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RESEARCH ARTICLE

Natural DENV-2 NS2B/NS3 protease inhibitors from *Myristica cinnamomea* King

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ABSTRACT

The NS2B/NS3 protease is crucial for the pathogenesis of the DENV. Therefore, the inhibition of this protease is considered to be the key strategy for the development of new antiviral drugs. In the present study, malabaricones C (**3**) and E (**4**), acylphenols from the fruits of *Myristica cinnamomea* King, have been respectively identified as moderate (27.33 ± 5.45 µM) and potent (7.55 ± 1.64 µM) DENV-2 NS2B/NS3 protease inhibitors, thus making this the first report on the DENV-2 NS2B/NS3 protease inhibitory activity of acylphenols. Based on the molecular docking studies, compounds **3** and **4** both have π-π interactions with Tyr161. While compound **3** has hydrogen bonding interactions with Gly151, Gly153 and Tyr161, compound **4** however, forms hydrogen bonds with Ser135, Asp129, Phe130 and Ile86 instead. The results from the present study suggests that malabaricones C (**3**) and E (**4**) could be employed as lead compounds for the development of new dengue antivirals from natural origin.

Keywords: *Myristica cinnamomea* King; acylphenols; malacaricone C; malabaricone E; DENV-2 NS2B/NS3 protease.

INTRODUCTION

Dengue is a viral disease which leads to considerable morbidity and mortality (Balasubramanian *et al.*, 2019; Dighe *et al.*, 2019). The dengue virus (DENV), one of the most rapidly spreading mosquito-borne viral pathogens in the tropical and subtropical regions of the world, is a member of the genus *Flavivirus* (family Flaviviridae). The DENV is transmitted to humans via the bite of infected *Aedes aegypti* and *Aedes albopictus* mosquitos. This virus causes a wide spectrum of infections ranging from a non-severe form, the dengue fever (DF), to more severe forms such as dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS) (Mir *et al.*, 2016; Weng *et al.*, 2017; Perera *et al.*, 2018; Balasubramanian *et al.*, 2019; Rozera *et al.*, 2019). There are five distinct, yet closely related, stereotypes of the DENV which include DENV-1, DENV-2, DENV-3, DENV-4, DENV-5 with DENV-2 being the most prevalent among the five (de Sousa *et al.*, 2015; Mir *et al.*, 2016; Aguilera-Pesantes *et al.*, 2017; Singh & Rawat, 2017; Weng *et al.*, 2017; Perera *et al.*, 2018).

The NS2B/NS3 protease is a two component non-structural protein in the genomic sequence of the DENV. This enzyme mediates the cleavage of polyproteins which subsequently releases functional proteins that are required for the production of mature DENV and for maintaining its infectivity. Hence, it can be concluded that the NS2B/NS3 protease plays an important role in the genome replication process of the DENV. The NS2B/NS3 protease is therefore an ideal target for drug discovery against dengue infection (de Sousa *et al.*, 2015; Timiri *et al.*, 2016; Aguilera-Pesantes *et al.*, 2017; Takagi *et al.*, 2017; Weng *et al.*, 2017).

A large number of medicinal plants have been used to alleviate dengue infection. As a result, the secondary metabolites derived from these plants could be potential sources of dengue antiviral agents. To date, flavonoids, chalcones, biflavonoids, phenolics, alkaloids and curcuminoids have been reported to inhibit the DENV-2 NS2B/NS3 protease (Abd Kadir *et al.*, 2013; Abubakr *et al.*, 2013; Teixeira *et al.*, 2014; de Sousa *et al.*, 2015; Mir *et al.*, 2016; Timiri *et al.*, 2016; Singh & Rawat, 2017; Perera *et al.*, 2018; Balasubramanian *et al.*, 2019; Dighe *et al.*, 2019).

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In search of new small molecule scaffolds as lead compounds for the inhibition of the DENV-2 NS2B/NS3 protease, we decided to investigate the inhibiting potential of acylphenols. Herein, we report on the isolation of malabaricones A–C (**1**–**3**) and E (**4**) from the fruits of *Myristica cinnamomea* King (Figure 1). Their structures were characterized by means of NMR and MS spectral analyses. These acylphenols were subsequently evaluated for their DENV-2 NS2B/NS3 protease inhibitory activity. Following this, molecular docking was performed in order to provide insights into the binding interactions between the most promising acylphenol(s) and the DENV-2 NS2B/NS3 protease.

