providing broad environment and good opportunity to drug discovery and development for dengue disease.

In addition, computational method has an apparent important role in drug discovery and development from the stage of target identification, lead discovery, and lead optimization, preclinical to clinical trials since 1980s (Ou Yang *et al.*, 2012). *In silico* methods decrease the amount of resources required for trial experiment, simulate the working environment and predict chemical interaction, and therefore aid in improving efficacy and efficiency of drug discovery (Kapetanovic, 2008).

Some commercial drugs begin their research with computer aided drug design method. Captopril is the first success drug candidate using structure based drug design. It is an angiotensin-converting enzyme (ACE) inhibitor used for hypertension and treat congestive heart failure (Cushman *et al.*, 1977). Dorzolamide is carbonic anhydrase inhibitor use in the treatment of the ocular disease, glaucoma (Greer *et al.*, 1994). Nelfinavir is HIV protease inhibitor (Wlodawer & Vondrasek, 1998). Zanamivir demonstrate the power of rational, structure-based and computer assisted drug design. It is neuraminidase inhibitor received US FDA approval in 1999 for the treatment of the influenza A and B viruses. (von Itzstein *et al.*, 1993). Imatinib is tyrosine kinase inhibitor for bcr-abl fusion protein (Philadelphia chromosome-positive leukemias) with the help of rational drug design approach (Nagar *et al.*, 2002).

In this study, the main aim was to utilise local natural product as the sources of candidate for dengue inhibitors confirmed by the *in vitro* enzymatic assay. The mechanism of action of inhibitor will then be investigated thoroughly using *in silico* molecular docking study.

## 1.2 Objectives and Scopes of Research

This study involved the screening of the inhibitory activity of selected Malaysian culinary plants using *in vitro* dengue enzyme bioassay. Seven plants were selected for extraction, separation and characterisation of the natural products. Each extract's fraction was evaluated in dengue enzymatic bioassay system and the active fraction was further sub-fractionated or isolated. Structure elucidation of chemical constituent was carried out with High Performance Liquid Chromatography (HPLC), Fourier Transform Infrared Spectroscopy (FTIR), Quadrupole Time-of-flight Mass Spectrometry (QTof-MS) and Nuclear Magnetic Resonance (NMR). Bioactivity of chemical constituent was evaluated using dengue enzymatic bioassay. Molecular interaction between chemical constituent and protein structure was studied with *in silico* method using software Autodock 4.2.

Specifically, the objectives of this study are:

1. To determine the inhibition activity of fractions from Malaysian culinary plants towards dengue protease NS2B-NS3 using *in vitro* method.
2. To characterise chemical constituents from Malaysian culinary plants with potential anti dengue property.
3. To study the interaction between chemical constituent and dengue protease using *in silico* docking method.

## 2.3 Natural Product in Drug Discovery

Plants are used as medicine by folks since ancient time (Weldegerima, 2009). Traditionally, plants with fewer side effects are the major driver in the study of phytochemical of natural products (Phillipson, 2001). Sources of natural products however are not only limited to higher plant species but also include terrestrial plants, microorganisms, marine organisms, vertebrates and invertebrates. The traditional way of drug discovery involving natural product is a high cost and time consuming. Thus, in modern days, different kinds of strategies such as genetic engineering, high speed dereplication and advanced methods in separation are used to expand the usage of natural products in drug discovery (Harvey, 2000). Among the families of secondary metabolites, nitrogen containing alkaloids are the largest group of drug (Raskin *et al.*, 2002) while terpenoids make important contribution as well (Cragg, 1998).

Drug discovery nowadays encompasses multidisciplinary approach from natural product research, combinatorial synthetic chemistry to biosynthetic pathways research and proteomics or genomics research. Drug discovery has shifted from natural product to microorganism field when the first antibiotic penicillin was derived from fungi at 1920's. Natural products derived from microbes are the outcome of genes or non-ribosomal peptide synthetases or pathways (Baker *et al.*, 2007). Antibiotics such as erythromycin, chloramphenicol and streptomycin isolated from various microbes are still being used as drugs today. Over sixty percent of drug in the market today have history related to natural product or natural product inspired. Among the 175 small molecules discovered in cancer research between 1940s and 2010, 48.6% are natural products or their derivatives. This proves the effectiveness of natural product in treating diseases and nature is shown to have important role in providing new scaffold for diseases (Newman & Cragg, 2012).

Big pharmaceutical companies emphasize the research focusing on synthetic chemistry, combinatorial chemistry and genomics over the past decades. Synthetic chemistry

and combinatorial chemistry have been at the forefront of the development of drug discovery for decades but there are limitations. Synthetic drug discovery output lacked new lead compounds from its library as the time passed by (Lee & Schneider, 2001). Combinatorial chemistry research is the combination of all possible chemical building blocks but the outcome of the huge library of compounds brings unfruitful effect. Sorafenib from Bayer as anti-tumor compound is the only new chemical entity as the product of combinatorial chemistry research (Newman & Cragg, 2007). Bioassay-guided extraction, separation and isolation with various types of chromatography technology are widely used nowadays (Sticher, 2008). A successful example for bioassay guided isolation method is the discovery of novel HIV inhibitor betulinic acid and derivatives (Itokawa *et al.*, 2008).

