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ORIGINAL ARTICLE

Metabolite Profiling and *in vitro* Evaluation of *Lepisanthes fruticosa* Fruit Pulp Extract as Inhibitor against Dengue and West Nile Virus NS2B-NS3 Proteases

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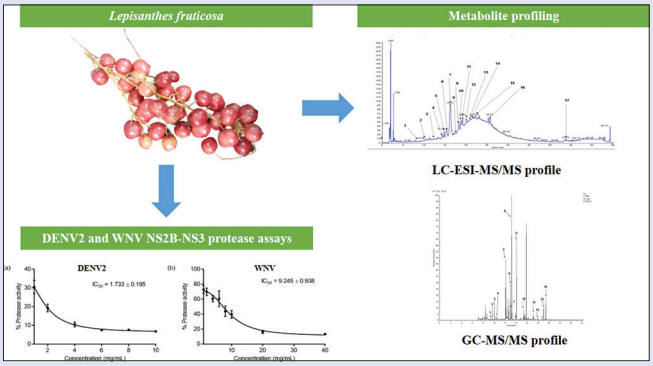
ABSTRACT

Background: Dengue virus serotype 2 (DENV2) and West Nile virus (WNV) fevers are mosquito-borne diseases with no effective treatment at present. In recent years, the development of plant-based antivirals targeting the viral NS2B-NS3 serine proteases has been the main focus as the synthetic antivirals available are not specific and less safe. **Objectives:** To evaluate the inhibitory activity of *Lepisanthes fruticosa* pulp extract against NS2B-NS3 proteases from DENV2 and WNV and identify the metabolites from this fruit extract. **Materials and Methods:** *In vitro* DENV2 and WNV NS2B-NS3 proteases assays were carried out using the methanolic extract of *L. fruticosa* pulp. Liquid chromatography-electron spray ionization-mass spectrometry/mass spectrometry (LC-ESI-MS/MS) and gas chromatography-mass spectrometry/mass spectrometry (GC-MS/MS) were performed to determine the metabolites present in this fruit species extract. **Results:** *L. fruticosa* extract exhibited inhibitory activity toward DENV2 and WNV NS2B-NS3 proteases with 50% inhibitory concentration value of 1.733 ± 0.195 and 9.245 ± 0.938 mg/mL, respectively. LC-ESI-MS/MS of *L. fruticosa* extract identified epigallocatechin-catechin, epigallocatechin, epicatechin, catechin, cyanidin rutinoside, procyanidin trimer, rutin, myricetin rhamnohexoside, luteolin glucoside and its derivative which were from the flavonoid group. In addition, GC-MS/MS identified fatty acids and sterols. **Conclusion:** The inhibitory activity of *L. fruticosa* pulp extract toward NS2B-NS3 proteases from DENV2 and WNV suggests this fruit species as a potential source for the development of antiviral. Metabolites from the groups of flavonols, flavones, and sterols identified in *L. fruticosa* pulp may contribute to the inhibitory properties of *L. fruticosa*.

Key words: Antiviral, dengue virus serotype 2, *Lepisanthes fruticosa*, NS2B-NS3 protease, West Nile virus

SUMMARY

- This study revealed the inhibitory activity *Lepisanthes fruticosa* pulp extract towards dengue virus serotype 2 and West Nile virus NS2B-NS3 proteases with 50% inhibitory concentration value of 1.733 ± 0.195 and 9.245 ± 0.938 mg/mL, respectively
- Metabolites identified by liquid chromatography-electron spray ionization-mass spectrometry/mass spectrometry from *L. fruticosa* pulp extract were epigallocatechin-catechin, epigallocatechin, epicatechin, catechin, cyanidin rutinoside, procyanidin trimer, rutin, myricetin rhamnohexoside, luteolin glucoside and its derivative
- Metabolites identified by gas chromatography-mass spectrometry/mass spectrometry were fatty acids and sterols.



Abbreviations Used: DENV2: Dengue virus serotype 2; WNV: West Nile virus; NS: Nonstructural; LC-ESI-MS/MS: Liquid chromatography-electron spray ionization-mass spectrometry/mass spectrometry; GC-MS/MS: Gas chromatography-mass spectrometry/mass spectrometry.

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INTRODUCTION

Flavivirus diseases such as dengue and West Nile Viral (WNV) fever have severely impacted and become widespread in the tropical and subtropical regions of the world. The incidence of these mosquito-borne diseases has increased dramatically every year and become a leading cause of hospitalization with estimated ambulatory and hospitalized costs of US$ 514–1394 and 20,000 cases of fatality among children and adults within these regions.[1] The high impact of this disease on global health has increased public attention in many aspects.[2] Even though a vaccine against dengue, known as Dengvaxia (CYD-TDV) has been recently developed to prevent this disease, the efficacy, however, is limited to a particular group of people and certain virus serotypes.[3] Therefore, an antiviral which can be used safely to reduce the disease

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severity in affected patients is much needed.[4] Hence, the development of antivirals against these viral diseases has been actively carried out.