MATERIALS AND METHODS

General experimental procedures

Analytical and preparative TLC was carried out on Merck 60 F_{254} silica gel plates (absorbent thickness: 0.25 and 0.50 mm, respectively) (Merck, Germany). Column chromatography (CC) was performed using silica gel (230-400 mesh, ASTM) (Merck, Germany) and Sephadex LH-20 (Sigma-Aldrich, USA). IR spectra were recorded using a Perkin-Elmer Spectrum 400 FT-IR Spectrometer. NMR spectra were acquired in CD_3OD (Merck, Germany) using a JOEL ECA 400 MHz NMR Spectrometer. LCMS-IT-TOF spectra were obtained using an Agilent 6530 Accurate-Mass Q-TOF LC/MS system. UV spectra were recorded using a Shimadzu 1650 PC UV-Vis Spectrophotometer. All solvents were of analytical grade and were distilled prior to use. DMSO and Tris-HCl buffer were respectively purchased from Merck (Germany) and Sigma-Aldrich (USA) while Boc-Gly-Arg-Arg-MCA was purchased from Peptide Institute, Inc (Japan).

Plant Material

M. cinnamomea was collected from Johor in 2003. The plant was identified by Mr. Teo Leong Eng and a voucher specimen (KL 5043) has been deposited with the University of Malaya herbarium.

*Isolation and characterization of compounds **1**-**4***

Dried powdered fruits (1.5 kg) of *M. cinnamomea* were extracted with ethyl acetate (5.0 L, 2x) at room temperature, yielding 262.99 g of extract. The ethyl acetate extract (30 g) was chromatographed on a silica gel column (625 g, 7.2 cm x 63 cm). Elution was carried out using mixtures of dichloromethane : acetone in proportions of 90:10 (v/v, 3 L), 85:15 (v/v, 5 L), 80:20 (v/v, 5 L) and 75:25 (v/v, 3 L) sequentially to afford fractions FA (5.73 g), FB (2.44 g), FC (8.50 g) and FD (6.25 g), respectively. FA was fractionated on a silica gel column (180 g, 2.5 cm x 72 cm), eluted using dichloromethane (2 L) to afford sub-fraction FA 1 (3.24 g), following which FA 1 was re-chromatographed over a Sephadex LH-20 column (25 g) using methanol (1 L) as the eluent to afford **1** (2.70 g). FB was subjected to column chromatography over 60 g of silica gel (2.5 cm x 64.5 cm) with an isocratic solvent system of dichloromethane : acetone (95:5 v/v, 1 L) to provide sub-fraction FB 1 (1.86 g). FB 1 was further purified by passing it through a Sephadex LH-20 column (25 g) with methanol (500 mL) to yield **2** (1.29 g). Column chromatography of FC over silica gel (270 g, 5.0 cm x 72 cm) to afford sub-fraction FC 1 (6.50 g) was achieved using dichloromethane : acetone (90:10 v/v, 1 L). Next, the purification of FC 1 to provide **3** (5.18 g) was carried out over a Sephadex LH-20 column (25 g) using methanol (1 L) as the eluting solvent. Column chromatography of FD over silica gel (180 g, 2.5 cm x 72 cm) with successive elutions using dichloromethane : acetone in ratios of (85:15 v/v, 300 mL) and (80:20 v/v, 500 mL) afforded sub-fractions FD 1 (3.14 g) and FD 2 (1.25 g), respectively. FD 1 was re-chromatographed over a silica gel column (120 g, 2.5 cm x 72 cm) with an isocratic solvent system of dichloromethane : acetone (80:20 v/v, 1.5 L) to give sub-fractions FD 1.1 (0.09 g), FD 1.2 (0.08 g) and FD 1.3 (1.05 g). FD 1.2 was purified over a Sephadex LH-20 column (25 g) using methanol (500 mL) as the eluent to afford sub-fraction FD 1.2.1 (0.02 g) which was later purified via repetitive preparative TLC with dichloromethane : acetone [75:25 v/v] to yield **4** (4.6 mg). The degree of the purity of the isolated compounds was determined by ^{1}H NMR spectroscopy and was found to be 98%-99% pure.

