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Aporphine Alkaloids Isolated from the Cardiovascular Active Fraction of *Tinospora crispa*

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ABSTRACT Preliminary screening using isolated rats' aortic strip and atria showed that the chloroform extract of *Tinospora crispa*, Miers possessed potent cardiovascular activity. This prompted us to undertake an investigation on the constituents present in CF3 fraction, i.e. the most active fraction of the chloroform extract [1]. Among the chemical constituents isolated from this fraction are aporphine alkaloids, which include two *N*-acylaporphinoids: *N*-formylnornuciferine (**1**) as the major compounds and *N*-acetylnornuciferine (**2**), together with a small amount of an oxoaporphine alkaloid; lysicamine (**3**). Structural identification of the compounds is based on spectral analysis. Compounds (**1**) and (**2**) present as a mixture of *Z* and *E* isomers where the ratio of *Z/E* isomer in each compound are 1.7/1.0 and 1.3/1.0 respectively, based on the intensity of the signal for one proton in ^{1}H-NMR spectrum. Isolation of (**3**) is reported the first time in *Tinospora* genus.

Keyword: *Tinospora crispa, N*-formylnornuciferine, *N*-acetylnornuciferine, lysicamine

ABSTRAK Penyaringan awal menggunakan kaedah sediaan terasing jalur aorta dan atrium tikus menunjukkan bahawa ekstrak kloroform *Tinospora crispa*, Miers mempunyai aktiviti kardiovaskular yang poten. Ini mendorong kami untuk menyelidiki sebatian-sebatian kimia yang hadir di dalam fraksi CF3, iaitu fraksi yang paling aktif dalam ekstrak kloroform [1]. Antara sebatian kimia yang diasingkan daripada fraksi ini adalah alkaloid aporfin, termasuk dua *N*-asilaporfin iaitu *N*-formilnornusiferin (1) sebagai sebatian major dan *N*-asetilnornusiferin (2), bersama-sama dengan sedikit sebatian alkaloid oksoaporfin; laisikamin (3). Struktur sebatian-sebatian dikenalpasti berdasarkan kajian spektroskopi. Sebatian (1) dan (2) masing-masing hadir dalam bentuk campuran isomer *Z* dan *E* dengan nisbah *Z/E* di dalam setiap sebatian adalah 1.7/1.0 dan 1.3/1.0, berdasarkan kepada keamatan puncak bagi satu proton dalam spektrum ^{1}H-NMR. Pengasingan (3) dilaporkan pertama kali bagi genus *Tinospora*.

INTRODUCTION

Tinospora crispa Miers (Synonym *T. tuberculata* Buemee and *T. rumphii* Boerl.) known as Putarwali or Seruntum in Malaysia belongs to the family of Menispermaceae. This plant is a climbing, dioecious vine reaching a height of four to twenty meters. The stems are up to one centimeter thick and somewhat fleshy, with scattered and numerous protuberances. This bitter tasting plant is widely distributed from the southwestern part of China to South East Asian countries including Vietnam, Thailand, Malaysia, Indonesia and also India. *T. crispa* extract has been used for the treatment of stomach troubles, ulcers and fevers, as a tonic and febrifuge for malaria and smallpox, as a vulnerary for itches and wounds and as an oral hypoglycaemic agent [2]. A number of chemical constituents have already been isolated from this plant, e.g. phenolic acid amide [3], aporphine and quaternary alkaloids [4,5] and some furano diterpenes and furano diterpene glucosides of the clerodane type [6,7,8].

The objective of this study was to isolate the cardiovascular active compounds of this plant extract using a bioassay-guided fractionation procedure. Preliminary screening using isolated rats' atria and aortic strips showed that the chloroform extract of *T. crispa* possessed potent cardiovascular activity [1]. The most active fraction of this extract which inhibited significantly the isoprenaline-induced contraction of rats' left atria and noradrenaline-induced contraction of rats' aortic strips was fraction 3 (CF3). Hence, isolation and identification of the constituents present in the fraction were then

carried out. In this paper, we report the isolation and structural elucidation of three aporphine alkaloids from the cardioactive fraction of *T. crispa*. Lysicamine (**3**), an oxoaporphine alkaloid, is reported for the first time for the genus of *Tinospora*.

