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A new naphthoquinone analogue and antiviral constituents from the root of *Rhinacanthus nasutus*

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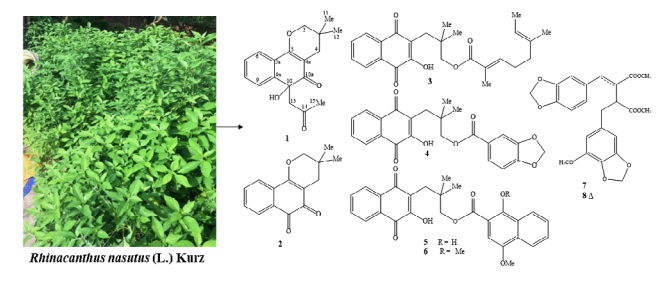
ABSTRACT

Rhinacanthus nasutus (L.) Kurz (Acanthaceae) is known as traditional medicine for the treatment of fungal and herpes virus infections. A new naphthoquinone racemate, rhinacasutone (**1**) together with seven known compounds, rhinacanthone (**2**), rhinacanthins C, D, N, Q, and E (**3**–**7**), and heliobuphthalmin (**8**) were isolated from root of *R. nasutus*. Their structures were determined on the basis of extensive spectroscopic methods, including 1D-, 2D-NMR and MS data. All the isolated compounds were tested for their antiviral activities against PR8, HRV1B, and CVB3-infected vero cells. Compounds **3**–**6** exhibited significant antiviral activities with the IC_{50} value ranging from 0.03 to 23.7 μM in all three infections.



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1. Introduction

Rhinacanthus nasutus (L.) Kurz (Acanthaceae) is a traditional South-East Asian countries medicine for the treatment of fungal and herpes virus infections. In these reason, many studies have been performed to find antiviral compounds from various parts of *R. nasutus*. Previous

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phytochemical investigations verified that lignans and naphthoquinones are major antiviral components of *R. nasutus* (Sendl et al. 1996; Kernan et al. 1997; Thongchuai et al. 2015). In our previous research, the isolated compounds from the aerial parts of *R. nasutus* significantly inhibited the activity of neuraminidase which plays an important role in influenza virus infection (Kwak et al. 2017). In a continuing project to identify plant natural products that exhibit anti-influenza virus effect, phytochemical investigation of roots of *R. nasutus* were performed in the present study and led to the isolation of one new naphthoquinone analogue, rhinacasutone (**1**), together with seven known compounds (**2**–**8**) (Figure 1). In addition, the isolated compounds were tested against human rhinovirus and coxsackievirus, which are the most common viral infectious agent in human. This paper mainly deals with the isolation, structural characterization and evaluation of the isolated compounds as antiviral agents.

2. Results and discussion

Compound **1** was obtained as a pale yellow amorphous powder. Its molecular formula was deduced to be $C_{18}H_{20}O_4$ from a pseudo-ion peak *m/z* 323.1257 $[M + Na]^+$ (Cald. for $[C_{18}H_{20}O_4Na]^+$, 323.1254) in the HR-ESI-MS spectrum. The ^{1}H-NMR and HSQC spectra of **1** presented signals of a 1,2-disubtituted benzene ring [δ_H 7.37 (1H, t, J = 7.5 Hz), 7.46 (1H, t, J = 7.5 Hz), 7.65 (1H, d, J = 7.5 Hz), and 7.79 (1H, d, J = 7.5 Hz)], oxygenated methylene protons [δ_H 3.92 (1H, d, J = 10.5 Hz) and 3.97 (1H, d, J = 10.5 Hz)], two methylene groups [δ_H 2.18 (1H, d, J = 16.8 Hz)/ 2.40 (1H, d, J = 16.8 Hz) and 2.88 (1H, d, J = 12.9 Hz)/ 2.92 (1H, d, J = 12.9 Hz)], and three methyl groups [δ_H 1.00 (3H, s), 1.10 (3H, s), 2.06 (3H, s). The ^{13}C-NMR spectra of **1** revealed the signals of 18 carbons, including eight non-protonated carbons, four methines, three methylenes, and three methyl carbons. The ^{1}H- and ^{13}C-NMR data of **1** suggested they