The development of antivirals can benefit from the availability of structural information on the dengue virus as well as WNV. These viruses contain a long polyprotein precursor that is broken down into nonstructural (NS) and structural proteins. One of the NS proteins is NS3 serine protease that requires NS2B, a 14-kDa protein, as a cofactor to form an NS2B-NS3 stable complex which assists the replication of the virus. The inhibition of this protein complex disrupts the process of viral replication and maturation.[5] Therefore, this protein has been a prime target in the development of antiviral drugs.[6,7]

Recently, the global pharmaceutical industry has turned their attention to medicinal crops in the development of drugs. So far, out of 150 prescription drugs, at least 113 of them are from natural sources with 74% derived from plants.[8] The demand for plant-based drugs has been increasing as they are considered safer with lesser side effects in a cost-effective manner compared to synthetic drugs.[9,10] Moreover, they are rich in essential vitamins and metabolites, which are powerful substances in maintaining human wellness and treating diseases including viral diseases. Numerous metabolites have been considered and utilized in lead optimization and synthesis.[11] Hence, the exploration and screening of underutilized plants with medicinal values are important and worth to develop life-saving cures.

Lepisanthes fruticosa, or locally known as Ceri Terengganu, is an under-researched and underutilized fruit species in Malaysia that has been only used as traditional medicine by rural folks. They use this fruit species to lower the body temperature during viral fever as well as to relieve itching.[12] This fruit species possesses strong antioxidant properties and high total phenolic contents.[13] Flavonoids and tannins with antioxidant and antidiabetic activities were found in the ethanolic crude extract of *L. fruticosa* seed.[14] However, the metabolite composition and medicinal effect of its pulp has not been evaluated.

Therefore, in the present study, we evaluated the therapeutic effect of *L. fruticosa* pulp extract in inhibiting the activity of NS2B-NS3 proteases from dengue virus serotype 2 (DENV2) and WNV. We also determined the metabolite composition of the pulp extract through the combination of liquid chromatography-electron spray ionisation-mass spectrometry/mass spectrometry (LC-ESI-MS/MS) and gas chromatography-mass spectrometry/mass spectrometry (GC-MS/MS) approaches.

MATERIALS AND METHODS

Chemicals and reagents

Analytical grade chemicals and reagents from Sigma-Aldrich, USA were used for LC-ESI-MS/MS and GC-MS/MS. Difco™ Luria-Bertani broth and ampicillin were purchased from Fischer Scientific (Fischer Scientific, USA) and Sigma-Aldrich, respectively.

Plant material and extraction

L. fruticosa fruits were collected from Ceri Terengganu Plot, Genebank and Seed Center at Malaysian Agricultural Research and Development Institute (MARDI), Serdang, Selangor, Malaysia. Plant identification was conducted by Dr. Mohd Norfaizal Ghazalli (MARDI) and a voucher specimen of *L. fruticosa* (MDI 12809) was deposited in MDI Herbarium, MyGenebank™ Complex, MARDI. Fully bright red *L. fruticosa* fruits at a maturity index of 8 were used throughout the experiments.[13] Before use, *L. fruticosa* fruits were washed thoroughly under running tap water and dried at room temperature. Pulp and seed were separated before the pulp was freeze-dried and grounded into powder.

Methanolic crude extraction was performed using powder samples (0.5 g) in 20 mL of 20% methanol. The mixture was homogenized for 1 min and vortexed for 15 min before centrifugation at 8,900 rpm for 5 min at 4°C. The supernatants were collected whereas the leftover residues were reextracted twice using 10 mL of the same extraction solvent. All collected supernatants were combined and filtered using Whatman® cellulose filter papers 11 µm (Merck, Darmstadt, Germany) to remove any carry-over residues. The supernatants were then concentrated and dried via a concentrator at ambient temperature. All extractions were done in triplicate.