*Malabaricone A (**1**)*

Pale yellow amorphous powder. UV (MeOH): 342 (3.07), 269 (3.67), 214 (3.78). IR (NaCl): 3583, 3271, 2920, 2851, 1714, 1589, 1511. ^{1}H-NMR (CD_3OD, 400 MHz): 7.23 (2H, m, H-12 & H-14), 7.21 (1H, t, *J* = 8.0, H-19), 7.17 (2H, m, H-11 & H-15), 7.12 (1H, m, H-13), 6.34 (2H, d, *J* = 8.0, H-18 & H-20), 3.10 (2H, t, *J* = 8.0, H-2), 2.58 (2H, t, *J* = 8.0, H-9), 1.66 (2H, brt, *J* = 8.0, H-3), 1.58 (2H, brt, *J* = 8.0, H-8), 1.33 (8H, brs, H-4-H-7). ^{13}C-NMR (CD_3OD, 400 MHz): 209.8 (C-1), 163.5 (C-17 & C-21), 144.1 (C-10), 136.9 (C-19), 129.5 (C-11 & C-15), 129.4 (C-12 & C-14), 126.7 (C-13), 111.5 (C-16), 108.5 (C-18 & C-20), 45.9 (C-2), 37.1 (C-9), 32.9 (C-8), 30.7 (C-4), 30.6 (C-5 & C-6),

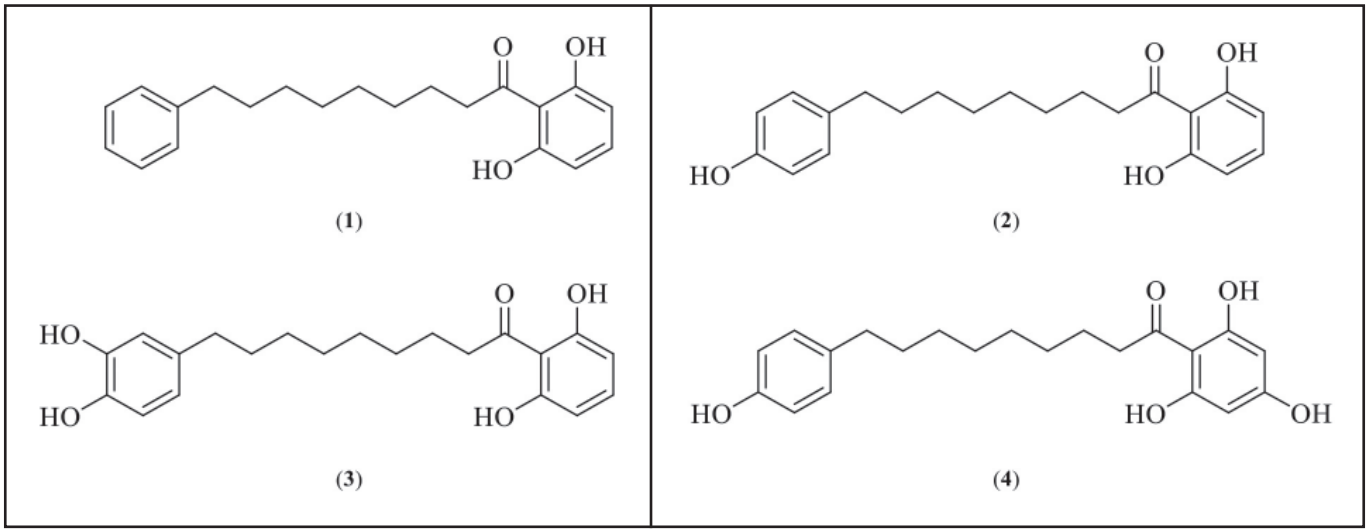

Figure 1. Structures of compounds **1**-**4**.

30.4 (C-7). 25.9 (C-3). LC/MS-IT-TOF: 349.1774 ([M + Na]$^+$; calc, $C_{21}H_{26}O_3Na$ 349.1774).