MATERIALS AND METHODS

General experimental procedures

Melting points were recorded on a Gallenkamp (England) melting point apparatus and were uncorrected. UV and IR spectra were obtained on Hitachi U-2000 spectrophotometer and Nexus FT-IR, respectively. ^{1}H-NMR and ^{13}C-NMR were recorded on Bruker 400 MHz and 300 MHz respectively using $CDCl_3$ as solvent. GC-MS spectra were obtained using Agilent 5793N/6980 GC-MS system. TLC analysis of the compound which was performed on pre-coated silica gel plates (F254, 0.2 mm, Merck) was used routinely for monitoring chromatographic fractions. TLC spots were detected by spraying with Dragendorff's reagent. Dry-column flash chromatography with silica gel (Art No. 7730, Merck) was used to fractionate the crude extract into fractions. The CF3 fraction was rechromatographed on gravity silica gel (Art No. 9385, Merck) for further isolation process.

Semi-preparative column (Whatman Partisil-10 ODS-3, 9.4 x 250 mm) was used for HPLC analysis. The HPLC system consisted of a Waters 510 (Massachusetts, USA) delivery pump connected to a Rheodyne 7125 (California, USA) injector containing a 2.0 mL sample loop, a Waters 486 UV/VIS detector and a Hitachi D-2500 (Tokyo) chromato-integrator. The eluent from the column was monitored at 270 nm.

Reagents

Acetonitrile (LiChrosolv grade) was purchased from Merck, Germany. Methanol (HPLC grade) was purchased from BDH Supplies, England. Freshly prepared distilled water was used for HPLC analysis. $CDCl_3$ for NMR analysis and Dragendorff's reagent were purchased from Sigma-Aldrich Co., USA.

Plant Materials

Fresh stems of *T. crispa* (20.0 Kg) were collected from Penang Island, Malaysia. A voucher specimen has been deposited at the herbarium, School of Pharmaceutical Sciences, University Science of Malaysia, Penang.

Extraction and Isolation

The air-dried *T. crispa* stems (4.4 Kg) were ground into powder and then macerated for 18 hours with petroleum ether (3x12.5L) in a hot water bath (60-70°C). The petroleum ether solution was filtered and concentrated using rotary evaporator to give crude petroleum ether extract (62.84 g). The marc obtained after petroleum ether extraction, was further macerated for 18 hours with chloroform (3x12.5L). The solution was filtered and concentrated in vacuo to give the crude chloroform extract as a dark solid (138.25 g). The crude chloroform extract (10.0 g) was subjected to a dry-column flash chromatography [9] on silica gel (70 g). The column was eluted with hexane, followed by a gradient mobile phase consisting of a mixture with increasing polarity of hexane-chloroform and chloroform-ethanol and finally with methanol which afforded 22 fractions of 100 ml each. The fractions were then combined into four fractions (CF1-CF4) based on the similarity of the TLC profiles. The most potent cardioactive fraction, CF3 (1.2 g) was subjected to silica gel column chromatography (100 g) and eluted with gradient mobile phase consisting a mixture of chloroform-ethanol with increasing ethanol content to give 20 subfractions. Subfractions F_{12}-F_{13} (0.1 g) was rechromatographed on silica gel (20 g) column chromatography, eluted with petroleum ether-ethyl acetate-methanol (35:60:5) to give five combined fractions. Fraction 3 which was a colourless solution, gave CF3B1 (50.2 mg) after concentration. CF3B1 (25 mg) which was further separated by semi-preparative reverse phase HPLC (acetonitrile-water 40:60) at a flow rate of 4.0 mL/min yielded *N*-formylnornuciferine (**1**) (t_R 43.07 minute, 18.9 mg) and *N*-acetylnornuciferine (**2**) (t_R 48.11 minute, 4.4 mg). The samples collected were concentrated in vacuo at 50°C. Fraction 4, which was a yellowish solution, was concentrated to afford Lysicamine (**3**, 2.5 mg).

