Isolation of Alkaloids from *Artabotrys suaveolens* and Their Cytotoxic Activity

Pengasingan Alkaloid dari *Artabotrys suaveolens* dan Aktiviti Sitotoksiknya

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Abstract

A phytochemical investigation on the stem bark of *Artabotrys suaveolens* has led to the isolation of an azaanthraquinone, cleistopholine (**1**) a dioxoaporphine alkaloid, artabotrine (**2**) and a benzylisoquinoline alkaloid, reticuline (**3**) The compounds were isolated using various chromatographic methods. The structures were mainly determined by spectroscopic methods such as ^{1}H and ^{13}C NMR, mass spectrometry and also by comparison with previous data. The compounds (**1-3**) have been isolated from others *Atrabotrys* species but here we are reporting for the first time from *Artabotrys suaveolens*. Meanwhile, artabotrine (**2**) showed a moderate cytotoxicity against human cancer cell lines, HL60, HCT116, RPMI8226 and MCF 7.

Keywords *Artabotrys suaveolens*, Annonaceae, alkaloids, cytotoxicity, phytochemical investigation

Abstrak

Kajian fitokimia terhadap kulit pokok *Artabotrys suaveolens* telah membawa kepada pengasingan sebatian azaantrakuinon, cleistopholina (1); alkaloid dioksoaporfina, artabotrina (2); dan alkaloid benzilisokuinolina, retikulina (3). Sebatian-sebatian ini diasingkan melalui pelbagai kaedah kromatografi. Penentuan struktur dijalankan terutamanya melalui kaedah spektroskopi seperti ^{1}H dan ^{13}C NMR, spektrometri jisim dan perbandingan dengan data kajian terdahulu. Sebatian (1-3) telah dijumpai daripada species lain *Artabotrys* tetapi ini adalah kali pertama kami melaporkan dari spesis *Artabotrys suaveolens*. Sementara itu, artabotrina (2) menunjukkan aktiviti sitotoksik yang sederhana terhadap sel kanser manusia, HL60, HCT116, RPMI8226 dan MCF 7.

Kata kunci *Artabotrys suaveolens*, Annonaceae, alkaloid, sitotoksik, kajian fitokimia

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Introduction

The genus *Artabotrys* contains more than 100 species throughout tropical Africa and East Asia (Sagen *et al*., 2003). In Malaysia, 13 species were reported; *A. grandifolius, A. scortechinii, A. pleurocarpus, A. venustus, A. wrayi, A. crassifolius, A. oblongus, A. lowianus, A. oxycarpus, A. gracilis, A. maingayi, A. suaveolens* and *A. costatus* (Sinclair, 1955). Among them six species have been studied before, i.e.; *A. grandifolius*, *A. maingayi, A. suaveolens, A. pleurocarpus, A. venustus* and *A. crassifolius* (Chan *et al*., 1987; Cortes *et al*., 1990; Cave *et al*., 1986; Barger *et al*., 1939). *Artabotrys suaveolens* Blume (Annonaceae), locally known as *akar chenana* or *akar larak* is a woody climber which is widely distributed in Chittagong, Mergui, Burma and Peninsula Malaysia (Burkill, 1985). In Philippines it is known under various names such as, bahaibalagan (C. Bis.), kintubo (Sub.) and susong damulag. It is a woody climber about 12 meters in length and about 10 cm in diameters. The young branches are glabrous, slightly pubescent, striate and dark coloured. The leaves are coriaceous, oblong-lanceolate to ovate lanceolate acute and both surface are dark green and shining. The flowers are about 1 cm long creamy-white, tomentose and fragrant. In Philiphines, *A.odoratissimus* R. Brown and *A. suaveolens* Blume were reported to contain active principles which act as stimulants. The bark of *A. suaveolens* is used for fishing-lines and the young leaves as cattle-food. Meanwhile in India, the leaves are used for treatment of cholera (Maranon, 1929).

