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Chemical Constituents From *Endiandra kingiana* (Lauraceae) as Potential Inhibitors for Dengue Type 2 NS2B/NS3 Serine Protease and its Molecular Docking

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Abstract

A phytochemical study on the bark of *Endiandra kingiana* Gamble (Lauraceae) led to the isolation of a new benzofuranone, 4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)-2(3*H*)-benzofuranone (**1**), together with 6 known compounds. Their structures were established on the basis of detailed spectroscopic analysis, including one- and two-dimensional nuclear magnetic resonance (NMR) and electrospray Ionisation mass spectrometry techniques. Compounds **1**-**3** showed moderate inhibition against dengue virus type 2 NS2B/NS3 protease with percentage inhibitions of 61.23 ± 6.96, 69.92 ± 3.34, and 62.02 ± 6.19, respectively. Molecular docking was performed to predict the binding mode of all protease inhibitor models and the results revealed that most of the essential amino acid residues such as Asp129, Ser135, Tyr161, Asn152, and His51 significantly interact with the ligands.

Keywords

Endiandra kingiana, Lauraceae, benzofuranone, dengue virus type 2, NS2B/NS3 protease, molecular docking

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Endiandra, a genus belonging to the family Lauraceae, comprises approximately 125 species found throughout the tropics.[1] Only 8 species, *Endiandra kingiana*, *Endiandra anthropophagorum*, *Endiandra oligandra*, *Endiandra introrsa*, *Endiandra jonesii*, *Endiandra monothyra*, *Endiandra baiilonii*, and *Endiandra xanthocarpa,* had undergone scientific studies that resulted in the isolation of endiandric acids, lignans, neolignans, and sesquiterpenes.[1-4] The previous studies on *E. kingiana* by Azmi et. al[5] led to a series of endiandric acid analogs named kingianic acids A to I. These compounds were screened for Bcl-xL and Mcl-1 binding affinities, as well as cytotoxic activity on various cancer cell lines.[6]

To the best of the authors' knowledge, there is no scientific report as yet on this species regarding dengue virus related studies. Dengue virus is among the most widespread mosquito-borne diseases and is endemic in most tropical and subtropical parts of the world.[7,8] Malaysia with a population of 31.1 million and a population density of 95 per km^2 has seen increasing cases of dengue over the past two decades with 49 726 cases.[9] There are 4 serotypes of dengue viruses (DENV1, DENV2, DENV3, and DENV4) that cause dengue hemorrhagic fever. Dengue virus type 2 is the most prevalent one of all serotypes in Malaysia.[9]

In our search for bioactive compounds from *Endiandra* species against dengue virus, we carried out a phytochemical study of the bark of *E. kingiana*. A new benzofuranone (**1**), along with 6 known compounds (−)-epicatechin (**2**),[10] (+)-catechin (**3**),[10] methyl orsellinate (**4**),[11] vanillic acid (**5**),[12] vanillin (**6**),[13] and cinnamtannin B1 (**7**),[14] was characterized by comparison of the spectroscopic data with that in the literature (Figure 1).

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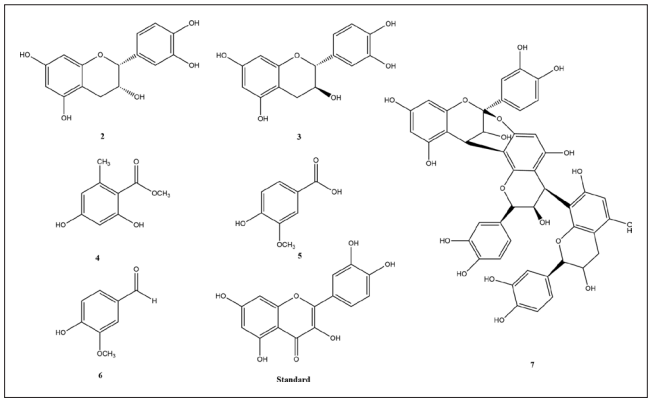

Figure 1. Molecular structures of the isolated compounds from *Endiandra kingiana*.[10-14]

These compounds were evaluated against dengue virus type 2 NS2B/NS3 serine protease. In addition, molecular docking simulations were implemented to probe the binding modes of the active ligands to the amino acids in the active site of serine protease. Herein, we report the isolation and structural elucidation of these compounds, their dengue inhibitions, and their molecular docking.