*Malabaricone B (**2**)*
Yellow amorphous powder. UV (MeOH): 341 (2.83), 270 (3.46), 206 (3.56). IR (NaCl): 3584, 3339, 2927, 2854, 1708, 1596, 1514. ^{1}H-NMR (CD_3OD, 400 MHz): 7.17 (1H, t, *J* = 8.0, H-19), 6.96 (2H, d, *J* = 8.0, H-11 & H-15), 6.67 (2H, d, *J* = 8.0, H-12 & H-14), 6.33 (2H, d, *J* = 8.0 Hz, H-18 & H-20), 3.10 (2H, t, *J* = 8.0, H-2), 2.47 (2H, t, *J* = 8.0, H-9), 1.66 (2H, p, *J* = 8.0, H-3), 1.56 (2H, p, *J* = 8.0, H-8), 1.31 (8H, brs, H-4-H-7). ^{13}C-NMR (CD_3OD, 400 MHz): 209.8 (C-1), 163.5 (C-17 & C-21), 156.3 (C-13), 137.0 (C-19), 135.0 (C-10), 130.4 (C-11 & C-15), 116.1 (C-12 & C-14), 111.5 (C-16), 108.5 (C-18 & C-20), 45.9 (C-2), 36.2 (C-9), 33.2 (C-8), 30.7 (C-4), 30.6 (C-5), 30.4 (C-6 & C-7), 25.9 (C-3). LC/MS-IT-TOF: 365.1717 ([M + Na]$^+$; calc. $C_{21}H_{26}O_4Na$ 365.1723).

*Malabaricone C (**3**)*
Yellow amorphous powder. UV (MeOH): 338 (2.80), 269 (3.36), 222 (3.50), 204 (3.70). IR (NaCl): 3583, 3343, 2927, 2854, 1714, 1589, 1516. ^{1}H-NMR (CD_3OD, 400 MHz): 7.17 (1H, t, *J* = 8.0, H-19), 6.33 (2H, d, *J* = 8.0, H-18 & H-20), 6.65 (1H, d, *J* = 8.0, H-14), 6.60 (1H, d, *J* = 8.0, H-11), 6.46 (1H, dd, *J* = 8.0, 1.8, H-15), 3.10 (2H, t, *J* = 8.0, H-2), 2.43 (2H, t, *J* = 8.0, H-9), 1.65 (2H, p, *J* = 8.0, H-3), 1.53 (2H, brt, *J* = 8.0, H-8), 1.31 (8H, brs, H-4-H-7). ^{13}C-NMR (CD_3OD, 400 MHz): 209.8 (C-1), 163.5 (C-17 & C-21), 146.1 (C-12), 144.1 (C-13), 137.0 (C-19), 136.0 (C-10), 120.8 (C-15), 116.6 (C-11), 116.3 (C-14), 111.5 (C-16), 108.5 (C-18 & C-20), 45.9 (C-2), 36.4 (C-9), 33.1 (C-8), 30.7 (C-4), 30.5 (C-5 & C-6), 30.4 (C-7), 25.9 (C-3). LC/MS-IT-TOF: 359.1860 ([M + H]$^+$; calc. $C_{21}H_{27}O_5$ 359.1853).

*Malabaricone E (**4**)*
Yellow amorphous powder. UV (MeOH): 341 (2.83), 270 (3.46), 224 (3.67). IR (NaCl): 3583, 3272, 2924, 2853, 1611, 1515. ^{1}H-NMR (CD_3OD, 400 MHz): 6.97 (2H, d, *J* = 8.0, H-11 & H-15), 6.67 (2H, d, *J* = 8.0, H-12 & H-14), 5.80 (2H, s, H-18 & H-20), 3.02 (2H, t, *J* = 8.0 Hz, H-2), 2.50 (2H, t, J = 8.0 Hz, H-9), 1.64 (2H, p, *J* = 8.0 Hz, H-3), 1.55 (2H, brt, *J* = 8.0, H-8), 1.33 (8H, *brs*, H-4-H-7). ^{13}C-NMR (CD_3OD, 400 MHz): 207.6 (C-1), 166.5 (C-19), 165.9 (C-17 & C-21), 156.3 (C-13), 135.0 (C-10), 130.3 (C-11 & C-15), 116.1 (C-12 & C-14), 105.0 (C-16), 95.8 (C-18 & C-20), 44.9 (C-2), 36.2 (C-9), 33.2 (C-8), 30.9 (C-4), 30.6 (C-5 & C-6), 30.4 (C-7), 26.3 (C-3). LC/MS-IT-TOF: 381.1675 ([M + Na]$^+$; calc. $C_{21}H_{26}O_5Na$ 381.1672).

Expression and purification of the DENV-2 NS2B/NS3 protease
The DENV-2 NS2B/NS3 protease was expressed and purified according to the method described by Hariono *et al*. (2019).

