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Inhibitory activity of cyclohexenyl chalcone derivatives and flavonoids of fingerroot, *Boesenbergia rotunda* (L.), towards dengue-2 virus NS3 protease

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Abstract—*Boesenbergia rotunda* (L.) cyclohexenyl chalcone derivatives, 4-hydroxypanduratin A and panduratin A, showed good competitive inhibitory activities towards dengue 2 virus NS3 protease with the K_i values of 21 and 25 μM, respectively, whilst those of pinostrobin and cardamonin were observed to be non-competitive. NMR and GCMS spectroscopic data formed the basis of assignment of structures of the six compounds isolated.

Anthropod-borne dengue fever (DF) and dengue haemorrhagic fever (DHF)/dengue shock syndrome (DSS) are caused by four closely related viruses (DEN-1, DEN-2, DEN-3 and DEN- 4) of the *Flaviviridae* family. According to WHO, DF and DHF/DSS are endemic in over 100 countries, with more than 2.5 billion people at risk for epidemic transmission and an estimated 50 million infections each year.

Typically of *Flaviviridae* family, dengue virus consists of a single positive-sense 1-kb RNA genome that translates into a single polyprotein comprising 3 structural (C, prM, E) and 7 non-structural (NS) proteins, of sequential order C-prM-E-NS1-NS2A-NS2B-NS3-NS4A-NS4B-NS5.[1] For gene expression, both co- and post-translational proteolytic processing of this polyprotein is required. The polyprotein precursor is cleaved by both host proteases and virus protease complex NS2B/NS3. Together with its cofactor NS2B, NS3pro catalyzes the *cis*-cleavage of NS2A/NS2B and NS2B/NS3,[2] as well as *trans*-cleavage of NS3/NS4A, and NS4B/NS5 junctions of the polyprotein.[1,3] Thus, the NS3pro is an attractive target for potential chemotherapeutic agents.

To date, there is no known vaccine licenced for human use against dengue, although the efficacy of a recently developed ChimeriVax vaccine is currently being evaluated.[4] In addition, there have been various reports recently of a work on therapeutic strategies for use against dengue.[5–7] However, all efforts thus far have not been successful. As part of our ongoing effort to search for a lead therapeutic agent for DF/DHF, the activity of some compounds extracted from fingerroot, *Boesenbergia rotunda* (L.) Mansf. Kulturpfl. (BR), for the inhibition of dengue virus protease has been tested.

Boesenbergia rotunda (L.) Mansf. Kulturpfl. (BR) is a common spice belonging to a member of the ginger family (Zingiberaceae). Several studies have shown some of the BR compounds, such as flavanoids and chalcones, to be pharmaceutically active. The chalcone, cardamonin, isolated from BR was recently reported to exhibit appreciable anti-HIV-1 protease inhibition.[8] In this paper, we described inhibitory activities of six compounds isolated from BR towards the DEN-2 virus NS3 protease.

Keywords: Boesenbergia rotunda (L.) extracts; Cyclohexenyl chalcone derivatives; Dengue 2; NS3 protease; Competitive inhibitory activities.
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For structural determination of BR extracts, nuclear magnetic resonance (^{1}H and ^{13}C NMR 400 MHz) and gas-chromatography/mass spectra (GCMS, AcqMethod NATPRO, HP 6890 series Mass Selective Detector) were used. Bioassays were carried out on RF5301PC Shimatzu Spectrofluorometer. The intensity of the fluorogenic moiety (7-amino-4-methylcoumarin, AMC) from the cleaved fluorogenic peptide substrates was set at the following wavelength: excitation $\lambda = 385$ nm and emission $\lambda = 465$ nm.

Fresh yellow rhizomes of BR from Thailand were purchased from Chow Kit Market (KL, Malaysia). The slurry liquid obtained after solvent evaporation of methanolic extract of dried rhizome powder was partitioned with ethyl acetate and water (1:1, v/v). The second slurry liquid obtained after ethyl acetate evaporation was subsequently eluted with 5% stepwise gradient of increasing polar solvent in the order of toluene, toluene/ ethyl acetate, ethyl acetate, ethyl acetate/ acetone, acetone, acetone/methanol and methanol using a silica gel chromatographic column. The structures of the six purified compounds (**1**–**6**) were identified using ^{1}H and ^{13}C NMR together with GCMS spectroscopic data.