N-formylnornuciferine (**1**)

Colourless needles, mp 141-143°C (lit. 140°C), IR (KBr) ν_{max} cm^{-1}: 1666 (C=O), UV λ_{max} (EtOH): 235, 270, 248(valley). EIMS *m/z*: 309 (M^+), 264, 251, 237, 178, 165. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 2.75-2.95 (7H, m, H-4, H-7 eq (*E, Z*); H-7ax, *Z*), 3.14 -3.25 (2H, m, H-5ax, H-7ax, *E*), 3.40 (1H, ddd, 12.0, 12.0, 3.0 Hz, H-5ax, *Z*), 3.69 (6H, s, C_1-OCH_3, *E, Z*), 3.85 (1H, m, H-5eq, *Z*), 3.93 (6H, s, C_2-OCH_3, *E, Z*), 4.41-4.53 (2H, m, H-6a, H-5eq, *E*), 4.95 (1H, dd,

13.6, 4.0 Hz, H-6a, *Z*), 6.68 (1H, s, H-3, *Z*), 6.71 (1H, s, H-3, *E*), 7.28-7.35 (6H, m, H-8, H-9, H-10, *E, Z*), 8.28 (2H, s, CHO, *E, Z*), 8.42 (1H, d, 11.8 Hz, H-11, *Z*), 8.44 (1H, d, 14.0 Hz, H-11, *E*). ^{13}C-NMR (300 MHz, $CDCl_3$): Table 1.

Table 1. ^{13}C-NMR data for **1** and **2** ($CDCl_3$, δ (ppm), 300 MHz)

C-atom	$\mathbf{1}_Z$	$\mathbf{1}_E$	$\mathbf{2}_Z$	$\mathbf{2}_E$
1	144.2	144.1	145.8	145.8
2	152.5	152.5	152.5	153.0
3	112.0	112.2	111.7	112.1
3a	125.5	125.5	126.6	125.2
4	31.4	30.0	31.2	30.1
5	42.4	36.5	42.4	36.8
6a	49.8	54.0	50.7	54.0
7	34.5	38.3	34.4	37.0
7a	136.5	136.0	137.0	136.5
8	129.0*	128.5*	128.8*	128.2*
9	128.2*	127.9*	127.9*	127.9*
10	127.5*	127.5*	127.4*	127.6*
11	128.8*	129.1*	128.3*	129.0*
11a	131.5	131.5	132.0	132.0
11b	128.2	127.5	127.4	126.5
11c	129.0	129.8	129.0	129.1
12	162.5	162.3	164.5	164.5
13	-	-	23.0	22.0
1-Ome	60.4	60.5	60.4	60.4
2-Ome	56.4	56.4	56.4	56.4

* These assignments may be interchanged.

N-acetylnornuciferine (**2**)
Colourless needles, mp 229-231°C (lit. 230°C), IR (KBr) ν_{max} cm^{-1}: 1633 (C=O), UV λ_{max} (EtOH): 231, 272, 246(valley). EIMS *m/z*: 323 (M^+), 264, 251, 237, 178, 165. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 2.19 (3H, s, $COCH_3$, *E*), 2.25 (3H, s, $COCH_3$, *Z*), 2.65-2.95 (6H, m, H-4, H-7eq, *E, Z*), 2.78 (1H, d, 14.3 Hz, H-7ax, *Z*), 3.06-3.31 (2H, m, H-5ax, H-7ax, *E*), 3.33 (1H, t, 12.0 Hz, H-5ax, *Z*), 3.69 (6H, s, C_1-OCH_3, *E, Z*), 3.93 (6H, s, C_2-OCH_3, *E, Z*), 4.03 (1H, d, 13.0 Hz, H-5eq, *Z*), 4.58 (1H, d, 15.0 Hz, H-6a, *E*), 5.00 (1H, d, 10.0 Hz, H-5eq, *E*), 5.12 (1H, d, 14.3 Hz, H-6a, *Z*), 6.65 (1H, s, H-3, *Z*), 6.72 (1H, s, H-3, *E*), 7.29-7.38 (6H, m, H-8, H-9, H-10, *E, Z*), 8.44 (1H, d, 7.8 Hz, H-11, *Z*), 8.48 (1H, dd, 11.0, 3.4 Hz, H-11, *E*). ^{13}C-NMR (300 MHz, $CDCl_3$): Table 1.

