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In vitro cytotoxic activity of isolated acridones alkaloids from *Zanthoxylum leprieurii* Guill. *et* Perr

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ABSTRACT

Chemical investigation of the roots and fruits of *Zanthoxylum leprieurii* Guill. *et* Perr. led to the isolation of three new alkaloids including two acridone derivatives, 3-hydroxy-1,4-dimethoxy-10-methyl-9-acridone (**2**) and 3-hydroxy-1,2-dimethoxy-10-methyl-9-acridone (**3**) named helebelicine A and B, respectively, and one secobenzo[*c*]phenantridine, 10-*O*-demethyl-12-*O*-methylarnottianamide (**10**), together with thirteen other compounds. The structures of compounds **2**, **3** and **10** as well as those of the known compounds were elucidated by using spectroscopic methods and by comparison with reported data. The brine-shrimp (*artemia salina*) lethality bioassay of the chloroform extract of the fruits showed modest cytotoxicity with LD_{50} at 13.1 μg/mL. Isolated compounds **1**, **4**–**6** were found to be moderately active against lung carcinoma cells (A549), colorectal adenocarcinoma cells (DLD-1) and normal cells (WS1) with IC_{50} values ranging from 27 to 77 μM. In contrast to the positive control etoposide used, the cytotoxicity of the most active compound **4** was found to be selective against cancer cells in comparison to normal cells WS1 with IC_{50} of 51 ± 8 μM and 4.3 ± 0.4 μM, respectively.

1. Introduction

Formerly known under the generic name *Fagara* Linn., *Zanthoxylum* belong to the sub-family of Rutoideae, tribe Zanthoxyleae in the Rutaceae family.[1] Like other African *Zanthoxylum* species, *Zanthoxylum leprieurii* is distinguishable by its taxonomic characteristics such as the anatomical and morphological features of the roots, leaves, and fruits.[1] A major characteristic is that the trunks, branches, leaf stalks and inflorescence axes of all these species are covered by prickles or what others describe as spines.[2] Traditional healers throughout West and Central Africa used species of the *Zanthoxylum* for the treatment of a wide range of disorders, including toothache, urinary and venereal diseases, rheumatism and lumbago.[2] Dried fruits of *Z. leprieurii* are used as spices by local population from the West province of Cameroon, particularly in two famous soups called «nkui» and «nah poh».[3] The genus *Zanthoxylum* is very well known[4,5] for its diversified chemistry, particularly by the presence of alkaloids, aromatic and aliphatic amides, sterols and phenylpropanoids-lignans and coumarins. These secondary metabolites are well distributed within the *Zanthoxylum* species investigated to date and among them, benzophenanthridines and acridones are particularly abundant.[6–12] Some of the metabolites have shown cytotoxic, molluscicidal, anticonvulsant, anti-sickling, anesthetic, antibacterial, anti-hypertensive and anti-inflammatory properties.[13–15]

In the present work, our interest in the research of active anticancer molecules, led us to investigate the roots and fruits of *Z. leprieurii*. Despite the fact that there are no known folkloric reports of their use in the management or cure of tumors and cancerous conditions, we have been attracted as some other scientists because of the anti-leukemic properties and other values of the isolates from this plant.

2. Results and discussion

Fractionation and purification of the chloroform extract of air-dried roots and fruits of *Z. leprieurii* were made using flash and nor-

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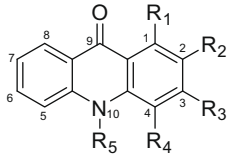
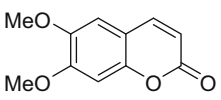
mal column chromatography under silica gel followed by Sephadex LH-20 separation as described in the experimental section. From the fruits of *Z. leprieurii*, two new acridone derivatives, 3-hydroxy-1,4-dimethoxy-10-methyl-9-acridone (**2**) and 3-hydroxy-1,2-dimethoxy-10-methyl-9-acridone (**3**) named helebelicine A and B, respectively, were isolated and characterized while one new seco-benzo[*c*]phenantridine, 10-*O*-demethyl-12-*O*-methylarnottianamide (**10**) was obtained from their roots (Fig. 1). Thirteen known compounds were similarly identified such as: 3-hydroxy-1-methoxy-10-methyl-9-acridone (**1**), 1-hydroxy-3-methoxy-10-methyl-9-acridone (**4**), 1-hydroxy-2,3-dimethoxy-10-methyl-9-acridone (**5**), 1,3-dihydroxy-2-methoxy-10-methyl-9-acridone (**6**), 1,2-dihydroxy-3-methoxy-10-methyl-9-acridone (**7**), 1-hydroxy-2,3-dimethoxy-9-acridone (**8**), 6,7-dimethylcoumarine (**9**), 10-*O*-demethyl-12-*O*-methyl isoarnottianamide (**11**), hesperidine (**12**), saccharose, D-glucose, nitidine, angoline, lupeol, dehydronitidine, decarine and sesamine.[16,17] The new compounds **2**, **3** and **10,** were fully characterized using mass spectra, 1D and 2D spectroscopic data as well as HMQC, HMBC and ^{1}H–^{1}H COSY spectra while the known compounds were identified by direct comparison of their physical data with those of the literature and from spectroscopic evidences.

