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### Abstract

The phytochemical investigation of an alkaloidal extract of *Holarrhena pubescens* roots led to the isolation and identification of a new pregnene-type alkaloid, mokluangin D (1), together with nine known steroidal alkaloids (2-10). The structure of the new metabolite was determined on the basis of spectroscopic analyses including 1D- and 2D-NMR spectroscopy and mass spectrometry. Compounds 3 and 4 showed potent antimalarial activity against *Plasmodium falciparum* K1 stain with IC<sub>50</sub> values of 1.2 and 2.0 μM, respectively, and showed weak cytotoxic activity against the NCI-H187 cell line with IC<sub>50</sub> values of 27.7 and 30.6 μM, respectively. The substituent groups at C-3 and the carbonyl group at C-18 are important for the activity against the *P. falciparum* K1 stain.

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**Antimalarial and cytotoxic activities of pregnene-type steroidal alkaloids  
from *Holarrhena pubescens* roots**

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# Antimalarial and cytotoxic activities of pregnene-type steroidal alkaloids from *Holarrhena pubescens* roots

## ABSTRACT

The phytochemical investigation of an alkaloidal extract of *Holarrhena pubescens* roots led to the isolation and identification of a new pregnene-type alkaloid, mokluangin D (**1**), together with nine known steroidal alkaloids (**2-10**). The structure of the new metabolite was determined on the basis of spectroscopic analyses including 1D- and 2D-NMR spectroscopy and mass spectrometry. Compounds **3** and **4** showed potent anti-malarial activity against *P. falciparum* K1 stain with IC<sub>50</sub> values of 1.2 and 2.0 μM, respectively, and showed weak cytotoxic activity against the NCI-H187 cell line with IC<sub>50</sub> values of 27.7 and 30.6 μM, respectively. The substituent groups at C-3 and the carbonyl group at C-18 are important for the activity against the *P. falciparum* K1 stain.

**Keywords:** *Holarrhena pubescens*; pregnene-type steroidal alkaloids; cytotoxicity; anti-malarial activity

## 1. Introduction

*Holarrhena pubescens* (Apocynaceae) (synonym: *H. antidysenterica*) is a shrub, widely distributed throughout in the tropics, including Thailand (Middleton 1999). The bark of this plant has been used in traditional medicine as in astringent, anthelmintic, stomachic and febrifugal and for its tonic properties (Middleton 1999; Chouhan et al. 2017). Pharmacological studies demonstrated that *H. pubescens* extracts possessed anti-bacterial (Siddiqui et al. 2012; Kaundal and Sagar 2016), anti-inflammatory (Darji et al. 2012), acetylcholinesterase inhibitory (Yang et al. 2012; Cheenpracha et al. 2016), analgesic (Shwetha et al. 2014), and anti-diabetic activities (Umashanker et al. 2012). Previous chemical investigations have demonstrated the presence of steroidal alkaloids (Kumar A and

Ali 2000; Kumar et al. 2007; Yang et al. 2012; Cheenpracha et al. 2016) and furfuran lignan (Xie et al. 2017). In our ongoing search for bioactive natural products from Thai medicinal plants (Cheenpracha et al. 2016), we herein report the isolation and structural elucidation of a new pregnene-type steroidal alkaloid, mokluangin D (**1**), along with the isolation of nine known alkaloids (**2–10**), two of which showed potent anti-malarial activity, which having weak cytotoxicity against the NCI-H187 cell line.

