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ANTITUMORAL ALKALOIDS FROM *CLAUSENA LANSIUM*

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Abstract – Three new carbazole alkaloids, mafaicheenamine A-C (**1-3**), along with five know compounds (**4-8**) were isolated from the twigs of *Clausena lansium*. All compounds were characterized by the analysis of spectroscopic methods. In addition, the evaluation of antitumoral activity against three human cancer cell lines, KB, MCF-7 and NCI-H187, of compounds **1**, **2** and **4-8** were also reported.

INTRODUCTION

A number of carbazole alkaloids have been isolated from Rutaceae plants, especially in the genus of *Clausena.*[1-4] Many of them had interesting pharmacological activity, such as anti cancer, anti bacterial and anti HIV activities.[4-6] *Clausena lansium* or "mafaicheen" in local Thai name is one of the Rutaceae plants that has been known as a folk medicine in many countries.[7,8] Different parts of the plant are used for the treatment of several diseases, for example in China and Taiwan, the leaves have been used for the treatment of coughs, asthma and gastro-intestinal diseases and the seeds for acute and chronic gastro-intestinal inflammation and ulcers.[7] Moreover, the fruits are used for influenza, colds and abdominal colic pains in Philippines.[8] Recently, the seed extract of *C. lansium* was found to exhibit antifungal, antiproliferative, and HIV reverse transcriptase-inhibitory activities.[9] Previous chemical investigations of this plant, we described the isolation and cytotoxicity of coumarins.[10] Further investigation of the dichloromethane and acetone extracts from the twigs of the same plant, we describe herein the isolation and characterization of three new carbazole alkaloids and five known alkaloids (Figure 1). The cytotoxicity against oral human epidermal carcinoma (KB), breast cancer (MCF7) and small cell lung cancer (NCI-H187) cell lines was also reported.

RESULTS AND DISCUSSION

The combination of dichloromethane and acetone extracts of *C. lansium* twigs was subjected to silica gel column chromatography to yield three new carbazole alkaloids (**1-3**) along with five known alkaloids

(**4**-**8**). All new compounds isolated from twigs of *C. lansium* were 1-methoxyl carbazole alkaloids with a lactone ring or ketone ring moiety attached at C-2 and C-3. Compounds of this type showed common signals in ^{1}H NMR spectra for N*H* signal ca. δ 8.6-10.9, a methoxyl group at ca. δ 4.0 (1-OMe) and a set of four spin proton signals of ring A at ca. δ 8.0 (H-5), 7.2 (H-6), 7.4 (H-7) and 7.5 (H-8).

Mafaicheenamine A (**1**) was obtained as brown solid, $[\alpha]_D^{26}$ +81.37 (*c* 0.02, MeOH). The molecular formula of $C_{19}H_{19}NO_4$ was determined by a molecular ion peak at $[M]^+$ *m/z* 325.1315 (calcd. for $C_{19}H_{19}NO_4$, 325.1314) in HRMS. By comparison the ^{1}H and ^{13}C NMR spectral data (Table 1) of **1** with that of clausevatine D,[11] which isolated from the roots of *C. excavata*, both of them showed similar ^{1}H and ^{13}C NMR signals, indicating that compound **1** was a lactonic carbazole alkaloid skeleton which appeared ^{1}H NMR signals of a four mutually coupling aromatic protons of ring A at δ 8.23 (1H, d, 8.0 Hz, H-5), 7.56 (1H, d, 8.0 Hz, H-8), 7.46 (1H, ddd, 8.0, 7.2, 1.2 Hz, H-7) and 7.26 (1H, ddd, 8.0, 7.2, 1.2 Hz, H-6) and a lactonic moiety at δ 3.47 (1H, dd, 16.4, 2.4 Hz, H-1′a), 3.02 (1H, dd, 16.4, 12.4 Hz, 1′b), 4.29 (1H, dd, 12.4, 2.4 Hz, H-2′), 1.37 (6H, s, H_3-4′ and H_3-5′). However, two main differences were observed in ^{1}H NMR spectrum. Firstly, an additional methoxyl group was observed at δ 3.99 which placed on C-1 due to the HMBC correlations between proton H-1′ (δ 3.47 and 3.02) and methoxyl protons (δ 3.99) with C-1 (δ 142.1). Secondly, the singlet aromatic proton on ring C was shifted from δ 7.55 (for clausevatine D, acetone-d_6)[11] to δ 8.59 (for carbazole **1**, acetone-d_6). These results implied that the lactonic ring of **1** should be placed on C-2 and C-3 instead C-3 and C-4 as appeared in clausevatine D. Therefore, the proton signal at δ 8.59 was identified to H-4 in which showed ^2J and ^3J correlations with C-4a (δ 124.4) and C-4b (δ 124.9), C-10 (166.4) in HMBC spectrum. Thus, the structure of **1** was indentified to be mafaicheenamine A.