Compound **2** was isolated as red crystals (mp 122–123 °C) from methanol. It was characterized as an alkaloid by its positive reaction with Draggendorf's and Mayer's tests and by its odd molecular mass (*m*/*z* 285) which showed $[M+H]^+$ signals at 286 by both ESI and HRESIMS. The molecular formula $C_{16}H_{15}NO_4$, containing ten unsaturations, has been deducted and was in good agreement with these results. The ^{1}H NMR spectrum exhibited eight clearly visible signals of 14 protons, with five aromatic signals at δ 6.43 (1H, s); 7.27 (1H, m); 7.48 (1H, br d, J = 9.0); 7.73 (1H, ddd, J = 1.8; 8.0 and 9.0) and at 8.38 (1H, dd, J = 1.8 and 8.0), together with three methyl signals at δ 3.72 (3H, s); 3.98 (3H, s) and at 4.05 (3H, s). Among the five aromatic signals, it was obviously observed in the ^{1}H–^{1}H COSY spectrum that they were belonging to two different aromatic rings which means two different systems of protons: one isolated proton at δ 6.43 (H-2) and four other protons at δ 7.48 (H-5), 7.73 (H-6), 7.27 (H-7) and at 8.38 (H-8) which correlated together as showed in Figure 2. The ^{13}C NMR spectrum (Table 1) of compound **2** exhibited 16 carbon signals among which 8 quaternary carbons at δ 106.2 (C-9a), 121.4 (C-8a), 129.1 (C-4), 138.6 (C-4a), 145.4 (C-5a), 160.2 (C-1), 161.3 (C-3) and 181.6 (C-9). Since C-9 was identified as a carbonyl, the remaining one unsaturation should be an additional ring containing the nitrogen atom. The presence of three methyl carbons was inferred from signals at δ 40.6, 56.4 and 61.7 corresponding to one *N*-methyl group and two *O*-methyl groups located at C-1 and C-4, respectively, by using HMBC experiment (Table 1). In fact, the proton signals at δ 3.72 and 3.98 directly connected to carbons at δ 61.7 and 56.4, respectively, as shown from the HMQC spectrum, which each exhibiting only one cross peak with carbons C-4 (δ 129.1) and C-1 (δ 160.2), in the HMBC experiment. In contrary, the methyl protons at δ 4.05 linked to the carbon at δ 40.6, exhibited two cross-peaks with carbons at δ 138.6 (C-4a) and 145.4 (C-5a). These HMBC correlations of the *N*-methyl group, added to that of H-8 with C-9, were good justifications for the presence of the third ring mentioned. Furthermore, the main hydroxyl group of compound **2** was located at C-3 (δ 161.3) on the basis of the weak ^2J correlations (Fig. 2), exhibited in the HMBC spectrum, between the singlet at δ 6.43 (H-2) and two carbon signals at δ 160.2 (C-1) and δ 161.3 (C-3). Added to the lack of chelated proton in the ^{1}H NMR spectrum, all physical data and spectral evidences led us to conclude that compound **2** was a 3-hydroxy-1,4-dimethoxy-10-methyl-9-acridone, a new natural acridone derivative named helebelicine A, reported in the literature for the first time.

Compound **3** was isolated as an amorphous yellow powder from methanol. It was characterized as an alkaloid by its positive reaction with Draggendorf's and Mayer's tests and by its odd molecular mass *m*/*z* 285 shown as its $[M+H]^+$ pic at 286 in both ESI and HRESIMS. The ^{1}H and ^{13}C NMR data of **3** were similar to those of helebelicine A (**2**). They showed 8 ^{1}H and 16 ^{13}C signals, respectively, as in **2** but since they were recorded under identical conditions, the small difference found in their chemical shift values