Dengue virus serotype 2 NS2B-NS3 protease production, purification and assay

Recombinant *Escherichia coli* harboring pQE30-cNSB-(G4TG4)-NS3 plasmid was grown in Luria-Bertani broth supplemented with 100 mg/L ampicillin at 37°C for overnight with agitation.[15] This starter culture was then added to 1000 mL of the same medium and grown under the same conditions until reaching 0.5 of the optical density at 600 nm. Isopropylthio-β-D-galactoside at a final concentration of 0.5 mM was subsequently added to induce protein expression and the culture was grown for additional 5 h. Bacterial cells were harvested by centrifugation at 4000 rpm for 15 min at 4°C. The protease was produced in the supernatant as soluble protein. Partial purification of the protein was carried out using His GraviTrap Flow precharged Ni Sepharose 6 Fast column (GE Healthcare, Chicago, Illinois, United States) using the method described previously.[15] The column was equilibrated with an equilibration buffer (20 mM sodium phosphate and 500 mM sodium chloride at pH 7.4). The protein sample was loaded into the column before washing with binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 20 mM imidazole at pH 7.4). Elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 200 mM imidazole at pH 7.4) was then added to elute the protein.

DENV2 assay was performed using the method described by Rothan *et al.*[16] Before the assay, the dried extract was reconstituted with 0.1% dimethylsulfoxide (DMSO). In 200 µL end-point reaction, the extract was added with 2 µM NS2B-NS3 protease and buffered at pH 8.5 with 200 mM Tris-HCl. The reaction mixture was incubated at 37°C for 30 min. Subsequently, 20 µM fluorogenic peptide substrate (Boc-Gly-Arg-Arg-AMC) was added and the mixture was further incubated at the same temperature for 30 min. Measurements were performed in triplicates and read using EnSpire Multimode Plate Reader (PerkinElmer, Waltham, Massachusetts, United States). Substrate cleavage was optimized at the emission at 440 nm upon the excitation at 350 nm and normalized to the negative control (reaction mixture without substrate). The protease activity was calculated based on the cleavage of fluorogenic peptide substrate which provided the 50% inhibitory concentration (IC_{50}) of the extract using nonregression linear model in GraphPad Prism version 7.0 (GraphPad Software, San Diego, California, United States).

West Nile Virus NS2B-NS3 protease assay

WNV NS2B-NS3 protease assay was performed using SensoLyte® 440 West Nile Virus Protease Assay Kit (Anaspec, Fremont, California, United States) according to the manufacturer's instructions. The activity of recombinant WNV NS3 protease was determined using the fluorogenic Pyr-RTKR-AMC substrate in the presence of the extract reconstituted with 0.1% DMSO at 37°C for 30 min. The generated AMC fluorophore upon NS3 protease cleavage was detected with an excitation at 354 nm and emission at 442 nm using EnSpire Multimode Plate Reader. Measurements were performed in triplicate and normalized to the negative control (reaction mixture without substrate). The IC_{50} value of the extract was calculated from the readings using the nonregression

linear model in GraphPad Prism version 7.0. (GraphPad Software, San Diego, California, USA).

Metabolite identification via liquid chromatography-electron spray ionisation-mass spectrometry/mass spectrometry

The separation and relative identification of metabolites were achieved using a 1200 series high-performance liquid chromatography (HPLC) unit (Agilent Technologies, Santa Clara, California, United States) coupled with a 3200 QTrap MS/MS (AB Sciex, USA). An HPLC column (C18 Thermo Hypersil Gold, 5 µm, 150 mm × 4.6 mm) (Thermo Fisher Scientific, Waltham, Massachusetts, United States) preceded with a guard column (5 µm, 10 mm × 4.6 mm) was used for the separation of compounds. Solvent A (0.5% formic acid) and solvent B (0.5% formic acid in acetonitrile) were used as the mobile phases. The gradient setting was set as follows: equilibration of the column for 5 min at 95% A; gradual decrement of A until 70% for 40 min; fast decrement of A to 5% in 5 min and further maintaining the same concentration of A for another 5 min; fast increment of A to 95% within a minute and maintaining the same concentration of A for 4 min. The extract (20 mg/mL in 30% methanol) at 20 µL was injected into the HPLC with a flow rate at 1000 µL/min. Phenolic acids were monitored at 280 nm and 360 nm while anthocyanins at 480 nm and 540 nm. The temperature in MS was set at 500°C in negative ionization. Collision energy, de-clustering potential, and entrance potential were set at-25.0,-40.0 and-10.0 volt, respectively. Analyst Software version 1.4.2 (AB Sciex, USA) was used for data analysis. Metabolite identification was performed by comparing the mass fragmentations and retention time from past studies.