Lysicamine (**3**)
Yellow powder (2.5 mg), mp 183-185°C (lit. 185-187°C), IR (KBr) ν_{max} cm^{-1}: 1677 (C=O), UV λ_{max} (EtOH): 252, 268, 344, 404 nm. EIMS *m/z*: 291 (M^+), 248, 233, 177. ^{1}H-NMR (400 MHz, $CDCl_3$) δ ppm: 4.03 (3H, s, C_1-OCH_3), 4.11 (3H, s, C_2-OCH_3), 7.25 (1H, s, H-3), 7.60 (1H, t, 8.0 Hz, H-9), 7.78 (1H, d, 8.0Hz, H-10), 7.82 (1H, d, 5.0 Hz, H-4), 8.59 (1H, d, 8.0 Hz, H-8), 8.93 (1H, d, 5.0 Hz, H-5), 9.19 (1H, d, 8.0 Hz, H-11).

RESULTS AND DISCUSSION

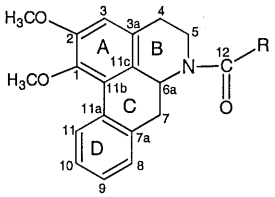
Isolation of the cardioactive fraction of *T. crispa* by extensive chromatographic techniques resulted in the isolation of two *N*-acylaporphine and one oxoaporphine alkaloids. Their structures were established by spectral methods and then compared with that in the published literature [5,10,11,12].

N-formylnornuciferine (**1**) was obtained as colourless needles with a melting point of 141–143°C (lit. 140°C, [5]). The UV spectrum showed strong absorption at λ_{max} (EtOH) 235, 270 and 248(valley) nm and the IR spectrum exhibited a strong absorption band at 1666 cm^{-1} indicating the presence of a carbonyl group. The mass spectrum showed a strong molecular ion peak at *m/z* 309, which corresponds to the molecular formula $C_{19}H_{19}NO_3$. *N*-formylnornuciferine was found to be a mixture of *Z* ($\mathbf{1}_Z$) and *E* ($\mathbf{1}_E$) isomers as was reported before [5]. The ratio of *Z*: *E* isomer was 1.7:1.0, based on the intensity of proton H-3 which resonated at δ 6.68 and 6.71 ppm respectively. The ^{1}H-NMR spectrum exhibited two singlets at 3.69 and 3.93 ppm, corresponding to two methoxyl groups at C-1 and C-2 respectively. A singlet at 8.28 ppm was assigned for the aldehyde proton at C-12 and the multiplet at 7.28 – 7.35 ppm was assigned for the three aryl protons, H-8, H-9 and H-10. Another aryl proton (H-11), was resonated as a doublet at 8.42 ppm (J=11.8 Hz, *Z* isomer) and 8.44 ppm (J=14.0 Hz, *E* isomer). A doublet-doublet at 4.95 ppm (J=13.6, 4.0 Hz) was attributed to H-6a of *Z* isomer and a multiplet at 4.41-4.53 ppm were assigned for H-6a and H-5eq of *E* isomer. Proton H-5eq of *Z* isomer was resonated as a multiplet centered at 3.85 ppm. Proton H-5ax and H-7ax of *E* isomer were resonated as a multiplet at 3.14-3.25 ppm. Proton H-5ax of *Z* isomer was resonated at 3.40 ppm (ddd, 12.0, 12.0, 3.0 Hz). The multiplet peaks centered at 2.85 ppm was assigned for the remaining protons at H-4 (*E, Z*

isomers), H-7eq (*E, Z* isomers) and H-7ax (*Z* isomer). The ^{13}C-NMR chemical shift of both isomers of *N*-formylnornuciferine (**1**$_Z$, *Z* isomers; **1**$_E$, *E* isomer) is summarized in Table1.