DENV-2 NS2B/NS3 protease inhibition assay
The *in vitro* protease inhibition assay was carried out using purified DENV-2 NS2B/NS3 protease as the enzyme and Boc-Gly-Arg-Arg-MCA as the substrate according to the method previously described by our group (Salleh *et al.*, 2019). The concentration of the enzyme and the substrate were 0.5 µM and 10mM, respectively in 200 mM of Tris-HCl buffer (pH 8.5) while the concentration of the inhibitor was 200 µg/mL. All of the tests were performed in quadruplicates. Firstly, the Tris-HCl buffer (pH 8.5) was pipetted into the wells, followed by 1 µL of the inhibitor and 3.1 µL of the enzyme. Before adding the substrate, the enzyme and the inhibitor were incubated at 37°C for 10 minutes. After adding the substrate, the reaction mixture was incubated at 37°C for 60 minutes. All of the reactions were performed in 96-well plates with a final volume of 100 µL per well. Fluorescence was detected using a Promega Glomax Multi Detection System microplate reader with the excitation and emission wavelengths at 365 and 410-460 nm, respectively. In order to determine the IC_{50} values, the same protocol was used as described above with serial dilutions of the inhibitors with concentrations in the range of 1.5625 to 200 µg/mL.

Molecular docking
Molecular docking was performed using AutoDock4.2 along with AutoDockTools (ADT) following the method described by Hariono *et al*. (2019) with some modifications (Hariono *et al*., 2019; Morris *et al.*, 2009). The protein was prepared as described by Hariono *et al*. (2019) whereby the DENV-2 NS2B/NS3pro model was built based on the DENV-2 complex cofactor-protease homology model using the crystal structure of the NS2B/NS3pro West Nile Virus (WNV) in complex with peptidic inhibitor and DENV-2 NS2B/NS3pro apoprotein as the template (Hariono *et al*., 2019; Wichapong *et al.*, 2010). The structures of the compounds were constructed and energy minimization was performed using Hyperchem 8.0 (HyperChem (TM), Profesional 8.0, Hypercube, Inc). The minimized structures of the ligand and the protein were subjected to molecular docking using AutoDock4.2. Polar hydrogen and Kollman charges were added to the protein. For the ligands, the non-polar hydrogen atoms were merged and Gasteiger charges were assigned. A grid box with a dimension of 60 × 60 × 60 and with a grid spacing of 0.375 Å was positioned around the catalytic triad with the centre set at x = 21.517, y = 43.428 and z = -1.743. The dockings were carried out with the Lamarckian Genetics Algorithm (GA) search program applied to generate 250 runs. The conformation with the lowest binding energy in the most populated cluster was selected. The conformations from the docking experiments were analyzed and visualized using Discovery Studio 3.5 (www.accelrys.com).

RESULTS AND DISCUSSION

Preliminary screening of the ethyl acetate and methanol extracts of the fruits of *M. cinnamomea* at a concentration of 200 µg/mL proved that the extracts were potential inhibitors (96.69% and 70.43%, respectively) of the DENV-2 NS2B/NS3 protease. The ethyl acetate extract was subsequently subjected to repeated silica gel column chromatography, Sephadex LH-20 and preparative thin layer chromatography to yield four acylphenols, identified as malabaricone A (**1**), malabaricone B (**2**), malabaricone C (**3**) and malabaricone E (**4**) (**S1**) upon comparison of their spectroscopic data with those previously reported by our group (Abdul Wahab *et al.*, 2016; Sivasothy *et al.*, 2016; Othman *et al.*, 2019).

The initial DENV-2 NS2B/NS3 protease inhibitory activities of compounds **1**–**4** were assayed at 200 µg/mL (Table 1). Since compounds **3** and **4** exhibited more that 70% inhibition towards the DENV-2 NS2B/NS3 protease, these compounds were further evaluated in order to determine

Table 1. Inhibition of the DENV-2 NS2B/NS3 protease by compounds **1**-**4** and Quercetin (standard)

Compounds	Percentage inhibition at 200 µg/mL	IC_{50} (µM)[a]
1	31.92	–
2	67.70	–
3	98.54	27.33 ± 5.45
4	99.91	7.55 ± 1.64
Quercetin (standard)	–	10.48 ± 2.14

[a] Data presented as Mean ± SD (n = 4).

their respective IC_{50} values. The IC_{50} values of compounds **3** and **4** along with the reference standard employed in this study are summarized in Table 1. Compounds **3** (27.33 ± 5.45 µM) and **4** (7.55 ± 1.64 µM) both inhibited the DENV-2 NS2B/NS3 protease with the latter being the stronger inhibitor among the two and around 1.5 times more potent than that of quercetin (10.48 ± 2.14 µM) itself.