Expression and purification of DEN-2 NS2B/NS3 protease were similar to Ref. 9, whilst the bioassay protocol used was modified from that established in Ref. 9. The standard 100 μL reaction mixtures comprised 100 μM fluorogenic peptide substrate Boc-Gly-Arg-Arg-MCA (S1), 2 μM Den-2 protease complex and with or without BR extract/compounds of varying concentrations buffered at pH 8.5 by 200 mM Tris–HCl. Each extract/compound was assayed at three different concentrations; 120, 240 and 400 ppm, except for compounds **5** and **6** which were assayed at six different concentrations ranging from 40 ppm to 400 ppm. Each test was done in quadruplicate. Four readings were taken each at a time interval of 5 s per sample and the three most consistent readings (% standard deviation <5%) were accepted.

The most inhibiting of the BR compounds were subsequently bio-assayed for *trans* cleavage inhibition using a second fluorogenic peptide substrate Ac-Thr-Arg-Arg-MCA (S2) which tripeptide residue was modelled after the NS4B/NS5 cleavage site of DEN-2 polyprotein. All solutions bio-assayed were prepared in methanol (prior tests showed the absence of methanolic inhibition even at 30% (v/v)).

Spectroscopic data (^{1}H NMR, ^{13}C NMR and MS) of the six compounds extracted from BR that have shown inhibitory activities towards dengue virus protease were in accordance with those reported previously.[10–13] These compounds were identified as pinostrobin (**1**), pinocembrin (**2**), alpinetin (**3**), cardamonin (**4**), panduratin A (**5**) and 4-hydroxypanduratin A (**6**) (Fig. 1).

Protease assay was performed using a fluorogenic peptide substrate Boc-Gly-Arg-Arg-MCA (S1), which has been shown to be an active substrate towards DEN-2 NS3 protease.

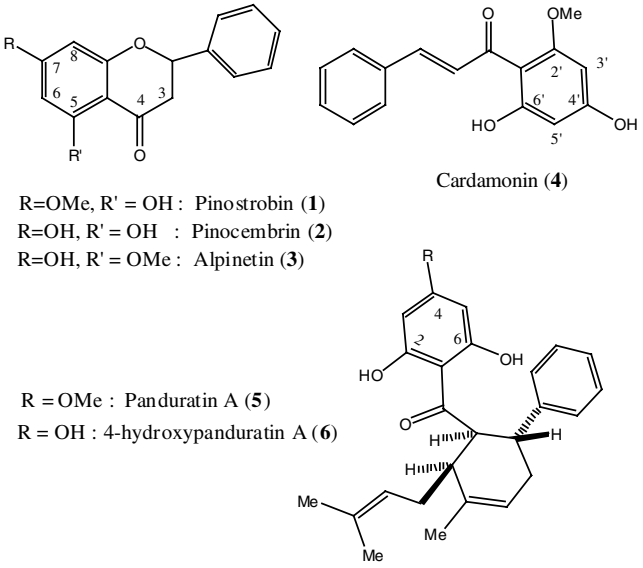

Figure 1. The structures of the six compounds extracted from *Boesenbergia rotunda* (L.).

Inhibitory activities of DEN-2 NS2B/NS3pro cleavage of S1, the most active substrate, were carried out with the six compounds described above.[9] Figure 2 shows the percentage of inhibition of the compounds tested at three different concentrations. The results indicated an increase in inhibitory activities with increasing concentration of compounds tested.

Of all the six compounds tested, panduratin A (**5**) and 4-hydroxypanduratin A (**6**) exhibited inhibitory activities even at low concentration tested where inhibitory activities of more than 65% were observed at 80 ppm (Table 1). 4-Hydroxypanduratin A showed better inhibition than panduratin A at an even lower concentration of 40 ppm (Table 1).

Although pinocembrin (**2**) and cardamonin (**4**) individually showed low inhibitory activities at all concentrations tested, marked increase in activity was observed when both these compounds were combined in equal portion (1:1, w/w) and bio-assayed (Table 2). This seems to suggest some synergistic effect on the activities of pinocembrin (**2**) and cardamonin (**4**) against the

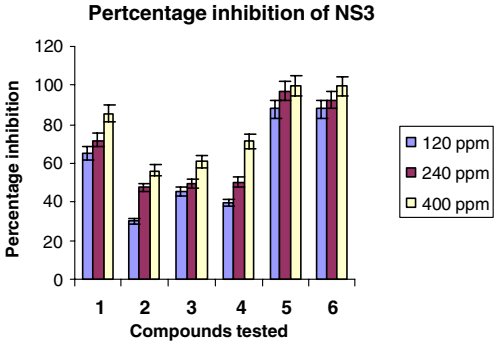

Figure 2. Percentage inhibition of NS3 cleavage of S1 by BR compounds.