## 2. Results and discussion

The MeOH extract of the air-dried roots of *H. pubescens* was partitioned between EtOAc and 1% HCl. The aqueous layer was adjusted to pH 8–9 with saturated NH<sub>3</sub>, and further extracted with EtOAc to give the alkaloidal extract. A new pregnene alkaloid, mokluangin D (**1**), and nine known compounds including, malouetafrine (**2**) (Khuong-Huu et al. 1973), irehline (**3**) (Janot et al. 1963), mokluangin A (**4**) (Cheenpracha et al. 2016), conessine (**5**) (Zirihi et al. 2005), conimin (**6**) (Yang et al. 2012), holaphyllaminol (**7**), holaphylline (**8**) (Dadoun et al. 1973), holaphyllamine (**9**) (Cheenpracha et al. 2016) and methylholaphyllamine (**10**) (Cheenpracha et al. 2016) (Figure 1) were obtained through a series of column chromatographic purifications. All isolated structures were elucidated by spectroscopic evidence including 1D and 2D experiments and physical data.

Mokluangin D (**1**) was isolated as a white solid with the molecular formula as C<sub>21</sub>H<sub>27</sub>NO from the pseudomolecular ion at  $m/z$  310.2162 [M+H]<sup>+</sup> in the HRESIMS spectrum, indicating nine degrees of unsaturation in combination with interpretation of <sup>13</sup>C NMR data. The four degrees of unsaturation could be accounted for by two carbon-carbon double bonds, one carbonyl group and one imine group, as indicated for <sup>13</sup>C NMR spectroscopic analysis ( $\delta_C$  200.6, 171.2, 161.5, 137.2, 127.8 and 124.0). The five remaining degrees were due to a pentacyclic structure related to mokluangin A (Cheenpracha et al. 2016). The UV spectrum displayed an absorption band at  $\lambda_{max}$  277 nm, characteristic of a

conjugated ketone. Its IR spectrum showed an imino group at  $3419\text{ cm}^{-1}$  and the carbonyl stretching at  $1655\text{ cm}^{-1}$ . The  $^{13}\text{C}$  NMR and DEPT data of **1** displayed 21 carbon resonances that were identified as two methyls ( $\delta_{\text{C}}$  16.8, 16.7), six methylenes ( $\delta_{\text{C}}$  33.1, 32.4, 31.7, 24.3, 23.5, 22.3), nine methines (five  $sp^3$  methines ( $\delta_{\text{C}}$  68.3, 49.9, 49.1, 47.5, 47.3) and four  $sp^2$  methines ( $\delta_{\text{C}}$  171.2, 137.2, 127.8, 124.0)), three quaternary carbons ( $\delta_{\text{C}}$  161.5, 67.1, 36.6) and a carbonyl carbon ( $\delta_{\text{C}}$  200.6). The assignments of the  $^{13}\text{C}$  NMR chemical shifts are based on HMQC and HMBC experiments. The  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data indicated the presence of a pregnene-type steroidal alkaloid according to two methyl proton resonances at  $\delta_{\text{H}}$  1.39 (3H, d,  $J = 6.0$  Hz, H<sub>3</sub>-21) and 1.18 (3H, s, H<sub>3</sub>-19) (Phi et al. 2011). The downfield signal at  $\delta_{\text{H}}$  7.59 (1H, br d,  $J = 3.0$  Hz, H-18), attributable to an imino methine, was attached to a carbon resonating at  $\delta_{\text{C}}$  171.2 (C-18) from the HMQC experiment. In the  $^1\text{H}$  NMR spectrum, three olefinic proton signals resonated at  $\delta_{\text{H}}$  6.22 (1H, m, H-3), 6.13 (br dd,  $J = 9.7, 1.4$  Hz, H-4) and 5.66 (1H, s, H-6), revealing that the pregnane moiety in **1** was dehydrogenated to form a conjugated system comprising two double bonds. In the HMBC spectrum, the correlations from H-3 ( $\delta_{\text{H}}$  6.22) to C-1 ( $\delta_{\text{C}}$  33.1), C-2 ( $\delta_{\text{C}}$  23.5) and C-5 ( $\delta_{\text{C}}$  161.5), of H-4 ( $\delta_{\text{H}}$  6.13) to C-2 ( $\delta_{\text{C}}$  23.5), C-5 ( $\delta_{\text{C}}$  161.5), C-6 ( $\delta_{\text{C}}$  124.0) and C-10 ( $\delta_{\text{C}}$  36.6), of H-6 ( $\delta_{\text{H}}$  5.66) to C-4 ( $\delta_{\text{C}}$  127.8), C-8 ( $\delta_{\text{C}}$  47.5) and C-10 ( $\delta_{\text{C}}$  36.6), of H-8 ( $\delta_{\text{H}}$  2.65) to C-6 ( $\delta_{\text{C}}$  124.0), C-7 ( $\delta_{\text{C}}$  200.6), C-9 ( $\delta_{\text{C}}$  49.9), C-10 ( $\delta_{\text{C}}$  36.6) and C-14 ( $\delta_{\text{C}}$  49.1), and of H<sub>3</sub>-19 ( $\delta_{\text{H}}$  1.18) to C-1 ( $\delta_{\text{C}}$  33.1), C-5 ( $\delta_{\text{C}}$  161.5), C-9 ( $\delta_{\text{C}}$  49.9) and C-10 ( $\delta_{\text{C}}$  36.6) confirmed the location of the two conjugated double bonds at C-3–C-4–C-5–C-6 and the ketone group at C-7. Comparison of the  $^1\text{H}$  and  $^{13}\text{C}$  NMR spectral data between compound **1** and kibalaurifenone (Phi et al. 2011) revealed a close structural similarity, except for the replacement of a lactone ring with a 1-pyrroline ring, in which the imino proton ( $\delta_{\text{H}}$  7.59) at C-18 was determined from the HMBC correlations of H-18 to C-13 ( $\delta_{\text{C}}$  67.1), and C-20 ( $\delta_{\text{C}}$  68.3). The NOESY cross peaks were observed for H-8/H<sub>3</sub>-19 and H-17/H-20, indicating that H-20 is  $\alpha$  and the methyl groups (H<sub>3</sub>-