Mafaicheenamine B (**2**) was isolated as brown viscous, $[\alpha]_D^{24}$ +32.47 (*c* 0.02, MeOH), for which the molecular formula of $C_{19}H_{21}NO_5$ was inferred by HRMS (*m/z* 310.1436 $[M-H_2O_2]^+$, calcd. for $C_{19}H_{21}NO_5$, 310.1443). The ^{1}H and ^{13}C NMR spectral data (Table 1) of **2** were almost identical to those of **1** except compound **2** was not observed the lactonic carbonyl carbon in ^{13}C NMR spectrum. In addition, the ^{1}H NMR spectrum of **2** also appeared an additional oxymethine proton at δ 6.16 (1H, s) which connected to carbon at δ 101.6 in HMQC experiment. These results could be concluded that the carbonyl functionality of **1** was reduced to an alcohol. Thus, the proton signal at δ 6.16 was identified to H-10 which showed ^2J and ^3J cross peaks with C-2 (130.8), C-3 (120.4), and C-4 (112.3) in HMBC experiment. Moreover, the characteristic of quaternary carbon of C-3′ which connected to hydroperoxy moiety was also observed at δ 80.1.[12] Therefore, the structure of **2** was indentified to be mafaicheenamine B.

Mafaicheenamine C (**3**) was obtained as brown solid, $[\alpha]_D^{26}$ +64.25 (*c* 0.02, MeOH). It showed molecular

ion peak at $[M]^+$ *m/z* 309.1364 (calcd. for $C_{19}H_{19}NO_3$, 309.1365) in HRMS. The ^1H NMR signals of **3** were similar to those of **1** but differed in the higher field shift of non oxygenated proton H-2′ which appeared at δ 2.95 instead of an oxymethine proton at δ 4.29. In addition, the ^{13}C NMR signal of C-10 of **3** (δ 208.5) also resonated lower field than those of **1** (δ 166.4). This result implied that the lactonic ring of **1** was replaced by the five membered ring of ketone. Finally, the structure of **3** was confirmed by HMBC correlation as shown in Figure 2. Therefore, the structure of **3** was identified to be mafaicheenamine C.

The remaining known alkaloids were characterized as indizoline (**4**),[2] lansin (**5**),[13] glycozolidal (**6**),[2] murrayanine (**7**)[2] and daurine (**8**)[14] by 1D and 2D NMR spectroscopic data.