Table 1. Percentage inhibition of dengue 2 NS2B/3 virus protease cleavage of substrate S1 by **5** and **6** ± standard error

Compound	Percentage inhibition of dengue 2 virus protease NS3 concentration used (ppm)					
	40	80	120	160	240	400
5	27.1 ± 4.8	66.7 ± 0.1	87.7 ± 0.6	93.7 ± 0.5	92.2 ± 1.2	99.8 ± 1.1
6	52.0 ± 1.1	78.1 ± 0.1	87.6 ± 0.4	96.0 ± 0.5	97.3 ± 0.3	99.8 ± 0.3

Table 2. Percentage inhibition of DEN-2 NS2B/3 virus protease cleavage of substrate S1 by **2**, **4** and 1:1 (w/w) combination of **2** and **4** ±standard error

Compound	Percentage inhibition of DEN-2 NS3 concentration used (ppm)		
	120	240	400
2	30.1 ± 0.5	47.3 ± 0.5	56.1 ± 0.4
4	39.4 ± 0.6	50.1 ± 0.4	71.3 ± 0.3
2 + **4**	52.6 ± 0.4	63.5 ± 0.5	81.8 ± 0.3

Table 3. Percentage inhibition of dengue 2 NS2B/3 virus protease cleavage of substrate S2 by **1**, **5** and **6** ±standard error

Compound	Percentage inhibition of DEN-2 NS3 concentration used (ppm)		
	120	240	400
1	76.7 ± 0.5	80.7 ± 0.4	88.7 ± 0.3
5	47.5 ± 9.1	59.6 ± 7.9	90.4 ± 2.3
6	90.4 ± 0.3	96.1 ± 0.3	99.2 ± 0.3

DEN-2 virus protease. Studies are being carried out to gain further insight into their combined mode of inhibition.

The inhibition for cleavage of S2 showed a similar increase in inhibitory activities when the concentration of the compounds tested was increased (Table 3). With the exception of panduratin A (**5**), the percentage inhibitions for the cleavage of S2 for all other compounds tested were observed to be comparable to those of the first substrate, S1. At 120 ppm, 4-hydroxypanduratin A (**6**) showed a comparatively higher inhibitory activity than those of pinostrobin (**1**) and panduratin A (**5**). The second set of inhibition results using S2 showed that these BR compounds could also inhibit the cleavage of site modelled after the actual NS4B/NS5 cleavage junction of the dengue virus polyprotein.

The K_i for all compounds tested were calculated from the Lineweaver–Burk plot. Figure 3 is a Lineweaver–Burk plot using cardamonin (**4**) as an example of inhibitory activity. Similar plots were obtained for all other BR flavanoids (**1**, **5** and **6**). Activities of **4** indicate non-competitive inhibition towards DEN-2 protease NS3.

A graph of reciprocal of apparent maximum velocity to inhibitor concentration was plotted and the K_i value was calculated from the gradient ($1/(V_{max}\ K_i)$). The K_i value for cardamonin was determined to be 377 ± 77 μM (Fig. 4).

The Lineweaver–Burk plots for **5** and **6** intersected on the *y*-axis indicating a competitive inhibition with the

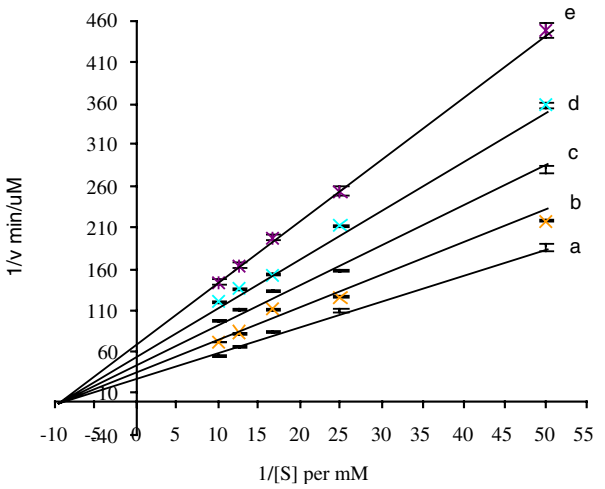

Figure 3. Lineweaver–Burk plot of inhibitor compound **4**. Plots: a, absence of inhibitor; b, [I] = 148 μM (40 ppm); c, [I] = 297 μM (80 ppm); d, [I] = 445 μM (120 ppm); e, [I] = 593 μM (160 ppm).