Compound **1** was obtained as a pale-yellow amorphous powder. The molecular formula was determined as $C_{23}H_{30}O_3$ by positive mode liquid chromatography mass spectrometry-Ion trap-time of fligh (LCMS-IT-TOF), which provided a molecular ion peak at m/z 355.2234 $[M+H]^+$ (calcd. m/z at 355.2228) corresponding to 9 degrees of unsaturation. The infrared (IR) spectrum showed strong absorption bands for a highly conjugated system (1470 and 1457 cm^{-1}), hydroxyl (3402 cm^{-1}), and conjugated carbonyl (1725 cm^{-1}) functionalities.[15]

The ^{1}H NMR spectrum of **1** established the existence of 2 aromatic signals, 3 methine protons, 6 methylene protons, and 4 methyl groups. The aromatic protons, H-3 and H-5, appeared as singlets at δ 6.58 and 6.61, while 3 methine protons, H-8, H-12, and H-16, gave signals at δ 5.30 (d, J = 7.3 Hz), 5.09 to 5.11 (m), and 5.09 to 5.11 (m), respectively. The spectrum also showed 4 methyl singlets at δ 1.71 (Me-9), 1.59 (Me-13), 1.59 (Me-17), and 1.67 (Me-18). The methylene protons at δ 3.34 (d, H-7) with a coupling constant of 7.3 Hz indicated that it is at a vicinal position with H-8. The more upfield chemical shifts were assignable to the remainder of the methylene protons: H-10, H-11, H-14, and H-15 at δ 1.96 to 2.03 (m). The ^{13}C NMR spectrum coupled with heteronuclear single quantum coherence spectroscopy (HSQC) analysis revealed the presence of 23 carbons, among which 4 were methyl carbons at δ 16.4 (Me-9), 16.2 (Me-13 and Me-17), 25.8 (Me-18); 3 were methine carbons at δ 120.6 (C-8), 124.5 (C-12), and 124.1 (C-16); 3 were olefinic quaternary carbons at δ 137.9 (C-9), 135.3 (C-13), and 135.5 (C-17); and 1 conjugated carbonyl carbon at δ174.6 (C-1').

In the correlated spectroscopy (COSY) experiment (Figure 2), the methine proton of H-8 was cross-correlated to

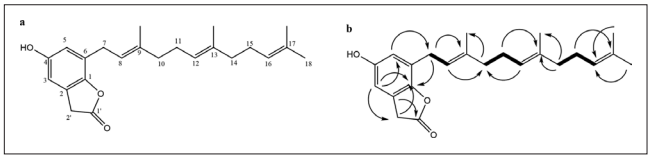

Figure 2. (a) Structure of **1** from the bark of *Endiandra kingiana*, (b) selected HMBC (arrows) and COSY (bold lines) correlations of **1**.

the methylene protons of H-7, suggesting that they were adjacent to each other. The in-depth analysis of HMBC experiments supported the location of 4 isoprene units which are connected to the aromatic ring through the correlation of H-7 with C-9, C-5, and C-1. The HMBC correlations of H-3 with C-2' confirmed the presence of the furanone group which is fused to the aromatic ring through C-1 and C-2. Compound **1** showed some similarities with a known compound, 5-hydroxy-7-(3,7,11,15-tetramethylhexadeca-2,6,10,11-tetraenyl)-2(3*H*)-benzofuranone, which was previously isolated from *Rhus chinensis*.[15] However, compound **1** lacks 1 isoprene unit in comparison to the known compound. Based on the complete assignments of NMR spectra and other spectroscopic data, compound **1** was identified as a new benzofuranone named 4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)-2(3*H*)-benzofuranone.

Compounds **1** to **4** and the crude extract of *E. kingiana* were tested *in-vitro* against NS2B/NS3 protease of DENV2. The crude extract showed moderate inhibition with 65.05% ± 3.7%. Compounds **1** to **3** gave moderate activities with more than 60% inhibition, while compound **4** showed weak inhibition at 16.42% ± 2.92%. The percentage inhibitions are presented in Table 1.

Subsequently, all compounds that exhibited more than 60% inhibition toward the DENV2 NS2B/NS3 protease were further evaluated to determine their respective IC_{50} values. The IC_{50} values showed that **1** (IC_{50} = 403.14 ± 33.03), **2** (IC_{50} = 170.10 ± 5.94), and **3** (IC_{50} = 184.13 ± 2.11) moderately inhibited the NS2B/NS3 protease of DENV2 with **2** being the most potent. One may observe that the inhibiting potential of **1** to **3** decreases with increasing number of hydroxyl groups. Furthermore, the position of these hydroxyl groups and the number of aromatic rings in the molecule could play an important role in influencing the activities.