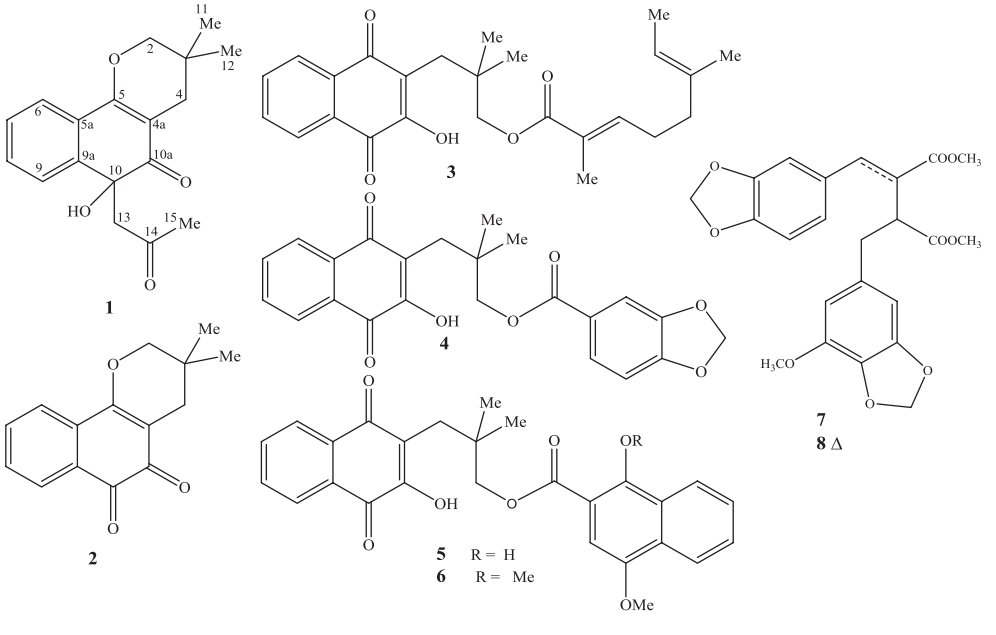

Figure 1. Chemical structures of compounds **1**–**8**.

are similar with those of rhinacanthone, a unique compound of *R. nasutus* (Kuwahara et al. 1995). Additional three carbon signals were assigned for the presence of a side-chain in **1**. The COSY cross peaks of H-6 (δ_H 7.79)/H-7 (δ_H 7.37)/H-8 (δ_H 7.46)/ H-9 (δ_H 7.65) were characterized for the connection of C-6/C-7/C-8/C-9 of rhinacanthone-skeleton. The HMBC correlations (Figure S1) from H-2 (δ_H 3.92 and 3.97)/H-6 (δ_H 7.79) to C-5 (δ_C 161.7) were confirmed the pyran-ring formation between C-2 and C-5. The HMBC correlations from H-11 (δ_H 1.10)/H-12 (δ_H 1.00) to C-2 (δ_C 76.8)/C-3 (δ_C 28.0) /C-4 (δ_C 31.9), from H-4 (δ_H 2.18, 2.40) to C-4a (δ_C 106.8)/C-5 (δ_C 161.6)/C-10a (δ_C 200.0) were supported rhinacanthone backbone. Besides, the chemical shift of C-10 (δ_C 76.0) and HMBC correlations between H-9 (δ_H 7.65) and C-10 (δ_C 76.0); between H-13 (δ_H 2.88 and 2.92) and C-9a (δ_C 141.0)/C-10 (δ_C 76.0)/C-10a (δ_C 200.0) indicated a hydroxyl group and side chain at C-10. Moreover, the down-field chemical shift of C-14 (δ_C 205.5) and the HMBC correlations from H-13 (δ_H 2.88 and 2.92)/H-15 (δ_H 2.06) to C-14 (δ_C 205.5); from H-15 (δ_H 2.06) to C-13 (δ_C 58.0) were proved the structure of side chain as prop-2-one-1-yl. The optical rotation of **1** ($[\alpha]_D^{25} = 0$ (*c* 0.1 $CHCl_3$) and CD spectrum of **1** has no positive/negative peaks may suggested **1** as racemic mixture. Consequently, chemical structure of **1** was completely established and named as rhinacasutone.