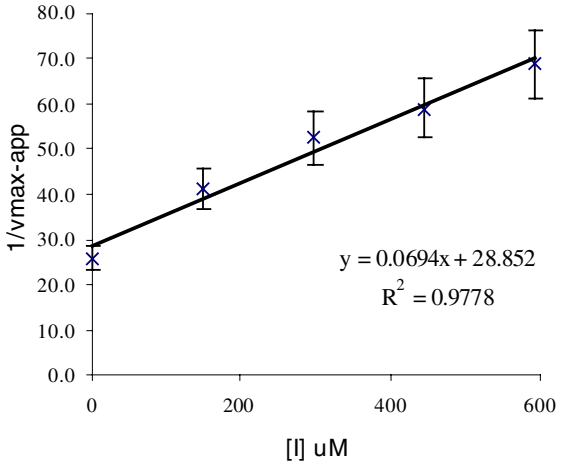

Figure 4. Plot of reciprocal of apparent maximum velocity, $1/v_{max\text{-}app}$ ($v_{max\text{-}app}$ in mM/min), to inhibitor compound **4** concentration, [I] μM.

substrate S1 towards dengue protease. Figure 5 shows the Lineweaver–Burk plot for 4-hydroxypanduratin A. A further plot reciprocal of the apparent K_m to the inhibitor **6** concentration was used to calculate the K_i for **6** (Fig. 6). Table 4 summarizes the K_i values for pinostrobin (**1**), cardomonin (**4**), panduratin A (**5**) and 4-hydroxypanduratin A (**6**).

The small K_i values of the competitive inhibitors for the CCD, especially 4-hydroxypanduratin A, show the potential of the CCD compounds as in vitro inhibitors for the DEN-2 NS2B/NS3 protease. The inhibitor dissociation constants of these two natural compounds were comparable to the best of the small

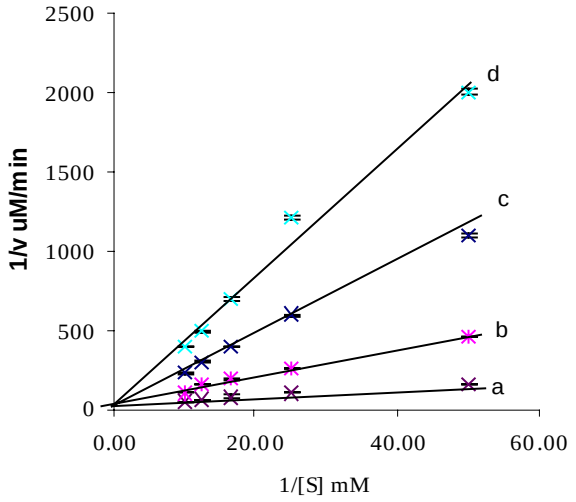

Figure 5. Lineweaver–Burk plot of inhibitor compound **6**, 4-hydroxypanduratin A. Plots: a, absence of inhibitor; b, [I] = 102 μM (40 ppm); c, [I] = 204 μM (80 ppm); d, [I] = 306 μM (120 ppm).

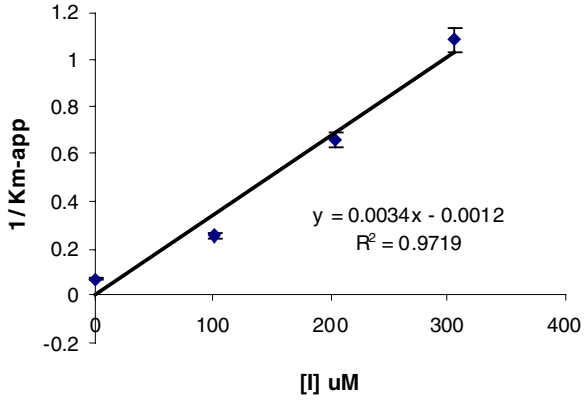

Figure 6. Plot of reciprocal of apparent K_m, $1/K_{m\text{-app}}$, ($K_{m\text{-app}}$ in mM), to inhibitor compound **6** (4-hydroxypanduratin A) concentration, [I] μM.

Table 4. K_i values of inhibitors of NS3 protease

Compound	K_i (μM)	Inhibition mechanism
1	345 ± 70	Non-competitive
4	377 ± 77	Non-competitive
5	25 ± 8	Competitive
6	21 ± 6	Competitive

synthetic peptide inhibitors assayed.[14] Presumably, the inhibitory activity of the CCD over the other similarly configured chalcones and flavanones extracted is in some ways influenced by the unique cyclohexenyl structure in the CCD. Further studies are being carried out in order to understand the structure-activity relationship of these compounds towards the DEN-2 virus protease.

Two types of natural products were extracted from BR, that is, flavanones and chalcones. Flavanone pinostrobin showed non-competitive inhibition towards DEN-2 protease. However, unlike chalcone cardamonin which also showed non-competitive inhibition towards DEN-2 protease, cyclohexenyl chalcone derivatives (CCD) showed competitive inhibition. The low K_i CCD calculated values indicated that these compounds have good potentials as in vitro inhibitors for DEN-2 NS2B/NS3 protease.

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