19 and H<sub>3</sub>-21) have  $\beta$  orientation. Thus, compound **1** was assigned as mokluangin D as shown in Figure 1.

Some of the isolated compounds were evaluated for their anti-malarial activity against *Plasmodium falciparum* (K1, a multidrug resistant strain) and cytotoxicity against the NCI-H187 (small cell lung cancer) cell line and the results are shown in Table 1. Compounds **3** and **4** showed significant anti-malarial activity against the *P. falciparum* K1 stain with IC<sub>50</sub> values of 1.2 and 2.0  $\mu$ M, respectively. These compounds showed weakly cytotoxic activity against the NCI-H187 cell line with IC<sub>50</sub> values of 27.7 and 30.6  $\mu$ M, respectively. Compounds **5-8** and **10** exhibited strong anti-malarial activity against the *P. falciparum* K1 stain with IC<sub>50</sub> values ranging from 4.1–11.7  $\mu$ M. Compounds **7** and **8** were weakly cytotoxic against the NCI-H187 cell line with IC<sub>50</sub> values ranging from 18.2–29.5  $\mu$ M, whereas compounds **5**, **6** and **10** were not cytotoxic. Because of the sample shortage, compounds **1** and **2** were not tested. The results indicated that substitution of the C-3 amino group and the nature of the E ring may influence anti-malarial activity. Importantly, these compounds show relating weak cytotoxicity, indicating the plants potential of a therapeutic plant to treat malaria.

