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Chemical constituents and antiviral study *of Goniothalamus velutinus*

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ABSTRACT

Phytochemical study on the stem barks of *Goniothalamus velutinus* resulted in the isolation of three compounds identified as goniothalamin (1), pinocembrine (2) and naringenin (3). Their structures were elucidated using various spectroscopic methods especially Fourier Transform Infrared (FTIR), Nuclear Magnetic Resonance (NMR) and gas chromatography-mass spectrometry (GC-MS). Goniothalamin (1) has been isolated from several *Goniothalamus* spp. but no report on the isolation of (1) from *Goniothalamus velutinus*. Antiviral properties of (1) against Measles virus showed weak activity compared to positive control, ribavirin.

| *Goniothalamus velutinus* | goniothalamin | pinocembrine | naringenin | antiviral |

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1. INTRODUCTION

Goniothalamus is a genus of shrubs and aromatic trees belongs to the Annonaceae family with approximately 160 species distributed in South Eastern Asia and throughout Malaysia [1]. The stem bark is aromatic and grows up to about 2 m tall. This genus is widely used in traditional medicines by natives especially for abortion and post partum treatment. For example, decoctions of *G. macrophyllus* and *G. scortechnii* are used as a post partum protective remedy while the roots of *G. tapis* and *G. giganteus* are used for abortion during early month of pregnancy [2]. Phytochemical investigations of *Goniothalamus* spp. resulted in the isolation of acetogenins, styryl lactones and alkaloids with significant cytotoxic, insecticidal and antimicrobial activities [3]. *G. velutinus*, locally known as 'Kayu Hujan Panas' is one of the interesting endemic *Goniothalamus* spp. of Borneo. It is a small tree up to 3 m tall and 3 cm diameter. The specific medicinal uses are not clearly described but the natives of Sabah and Sarawak use the roots decoction for the treatment of headaches and food poisoning [4,5]. The strong smell from the stem bark used as a mosquito repellent [5]. Previous study on *G. velutinus* resulted in the isolation of two alkaloids which have been identified as phenanthrene lactam and aristolactam BII [4]. This paper will discuss the isolation of goniothalamin (**1**), pinocembrine (**2**) and naringenin (**3**) from the stem barks of *G. velutinus*.

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2. EXPERIMENTAL

2.1 General.

IR: KBr discs and was recorded using Shimadzu FTIR-8201 PC spectrometer. ^{1}H and ^{13}C-NMR were recorded on a JEOL 500 MHz NMR spectrometer: 500MHz for ^{1}H and 125MHz for ^{13}C, with $CDCl_3$ and acetone-D_6 as solvents. Melting point was measured using STUART smp 3 instrument and was uncorrected. GC-MS analysis was performed using Hewlett Packard 6890 instrument.

2.2 Plant material.

Goniothalamus velutinus Airy-Shaw was collected from Limbang, Sarawak. All parts were divided into leaves, stem barks and roots, air dried and ground to fine powder. A voucher specimen (HUMS0061) was deposited at Universiti Malaysia Sarawak Herbarium.

2.3 Extraction and isolation.

Stem barks powder (2.0 kg) was extracted with hexane followed by dichloromethane (CH_2Cl_2), chloroform ($CHCl_3$), ethyl acetate (EtOAc) and methanol each (MeOH) for three days at room temperature. Each extract was evaporated to dryness to give 4.70 g (0.24%), 33.70 g (1.67%), 12.10 g (0.60%), 6.40 g (0.32%) and 16.30 g (0.82%) of hexane, CH_2Cl_2, $CHCl_3$, EtOAc and MeOH, crude extract respectively. The CH_2Cl_2 extract afforded 0.70 g of white precipitate which was labelled as NKNGV-G1. NKNGV-G1 was dissolved in CH_2Cl_2 followed by

addition of hexane drop wise to form cloudy solution and left at room temperature. The precipitates were filtered and dried at room temperature. This method was repeated several times to give 91 mg of compound (**1**) with R_f 0.6 (hexane: CH_2Cl_2; 1:4). In order to form a perfect crystal shape for X-ray crystallography analysis, 10 mg of compound (**1**) was redissolved in 20 mL of $CHCl_3$ and allowed to evaporate slowly in a test tube.

The MeOH crude extract of the stem barks of *G. velutinus* afforded 30 mg precipitate each and labelled as S1 and S2. Exactly 25 mg of S1 was further purified using prep. TLC in $CHCl_3$:acetone (4:1) to give 15 mg of compound (**2**). Using prep. TLC in similar solvent system, 25 mg of S2 afforded 12 mg of compound (**3**). All compounds were identified as goniothalamin (**1**), pinocembrine (**2**) and naringenin (**3**) based on spectroscopic data and comparison with published information.