Molecular docking studies were also attempted in order to better understand how the chemical groups in these molecules (**1-3**) may have influenced their activities. Based on molecular docking studies (Figure 3), diastereomer **2** was shown to be slightly more active than diastereomer **3**, which may be attributed to the type of bonding with Tyr161 of the DENV2 NS2B/NS3 protease at the S1 pocket. Compound **2** showed 4 hydrogen bonding interactions of hydroxyl groups, while **3** has 3 hydrogen bondings and 1 π-π stacking interaction with the DENV2 NS2B/NS3 serine protease. Having more hydrogen bonding interactions resulted in **2** being more active than **3**, due to the fact that hydrogen bonding

Table 1. Percentage Inhibition of the Crude Extract and Compounds Isolated From *Endiandra kingiana* Against DENV2 NS2B/NS3 Serine Protease.

Compounds	Percentage inhibition
Crude extract	65.05 ± 3.73
4-Hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)2(3*H*)-benzofuranone **1**	61.23 ± 6.96
(−)-Epicatechin **2**	69.92 ± 3.34
(+)-Catechin **3**	62.02 ± 6.19
Methyl orsellinate **4**	16.42 ± 2.92
Quercetin (standard)	90.90 ± 9.13

is more stable than π-π stacking interaction. Compound **1** was the least potent one, as it only showed 2 hydrogen bonds with Asp129 and Ser135 at the S1 pocket.

In conclusion, the phytochemical investigation of *E. kingiana* bark extract led to the isolation of 1 new benzofuranone compound, 4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)-2(3*H*)-benzofuranone (**1**), together with 6 known compounds (−)-epicatechin (**2**), (+)-catechin (**3**), methyl orsellinate (**4**), vanillic acid (**5**), vanillin (**6**), and cinnamtannin B1 (**7**). Compounds **1** to **4** and the crude extract of *E. kingiana* were tested *in-vitro* against NS2B/NS3 protease of DENV2. Compounds **1** to **3** had moderate activity with more than 60% inhibition. This is the first report on an *Endiandra* species possessing dengue inhibitors.

Experimental

General

Preparative thin layer chromatography (PTLC) and analytical thin layer chromatography (TLC) were performed on commercially precoated aluminum supported silica gel 60 $_{F254}$ TLC sheets (Merck 5554). The NMR spectra were recorded on BRUKER Advance III NMR spectrometers (400 MHz). The mass spectra were performed on Agilent 6530 LCMS-IT-TOF. The UV spectra were obtained on Jasco V530 UV-Vis spectrophotometer. The IR spectra were recorded on a Perkin-Elmer System 400 Fourier Transform Infrared Spectroscopy with methanol.

Plant

The bark of *E. kingiana* was collected from Kuala Lipis, Pahang. The identity of the plant was determined by Mr Teo Leong Eng and Mr Din Mat Nor, University of Malaya. Voucher specimens were numbered as KL4828 and deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur, Malaysia.

Extraction and Isolation

The extraction process was carried out using simple maceration method. The dried, ground bark of *E. kingiana* was first defatted with hexane for 3 days. The hexane extract was filtered and dried on the rotary evaporator. The plant material was dried followed by extraction with dichloromethane (CH_2Cl_2) for 48 hours and repeated twice. The liquid extracts were dried under reduced pressure using a rotary evaporator to get brown crude extract (15.2 g). The crude extract (8.0 g) was purified by an open column chromatography (CC) eluting with gradient solvent system 90:10 to 30:70 CH_2Cl_2:CH_3OH to give 5 major fractions (A-E). Fraction A (52 mg) was separated by PTLC eluted with a CH_2Cl_2: CH_3OH (98:2) solvent system to furnish vanillic acid (1.1 mg) and vanillin (1.0 mg). Fraction B (162.0 mg) was also purified by PTLC eluted with a CH_2Cl_2:CH_3OH (97:3) solvent system to furnish methyl orsellinate (**4**) (2.4 mg) and 4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)−2(3 H)-benzofuranone (**1**) (2.6 mg). For fraction C (77.0 mg), high performance liquid chromatography (HPLC) was used during purification. In this stage, reverse phase analytical and semipreparative columns were used, and the compounds eluted with a mixture of CH_3OH/H_2O in the presence of 0.1% formic acid as a buffer to give (−)-epicatechin (**2**) (1.6 mg) and (+)-catechin (**3**) (2.7 mg).

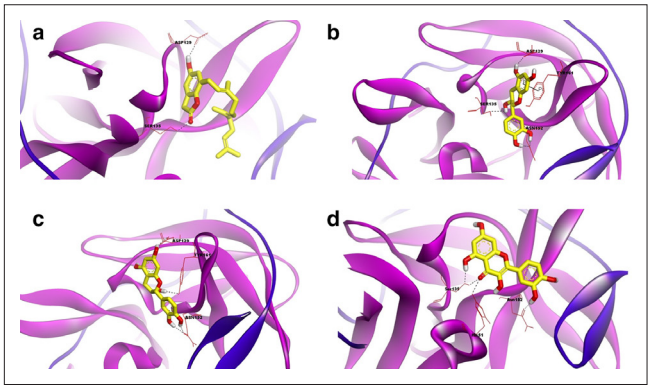

Figure 3. Molecular docking of the active compounds with the DENV2 NS2B/NS3 serine protease: (a) 4-hydroxy-6-(9,13,17-trimethyldodeca-8,12,16-trienyl)-2(3*H*)-benzofuranone **1**, (b) (−)-epicatechin **2**, (c) (+)-catechin **3**, and (d) quercetin (standard).