Metabolite extraction and identification via gas chromatography-mass spectrometry/mass spectrometry

Unlike LC-ESI-MS/MS, the metabolite extraction and derivatization for GC-MS/MS were performed using a two-phase methanol-chloroform method according to Roessner *et al.*[17] with a slight modification. Methyl nonadecanoate was used as an internal standard for nonpolar compounds. Methanol (8 mL) was added to powder samples (1.0 g) and incubated for 15 min at 70°C in a water bath. The samples were then mixed vigorously with 8 mL volume of distilled water. The mixtures were added with chloroform prior to phase separation by centrifugation. Nonpolar supernatants were collected and dried in a vacuum concentrator for 2–6 h. A silylation step was performed by adding 250 µL N-methyl-N-TMS trifluoroacetamide (Sigma Aldrich, St. Louis, Missouri, United States) to the extracts followed by incubation for 1 h at 37°C in a water bath. The extracts were chilled down at room temperature for at least 1 h before the GC-MS/MS injection. Samples at 1 µL were injected using a splitless mode into a GC-MS/MS system consisting of TSQ Quantum XLS GC-MS/MS (Thermo Fisher Scientific, Waltham, Massachusetts, United States). The GC column utilized for the analysis was TG-5MS with an inner diameter of 0.25 mm, 30 m length, and 0.25 µm film thicknesses. Helium gas at a flow rate of 1 mL/min was used as a carrier gas. The samples using M/C technique were analyzed using the following oven temperature program: injection at 70°C, increase to 76°C at 1°C/min, increase to 330°C at 6°C/min and 10 min isothermal at 330°C. Mass spectra were attained using the full scan monitoring mode with a mass scan range of 50–700 *m/z*. XCalibur™ software (Thermo Fisher Scientific, Waltham, Massachusetts, United States) provided in the GC-MS/MS system was used to evaluate the chromatogram and mass spectra. Metabolite identification was performed by comparing the mass spectra with the spectrum available in NIST 98 mass spectral library. The metabolites were characterized on the basis of their molecular formula, retention time, and total ion chromatogram.

RESULTS

Inhibitory activity of *Lepisanthes fruticosa* extract against NS2B-NS3 proteases from dengue virus serotype 2 and West Nile virus

The recombinant NS2B-NS3 protease from DENV2 with the size of ~34 kDa was produced in *E. coli* as soluble protein and partially purified using a nickel column [Figure 1]. The inhibition of DENV2 protease activity by *L. fruticosa* pulp extract was proven in a dose-dependent manner [Figure 2a]. At the lowest concentration tested of 1 mg/mL, the extract reduced the DENV2 activity by 70%. A maximal inhibition of DENV2 activity by the extract was approximately 93% at the concentration of 10 mg/mL. The IC_{50} of the extract against DENV2 was observed at 1.733 ± 0.195 mg/mL.

L. fruticosa pulp extract was also able to inhibit WNV NS2B-NS3 protease activity [Figure 2b]. The extract inhibited WNV protease activity by up to 28% at the concentration of 1 mg/mL. Similar to DENV2, this protease activity was maximally inhibited by 87% but at a higher concentration of 40 mg/mL. The IC_{50} of the extract against WNV was observed at 9.245 ± 0.938 mg/mL.

Metabolite identification and characterization via liquid chromatography-electron spray ionisation-mass spectrometry/mass spectrometry

The mass spectrometer of *L. fruticosa* pulp extract detected ten peaks identified with known metabolites based on previously reported mass fragmentation for the same genus and other herbs [Figure 3]. Based on this data, *L. fruticosa* pulp extract was found rich in flavonoids. Epigallocatechin, epicatechin, catechin, luteolin, and cyanidin, which belong to this group, were present in this pulp extract [Table 1].

Peak 6, which registered for $(M-H)^-$ 289, produced a product ion *m/z* 245 indicating a loss in the carboxyl group $(M-H-COO)^-$. Further

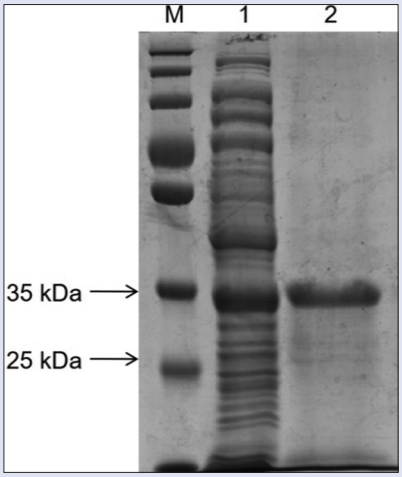

Figure 1: Expression and partial purification of the recombinant dengue virus serotype 2 NS2B-NS3 protease. The recombinant protein at ~ 34 kDa was obtained. Lane M, PageRuler prestained protein ladder (Promega, USA), lane 1, the crude extracts of *Escherichia coli* cells containing dengue virus serotype 2 NS2B-NS3 protease, and lane 2, the purified dengue virus serotype 2 NS2B-NS3 protease