2.4 Goniothalamin (1):

Colourless needle crystals; m.p. 83-84 ^oC; (lit [7] 80-82^oC); IR υ_{max} cm^{-1} 1720, 1703, 1388 and 1249; MS m/z (%): 200 [M^+, $C_{13}H_{12}O_2$] (82%), 172 [M^+ - CO] (29%), 131 $[C_9H_7O]^+$ (26%), 115 $[C_9H_7]^+$ (24%), 104 $[C_8H_8]^+$ (100%), 91$[C_7H_7]^+$ (50%), 68$[C_4H_4O]^+$ (95%); ^{1}H NMR (500 MHz) ($CDCl_3$) ppm: δ 7.31 (m, 5H, aromatic protons), δ 6.92 (dt, 9.8Hz, 4.3Hz, 1H, H-3), δ 6.73 (d, 16.0 Hz, 1H, H-8), δ 6.29 (dd, 16.0 Hz, 6.3 Hz, 1H, H-7), δ 6.09 (dt, 9.8 Hz, 1.6 Hz, 1H, H-4), δ 5.11 (m, 1H, H-6), δ 2.57 (m, 2H, H-5); ^{13}C NMR (125 MHz) ($CDCl_3$) : δ 29.9 (C-5), δ 77.9 (C-6), δ 121.7 (C-3), δ 125.6 (C-7), δ 126.7 (C-10 & C-14), δ 128.3 (C-12), δ 128.7 (C-11 & C-13), δ 133.1 (C-8), δ 135.8 (C-9), δ 144.6 (C-4), δ 163.8 (C-2).

2.5 Pinocembrine (2):

Yellowish amorphous solid; m.p 196.9-197.7^oC; (lit [8] 191-192^oC); IR ν_{max} cm^{-1} 3090, 2890, 1631, 1604, 1487 and 1169; MS m/z (%): 256 [M^+, $C_{15}H_{12}O_4$] (91 %), 238 (10.5%), 179 (98.2%), 152 (100%), 124 (73.7%) and 91 (8.7%); ^{1}H NMR (500 MHz) ($CDCl_3$) ppm: δ 5.43 (dd, 3.2 Hz, 12.8 Hz, 1H, H-2), δ 3.11 (dd, 12.8 Hz, 17.2 Hz, 1H, Hα-3), δ 2.84 (dd, 3.2 Hz, 17.2 Hz, 1H, Hβ-3), δ 6.00 (s, 1H, H-6), δ 6.00 (s, 1H, H-8), δ 7.37-7.44 (m, 5H, aromatic protons), δ 12.02 (s, 1H, OH); ^{13}C NMR (125 MHz) ($CDCl_3$) ppm: δ 79.20 (C-2), δ 43.20 (C-3), δ 195.80 (C-4), δ 163.18 (C-5), δ 96.76 (C-6), δ 164.55 (C-7), δ 95.49 (C-8), δ 164.36 (C-9), δ 103.24 (C-10), δ 138.28 (C-1'), δ 128.88 (C-4'), δ 126.13 (C-2' & C-6') and δ 128.91 (C-3' & C-5').

2.6 Naringenin (3):

Yellowish amorphous solid; m.p. 250-251^oC; (lit [9] 249-252^oC); IR ν_{max} cm^{-1} 3124, 2832, 1630, 1603, 1463 and 1158; MS m/z (%): 272 [M^+, $C_{15}H_{12}O_5$] (60%), 179 (26.7%), 166 (27.7%), 153 (100%), 120 (61.4%), 107 (17.8%) and 91 (23.8%); ^{1}H NMR (500 MHz) (acetone-D_6) ppm: δ 5.45 (d, 13.0 Hz, 1H, H-2), δ 2.73 (d, 17.0 Hz, 1H, Hα-3), δ 3.17 (dd, 17.0 Hz, 13.0 Hz, 1H, Hβ-3), δ 5.96 (s, 1H), δ 5.96 (s, 1H, H-6), δ 7.40 (d, 7.6 Hz, 1H, H-2'), δ 6.90 (d, 7.6 Hz, 1H, H-3'), δ 6.90 (d, 7.6 Hz, 1H, H-5'), δ 7.40 (d, 7.6 Hz, 1H, H-6'), δ 12.18 (s, 1H, OH); ^{13}C NMR (125 MHz) (acetone-D_6) ppm: δ 79.9 (C-2), δ 43.4 (C-3), δ 197.1 (C-4), δ 165.3 (C-5), δ 95.9 (C-6), δ 167.7 (C-7), δ 96.8 (C-8), δ 164.3 (C-9), δ 103.0 (C-10), δ 128.9 (C-1'), δ 130.7 (C-2' & C-6'), δ 116.1 (C-3' & C-5'), δ 158.7 (C-4').