1 R_1 = OMe; R_2 = H; R_3 = OH; R_4 = H; R_5 = Me
2 R_1 = OMe; R_2 = H; R_3 = OH; R_4 = OMe; R_5 = Me
3 R_1 = OMe; R_2 = OMe; R_3 = OH; R_4 = H; R_5 = Me
4 R_1 = OH; R_2 = H; R_3 = OMe; R_4 = H; R_5 = Me
5 R_1 = OH; R_2 = OMe; R_3 = OMe; R_4 = H; R_5= Me
6 R_1 = OH; R_2 = OMe; R_3 = OH; R_4 = H; R_5 = Me
7 R_1 = OH; R_2 = OH; R_3 = OMe; R_4 = H; R_5 = Me
8 R_1 = OH; R_2 = OMe; R_3 = OMe; R_4 = H; R_5 = H



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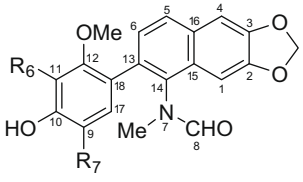
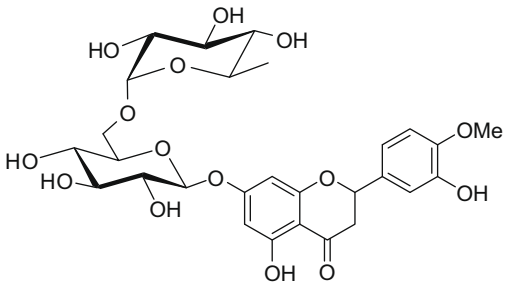


10 R_6 = OMe; R_7 = H
11 R_6 = H; R_7 = OMe



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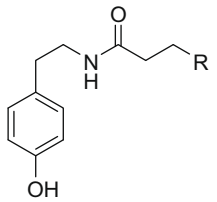

13 R = $(CH_2)_{18}CH_3$
14 R = $(CH_2)_{20}CH_3$

Figure 1. Some isolated compounds from *Z. leprieurii* and *Z. lemairei*.

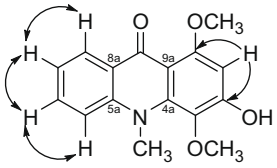

Figure 2. Key COSY ( ) and HMBC ( ) correlations of compound **2**.

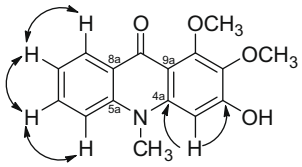

Figure 3. Key COSY ( ) and HMBC ( ) correlations of compound **3**.

listed in Table 1 was the key to its structural elucidation. In fact, the observed difference in **3**, was attributed to the substitution pattern of the main aromatic ring where the singlet of one proton at δ 6.11 (δ 6.43 in **2**) was linked to C-4 (δ 86.8) instead of C-2 (δ 94.0), as in **2**. This was supported by the main ^{2}J correlations of compound **3**, in the HMBC experiment, between H-4 (δ 6.11) and carbons C-3 (δ 156.0) and C-4a (δ 140.4), as presented in Figure 3. Also, compared to compound **6** containing a hydroxyl group in C-1, isolated together with these 1-*O*-methyl group's compounds **1–3**, there was an absence of the chelated proton. On the basis of the above evidence, compound **3** was characterized as 3-hydroxy-1,2-dimethoxy-10-methyl-9-acridone and named helebelicine B.

Compound **10** was isolated as a brown amorphous powder. The high-resolution electrospray ionization mass spectrometry (HRESIMS) gave an $[M+H]^+$ ion at *m*/*z* 382.1285 (calcd for $C_{21}H_{20}O_6N$, 382.1288), consistent with a molecular formula of $C_{21}H_{20}O_6N$. The odd mass at *m*/*z* 381 indicated the presence of a nitrogen atom in **10** and its possible alkaloid nature which was confirmed by the positive response to the Draggendorf's and Mayer's tests. Also, its positive reaction with $FeCl_3$ indicated the presence of a free hydroxyl group as shown in the IR spectrum with an absorption band at 3341 cm^{-1}. The observed IR absorption at 1650 cm^{-1} was also an indication of the presence of a carbonyl function. Absorption maxima at 232, 290 and 329 nm exhibited by the UV spectrum of compound **10** were similar to those of 10-*O*-demethyl-17-*O*-methyl arnottianamide[16] and suggested that alkaloid **10** contains a secobenzo[*c*]phenanthridine moiety.[16–18] The ^{1}H NMR spectrum showed four aromatic signals at δ 6.61 (1H, d, *J* = 8.2 Hz), 7.01 (1H, d, *J* = 8.2 Hz), 7.58 (1H, d, *J* = 8.7 Hz) and at 7.82 (1H, d, *J* = 8.7 Hz), characteristic of two *ortho* system of protons (Table 2). This was confirmed in the ^{1}H–^{1}H COSY spectrum by two separated system of correlations observed between signals at δ 6.61 (H-9)–7.01 (H-17) and δ 7.58 (H-6)–7.82 (H-5). The first singlet of two protons at δ 6.12 was typical of a methylene-dioxy group signal while the second at δ 7.38 was found to be that of two *para* protons, H-1 and H-4. The three singlets of three protons each exhibited by the ^{1}H NMR spectrum of compound **10** at δ 3.22, 3.79 and 3.81 with the corresponding carbon atoms at δ 33.0, 60.5 and 55.8, respectively, indicated the presence of one *N*-methyl and two *O*-methyl groups. The low-field proton signals at δ 8.63 (s) and 11.33 (s) were attributed, respectively, to the aldehyde function for which the corresponding carbon