Studied from the bark has found a poisonous bitter alkaloid that killed a guinea-pig by acting on the muscular system and arresting the respiration (Keng, 1990). Investigations on *A. suaveolens*, from the Philippines have reported the isolation of isoquinoline-derived alkaloids; isocorydine (Maranon, 1929) and suaveoline (Santos *et al*., 1932). Since this class of alkaloids showed promising and interesting activities therefore we are focused our studies on the isolation of this type of compounds. In earlier studies we have reported the isolation of three oxoaporphines alkaloids; liriodenine, lysicamine and atherospermidine together with an aporphine; isocorydine from the stem bark of Malaysian *Artabotrys suaveolens* (Azziz *et al*., 2006). Now, as part of a continuing investigation on the alkaloids content of this species, we now report the isolation and structural elucidation of three alkaloids from the stem bark of *Artabotrys suaveolens* namely cleistopholine (**1**), artabotrine (**2**) and reticuline (**3**). Since most of the alkaloids are prescribed as anticancer drugs for example vincristine, vinblastine, taxol and etc. (Terasaka, 2003) so we carried out the cytotoxic activity of the isolated compounds against selected human cancer cell lines hoping that they will show a potential anticancer agents in the future.

Experimental

General Procedures

The NMR (^{1}H, ^{13}C and 2D) spectra were recorded in deuterated chloroform on a JEOL JNM-FX100 (400 MHz). Chemical shifts were reported in ppm on δ scale and the coupling constants were given in Hz. The infrared spectra were obtained with chloroform as a solvent on a Perkin Elmer 2000 spectrometer. The ultraviolet absorption spectra were recorded on UV-VIS NIR Scanning Spectrophotometer (model Shimadzu UV-310 IPC) with methanol as solvent. Optical rotations were determined on Jasco (Japan) P1010 with tungsten lamp. Mass spectrum was obtained on a Jeol JMS 700 TZ spectrometer using *meta*-nitro-benzylalcohol (NBA) or glycerol as the matrix for FAB analysis and EIMS spectra were recorded on Shimadzu GC-MS-QP2000A Mass Spectrometer 70 eV. Column chromatography was carried out using silica gel 60, 230–400 Mesh ASTM (Merck 9385) and Thin Layer Chromatography (TLC) was performed on silica gel 60 F_{254}. TLC spots were visualized under ultraviolet light (254 and 365 nm) and by spraying with Dragendorff's reagent for alkaloid detection. Preparative thin layer chromatography was carried out by using silica gel 60 F_{254}.

Plant Material

The stem barks were collected from Mersing, Johore, Malaysia on 29 June 1989 by the phytochemical group of Chemistry Department, University of Malaya. Plant identification was verified by Teo Leong Eng (University Malaya). A voucher specimens (KL 3777) were deposited at the Herbarium of the Department of Chemistry, University of Malaya, Kuala Lumpur.

Extraction and Isolation

Dried and milled stem bark (2kg) of *A. suaveolens* were defatted with hexane. Then, the sample was dried and moistened with 10% ammonia solution followed by extraction with dichloromethane. The alkaloids were extracted using the classical method. A portion (3g) of the dichloromethane extract was subjected to column chromatography on silica gel, eluted with CH_2Cl_2, CH_2Cl_2-MeOH and MeOH to furnish 369 fractions. Fraction 60 showed single spot on TLC and identified as cleistopholine **1** (15.3 mg,CH_2Cl_2-MeOH, 99:1). In addition, six isoquinoline alkaloids; liriodenine, lysicamine, atherospermidine, isocorydine, anonaine and stephalagine were also isolated (Azziz *et al*., 2006). In our continuing search for new natural products, we repeated the extraction and isolation process with 2.6kg of air-dried and milled stem bark of the same species but this time without doing the acid-base extraction. Firstly, the sample was soaked overnight in hexane using cold extraction method and followed by dichloromethane for another 3 days. The dichloromethane extract was concentrated to give a residue (48.9g). Then 35g of dichloromethane extract were column chromatographed over silica gel eluted with CH_2Cl_2-MeOH for purification to afford 85 fractions. Fractions 12-19 (149 mg) were combined and further purified by preparative thin layer chromatography (PTLC) using solvent system of 99.5 CH_2Cl_2: 0.5 MeOH saturated with NH_4OH vapour to give cleistopholine **1** (4.5mg) and artabotrine **2** (13.5mg). Fractions 80-84 (104mg) were group together and repeatedly purified by column chromatography over silica gel using CH_2Cl_2-MeOH as eluent to yield 55 sub fractions. Fractions 22-40 (75 mg) were then purified by preparative thin layer chromatography using solvent system of 99 CH_2Cl_2: 1 MeOH saturated with NH_4OH vapour to give reticuline **3** (10.8mg).