### 3. Experimental

#### 3.1 General experimental procedures

Melting points were determined on a Buchi 535 melting point apparatus and are uncorrected. Optical rotations were determined on a Bellingham & Stanley ADP400 polarimeter. The UV and IR spectra were recorded on PerkinElmer UV–Vis spectrophotometer and PerkinElmer FTS FT-IR spectrometer. The 1D and 2D NMR spectra were recorded in CDCl<sub>3</sub> using TMS as internal standard on a 400 Bruker FTNMR Ultra

Shield spectrometer. Chemical shifts are reported in parts per million (d) and the coupling constants are in Hz. The TOFMS data was obtained from a Micromass LCT and MAT 95 XL mass spectrometer. Quick column chromatography (QCC) and column chromatography (CC) were carried out on silica gel 60 F<sub>254</sub> (Merck) and silica gel 100 (Merck), respectively. Precoated plates of silica gel 60 F<sub>254</sub> was used for analytical purposes. Spots were detected under UV light followed by heating after spraying with Dragendorff's reagent.

### 3.2 Plant material

The roots of *H. pubescens* were collected from Nan Province, northern Thailand, in April 2013. Botanical identification was achieved through comparison with the voucher specimen, number MFU-NPR0086, in the Natural Products Research Laboratory, School of Science, Mae Fah Luang University, Thailand by Mr. Martin van de Bult (Doi Tung Development Project, Chiang Rai, Thailand).

### 3.3 Extraction and isolation

Dried and chopped roots of *H. pubescens* (9.0 kg) were extracted with MeOH (2 x 13 L, 3 days) at room temperature to afford a dark green residue after removal of the solvent under reduced pressure. The residue was dissolved in 1% HCl (1 L). After extraction with ethyl acetate (2 x 1 L), the acidic layer was basified with saturated NH<sub>3</sub> to adjust the pH to 8–9 and extracted with EtOAc (2 x 2 L) to give an alkaloidal fraction (44.7 g) (Cheenpracha et al. 2016). The crude alkaloid was separated by silica gel QCC with hexanes/acetone gradient (1:0 – 0:1, v/v) to give eight fractions (F1–F8). Fraction F2 (282.8 mg) was subjected to silica gel CC using acetone–CH<sub>2</sub>Cl<sub>2</sub> (1:49, v/v) to afford five subfractions (F2a–F2e). Purification of subfraction F2c (31.5 mg) by silica gel CC eluting with diethyl ether–hexanes led to

compound **1** (3.9 mg). Fraction F3 (3.1 g) was fractionated by silica gel CC with MeOH–CH<sub>2</sub>Cl<sub>2</sub> (1:19, v/v, sat. NH<sub>3</sub>), followed by silica gel CC using acetone-hexanes (2:3, v/v, sat. NH<sub>3</sub>) to provide compounds **5** (4.5 mg), **6** (9.1 mg) and **7** (27.6 mg). Fraction F4 (1.10 g) was purified by CC over Sephadex LH-20 eluting with 100% MeOH and followed by silica gel CC with MeOH–CH<sub>2</sub>Cl<sub>2</sub> (1:19, v/v) to give compounds **9** (3.4 mg) and **10** (5.4 mg). Fraction F5 (8.18 g) was subjected to silica gel CC with MeOH–CH<sub>2</sub>Cl<sub>2</sub> (1:19, v/v, sat. NH<sub>3</sub>) providing seven subfractions (F5a–F5g). Compound **8** (227.5 mg) was isolated from subfraction F5b (400.7 mg) by silica gel CC eluting with acetone-hexanes (1:4, v/v) and followed by CC over Sephadex LH-20 with 100% MeOH. Subfraction F5c (10.5 mg) was further separated by silica gel CC with acetone-hexanes (1:4, v/v) giving compound **2** (0.5 mg). Compound **3** (18.7 mg) was purified from subfraction F5e (242.1 mg) by silica gel CC with acetone–hexanes (2:3, v/v, sat. NH<sub>3</sub>). Fraction F7 (8.5 g) was purified by silica gel CC with MeOH–CH<sub>2</sub>Cl<sub>2</sub> (0:1 – 1:1, v/v) to give eight subfractions (F7a–F7h). Subfraction F7e (273.6 mg) was further purified by silica gel CC using acetone–hexanes (2:3, v/v, sat. NH<sub>3</sub>) to afford compound **4** (22.5 mg).