2.7 Antiviral assay.

Vero cells were seeded onto 96 wells microtiter plate with a concentration of 1.0×10^5 cells per mL and a volume of 100 µL per well. Plate was then incubated at 37^oC with 5% CO_2 overnight to obtain confluent monolayer cells. After that, the growth medium in each well was discarded and cells were washed with sterile phosphate buffered saline (PBS). In order to determine the mode of antiviral action, cells and virus were incubated with compound (**1**) at different stages during the viral infection cycle.

a) Pre-infection protocol

The diluted compound (**1**) was added to cells and incubated at 37°C in CO_2 incubator for 24 hours. After this incubation, the compound was aspirated and replaced with 10 µL measles virus and growth medium before incubated again for 72 hours.

b) Virucidal protocol

Compound dilutions (200 µL) were prepared in 96 wells microtiter plate. Measles virus (10 µL) was added to each well (except for control) and the mixtures were left for an hour at 37°C. After that, the virus-compound mixtures were added to cells and plate was incubated at 37°C for 72 hours in CO_2 incubator [6].

c) Post-infection protocol

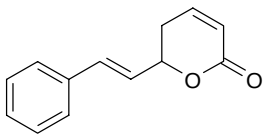
Cells were inoculated with measles virus (10 µL) first, followed by one hour incubation at 37°C in CO_2 incubator to allow the virus to absorb and penetrate the cells. Then, 200 µL diluted compound was added to the infected cells and plate was incubated at 37°C for 72 hours in CO_2 incubator.

There were four controls involved in this test; (i) cells inoculated with virus only, (ii) cells added with compound only, (iii) cells only and (iv) Dulbecco's modified Eagle's medium (DMEM) only. Ribavirin at concentration of 0.1 LC_{50} and 0.01 LC_{50} was used as positive control. After 72 hours, plate was processed using Eosin B assay and ELISA reader at 490nm.

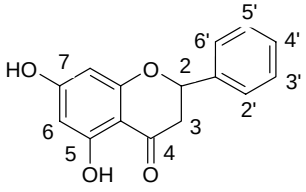
3. RESULTS & DISCUSSION

Three pure compounds identified as goniothalamin (**1**), two known flavonoids namely pinocembrine (**2**) and naringenin (**3**) have been isolated from *G. velutinus*.

Compound (**1**) was isolated from the dichloromethane extract of the stem bark of *G. velutinus*. It is known as embryotoxic styryl lactone which was previously isolated from several *Goniothalamus* spp. such as *G. macrophyllus*, *G. clemensii*, *G. andersonii* and *G. malayanus* [7, 10-12] but this is the first report of the isolation of (**1**) from *G. velutinus*. The ^{1}H NMR spectrum of (**1**) revealed the signal at δ 6.92 (dt, 9.8Hz, 4.3Hz) for H-3 and δ 6.09 (dt, 9.8 Hz, 1.6 Hz) for H-4 as olefinic proton of lactone skeleton. Two proton signal observed at δ 2.57 (m, 2H) for proton at H-5 appeared as multiplet. Another two olefinic protons appeared at δ 6.29 (dd, 16.0 Hz, 6.3 Hz) and δ 6.73 (d, 16.0 Hz) which were ascribed to protons H-7 and H-8 respectively. The signal which appeared at δ 5.11 (m, 1H) assignable to the proton H-6. The signal at δ 7.31 (m, 5H) was ascribed to proton of phenyl group in (**1**). The ^{13}C NMR spectrum gave signals at δ 126.7 (C-10 and C-14), δ 128.3 (C-12), δ 128.7 (C-11 and C-13) and δ 135.8 (C-9). These signals indicated the presence of mono substituted phenyl carbons which also supported by ^{1}H NMR which gave signal at 7.31 (m, 5H) assignable to the proton of an aromatic ring. The signal at δ163.8 was ascribed to C-2 indicated the presence of carbonyl group which also proved previously by IR spectrum at absorption band of 1719 cm^{-1}. The spectral data, melting point and physical properties of (**1**) were identical with goniothalamin that has been isolated from *G. macrophyllus* [7] thus (**1**) has been identified as goniothalamin. Previous studies reported that, goniothalamin was found to possess anticancer properties against colon cancer cell line (LS-174T and HT-29) and breast cancer cell line (MCF-7), lung carcinoma (COR-L23 and NCI.460), kidney tumor cell line (786-0) with IC_{50} values of 3.41 µg/mL, 12.62 µg/mL and 23.33 µg/mL and 4 nM respectively. It also showed cytotoxicity and genotoxicity against human HL-60 promyelocytic leukemia cells and CEM-SS T-lymphoblastic cells with IC_{50} values of 4.5 µg/mL and 2.4 µg/mL respectively [7, 13,14].