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Spectral data of the alkaloids

Compound **1**: Cleistopholine, pale yellow amorphous solid. EIMS *m/z*: 223 (100), 195 (76), $C_{14}H_9NO_2$. UV λ_{max} nm (CH_3OH): 320. IR ν_{max} cm^{-1} (liquid film): 1684, 1669, 1590. ^{1}HNMR ($CDCl_3$, 400 MHz) ppm: 8.88 (1H, *d*, *J* = 4.8 Hz, H-2), 7.48 (1H, *d*, *J* = 4.8 Hz, H-3), 8.35 (1H, *dd*, *J* = 9.0, 2.2 Hz, H-5), 8.25 (1H, *dd*, *J* = 9.0, 2.2 Hz, H-8), 7.81 (2H, *m*, H-6, H-7), 2.89 (3H, *s*, CH_3-4).^{13}CNMR ($CDCl_3$, 100 MHz) ppm: 184.7 (C-9), 181.8 (C-10), 153.4 (C-2), 151.5 (C-4), 150.1 (C-9a), 134.5 (C-7), 134.1 (C-6), 132.6 (C-10a), 131.2 (C-3), 129.1(C-4a), 127.4 (C-5), 127.2 (C-8), 127.1 (C-8a), 22.8 (4-CH_3)

Compound **2**: Artabotrine, orange-yellow powder. HRFAB *m/z*: 322 $[M+H]^+$; HRFABMS *m/z*: 322.1220 (calc. 322.1207), $C_{18}H_{11}NO_5$. UV λ_{max} nm (CH_3OH): 450, 430, 310, 300, 280, 235, 220. IR ν_{max} cm^{-1} (liquid film): 1691, 1660. ^{1}HNMR ($CDCl_3$, 400 MHz) ppm: 8.10 (1H, *s*, H-3), 7.79 (1H, *s*, H-7), 7.95 (1H, *m*, H-8), 7.71 (1H, *m*, H-9), 7.69 (1H, *m*, H-10), 8.99 (1H, *m*, H-11), 6.46 (2H, *s*, OCH_2O), 4.20 (3H, *s*, OCH_3).^{13}CNMR($CDCl_3$, 100 MHz) ppm. 175.4 (C-4), 152.0 (C-5), 151.5 (C-1), 148.0 (C-2), 131.4 (C-6a), 129.8 (C-7a), 128.9 (C-8), 128.4 (C-11), 127.9 (C-9), 126.8 (C-10), 125.9 (C-11a), 122.8 (C-3a), 118.9 (C-11c), 115.1 (C-11b), 112.4 (C-7), 109.2 (C-3), 103.2 (OCH_2O), 63.3 (OCH_3)