**Mokluangin D (1)**; white solid, m.p. 198-200 °C; [ $\alpha$ ]<sub>D</sub> -165.2 (c 0.01, CHCl<sub>3</sub>); UV (MeOH)  $\lambda_{\max}$  (log  $\epsilon$ ): 210 (3.10), 277 (4.01) nm; IR (neat)  $\nu_{\max}$ : 3419, 2928, 1655, 1594, 1418, 1118 cm<sup>-1</sup>; <sup>1</sup>H NMR (400 MHz, CDCl<sub>3</sub>):  $\delta_{\text{H}}$  7.59 (1H, br d,  $J$  = 3.0 Hz, H-18), 6.22 (1H, m, H-3), 6.13 (1H, brdd,  $J$  = 9.7, 1.4 Hz, H-4), 5.66 (1H, s, H-6), 4.12 (1H, m, H-20), 2.65 (1H, t,  $J$  = 12.0 Hz, H-8), 2.57 (2H, m, H-16), 2.33 (1H, m, H-2a), 2.31 (4H, m, H-11, H-15), 2.01 (1H, m, H-17), 1.99 (1H, m, H-1a), 1.93 (1H, m, H-1b), 1.88 (1H, m, H-2b), 1.86 (1H, m, H-9), 1.71 (1H, m, H-14), 1.56 (2H, m, H-12), 1.39 (3H, d,  $J$  = 6.0 Hz, H-21), 1.18 (3H, s, H-19); <sup>13</sup>C NMR (100 MHz, CDCl<sub>3</sub>) data:  $\delta_{\text{C}}$  200.6 (C-7), 171.2 (C-18), 161.5 (C-5), 137.2 (C-3), 127.8 (C-4), 124.0 (C-6), 68.3 (C-20), 67.1 (C-13), 49.9 (C-9), 49.1 (C-14), 47.5 (C-8), 47.3

(C-17), 36.6 (C-10), 33.1 (C-1), 32.4 (C-12), 31.7 (C-16), 24.3 (C-15), 23.5 (C-2), 22.3 (C-11), 16.8 (C-19), 16.7 (C-21). HRESIMS [M+H]<sup>+</sup> *m/z* 310.2162 (calcd 310.2171, C<sub>21</sub>H<sub>28</sub>NO).

### 3.4 Biological assays

The antimalarial activity against *Plasmodium falciparum* (K1, multidrug resistant strain) was performed using a modified Microdilution Radioisotope Technique (Yuthavong et al. 2000). Dihydroartemisinin (IC<sub>50</sub> = 3.74 nM) and mefloquine (IC<sub>50</sub> = 70.02 nM) were used as the reference substances. In vitro anticancer activity tests against NCI-H187 cells (small cell lung cancer) were evaluated using resazurine microplate assay (REMA) (Skehan et al. 1990). Doxorubicin (IC<sub>50</sub> = 0.079 ug/ml) and ellipticine (IC<sub>50</sub> = 0.079 ug/ml) were used as the standard compounds. Both assays were performed at the bioassay laboratory, BIOTEC, NSTDA, Thailand.

## 4. Conclusions

Recently, compound **5** was isolated from the barks of *H. antidysentrica* exhibited substantial antimalarial activity with slight cytotoxicity (Dua et al. 2013). We currently report a new pregnene alkaloid, mokluangin D (**1**), together with nine known steroidal alkaloids, isolated from the roots of *H. pubescens* and evaluated for their inhibitory activity against a *P. falciparum* K1 stain and NCI-H187 cell line. Compounds **3** and **4** showed potent anti-malarial activity against the *P. falciparum* K1 stain with the IC<sub>50</sub> values of 1.2 and 2.0 μM, respectively, compared to the control, dihydroartemisinin, which showed an IC<sub>50</sub> of 3.7 nM. The other compounds (**5-8**, **10**) showed anti-malarial activity against the *P. falciparum* K1 stain with the IC<sub>50</sub> values ranging from 4.1–11.7 μM. Importantly, compounds **3**, **4**, **7** and **8** have weakly cytotoxicity against the NCI-H187 cell line. Compounds **3** and **4** could potentially be chemically modified to improve their activities and the development of future

anti-malarial drug candidates.