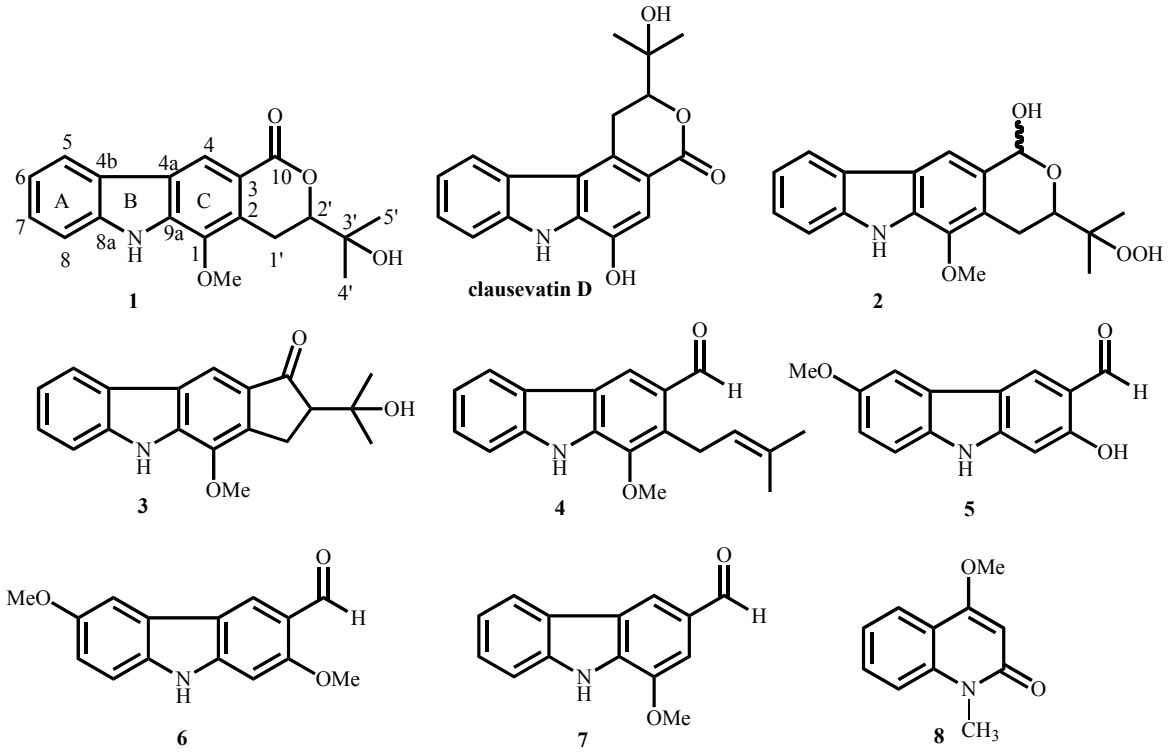


Figure 1. Structure of compounds **1-8**

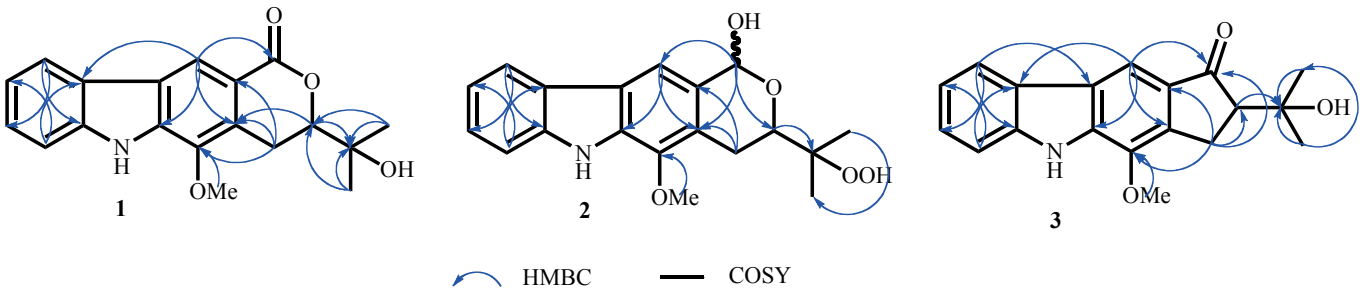
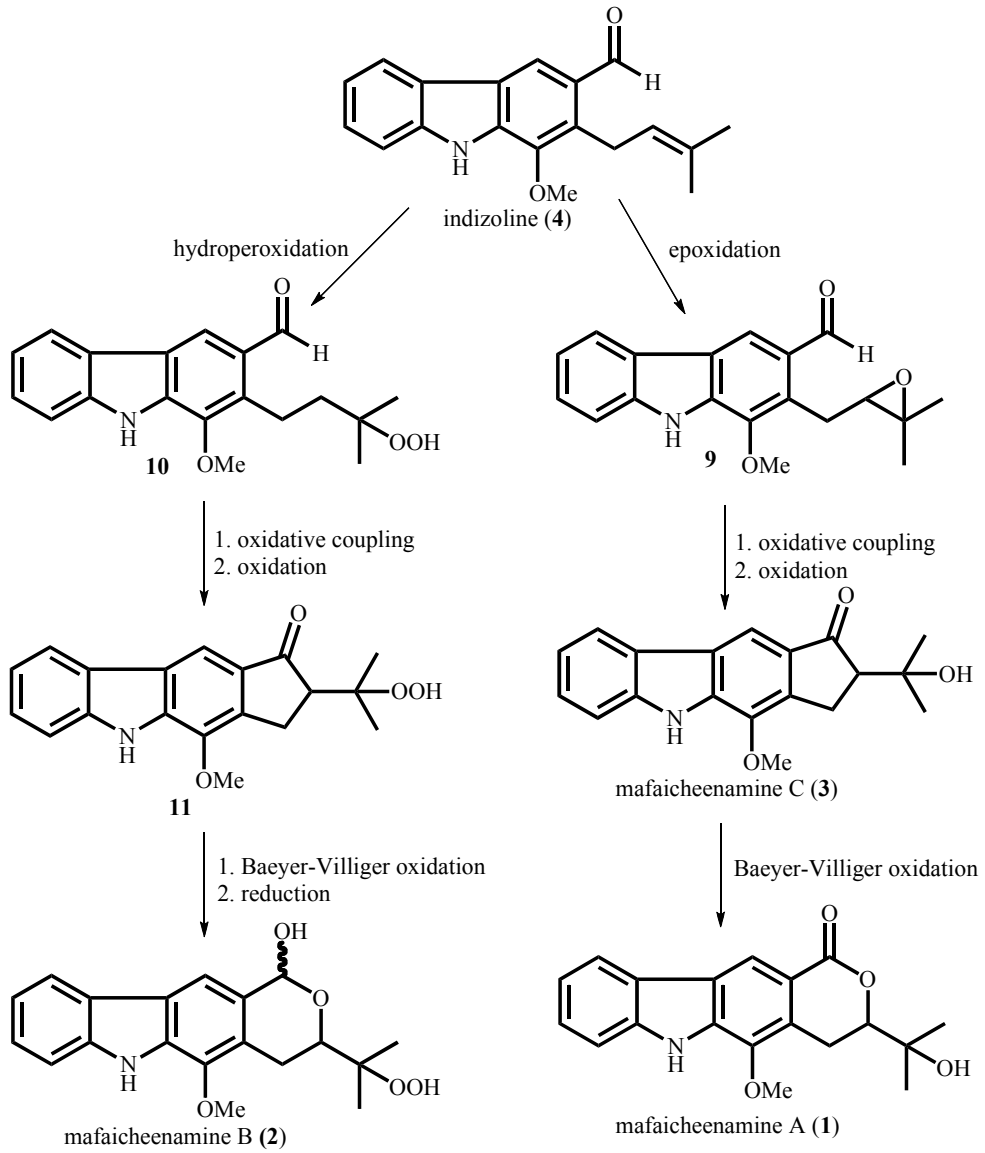


Figure 2. COSY and selective HMBC Correlations of **1-3**

Table 1 ^{1}H (400 MHz) and ^{13}C NMR (100 MHz) spectral data of compounds **1** – **3** and clausevatine D[11]