Based on the molecular docking studies (Table 2, Figures 2 and 3) the inhibition of the DENV-2 NS2B/NS3 protease by compound **4** was probably due to the hydrogen bonding interactions between the following functional groups in compound **4** and the acid amino residues in the protease: hydroxyl group at C-17 and the oxygen atom of the carbonyl group (C-1) with Ser 135 of the catalytic triad, between the hydroxyl group at C-19 with Asp129 and Phe130 in the S1 pocket and between the hydroxyl group at C-13 with Ile86 in the S3 pocket. In addition, there was also a π-π interaction between ring A with Tyr161 in the S1 pocket. As for compound **3**, the hydroxyl groups at C-13 and C-17 formed hydrogen bonds with Gly151 while that of C-12 formed hydrogen bonds with not only Gly151 but with Tyr161 in the S1 pocket and Gly153 as well. The π-π interaction between ring A with Tyr161 in the S1 pocket was also observed. The aforementioned interactions could have contributed to the activity of compound **3** (Table 2, Figures 2 and 3).

Table 2. Binding interaction data for compounds **3** and **4** with amino acid residues of the NS2B/NS3 protease

Ligand/Compound	Binding Energy (kcal/mol)	Residue	Type of Interaction	Distance (Å)	C10
Malabaricone C (**3**)	-5.47	Gly153	Hydrogen	2.23	Hydroxyl group (OH) at C-12
		Gly151	Hydrogen	2.16	Hydroxyl group (OH) at C-12
				2.08	Hydroxyl group (OH) at C-13
				2.03	Hydroxyl group (OH) at C-17
		Tyr161	Hydrogen	2.39	Hydroxyl group (OH) at C-12
			π-π	–	Aromatic ring A
Malabaricone E (**4**)	-5.89	Ser135	Hydrogen	2.02	Carbonyl group at C-1
			Hydrogen	1.80	Hydroxyl group (OH) at C-17
		Phe130	Hydrogen	2.40	Hydroxyl group (OH) at C-19
			Hydrogen	2.33	Hydroxyl group (OH) at C-19
		Asp129	Hydrogen	1.96	Hydroxyl group (OH) at C-19
		Ile86	Hydrogen	2.18	Hydroxyl group (OH) at C-13
		Tyr161	π-π	–	Aromatic ring A