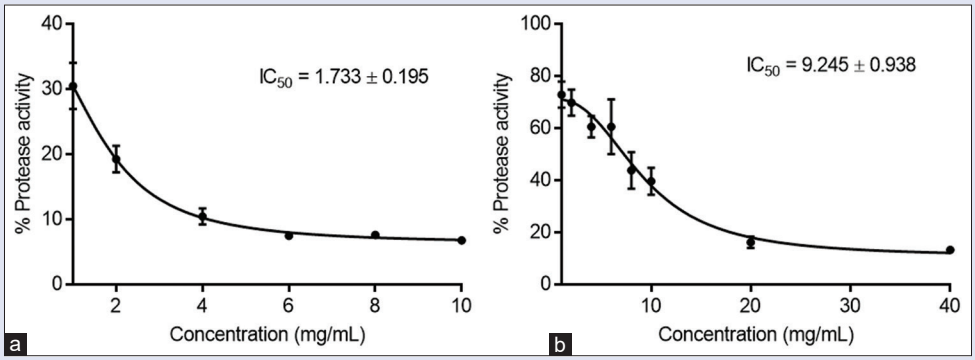

Figure 2: Inhibitory activity of *L. fruticosa* pulp extract against NS2B-NS3 proteases from dengue virus serotype 2 and West Nile virus. (a) Activity of dengue virus serotype 2 protease was measured based on the ability of the enzyme to cleave the fluorogenic peptide substrate (Boc-Gly-Arg-Arg-AMC) at 37°C. (b) Activity of West Nile virus protease was measured based on the ability of the enzyme to cleave the fluorogenic peptide substrate (Pyr-RTKR-AMC) at 37°C. For both assays, the 50% inhibitory concentration value was calculated from the readings using non-regression linear model in GraphPad Prism 7.0 software

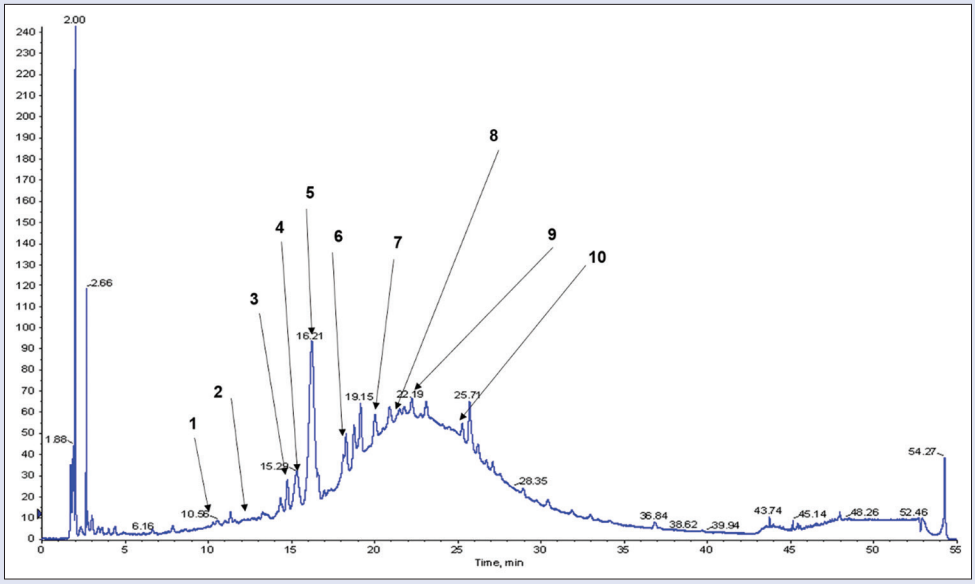

Figure 3: Liquid chromatography profile of *L. fruticosa* pulp extract. Peak number represents the metabolite identified as in Table 1

Table 1: The proposed metabolites identified in *Lepisanthes fruticosa* pulp extract obtained from liquid chromatography-electron spray ionisation-tandem mass spectrometry

Peak number	Metabolite	Retention time (min)	$[M-H]^-$ (*m/z*)	MS/MS (*m/z*)*
1	Epigallocatechin-catechin	10.3	593	467, **425**, **407**, **289**, **245**, 203, **177**
2	Epigallocatechin	12.6	305	287, **261**, **219**, **179**, **167**, **137**
3	Luteolin glucoside	15.3	447	299, **285**, 284, 240, 388, 357, 321
4	Luteolin derivative	16.0	611	**501**, **475**, 485, **285**, 241
5	Cyanidin rutinoside	16.4	593	285, 309, 339, 201, 173, 155
6	Epicatechin	18.2	289	**271**, **245**, 231, 227, 205, **203**, **187**, 179, 75, 167, **161**, 151, 149, 137
7	Procyanidin trimer	19.9	865	**739**, 713, **695**, **577**, **543**, **425**, **407**, **287**, 245
8	Catechin derivative	20.8	401	**289**, 257, **245**, 231, 215, 205, **203**, **161**, 195, 187, 173
9	Myricetin rhamno-hexoside	22.1	625	625, 607, 316
10	Rutin	25.2	609	**301**, 343