### Supplementary material

Supplementary material relating to this article is available online,

### Disclosure statement

No potential conflict of interest was reported by the authors.

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Figure 1

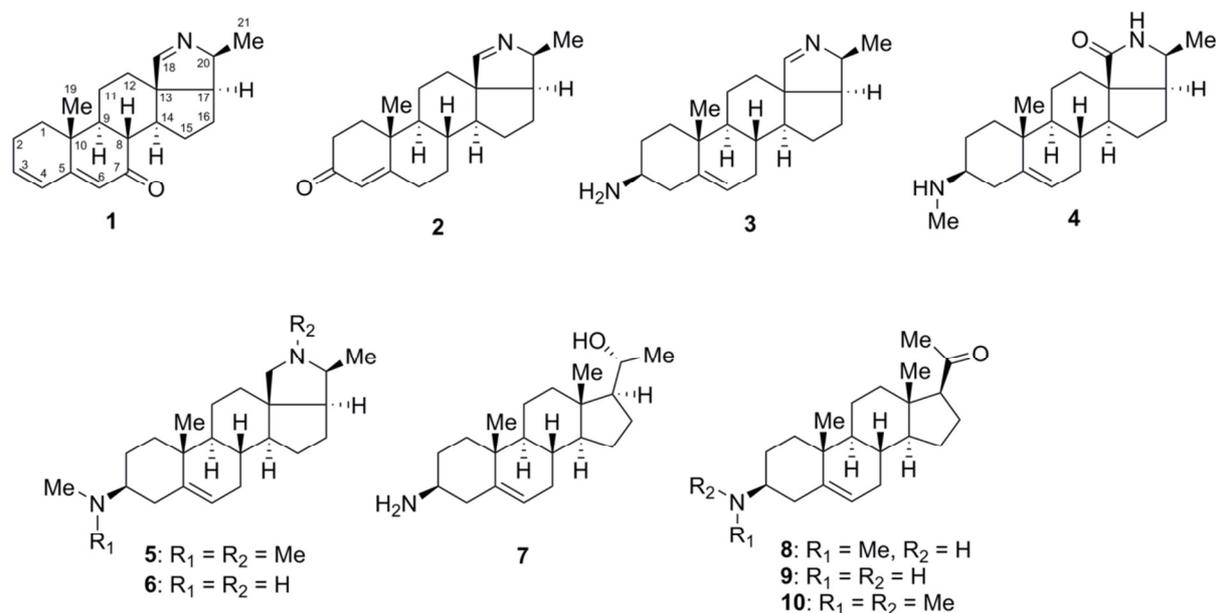


Table 1

Compounds	antimalarial	cytotoxicity
	(IC <sub>50</sub> , μM)	NCI-H187 <sup>a</sup> (IC <sub>50</sub> , μM)
<b>3</b>	1.2	27.7
<b>4</b>	2.0	30.6
<b>5</b>	5.9	inactive
<b>6</b>	8.0	inactive
<b>7</b>	11.7	29.5
<b>8</b>	4.1	18.2
<b>10</b>	10.6	inactive
Dihydroartemisinin	3.7 <sup>b</sup>	-
Mefloquine	70.0 <sup>b</sup>	-
Doxorubicin	-	0.2
Ellipticine	-	7.2

<sup>a</sup>Human small cell lung cancer; <sup>b</sup> IC<sub>50</sub> values in nM; Inactive at > 50 μM.

**Figure 1.** The structures of isolated compounds **1-10**

**Table 1.** Antimalarial and cytotoxic activities of some isolated compounds.