In-Vitro Analysis Toward DENV2 NS2B/NS3 Serine Protease

The protease activity assay was conducted at constant concentrations of the protease, substrate, and the compounds with the values of 0.5 μM, 10 mM in dimethyl sulfoxide (DMSO) solution, and 200 ppm, respectively. The reaction mixtures were prepared in black 96-well plates. Each reaction mixture consisted of 200 mM Tris buffer with the total volume of 100 μL. Tris buffer was pipetted to the wells, followed by compounds and by the enzyme. The mixtures were pre-incubated at 37°C, shaken at 200 rpm for 10 minutes, the substrate added to the wells and incubated at 37°C and shaken at 200 rpm for 60 minutes. After the incubation time was completed, the well plate was analyzed, and the fluorescence was detected using the

Table 2. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR Data of Compound **1** in $CDCl_3$.

No	δ_H (ppm), *J* (Hz)	δ_C (ppm)	No	δ_H (ppm), *J* (Hz)	δ_C (ppm)
1'	-	174.6	11	1.96-2.12 (m)	26.6
2'	3.69 (s)	34.0	12	5.09-5.11 (m)	124.5
1	-	147.0	13	-	135.3
2	-	123.8	14	1.96-2.03 (m)	39.8
3	6.58 (s)	109.3	15	1.96-2.03 (m)	25.8
4	-	152.4	16	5.09-5.11 (m)	124.1
5	6.61 (s)	115.5	17	-	135.5
6	-	126.1	18	1.67 (s)	25.8
7	3.34 (d, *J* = 7.3)	27.8	9-Me	1.71 (s)	16.4
8	5.30 (d, *J* = 7.3)	120.6	13-Me	1.59 (s)	16.2
9	-	137.9	17-Me	1.59 (s)	16.2
10	1.96-2.10 (m)	39.9			

Promega Glomax Multi Detection System microplate reader with excitation and emission wavelengths at 365 and 410 to 460 nm, respectively.

Molecular Docking

A homology model of the DENV2 NS2B/NS3 protease was obtained from the Research Collaboratory for Structural Bioinformatics-Protein Data Bank (RCSB-PDB) database for structure-based design purposes. The selected 3D structure was downloaded in a PDB format file. The enzyme was then prepared under the protein preparation protocol implemented in Discovery Studio 2.5 (Accelry Inc., CA, United States) suite of program. After that, the geometry optimization and energy minimization of DENV2 NS2B/NS3 protease was conducted by removing the water molecules. Then, the protonation of the DENV2 NS2B/NS3 protease was done through "Protonate 3D" feature, followed by optimization of the partial charge and energy minimization. The optimized, minimized 3D structure of DENV2 NS2B/NS3 protease was saved in the .pdbqt format. For the ligands, the two-dimensional structure of **1** to **3** was built using Hyperchem 8 and subjected to energy minimization with a convergence criterion of 0.05 kcal/(mol A). Nonpolar hydrogens and lone pairs were then merged, and each atom was assigned with Gasteiger partial charges. A grid box was generated at the center of the active site gorge with 60 × 60×60 points along the *x*, *y,* and *z* axes. The remaining parameters were at default setting. The molecular docking simulation of DENV2 NS2B/NS3 protease and the ligands used Autodock 3.0.5 software. The docking simulation process began by selecting "Dock" feature from "Compute" panel. The remaining parameters were at default setting. After the docking control, parameters and models to display were set to the receptor and ligand molecule. The output was set to predict 100 solutions. The docking result was saved in .mdb file format. The obtained log files were read in autoDockTools (ADT) to analyze the results of docking.

4-Hydroxy-6-(9,13,17-Trimethyldodeca-8,12,16-Trienyl)-2(3H)-Benzofuranone (1)

Pale-yellow amorphous powder.
IR (KBr): 3402, 1725, 1470, 1457 cm^{-1}.
UV/Vis λ_{max} (MeOH) nm (log ε): 210, 275.
^{1}H and ^{13}C NMR: Table 2.
LCMS-IT-TOF: *m/z* $[M+H]^+$ calcd. for $C_{23}H_{31}O_3$: 355.2228; found 355.2243

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Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Supplemental Material

Supplemental material for this article is available online.

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