*Bold numbers indicate the *m/z* values of the predominant ions. MS/MS: Tandem mass spectrometry

fragmentation of *m/z* 245 produced *m/z* 203, which corresponded to the loss of an acetyl group (M-H-COO-CH_3CO)$^-$. Other product ions include 187, 161, 137, which were in line with the fragmentation and retention time of epicatechin.[18,19] Peaks 1 and 2 were identified tentatively as epigallocatechin-catechin and epigallocatechin respectively. The former has a precursor ion $[M-H]^-$ 593 and product ions at $(M-H)^-$ 289 after the loss of a epigallocatechin unit $[M-H]^-$ 305. Peak 7 has a precursor ion $(M-H)^-$ 865 and product ions *m/z* at 577 and 287 suggesting the possible loss of two epicatechin moieties. Other product ions such as *m/z* 407 (M-H-epicatechin-galloyl)$^-$ confirmed

peak 7 as the procyanidin trimer.[19,20] Peak 8 was named as a derivative of catechin since its precursor ion $(M-H)^-$ 401 has product ions that similar to catechin $(M-H)^-$ 289 after a loss of unknown mass at *m/z* 112.

Peaks 3 and 4 shared a common product ion at *m/z* 285 indicating the presence of luteolin in the extract. Peak 3 has precursor ion $(M-H)^-$ 447 and subsequent loss of a glucose moiety produced luteolin as the product ion. Based on the ultraviolet (UV) absorption and retention time of peak 3, it was identified as luteolin glucoside.[21,22] Peak 4 was labeled as luteolin derivative due to the unknown loss of mass fragment at *m/z* 326 to produce the product ion 285 $[(M-H-326)^-$.

Peak 9 with $(M-H)^-$ at 625 has product ions at *m/z* 316 indicating a loss of glucose-rhamnoside moiety $(M-H-162-148)^-$. Based on the molecular weight of myricetin at 317, peak 9 could be myricetin rhamno-hexoside.[18] Peak 10 was confirmed as rutin where its precursor ion *m/z* 609 $(M-H)^-$ producing product ion at *m/z* 301 $(M-H)^-$ after the elimination of a rutinoside moiety.[22] Peak 5 has an intense product ion at *m/z* 285 indicating a loss of rutinoside moiety $(M-H-308)^-$. Further analysis on the UV absorbance indicated that peak 5 has UV_{max} at 540/480 nm, which confirmed the presence of cyanidin.[23] Hence, peak 5 was labelled as cyanidin rutinoside.

Metabolite profiling and identification via gas chromatography-mass spectrometry/mass spectrometry

GC-MS/MS chromatogram of non-polar extract of *L. fruticosa* pulp showed 14 peaks indicating the presence of 14 different metabolites [Figure 4]. The majority of these metabolites belong to fatty acids and plant sterols [Table 2]. Hexadecanoic acid was the most abundant metabolite found in this extract, which was categorized under the fatty acid group. The other identified metabolites which belong to this group were palmitelaidic acid, linoleic acid and stearic acid, whereas the metabolites belonging to plant sterols were stigmasterol, tocopherol and sitosterol. The sterols were eluted at the very end of the chromatogram. Other metabolites identified were acetopyruvic acid, succinic acid, and glutoconic acid which belong to organic acid groups and D-fructofuranose, mannopyranose and D-turanose which were sugars.

DISCUSSION

The demand of antivirals against dengue and WNV is globally high despite the availability of the vaccine. The antiviral can help in reducing the severe outcomes among dengue and WNV patients who currently only depend on supportive care. NS2B-NS3 proteases from flaviviruses have been the prime target for the development of antivirals.[6] Synthetic antivirals have been developed, but they cause viral resistance and pose side effects if they are consumed for a long period.[24] Thus, the development of plant-based antivirals as a potent and safe alternative against dengue and WNV is much needed.

In the present study, we proposed *L fruticosa* as a new inhibitor against NS2B-NS3 proteases that could be considered in the development of antivirals against DENV2 and WNV. Protease assay demonstrated the inhibitory activity of *L. fruticosa* pulp extract towards DENV2 and WNV NS2B-NS3 proteases. The crude extract was evaluated as to substitute traditional medicines in which whole plants or mixtures of plants are used rather than isolated single compounds or synthetic analogues. In other plants, the extracts often have a greater therapeutic effect *in vitro* and *in vivo* than the single compounds due to the synergistic action between the compounds in the extracts.[25] In the case of *L. fruticosa*, however, the differences in the inhibitory effect on NS2B-NS3 proteases between the extract and the isolated compounds have not been determined yet.