The known compounds, rhinacanthone (**2**) (Kuwahara et al. 1995), rhinacanthins C, D, N, Q, and E (**3**–**7**) (Sendl et al. 1996; Kernan et al. 1997; Wu, Hsu, Wu, Leu, et al. 1998; Wu, Hsu, Wu, Teng, et al. 1998), and heliobuphthalmin (**8**) (Abrantes et al. 2008), were identified by comparison of their NMR data with those reported in the literature.

The all isolated compounds were evaluated to find for new and effective antiviral compounds against PR8, HRV1B, and CVB3-infected cells. Since the isolated compounds from the aerial parts of *R. nasutus* significantly inhibited neuraminidase in our previous study (Kwak et al. 2017), we firstly evaluated compounds and the extract against PR8 cells. Compounds **3**–**6** exhibited IC_{50} values of 0.95–23.7 μM (Table 1). Compared to anti-influenza drug oseltamivir phosphate and oseltamivir carboxylate which showed IC_{50} values of more than 100 μM and 4.1 μM in PR8-infected MDCK cells, respectively, the isolated compounds showed very potent anti-influenza activity (Shin et al. 2017). These active compounds were additionally tested for antiviral effect against HRV1B and CVB3 infections. All the tested compounds showed significant antiviral activities in both infections (Table 2). In our previous study, the IC_{50} values of rupintrivir and ribavirin, were 0.12 μM and 48.07 μM against CVB3 and HRV1B, respectively (Ban et al. 2017). When compared with these potent antiviral compounds, the isolated compounds were effective against CVB3 or HRV1B infection at lower

Table 1. Antiviral activity of *R. nasutus* extract and compounds **1–8** towards influenza PR8 virus.

Compound/extract	CC_{50} (μM)	IC_{50} (μM)	TI
MeOH extract	>50	1.53 ± 0.16[a]	>32.68
1	>50	N.D	–
2	>50	N.D	–
3	25.89	0.30 ± 0.06	N.D
4	>50	0.95 ± 0.06	>52.63
5	>50	1.95 ± 0.46	>25.64
6	>50	23.7 ± 0.51	>2.11
7	>50	N.D	–
8	>50	N.D	–

Notes: CC_{50}: Concentration required to reduce cell growth by 50%; IC_{50}: Concentration required to inhibit virus-induced CPE by 50%; TI: Therapeutic index = CC_{50}/IC_{50}; N.D: not detected.

[a]μg/mL. Data represent the mean ± SD of at least three independent experiments, each experiment was performed in triplicate.

Table 2. Antiviral activity of compounds **3**–**6** against HRV1B and CVB3 virus.

	HRV1B			CVB3		
Compd.	CC_{50} (μM)	IC_{50} (μM)	TI	CC_{50} (μM)	IC_{50} (μM)	TI
3	29.98	0.29 ± 0.02	103.38	>50	0.03 ± 0.01	>1666.67
4	29.84	0.24 ± 0.01	124.33	>50	1.44 ± 0.23	>34.72
5	>50	0.97 ± 0.13	51.54	>50	1.50 ± 0.07	>33.33
6	>50	5.35 ± 0.21	9.34	>50	0.82 ± 0.40	>60.98

concentrations. Among the isolated compounds, only naphthoquinones with ester linkage showed antiviral activity. Anti-viral effects vary with the acid component of the ester.