N-acetylnornuciferine (**2**) was isolated as colourless needles with a melting point of 229-231°C (lit. 230°C, [5]) and had a molecular ion peak at *m/z* 323, consistent with $C_{20}H_{21}NO_3$. The IR spectrum showed the presence of a carbonyl group by a strong absorption at 1633 cm^{-1}. The UV spectrum of (**2**) resembles that of (**1**) with absorption at λ_{max} (EtOH) 231, 272 and 246(valley) nm. *N*-acetylnornuciferine was also found to be a mixture of *Z* and *E* isomers as was reported before [5]. The ratio of *Z*: *E* isomer was 1.3:1.0, based on the intensity of proton H-3 which resonated at 6.65 and 6.72 ppm respectively. The ^{1}H-NMR spectrum was also similar to (**1**), except there were significant two 3-H singlets at 2.19 and 2.25 ppm, which were due to the presence of the methyl group bonded to the carbonyl group in *E* and *Z* isomer respectively. The two methoxyl groups at C-1 and C-2 appeared as singlets at 3.69 and 3.93 ppm respectively. The aromatic signal at 8.48 ppm (dd, 11.0, 3.4 Hz) was assigned to H-11 of *E* isomer and another signal at 8.44 ppm (d, 7.8 Hz) was due to H-11 of *Z* isomer. A multiplet at 7.29-7.38 ppm was attributed to the three protons, H-8, H-9 and H-10 in ring D of both isomers. The methylene proton, H-5eq was resonated as a doublet at 4.03 ppm (13.0 Hz, *Z* isomer) and 5.00 ppm (10.0 Hz, *E* isomer). Signal for H-6a appeared as a doublet at 4.58 ppm (15.0 Hz, *E* isomer) and 5.12 ppm (14.3 Hz, *Z* isomer). A triplet at 3.33 ppm (J=12.0 Hz) was due to H-5ax of *Z* isomer while a multiplet at 3.06-3.31 ppm was attributed to H-5ax and H-7ax of *E* isomer. Another multiplet centered at 2.85 ppm was due to H-7eq and both of H-4 for *E* and *Z* isomer. A doublet at 2.78 ppm (J=14.3 Hz) was assigned for H-7ax of *Z* isomer. The above assignments were consistent with literature values [5, 10]. The ^{13}C-NMR chemical shift of both isomers of *N*-acetylnornuciferine (**2**$_Z$, *Z* isomers; **2**$_E$, *E* isomer) is summarized in Table1.

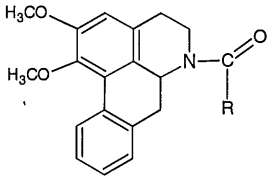
Lysicamine (**3**) was obtained as a yellow solid with a melting point of 183-185°C (lit. 185-187°C, [11]). The molecular formula is $C_{18}H_{13}NO_3$ as determined by its molecular ion peak, *m/z* of 291. The UV spectrum of (**3**) showed a complex pattern characteristic of a highly conjugated system, with absorption bands at λ_{max} (EtOH) 404, 268, 344 and 252 nm. The IR spectrum of (**3**) shows conjugated carbonyl absorption at 1677 cm^{-1}. The ^{1}H-NMR spectrum showed the presence of four aryl proton signals at 8.59 ppm (d, 8.0 Hz, H-8), 7.60 ppm (t, 8.0 Hz, H-9), 7.78 ppm (d, 8.0 Hz, H-10) and 9.19 ppm (d, 8.0 Hz, H-11), characteristic signals for oxoaporphines [12]. Another three aryl proton signals which were attributed to H-3, H-4 and H-5, appeared at 7.25 (s), 7.82 (d, 5.0Hz) and 8.93 (d, 5.0Hz) respectively. Two significant 3-H singlets at 4.03 ppm and 4.11 ppm were due to two methoxyl groups at C-1 and C-2 respectively. Due to the limitation of the sample amount, we were unable to get its ^{13}C-NMR spectrum. The mass fragmentation demonstrated 99% agreement with the library values for lysicamine, thus confirming its structure.

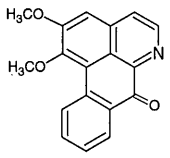


Z isomer

1$_Z$ R = H

2$_Z$ R = CH_3





E isomer (**3**)

1$_E$ R = H

2$_E$ R = CH_3

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