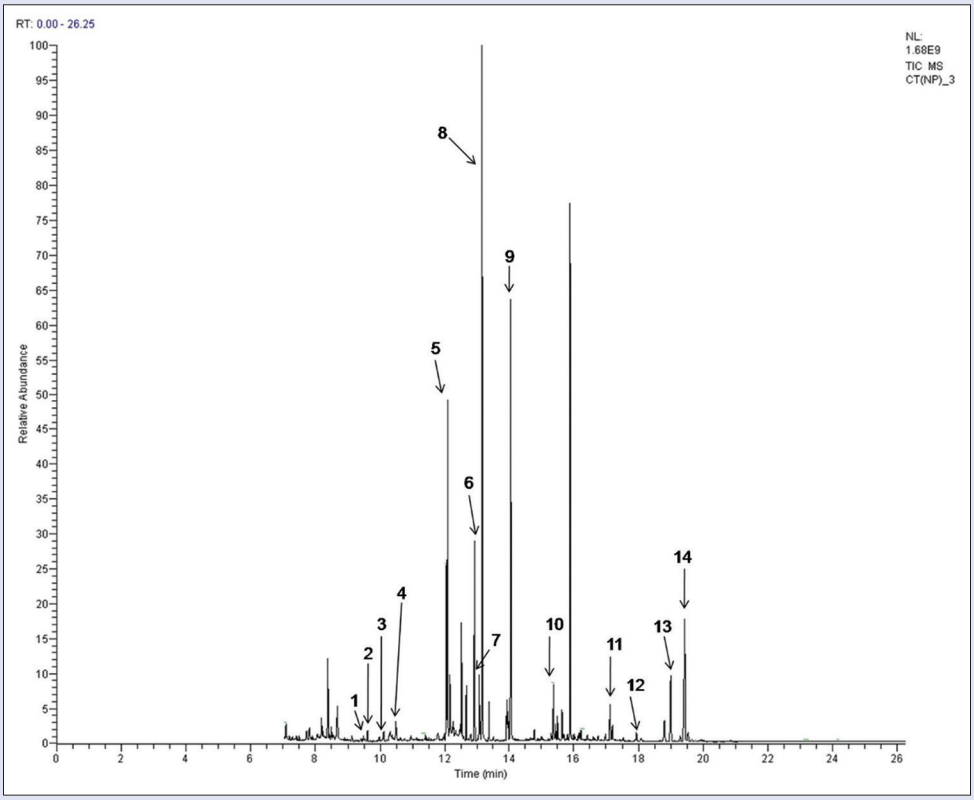

Figure 4: Gas chromatography profile of nonpolar extract from *L. fruticosa* pulp. Peak number represents the metabolite identified as in Table 2

Table 2: The proposed metabolites identified in *Lepisanthes fruticosa* pulp extract obtained from gas chromatography-mass spectrometry/mass spectrometry

Peak number	Metabolite	Molecular formula	Retention time (min)	Total composition/total ion chromatogram
1	Acetopyruvic acid	$C_{14}H_{30}O_4$	9.48	8.41×10^5
2	Glutaconic acid	$C_{14}H_{30}O_5$	9.61	1.36×10^5
3	Succinic acid	$C_{13}H_{30}O_5$	10.11	2.69×10^6
4	Benzoic acid esters	$C_{17}H_{26}O_3$	10.49	6.93×10^6
5	D-fructofuranose	$C_{21}H_{52}O_6$	12.08	1.03×10^8
6	Mannopyranose	$C_{21}H_{52}O_6$	12.91	4.69×10^7
7	Palmitelaidic acid	$C_{19}H_{38}O_2$	13.06	6.91×10^6
8	Hexadecanoic acid (palmitic acid)	$C_{19}H_{40}O_2$	13.15	1.13×10^8
9	Octadecadienoic acid (linoleic acid)	$C_{21}H_{44}O_2$	14.06	6.43×10^7
10	D-turanose	$C_{36}H_{86}O_{11}$	15.38	9.11×10^6
11	Octadecanoic acid (stearic acid)	$C_{32}H_{58}O_2$	17.12	4.97×10^6
12	Tocopherol	$C_{32}H_{58}O_2$	17.93	2.01×10^6
13	Stigmasterol	$C_{32}H_{58}O$	19.00	5.54×10^6
14	Sitosterol	$C_{29}H_{50}O$	19.42	1.16×10^7