Position	**1** (acetone-d_6)		**Clausevatine D** (acetone-d_6)[11]		**2** ($CDCl_3$)		**3** ($CDCl_3$)	
	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C	δ_H (mult., J in Hz)	δ_C
1	–	142.1	–	142.9	–	142.7	–	140.8
2	–	129.6	7.55	110.8	–	130.8	–	138.9
3	–	118.0	–	129.0	–	120.4	–	114.1
4	8.59 (s)	119.8	–	121.5	7.55 (s)	112.3	8.27 (s)	112.3
4a	–	124.4	–	116.9	–	123.4	–	123.8
4b	–	124.9	–	124.4	–	123.9	–	124.0
5	8.23 (d, 8.0)	121.5	8.21 (dd, 7.7, 10.0)	122.9	7.99 (d, 8.0)	120.3	8.07 (d, 7.6)	121.0
6	7.26 (ddd, 8.0, 7.2, 1.2)	121.0	7.27 (td, 7.7, 10.0)	120.7	7.21 (ddd, 8.0, 7.4, 1.2)	119.8	7.28 (ddd, 8.0, 7.6, 2.4)	120.7
7	7.46 (ddd, 8.0, 7.2, 1.2)	127.5	7.47 (td, 7.7, 10.0)	126.6	7.39 (ddd, 8.0, 7.4, 1.2)	125.8	7.47 (ddd, 8.0, 7.6, 2.4)	127.1
8	7.56 (d, 8.0)	112.5	7.68 (dd, 7.7, 10.0)	112.7	7.44 (d, 8.0)	110.8	7.48 (d, 7.6)	111.2
8a	–	141.6	–	141.4	–	139.4	–	140.2
9a	–	137.4	–	135.5	–	132.7	–	137.9
10	–	166.4	–	166.4	6.16 (s)	101.6	–	208.5
1′a	3.47 (dd, 16.4, 2.4)	23.2	3.42 (dd, 12.6, 16.5)	26.0	3.37 (dd, 17.6, 5.2)	25.9	3.52 (dd, 16.8, 8.0)	27.6
1′b	3.02 (dd, 16.4, 12.4)		3.78 (d, 12.6, 3.4)		3.11 (d, 17.6)		2.99 (dd, 16.8, 4.8)	
2′	4.29 (dd, 12.4, 2.4)	85.1	4.44 (d, 3.4, 12.6)	84.8	4.51 (d, 5.2)	80.5	2.95 (dd, 8.0, 4.8)	57.0
3′	–	71.2	–	71.3	–	80.1	–	72.9
4′	1.37 (s)	26.8	1.43 (s)	26.8	1.40 (s)	29.6	1.37 (s)	28.6
5′	1.37 (s)	25.3	1.43 (s)	25.3	1.26 (s)	23.9	1.16 (s)	24.4
1-OCH_3	3.99 (s)	61.3	–	–	3.96 (s)	60.0	4.13 (s)	60.2
-NH	10.96 (br s)	–	10.87 (br s)	–	8.15 (br s)	–	8.65 (br s)	–

It should be note that the plausible biogenetic pathway of mafiacheenamine A-C (**1-3**) could be derived from indizoline (**4**) (Scheme 1). The epoxidation of isoprenyl side chain of indizoline followed by oxidative coupling and oxidation produced mafiacheenamine C (**3**). Subsequent ring expansion via the Baeyer-Villinger oxidation gave mafiacheenamine A (**1**). We also suggested that mafiacheenamine B (**2**) could be derived from indizoline by similar pathway as shown in scheme 1.