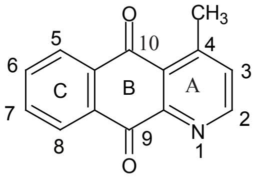
Compound **3**: Reticuline, white amorphous powder. EIMS *m/z*: 329, 192, 312, 296, 280, 207, 177, $C_{19}H_{23}NO_4$. UV λ_{max} nm (CH_3OH): 295. IRν_{max} cm^{-1} (liquid film): 3392, 2918, 1592. $[\alpha]_D^{26}$: + 300.0° (c = 7.59 x 10^{-5}, MeOH). ^{1}HNMR ($CDCl_3$, 400 MHz) ppm: 6.73 (1H, *d*, *J* = 1.9 Hz, H-2′), 6.70 (1H, *d*, *J* = 8.0 Hz, H-5′), 6.56 (1H, *dd*, *J* = 8.0, 1.9 Hz, H-6′), 6.51 (1H, *s*, H-5), 6.34 (1H, *s*, H-8), 3.83 (1H, *t*, H-1), 2.55-3.69 (3H, *m*, H-3, H-4, H- α), 3.83 (3H, *s*, OCH_3-4′), 3.82 (3H, *s*, OCH_3-6), 2.46 (3H, *s*, *N*-CH_3), ^{13}CNMR($CDCl_3$, 100 MHz) ppm. 145.3 (C-6), 145.3 (C-4′), 145.1 (C-3′), 143.4 (C-7), 132.8 (C-1′), 129.7 (C-4a), 124.8 (C-8a), 120.9 (C-2′), 115.7 (C-6′), 113.7 (C-5′), 110.6 (C-8), 110.5 (C-5), 64.4 (C-1), 55.9 (4′-OCH_3), 55.8 (6-OCH_3), 46.5 (C-3), 42.0 (*N*-CH_3), 40.9 (C- α), 24.7 (C-4).

Cytotoxic Activity

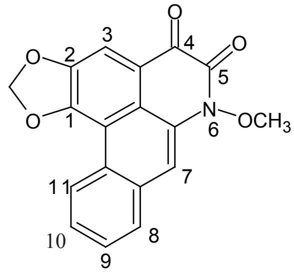
Each cell line [HL-60 (human blood premyelocytic leukemia), RPMI8226 (multiple myeloma), HCT-116 (human colon cancer) cells] was seeded onto 96-well microtiter plates at 1 × 10^4 cells per well for HL-60 and RPMI8226 and 5 × 10^3 cells per well for HCT-116. Cells were preincubated for 24 hours at 37°C in humidified atmosphere of 5% CO_2. Different concentrations of each compound (10µL) were added to the cultures, and then the cells were incubated at 37°C for 48 h. On the third day, 15µL MTT solution (5mg/mL) was added into each well of the cultured medium. After further 2 hours of incubation, 100µL of 10% SDS-0.01NHCl solution was added to each well and the formazan crystals in each well were dissolved by stirring with a pipette. The optical density measurements were made using a micropipette reader (Benchmark Plus microplate spectrometer, BIO-RAD) equipped with a two wavelengths system (550 and 700nm). In each experiment, three replicate of wells were prepared for each sample. The ratio of the living cells was determined based on the difference of the absorbance between those of samples and controls. These differences are expressed in percentage and cytotoxic activity was indicated as an IC_{50} value.

Results and Discussion

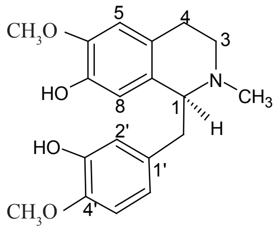
Extraction followed by chromatographic fractionation on the stem bark crude extract of *Artabotrys suaveolens* afforded three alkaloids; cleistopholine (**1**), artabotrine (**2**) and reticuline (**3**). Out of these three alkaloids, compound (**1**) has never been reported previously from any *Artabotrys* species whereas compound (**2**) and (**3**) were isolated for the first time from this species.



(1)



(2)



(3)