Table 2
^{1}H, ^{13}C chemical shifts and HMBC correlations of compound **10**

10-*O*-Demethyl-12-*O*-methylarnottianamide (**10**)			
	δ_c	δ_H (Mult., *J* in Hz)	HMBC
1	99.8	7.38 (s)	3, 14, 16
2	148.5		
3	149.6		
4	103.7	7.38 (s)	2, 5, 15
5	127.6	7.82 (d, 8.7)	4, 13, 15
6	128.5	7.58 (d, 8.7)	14, 16
7			
8	164.3	8.63 (s)	NCH_3
9	104.7	6.61 (d, 8.2)	10, 11, 18
10	149.7		
11	137.7		
12	153.6		
13	135.6		
14	136.7		
15	129.4		
16	131.7		
17	123.8	7.01 (d, 8.2)	3, 10, 12, 13
18	125.6		
N–CH_3	33.0	3.22 (s)	8, 14
–OCH_2O–	102.2	6.12 (s)	2, 3
11-OCH_3	60.5	3.79 (s)	11
12-OCH_3	55.8	3.81 (s)	12
10-OH		11.33 (s)	

Table 1
^{1}H, ^{13}C chemical shifts and HMBC correlations of compounds **2** and **3**

	Helebelicine A (**2**)			Helebelicine B (**3**)		
	δ_c	δ_H (Mult., *J* in Hz)	HMBC	δ_c	δ_H (Mult., *J* in Hz)	HMBC
1	160.2			159.4		
2	94.0	6.43 (s)	1_w,3_w,4,9a	130.2		
3	161.3			156.0		
4	129.1			86.8	6.11 (s)	9a,2,3_w,$4a_w$
5	115.7	7.48 (br d, 9.0)	7,8a	114.7	7.40 (br d, 9.0)	7,8a
6	134.4	7.73 (ddd, 1.8; 8.0 and 9.0)	5a,8	134.0	7.63 (ddd, 1.5; 7.5 and 9.0)	5a,8
7	121.7	7.27 (m)	5,8a	126.4	7.19 (br t, 7.5)	5,8a
8	126.5	8.38 (dd, 1.8 and 8.0)	5a,6,9	126.4	8.27 (dd, 1.5 and 7.5)	5a,6,9
9	181.6			180.7		
9a	106.2			105.7		
4a	138.6			140.4		
5a	145.4			141.9		
8a	121.4			120.6		
N–CH_3	40.6	4.05 (s)	4a,5a	34.2	3.96 (s)	4a,5a
1-OCH_3	56.4	3.98 (s)	1	60.9	3.71 (s)	1
2-OCH_3				56.1	3.90 (s)	2
4-OCH_3	61.7	3.72 (s)	4			

w: weak (^2J correlation).

appeared at δ 164.3, from its HMQC spectrum, and the hydroxyl group. The position of all these groups and/or functions as well as that of the two pair of doublets were inferred from different cross-peaks presented by the HMBC spectrum as shown in Table 2 or drawn in Figure 4. Then, the signals attributed to the methylene-dioxy group was easily located on C-2 (148.5) and C-3 (149.6), the two *O*-methyl groups in *ortho* position on C-11 (δ 137.7) and C-12 (δ 153.6) and the hydroxyl in C-10 (δ 149.7). The above evidences and the related secobenzo[*c*]phenantridine,[16–18] already isolated from the *Fagara* genus, led us to conclude that compound **10**, 10-*O*-demethyl-12-*O*-methylarnottianamide, is a new natural benzophenantridine derivative.

2.1. Brine shrimp lethality bioassay

LD_{50} measurement of the chloroform extract of *Z. leprieurii* was evaluated against *Artemia salina* brine-shrimp eggs (Table 3). It was evident from the results that the extract was significant lethal with LD_{50} value of 13.1 μg/mL.