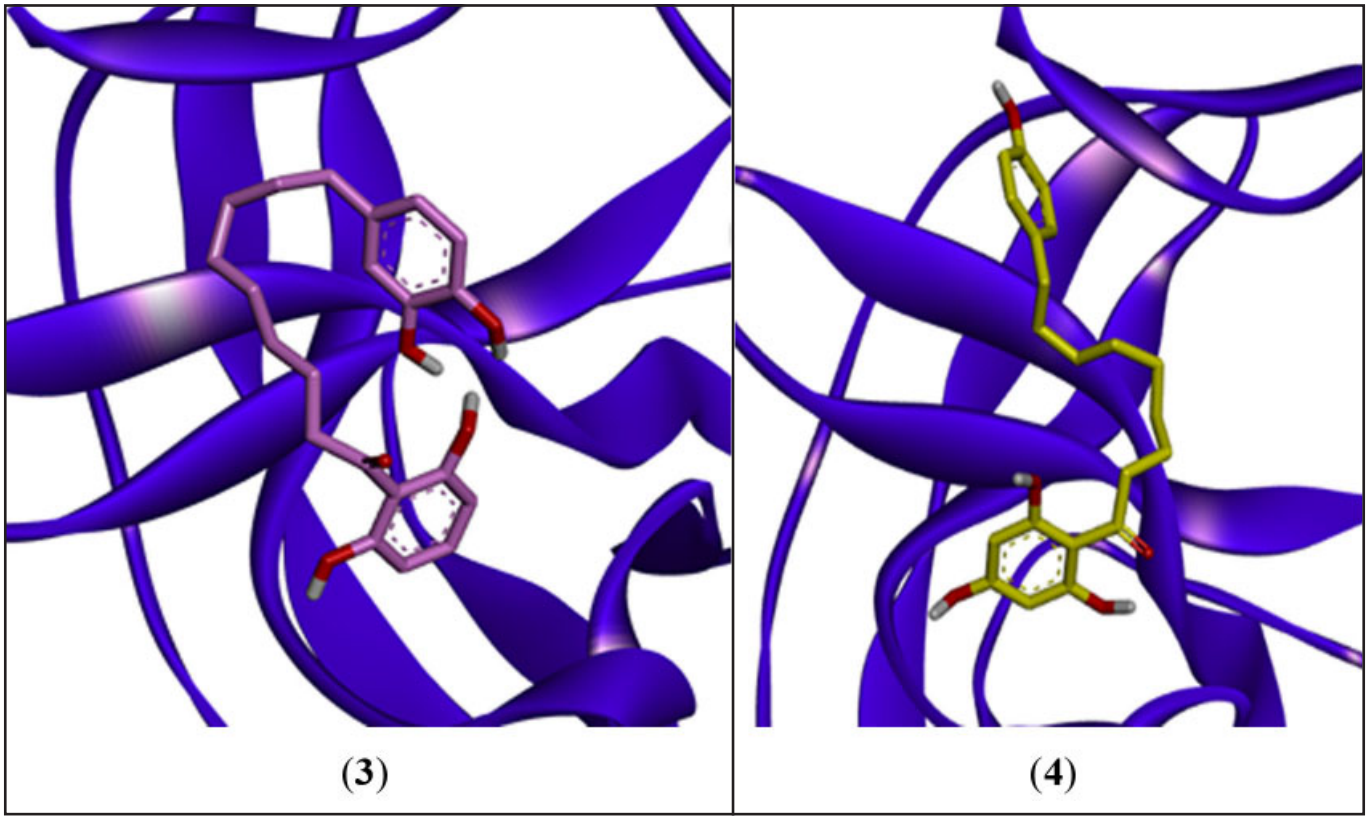

Figure 2. Visualization of malabaricone C (**3**) and malabaricone E (**4**) at the binding site of the NS2B/NS3 protease where the protein structures are represented by solid ribbon style.