L. fruticosa pulp extract exhibited a stronger preference of inhibition toward DENV2 protease than WNV protease. This could be due to the difference in the structure flexibility of their cofactor NS2Bs.[26] Although these proteins are highly homologous among flaviviruses, the NMR analysis indicated that the C-terminal segment of NS2B cofactor from DENV2, which contributes to the important residues in the active site, is susceptible to dissociation from NS3 compared to those in WNV protease.[27] This condition benefits the protease inhibitors where it prevents the correct association of NS2B cofactor to NS3 that leads to the suppression of protease activity in DENV2.[27]

The inhibitory effect of *L. fruticosa* pulp extract on DENV2 and WNV proteases could be contributed by the metabolites present in the extract. Both LC-ESI-MS/MS and GC-MS/MS systems were utilized to identify secondary metabolites from different types either they are volatiles or nonvolatiles. Through LC-ESI-MS/MS profiling, ten metabolites identified were mainly flavonoids. Catechin, epichatechin, and epigallocatechin, which belong to this group, have been known to possess antiviral effects against a broad spectrum of viruses including flaviviruses.[28] Catechin and its derivative from *Endiandra kingiana* exerted moderate inhibitory activities against DENV2 NS2B-NS3 protease and their activities were influenced by the hydrogen bonding formed with the important residue, Tyr161 within the pocket of the protease.[29] Raekiansyah *et al.*[30] also found that these polyphenols showed a strong inhibitory effect on DENV2 growth. Hence, the presence of these polyphenols in *L. fruticosa* is interesting as it could be the key component that may have the potential to contribute to the inhibitory effect of this fruit extract against DENV2 and WNV NS2B-NS3 proteases.

Moreover, *L fruticosa* pulp is also rich in luteolin, which could act as an antiviral. Luteolin is a natural flavone possessing strong antioxidant activity with numerous therapeutic effects including dengue fever.[31] Dwivedi *et al.*[32] revealed that luteolin inhibits DENV2 NS2B-NS3 protease by interacting with Asp75 and Ser135 residues at the catalytic triad of the protein that possibly changes the protein conformation leading to the alteration in the functional attribute of the protein. In addition, luteolin from *Viola yedoensis Makino* extract was also found to effectively reduce dengue infection by inhibiting the maturation process of the virus.[33] Similar to luteolin, myricetin was also reported to be an inhibitor of DENV2 NS2B-NS3 protease.[34] Interestingly, myricetin derivative has been also identified in *L. fruticosa* pulp extract which could provide the inhibitory effect of this extract towards DENV2 and WNV proteases.

Other than flavonols and flavones, an anthocyanidin, cyanidin rutinoside was also present abundantly in *L. fruticosa* pulp extract. This compound usually provides natural color in fruits and vegetables and demonstrates high medicinal value with strong antioxidant and anti-tumor activities.[35] Hence, this compound could be the main contributor of red purplish color in mature *L. fruticosa* fruit, which could serve as a natural colorant.[13] However, so far it has never been reported to possess antiviral activity against flavivirus diseases.

The employment of GC-MS/MS has enhanced the identification of small molecular weight metabolites from *L. fruticosa* pulp. This technique has revealed the abundance of fatty acids in this pulp. So far, there is no report on the inhibitory activity of fatty acids toward viral NS2B-NS3 proteases, but these organic compounds showed antiviral effect by affecting the viral envelope that subsequently causes a complete disintegration of the envelope and the viral particles.[36] Other than fatty acids, plant sterols, especially sitosterol have been proven to establish strong interaction with NS2B-NS3 protease of DENV2.[37] Likewise, stigmasterol was effective against dengue fever as proven in a traditional Chinese herb, *Isatis tinctoria.*[38] Tocopherol, a form of Vitamin E with strong antioxidant activity, also exhibited antiviral effects against few viruses including influenza[39] and hepatitis B[40] by preventing oxidative damage through its free-radical scavenging action.[41] Thus, the occurrence of these phytosterols in *L. fruticosa* may aid in the inhibitory effect towards DENV2 and WNV.

CONCLUSION

The present study revealed the inhibitory activity of *L. fruticosa* pulp against NS2B-NS3 proteases from DENV2 and WNV. Metabolites from the groups of flavonols, flavones and sterols were mostly present in *L. fruticosa* pulp that may contribute to the inhibitory properties of this fruit species. However, further analysis aiming at isolating and determining the inhibitory properties of the metabolites are required. The present study is also important in promoting this underutilized fruit species as a medicinal plant.

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Conflicts of interest

There are no conflicts of interest.

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