Discovery of new drug scaffold and pharmacophore from natural product offer new opportunity in the isolation of new bioactive compounds for lead discovery. So far, plants are still the majority sources for licensed drugs. For example, immunosuppressive agent such as cyclosporine A, antitumor agents such as paclitaxel and topotecan, anti-cholesterolemic agents such as lovastatin are derived from natural products (Bindseil *et al.*, 2001). Aspirin, morphine, quinine, paclitaxel and artemisinin are also the examples of drug developed from natural products (Cragg *et al.*, 1997).

On the other hand, the disadvantage of natural product is the difficulties for resupplying the same species of plant. The isolated compounds might be in small quantities or are not enough for study of lead optimization, lead development and clinical trials. However, many plant derived drugs cannot be fully synthesized due to its complexity and high cost such as atropine and reserpine. Thus, natural product drug discovery need collaboration from fields of natural product chemistry, pharmacognosy, pharmacology, ethnobotany and life sciences to get a novel marketed drug for future enhancement.

## 2.4 Malaysia Culinary Plant

Malaysia, located in the tropical area has rainforests which consist of 15,000 species of higher plants. According to literature review, *Cymbopogon citratus* (Cavalcanti *et al.*, 2004), *Curcuma longa* (Kalaivani *et al.*, 2012), *Ocimum basilicum* (Murugan *et al.*, 2007), *Pandanus amaryllifolius* (Pratama *et al.*, 2009), *Zingiber officinale* (Kalaivani *et al.*, 2012) and *Murraya koenigii* (Kovendan *et al.*, 2012) have potential inhibition activity towards larvicidal activity against *Aedes aegypti*. Previously, in a virtual screening study in our lab, some of these edible plants were found to have good inhibitions towards the dengue protease (PhDs, unpublished result).

Scientists have discovered that some Malaysian culinary plants were able to give promising vector control activity as shown in Table 2.2. Leave extracts of *Pandanus amaryllifolius* killed 100% of mosquito larvae of *Aedes aegypti* at 0.9% concentration (Pratama *et al.*, 2009). Leaf extracts of *Murraya koenigii* had larvicidal property against house-resting mosquitoes in the tropical country (Kovendan *et al.*, 2012). $LC_{50}$ value of *Ocimum basilicum* was 3.7 for larvae first instar, 4.1 for second instar, 4.6 for third instar and 5.1 for fourth instar suggesting that the plant can be used to control larvicidal activity for *A. aegypti*. Essential oil of *C. citratus* is also a promising larvicide against mosquitoes (Cavalcanti *et al.*, 2004), while the essential oil of *Z. officinale* and *C. longa* caused larval mortality within 24 hour at concentrations of 50.78 ppm and 192 ppm respectively (Kalaivani *et al.*, 2012). Only *P. odorata* did not have any literature review regarding its larvicidal activity. Thus, it would be interesting for these plants to be further investigated for anti dengue properties in enzymatic level.

**Table 2.2** The Selected Malaysia Culinary Plants and Their Scientific Names

| Scientific Name | Common Name | Malay Name | Extract | $LC_{50}$ (ppm) | Reference |
|---|---|---|---|---|---|
| *Curcuma longa* | Turmeric | Kunyit | Oil | 115.6 | (Kalaivani *et al.*, 2012) |
| *Cymbopogon citratus* | Lemon grass | Serai | Oil | 69 | (Cavalcanti *et al.*, 2004) |
| *Murraya koenigii* | Curry leaves | Daun kari | Hex | 963.53 | (Kovendan *et al.*, 2012) |
| *Ocimum basilicum* | Sweet basil | Daun selasih | Oil | 148.5 | (Kalaivani *et al.*, 2012) |
| *Pandanus amaryllifolius* | Screw pine | Daun pandan | $CHCl_3$ | 221.67 | (Mardiyah & Satoto, 2014) |
| *Persicaria odorata* | Vietnamese mint | Daun kesum | - | - | - |
| *Zingiber officinale* | Ginger | Halia | Oil | 40.5 | (Kalaivani *et al.*, 2012) |

### 2.4.1 *Curcuma longa*

In Chinese and Indian traditional medicines, *Curcuma longa* is used for the treatment of abdominal pains, sprains and swelling as well as stomach tonic and blood purifier. The common name for *C. longa* is turmeric and it is widely used as spice in food preparation. Table 2.3 shows the taxonomy of this plant.

**Table 2.3** Taxonomy of *Curcuma longa*

| Rank | Scientific Name | Common Name |
|---|---|---|
| **Kingdom** | Plantae | Plant |
| **Subkingdom** | Tracheobionta | Vascular plant |
| **Superdivision** | Spermatophyta | Seed plant |
| **Division** | Magnoliophyta | Flowering plant |
| **Class** | Liliopsida | Monocotyledons |
| **Order** | Zingiberales | - |
| **Family** | Zingiberaceae | Ginger family |
| **Genus** | *Curcuma* L. | - |
| **Species** | *Curcuma longa* L. | Turmeric |

The yellow pigment curcumin (diferuloylmethane) shows in Figure 2.7a is the major chemical constituent extracted from the rhizome of the plant. Essential oil extracted from rhizome contains borneol (0.5%), sabiene (0.6%), α-phellanderene (1%), cineol (1%), zingiberene (25%) and sesquiterpines (53%) (Jayaprakasha *et al.*, 2005). Ar-turmerone