3. Experimental

3.1. General

All NMR spectra were recorded on an Agilent 400 MHz (Agilent Technologies, Palo Alto, CA, USA). Data processing was carried out with the MestReNova ver. 6.0.2 program. HR-ESI-MS spectra were obtained using an AGILENT 6550 iFunnel Q-TOF LC/MS system. Preparative HPLC was carried out using an AGILENT 1200 HPLC system. Column chromatography (CC) was performed on silica-gel (Kieselgel 60, 70–230 mesh and 230–400 mesh, Merck) or RP-18 resins (30–50 μm, Fuji Silysia Chemical Ltd.). For thin layer chromatography (TLC), pre-coated silica-gel 60 F254 (0.25 mm, Merck) and RP-18 F254S (0.25 mm, Merck) plates were used.

3.2. Plant material

The roots of *R. nasutus* were collected at Longbien, Hanoi, Vietnam in December 2013, and identified by Pham Thanh Huyen, National Institute of Medicinal Materials, 3B Quang Trung, Hanoi, Vietnam. A voucher specimen (RN1312) was deposited at the National Institute of Medicinal Materials, 3B Quang Trung, Hanoi, Vietnam

3.3. Extraction and isolation

The dried powdered roots of *R. nasutus* (2.0 kg) were extracted with methanol three times (each 5 L, 5 h) under reflux condition and removed the solvent in *vacuo* to yield methanol extract (140.0 g). The methanol extract was suspended in H_2O (1.5 L) and successively partitioned with dichloromethane and EtOAc to yield corresponding dichloromethane (RND, 60.0 g), EtOAc (RNE, 8.5 g) residues, and water layer (RNW, 70.0 g). The RND was loaded on a silica gel column chromatography (CC) and eluted with increasing polarity solvent system of *n*-hexane/acetone (1/0 → 3/1, v/v) and then dichloromethane/methanol (10/1→0/1, v/v) to give six fractions, RND1-RND6. The RND2 fraction was repeatedly chromatographed on a silica gel CC, eluting with *n*-hexane/dichloromethane/methanol (2/2/0.1, v/v/v) to give two sub-fractions RND2A and RND2B. Purification of RND2A sub-fraction using an RP-18 column and eluent of methanol/acetone/water (10/1/0.1. v/v/v) obtained compound **3** (63 mg). The RND3 fraction was separated on a silica gel CC, eluting with *n*-hexane/dichloromethane/methanol (2/2/0.2, v/v/v) to give five fractions, RND3A-RND3E. Compound **5** (15 mg) was isolated from RND3A using RP-18 column and methanol/acetone/water (20/1/1,

v/v/v) as eluent. RND3C was subjected on a RP-18 column and eluted with methanol/acetone/water (10/1/1, v/v/v) to give compounds **2** (20 mg) and **6** (43 mg). Similarly, RND3D fraction was also purified by an RP-18 column, eluting with methanol/acetone/water (10/1/1, v/v/v) to give compounds **7** (19 mg) and **4** (36 mg). Next, RND4 fraction was separated on a silica gel CC, eluting with *n*-hexane/dichloromethane/methanol (1/1/0.1, v/v/v) to yield four fractions, RND4A-RND4D. Compounds **1** (27 mg) and **8** (24 mg) were isolated from RND4C using silica gel column and *n*-hexane/acetone (3/1, v/v) as an eluent. The purity of each compound was evaluated > 95% by HPLC.