Scheme 1. Plausible biogenetic pathway of mafiacheenamine A-C (**1-3**)

Compounds **1**, **2** and **4-8** were evaluated for their antitumoral activity against three human cancer cell lines including oral cavity cancer (KB), breast cancer (MCF7) and small cell lung cancer (NCI-H187). The results of cytotoxicity of the tested compounds are summarized in Table 2. All compounds were found to be active with three human cancer cell lines except for compound **8** was found to be in active

with MCF7 cancer cell line. Compounds **5** and **6** exhibited significant cytotoxic effect against MCF7 cancer cell line with the same IC_{50} value of 0.78 μg/ mL, higher active than that of doxorubicin, a standard drug (IC_{50} 1.25 μg/ mL). Compounds **1** and **7** were also found to be strongly active with IC_{50} 2.96 and 3.76 μg/mL, respectively, where as compounds **2** and **4** were weakly active with MCF7. Also, compounds **5** and **6** exhibited moderate activity with NCI-H187 cancer cell line where as all the rest of compounds were found to be weakly active. Only two compounds, **1** and **6**, showed moderate activity in KB cancer cell line.

Table 2. Antitumoral activity of compounds **1**, **2** and **4**-**8** isolated from the twigs of *C. lansium*

Compound	Antitumoral activity (IC_{50}, μg/mL)		
	KB[a]	MCF7[b]	NCI-H187[c]
1	7.68	2.96	13.27
2	14.94	23.41	19.65
4	26.50	11.46	12.50
5	6.84	0.78	7.74
6	10.02	0.78	4.17
7	19.34	3.76	10.72
8	28.41	inactive	35.38
elliticine	0.311	not tested	0.526
doxorubicin	0.180	1.25	0.077

[a] KB = oral cavity cancer; [b] MCF7 = breast cancer; [c] NCI-H187 = small cell lung cancer

It is worth noting that the genus of *Clausena* is known to be rich source of alkaloids especially carbazole alkaloids.[4,11,15] However, less than 10 compounds have been isolated from *C. lansium*. In this study, we also isolated three additional novel carbazole alkaloids from the twigs of *C. lansium* and all isolated alkaloids were reported for the first time as secondary metabolites of *C. lansium*. In addition, compounds **5** and **6** exhibited potent antitumoral activity against MCF7 cancer cell line.

EXPERIMENTAL

General

The optical rotation $[\alpha]_D$ values were determined with a Bellingham & Stanley ADP440 polarimeter. UV spectra were recorded with a PerkinElmer UV-Vis spectrophotometer. The IR spectra were recorded with a Perkin-Elmer FTS FT-IR spectrophotometer. The ^1H and ^{13}C NMR spectra were recorded using 400 MHz Bruker spectrometer. Chemical shifts were recorded in parts per million (δ) in $CDCl_3$ with tetramethylsilane (TMS) as an internal reference. The HRMS were obtained from MicroTOF, Bruker

Daltonics or MAT 95 XL mass spectrometers. Quick column chromatography (QCC) and column chromatography (CC) were carried out on silica gel 60 H (Merck, 5-40 μm) and silica gel 100 (Merck, 63-200 μm), respectively. Precoated plates of silica gel 60 F_{254} were used for analytical purposes.

Plant material

The twigs of *C. lansium* were collected from Nan Province, northern part of Thailand in April 2008. Botanical identification was achieved through comparison with a voucher specimen number QBG 25077 in the herbarium collection of Queen Sirikit Garden, Mae Rim District, Chiang Mai, Thailand.