1



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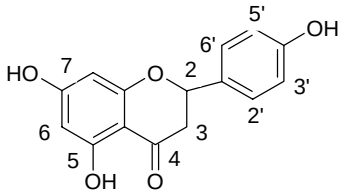

3

Compound (**2**) was isolated as yellowish amorphous solids, melting point 196.9-197.7^{o}C and gave IR absorptions at 3090, 2890, 1631, 1604, 1487 and 1169 cm^{-1} indicating the presence of hydroxyl, carbonyl and phenyl group. The gas chromatogram showed a molecular ion peak at *m/z* 256 which corresponding to a molecular formula of $C_{15}H_{12}O_4$. The ^{1}H NMR spectrum for (**2**) showed some similarities when compared to compound (**3**). Two signals were observed at H-3 and the proton of hydroxyl group appeared at δ 12.02 for both compounds. Two signals appeared as doublet of doublet at δ 3.11 (J = 12.8, 17.2 Hz) and δ 2.84 (J = 3.2, 17.2 Hz) were attributed to proton at H-3α and H-3β respectively. The multiplet signal within the range of δ 7.37-7.44 was assigned to aromatic protons. From the ^{13}C NMR spectrum, carbonyl group was observed by the peak appeared at δ 195.80 while the signals appeared at δ 128.88 and δ 138.28 were assigned to aromatic carbons at C-4' and C-1' respectively. Two aromatic carbons which are C-2' and C-6' were in the similar environment observed from the peak appeared at δ 126.13 while the signal at δ 128.91 was assigned to aromatic carbons at position C-3' and C-5'.

Compound (**3**) was isolated as yellow amorphous solid, melting point 250-251^{o}C and gave a molecular ion peak at *m/z* 272 which corresponding to a molecular formula $C_{15}H_{12}O_5$. A strong absorption at 1630 cm^{-1} was ascribed to carbonyl group due to conjugation with the aromatic system. The ^{1}H NMR spectrum for compound (**3**) revealed a singlet signal at δ 12.18 indicated the proton of hydroxyl group. The proton at position H-3 resonated as two signals at δ 2.73(d, 17.0 Hz) and δ 3.17 (dd, 17.0 Hz, 13.0 Hz) assignable to proton Hα-3 and Hβ-3 respectively. The doublet peaks appeared at δ 6.90 was attributed to aromatic protons at position H-3' and H-5' while the other doublet signal appeared at δ 7.40 was assigned to aromatic proton at H-2' and H-6'. The ^{13}C NMR spectrum showed a signal at δ 197.1 indicated the presence of carbonyl group at position C-4. Two signals observed at δ 165.3 and δ 167.7 were assigned to C-5 and C-7. The signal at δ 158.7 was attributed to aromatic carbon, C-4'. Two aromatic carbons were observed at δ 116.1 for C-3' and C-5'. The signal appeared at δ 130.7 were assigned to C-2' and C-6'.

Comparing the chemical shift of C-4' in (**2**) and (**3**), showed the value of C-4' in (**3**) was in the lower field due to the presence of hydroxyl substituent at C-4' of (**3**). Based on the ^{1}H and ^{13}C NMR spectra, (**2**) and (**3**) were elucidated as pinocembrine and naringenin, respectively. The melting point and the spectroscopic data of these compounds were in good agreement with the published data [8,9,15,16].

Pinocembrine (**2**) has been previously isolated from Chilean propolis, *Lippa gravolen*, *Galenia africana*, *Pinus strobes*, *G. scortechnii* and found to posses antifungal and antioxidant properties [8,16,17]. Naringenin (**3**) has been isolated from *Amygdalus lycioides* var. *horrida* and *Commiphora wightii* and reported to have antioxidant properties [15,18,19].

In this study, antiviral properties of compound (**1**) was tested against Measles virus and showed weak activity compared to positive control, ribavirin. Pretreatment of cells with (**1**) before addition of Measles virus caused a significant reduction of infectivity. Measles virus titer was reduced by more than 98% at a concentration of 0.1 LC_{50}. However, no significant effect on viral replication was detected when ribavirin was used for pretreatment of viruses or when added after the adsorption phase. The antiviral activity of (**1**) is summarized in Table 1.

Table 1. The effect of compound 1 against Measles virus compared to ribavirin

Concentration of pure compound (µg/mL)	Viral inhibition rate (%)		
	Pre-infection	Virucidal	Post-infection
0.1 LC_{50}	98.41	31.41	17.3
0.01 LC_{50}	22.4	-	-
Ribavirin (control)			
0.1 LC_{50}	100	55.08	23.79
0.01 LC_{50}	100	50.25	32.28

4. CONCLUSION

Three compounds identified as goniothalamin (**1**), pinocembrine (**2**) and naringenin (**3**) have been isolated from the stem barks of *G. velutinus*. Goniothalamin (**1**) inhibited 98% of viral replication before the addition of Measles virus at concentration of 0.1 LC_{50} however no significant activity was observed after the addition and adsorption phase of the virus to the cell.

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