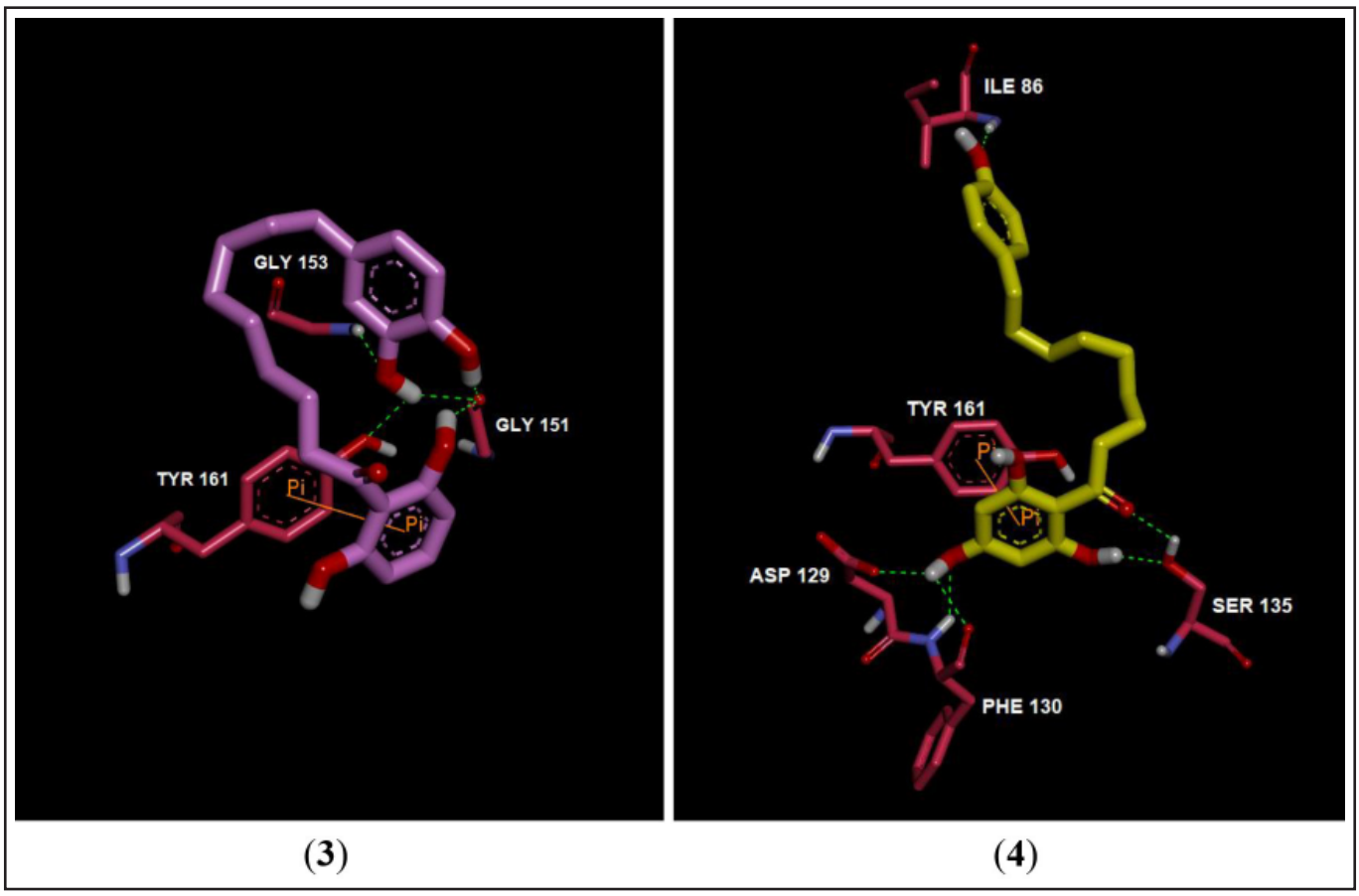

Figure 3. Simpliûed view of the binding interaction between malabaricone C (**3**) and malabaricone E (**4**) with the amino acid residues of the NS2B/NS3 protease. The hydrogen bonding of the ligands with the amino acid residues are shown in green dotted lines.

Upon comparison of the docking results obtained for both compounds, compound **4** was found to have a slightly lower binding energy (-5.89 kcal/mol) as compared to compound **3** (-5.47 kcal/mol) (Table 2). The catalytic triad of the DENV-2 NS2B/NS3 protease is made up of the His51, Asp75 and Ser135 amino acid residues and only compound **4** was found to have a hydrogen bonding interaction with Ser135 (*Hariono et al*., 2019; Yin *et al.*, 2006). Furthermore, the formation of a hydrogen bond with Asp129 was only observed for compound **4**. Asp129 which is located at the bottom of the S1 pocket is an important amino acid residue as a previous study has revealed that it is the key interaction for the substrate recognition in the S1 pocket of the protein (Frecer & Miertus, 2010). Hence, it would be reasonable to postulate that the above two interactions between compound **4** and the protease could be the reason behind the higher potency of compound **4** as compared to compound **3** towards the inhibition of the DENV-2 NS2B/NS3 protease.

CONCLUSIONS

Little is known about the potential usage of the genus *Myristica* in the treatment of dengue apart from a recent paper by Rosmalena *et al*. (2019). Rosmalena and her co-workers reported that the methanolic extract of *M. fatua* Houtt var. magnifica (Bedd.) Sinclair at a concentration of 20µg/mL was found to actively inhibit (122.7%) the DENV-2 NGC strain without any cytotoxic effect (Rosmalena *et al.*, 2019; Cao *et al*., 2013; Herath and Padmasiri, 1999; Herath and Priyadarshani, 1996; Herath and Priyadarshini, 1997; Maia *et al*., 2008; Pham *et al*., 2002).

The potent inhibitory activity of the ethyl acetate extract towards the DENV-2 NS2B/NS3 protease in the current investigation has provided scientific evidence for the possible usage of the fruits of *M. cinnamomea* to treat dengue. Moreover, since malabaricone C (**3**) which has been identified as a moderate inhibitor of the DENV-2 NS2B/NS3 protease is ubiquitous in many members of the genus *Myristica*, i.e *M. fragrans*, *M. malabarica*, *M. fatua*, *M. crassa*, *M. gigantea*, *M. maingayi* and *M. maxima*, therefore this makes the genus *Myristica* a promising and valuable source of dengue antivirals. On this basis, the *in vitro*, *in vivo* and *in silico* studies of compound **3** should be further investigated to facilitate the understanding of its mechanism of inhibition and to evaluate its effectiveness as an antiviral drug.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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