Extraction and Isolation

The air dried twigs (6.73 Kg) of *C. lansium* were extracted with CH_2Cl_2 and acetone, respectively, over a period of 3 days each at room temperature. The CH_2Cl_2 and acetone extracts were combined (34.02 g) and subjected to QCC over silica gel eluted with a gradient of hexane-acetone (100% hexane to 100% acetone) to provide seventeen fractions (A-Q). Fraction F (207.1 mg) was separated by CC with 20% EtOAc-hexane yielding compound **4** (13.5 mg). The isolation of fraction J (1.83 g) was performed by CC with 20% EtOAc-hexane to afford thirteen subfractions (J1-J13). Subfraction J3 (33.9 mg) was subjected to repeated CC with 65% CH_2Cl_2-hexane to afford compound **5** (4.2 mg). Subfraction J4 (173.3 mg) was separated by CC eluted with a gradient of 70% CH_2Cl_2-hexane to 2% MeOH-CH_2Cl_2, yielding compound **7** (2.6 mg) and fifteen fractions (J4a-J4O). Compound **2** (2.2 mg) was derived from fraction J2N (25.1 mg) by repeated CC with a gradient of 90% CH_2Cl_2-hexane to 10% EtOAc-CH_2Cl_2. Fraction K (806.5 mg) was performed by CC using a gradient of EtOAc-CH_2Cl_2 (5% EtOAc-CH_2Cl_2 to 100% EtOAc) to yield compound **6** (1.8 mg) and nine subfractions (K1-K9). Subfraction K6 (124.2 mg) was subjected to repeated CC with 2% acetone-CH_2Cl_2 to afford compound **3** (9.7 mg) while subfraction K8 was purefied by CC with 10% EtOAc-hexane to give compound **8** (16.2 mg). Purification of fraction M (806.5 mg) was performed by sephadex LH20 with 60% CH_2Cl_2-MeOH, yielding five subfractions (M1-M5). Subfraction M2 (199.9 mg) was further subjected to repeated CC with a gradient of $CHCl_3$-hexane (70% $CHCl_3$-hexane to 100% $CHCl_3$) to afford eleven subfractions (M2a-M2K). Compound **1** (9.8 mg) was derived from subfraction M2f (18.7 mg) by prep.TLC with 50% EtOAc-hexane.

Mafaicheenamine A (**1**); brown solid. $[\alpha]_D^{26}$ +81.37° (*c* 0.02, MeOH). UV (MeOH) (log ε): 234 (3.45), 244 (3.38), 267 (3.58), 282 (3.57), 319 (2.88), 322 (2.89) nm. IR (neat) ν_{max}: 3525, 2973, 1694, 1629, 1608 cm^{-1}. ^1H NMR (400 MHz, acetone-d_6) and ^{13}C NMR (100 MHz, acetone-d_6) see Table 1. HRMS m/z 325.1315 $[M]^+$ (calcd. for $C_{19}H_{19}NO_4$, 325.1314).

Mafaicheenamine B (**2**); brown viscous. $[\alpha]_D^{24}$ +32.47° (*c* 0.02, MeOH). UV (MeOH) (log ε): 239 (3.63), 249 (3.50), 258 (3.28), 295 (3.15), 320 (2.92), 331 (2.78) nm. IR (neat) ν_{max}: 3372, 2921, 2851, 1563 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1. HRMS m/z 310.1436

$[M-H_2O_2]^+$ (calcd. for $C_{19}H_{21}NO_5$, 310.1443).

Mafaicheenamine C (**3**); brown solid. $[\alpha]_D^{26}$ +64.25° (*c* 0.02, MeOH). UV (MeOH) (log ε): 232 (3.53), 245 (3.49), 269 (3.51), 291 (3.78), 332 (3.12), 347 (3.13) nm. IR (neat) ν_{max}: 3607, 2935, 1731, 1563 cm^{-1}. ^1H NMR (400 MHz, $CDCl_3$) and ^{13}C NMR (100 MHz, $CDCl_3$) see Table 1. HRMS m/z 309.1364 $[M]^+$ (calcd. for $C_{19}H_{19}NO_3$, 309.1365).

Cytotoxic assay

The procedures for cytotoxic assay were performed by resazurin microplate assay (REMA) which was a modified method of fluorescent dye for the mammalian cell cytotoxicity according to Brien *et al*.[16] In this study, three cancer cell lines, KB (oral cavity cancer), MCF7 (breast cancer) and NCI-H187 (small cell lung cancer) were used.

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