Compound **1** has a molecular formula of $C_{14}H_9NO_2$ as deduced from the EI-MS ion peak at *m/z* 223. Other significant fragmentation peaks were revealed at *m/z* 195 (76) $[M-28]^+$ indicating the loss of CO. The UV spectrum showed absorption maximum at 320 nm typical of the conjugated quinonoid moiety (Osashi *et al*., 1963). Moreover, the IR spectrum confirmed this evidence by showing the quinone carbonyl absorption at 1669 cm^{-1} (Shamma *et al*., 1969; Soonthornchareonnon *et al*., 1969). The integration of the ^1H NMR spectrum of **1** indicated the presence of nine protons. Two sets of doublet doublets (J = 9.0 Hz, J' = 2.2 Hz) at δ 8.35 and δ 8.25 which attributable to H-5 and H-8, respectively. A set of multiplet centered at δ 7.81 was assigned to H-6 and H-7 which proved that ring C was not substituted. A pair of doublets (J = 4.9 Hz) were present, each at δ 8.88 and δ 7.48, indicative of an *ortho* disubstituted aromatic ring. The former was assigned to H-2 that experienced a deshielding effect from the neighbouring nitrogen atom whereas the latter belonged to H-3. In addition, a singlet corresponding to three methyl hydrogens observed at δ 2.89 suggested that it was a 4-methylpyridine system (Tadic *et al*., 1987). The ^{13}C NMR spectrum showed the presence of fourteen carbon atoms. Two downfield signals at δ 181.8 and δ 184.7 belonged to the two carbonyl groups located at C-10 and C-9, respectively. The spectrum also gave one methyl, six methines and seven quartenary carbons. The long-range HMBC spectrum showed the correlations between H-8 (δ 8.25) and the methine carbons at δ 134.1 (C-6), δ 134.5 (C-7) and also with a quartenary carbon C-9 (δ 184.7). The methyl protons revealed correlations between the quartenary carbons at C-4 (δ 151.5), C-4a (δ 129.1) and a methine carbon, C-3 (δ 131.2). The investigation and comparison of the spectroscopic data to those obtained from the literature allowed the identification of compound **1** as cleistopholine which was classified as an azaanthraquinone-type of alkaloid. Cleistopholine was previously isolated from *Cleistopholine patens, Meiogyne virgata* and *Saprosma hainanense* MERR (Tadic *et al*., 1987; Waterman and Muhammad, 1985; Wang *et al*., 2011).

Compound **2** was obtained as an orange-yellow powder. The HRFABMS (+ve mode) exhibited an $[M+H]^+$ peak at 322.1210 (calc. 322.1207), indicating the molecular formula of $C_{18}H_{11}NO_5$. In addition, peaks at *m/z* 305 ($C_{18}H_{11}NO_4$) and 291 ($C_{17}H_9NO_4$) were due to the loss of oxygen and a molecule of formaldehyde from the molecular ion respectively. The UV spectrum showed absorptions at λ_{max} 450, 430, 310, 300, 280, 235 and 220 nm, some of which were characteristic of a 4,5-dioxoaporphine (Hocquemiller and Cave, 1981). The IR spectrum revealed two carbonyl bands at 1691 and 1660 cm^{-1}.The ^1H NMR spectrum showed two singlets at δ 7.79 and 8.10 which were assigned to the isolated aromatic protons, H-7 and H-3, respectively. In addition, three sets of multiplets appeared between δ 7.69 to 8.99 corresponding to four aromatic proton signals; H-10, H-9, H-8 and H-11, respectively. Hence, confirmed that ring D was unsubstituted. The spectrum also showed a singlet of two protons at δ 6.46, indicative of a methylenedioxy group. A three protons singlet at δ 4.20 was attributable to a methoxyl group. This was further support by NOE difference experiments. Irradiation of methoxyl signal at δ 4.20 showed a strong enhancement (1.09 %) of H-7, thus confirming it positioned was next to H-7. On the other hand, irradiation of H-7 at δ 7.79 showed enhancement of H-8 (8.95 %) and the OCH_3 (2.14 %), respectively. The COSY spectrum, showed the correlations between H-8/ H-9 and H-10/H-11. The ^{13}C NMR spectrum indicated the presence of eighteen carbon signals. In addition, the DEPT spectrum showed the presence of one methyl, one methylene, six methine and ten quartenary carbons. The ketone and amide carbonyl carbons in dioxoaporphines usually resonated at δ 178 and 157 (Achenbach *et al*., 1991) respectively and in this compound two signals occurred at δ 175.4 and 152.0. Compound **2** was identified as artabotrine based on the 1D and 2D NMR spectral data and comparison with literatures. The occurrence of this compound was also reported from *Artabotrys zeylanicus* (Wijeratna *et al*., 1995).