3.3.1. Rhinacasutone (1)

Pale yellow amorphous powder; $[\alpha]_D^{25} = 0$ (*c* 0.1 $CHCl_3$); HR-ESI-MS *m/z*: 323.1257 $[M + Na]^+$ (Calcd. for $[C_{18}H_{20}O_4Na]^+$, 323.1254); ^1H ($CDCl_3$, 400 MHz) *δ*: 7.79 (1H, d, *J* = 7.5 Hz, H-6), 7.65 (1H, d, *J* = 7.5 Hz, H-9′), 7.46 (1H, t, *J* = 7.5 Hz, H-8), 7.37 (1H, t, *J* = 7.5 Hz, H-7), 3.97 (1H, d, *J* = 10.5 Hz, H-2), 3.92 (1H, d, *J* = 10.5 Hz, H-2), 2.92 (1H, d, *J* = 12.9 Hz, H-13), 2.88 (1H, d, *J* = 12.9 Hz, H-13), 2.40 (1H, d, *J* = 16.8 Hz, H-4), 2.18 (1H, d, *J* = 16.8 Hz, H-4), 2.06 (1H, s, H-15), 1.10 (1H, s, H-11), 1.00 (1H, s, H-12); ^{13}C NMR ($CDCl_3$, 100 MHz) *δ*: 205.5 (C-14), 200.0 (C-10a), 161.7 (C-5), 141.0 (C-9a), 130.3 (C-8), 128.0 (C-7), 126.1 (C-5a), 125.6 (C-9), 123.7 (C-6), 106.8 (C-4a), 76.8 (C-2), 76.0 (C-10), 58.0 (C-13), 32.2 (C-15), 31.9 (C-4), 28.0 (C-3), 25.2 (C-11), 24.5 (C-12).

3.4. Antiviral activity assay

3.4.1. Cell culture

Coxsackievirus B3 (Korea Center Disease Control and Prevention, Chungcheongbuk, Korea) was propagated at 37 °C in vero cells (ATCC, Manassas, VA, USA), which are kidney epithelial cells that originated from an African green monkey. Human rhinovirus 1B (ATCC, Manassas, VA, USA) were propagated by infection of HeLa cells. Hela cells and Vero cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic-antimycotic solution (Invitrogen Life Technologies, Karlsruhe, Germany). Assays of antiviral activity and cytotoxicity were evaluated by the SRB method using cytopathic effect (CPE) induced by viral infection as previously reported (Song et al. 2014).

3.4.2. Antiviral assay

$$\text{Antiviral activity index} = \left[OD_t - OD_c\right] / \left[\left(OD_t\right)_{mock} - OD_c\right] \times 100\%.$$

The absorbance obtained from Vero cells infected with viruses at multiplicity of infection (MOI) of 0.06 were used as controls. OD_t is the optical density measured with a given test compound in virus-infected cells; OD_c is the optical density measured for the control untreated virus-infected cells; and $(OD_c)_{mock}$ is the optical density measured for control untreated non-infected cells. The concentration achieving 50% protection according to the formula above was defined as the 50% inhibitory concentration (IC_{50}). To calculate the IC_{50} values, the results were transformed to percentage of controls and the IC_{50} values were graphically obtained from the dose-response curves. The therapeutic index was defined as CC_{50}/IC_{50}.

3.4.3. Cytotoxicity assay

We seeded that Vero cells onto a 96-well culture plate at a concentration of 2×10^4 cells/well. The next day, medium was removed and the cells were washed with PBS. The cells were treated with compounds in maintenance medium for 48 h at 37°C, in parallel with the virus-infected cell cultures. For each compound, 3 wells were used as controls and were not treated with the compounds. After 48 h of incubation, cytotoxicity was evaluated using the SRB assay. Cytotoxicity is presented as % of control. To calculate the CC_{50} values, the results were transformed to percentage of controls and the CC_{50} values were graphically obtained from the dose-response curves.

4. Conclusion

Phytochemical study of the roots of *R. nasutus* resulted in isolation of one new naphthoquinone analogue, rhinacasutone (**1**), as well as seven previously reported compounds (**2**–**8**). All isolated compounds were tested for their antiviral activity against PR8, HRV1B, and CVB3-infected vero cells. Compounds **3–5** exhibited significant antiviral activities against all three infections with the IC_{50} value ranges of 0.03 to 1.95 μM. In addition, compound **6** showed IC_{50} value of 0.82 μM against CBV3-infected vero cells. This study demonstrated that the compounds isolated from the roots of *R. nasutus* as well as root extract could be effective antiviral agents.

Disclosure statement

No potential conflict of interest was reported by the authors.

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