Compound **3**, $[\alpha]_D^{26}$ + 300.0° (c = 7.59 x 10^{-5}, CH_3OH) was afforded as a white amorphous powder. The EI-MS spectrum showed the presence of a molecular ion peak at *m/z* 329 which was in agreement with the molecular formula of $C_{19}H_{23}NO_4$. In addition, the base peak appeared at *m/z* 192 $[M-137]^+$ corresponded to the loss of $[C_8H_9O_2]^+$ from the molecular ion, thus indicating the characteristic for the 1-benzyl 1, 2, 3, 4-tetrahydroisoquinoline skeleton (Chowdhury *et al*., 1976; Guinaudeau *et al*., 1975). IR spectrum showed strong absorptions at 3392 cm^{-1} and 2918 cm^{-1} due to the stretching of O-H and C-H aromatic. An absorption of the aromatic system (C=C stretching) was observed at 1592 cm^{-1}. The UV spectrum displayed an absorption maximum at 295 nm. The ^1H NMR spectrum exhibited five aromatic protons, two methoxyl groups and a methyl group attached to the nitrogen atom. Two singlets appeared at δ 6.34 and 6.51 were assigned to H-8 and H-5, respectively. The former was shifted to the upfield region due to the shielding effect by ring C. The spectrum also showed the resonances of three protons in ring C; H-2′, H-5′ and H-6′. Two sets of doublets at δ 6.73 (J = 1.9 Hz) and δ 6.70 (J = 8.0 Hz) were assigned to H-2′ and H-5′, respectively. Meanwhile, a doublet of doublets (J = 8.0, J' = 1.9 Hz) attributable to H-6′ was observed at δ 6.56. Two methoxyl signals were present at δ 3.82 and δ 3.83. The former was attached to C-6 while the latter to C-4′, respectively. A three-proton singlet at δ 2.46 was attributable to the *N*-methylated (*N*-Me) group. The six aliphatic protons corresponding to H-3, H-4 and H-α appeared as multiplets in the region of δ 2.55 – 3.69. A triplet belonging to H-1 appeared at δ 3.83. The ^{13}C NMR spectrum showed the presence of nineteen carbons which validated the molecular formula. Based on the spectroscopic information and comparison with those of literature (Martin *et al*., 1967; Stadler *et al*., 1987; Garcez *et al*., 1995) compound **3** was characterised as reticuline which was also previously isolated from *Artabotrys venustus*.

Table 1 showed that compound **2** exhibited moderate cytotoxicity against human cancer cell lines, HL60, HCT116, RPMI8226 and MCF7 as compare to compound **3**. Further examination about mode of action for cytotoxicity of compound **2** is still in progress. Previous study reported that compound **2** possessed notable inhibitory activity on P-388 leukemia cell lines of wild-type and camptothecin resistance type (Wijeratna *et al*., 1995). Besides, in 2006 Ding *et al*. had synthesized artabotrine and its analogue, *N*-methoxycepharadione B which both of them were cytotoxic to several tumor cell lines. Compound **1** did not show cytotoxicity activity (Wang *et al*., 2011) but positive as an antifungal agent.

Table 1 IC_{50} values of compounds **2** and **3** against human cancer cell lines, HL60, HCT116, RPMI8226 and MCF7

IC50 (µM)	HL60	HCT 116	RPMI 8226	MCF7
(2)	24.5	5.5	25.8	7.5
(3)	>50	>50	>50	-

HL60 and RPMI 8226 – leukemia
HCT116 – colon cancer
MCF7 – breast cancer

Conclusion

To the best of our knowledge, aporphinoids (aporphine and oxoaporhine) are among the largest class of compounds occurring in *Artabotrys* species. Our study, however, showed that azaanthraquinone-type of alkaloids cleistopholine, **1** were also found in this species. In addition, artabotrine, **2** showed potential cytotoxicity properties against four human cancer cell lines and further study need to carry out for the mode of its action.

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