2.2. Cytotoxic assay

Acridone alkaloids are known to have antiproliferative activity against most cancer cell lines.[19,20] In this study, we evaluated the cytotoxic activity of compounds **1**, **2**, **4–6**, **9**, **15** and **16** against lung carcinoma cells (A549), colorectal adenocarcinoma cells (DLD-1) and normal cells (WS1). The results presented in Table 4 are expressed as concentration inhibiting fifty percent of cell growth (IC_{50}). Etoposide was used as a positive control with IC_{50} ranging from 4 to 16 μM. Compounds **9**, **15** and **16** were found inactive against all the tested cell lines. The results showed that the isolated acridones **1**, **4–6** were found to be moderately active against both tumor cell lines with IC_{50} values ranging from 27 to 77 μM. To the best of our knowledge, this is the first time that the activity of these compounds is reported. The cytotoxic activity of compound **1**, **4–6** was similar against A549 cells with IC_{50} varying from 31 to 52 μM. However, the cytotoxic activity of compound **4** (IC_{50} 27 ± 1 μM) was higher in comparison with other acridones against DLD-1 cells (IC_{50} 71–77 μM). Unlike the other acridone tested, compound **2** was inactive with an IC_{50} higher than 100 μM. This result suggests that the *O*-methyl group located at the C-4 position of the acridone inhibited the cytotoxic activity. Interestingly, Braga et al.[20] reported that citrusinine-I, which is similar to compound **4** with the exception of the presence of *O*-methyl group at the C-4 position, was inactive against human lung carcinoma (COR-L23), human breast carcinoma (MCF-7), and human melanoma (C32). In contrast to the positive control etoposide used, the cytotoxicity of the most active compound **4** was found selective against cancer cells in comparison to normal cells, WS1 with IC_{50} of 51 ± 8 μM and 4.3 ± 0.4 μM, respectively. Work is now in progress to perform a deeper QSAR on this family of acridones and to modify the most active substances with other functionalities.

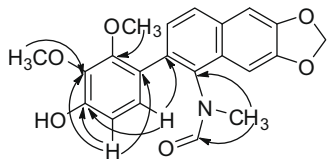

Figure 4. Some Key HMBC of compound **10**.

3. General experimental procedures (chemistry)

UV spectra were run in $CHCl_3$ on a Varian Cary 100 Bio UV–vis Spectrophotometer. IR spectra were run as KBr pellets on a Perkin–Elmer 1600 FTIR spectrophotometer. ^1H, ^{13}C NMR, ^1H–^1H COSY, HMQC and HMBC spectra were recorded in $CDCl_3$ on a Varian Gemini 2000 and Varian Inova AS600 spectrometer operating at 300 and 600 MHz for ^1H and 75 and 150 MHz for ^{13}C of **2**, **3** and **10**, respectively, with TMS as internal reference. High-resolution mass spectra (HRMS) were carried out by the analytical platform of UQAM (Université du Québec à Montréal, QC, Canada). Open column liquid chromatography was performed over silica gel (Merck, 60–200 mesh) and Sephadex LH-20. Analytical TLC was performed on precoated Merck glass sheets (Whatman K_6F Silica Gel 60°A), viewed under a UV lamp and sprayed with 50% H_2SO_4 solution and Draggendorf's reagent.

3.1. Plant material

Roots of *Z. leprieurii* were collected from South-West province of Cameroon, in june 2006 while the fruits were brought at Mbouda market, West Province of Cameroon in January 2007. The plants were identified by M. Nana, Botanist at the National Herbarium of Cameroon, where a voucher specimen (HNC No. 10669/SFR/CAM) was deposited.

3.2. Extraction and isolation

The air-dried powdered roots (1.5 kg) and fruits (7.2 kg) were extracted three times, at root temperature, with MeOH to provide 56 g and 1.05 kg (oily) crude material, respectively. One kg of fruits extract was further partitioned into two sub-extracts to afford 850 g of crude material from chloroform and 141 g from methanol. The

Table 4
Cytotoxic activity of isolated compounds against human lung carcinoma cells (A549), colorectal adenocarcinoma cells (DLD-1) and normal skin fibroblasts (WS1)

Compound	IC_{50}[a] (μM)		
	A549	DLD-1	WS1
1	36 ± 2	71 ± 12	42 ± 8
2	>100[b,c]	>100[b,c]	>100[b,c]
3	52 ± 7[b]	77 ± 7[b]	74 ± 4[b]
4	31 ± 4	27 ± 1	51 ± 8
5	35 ± 7	74 ± 7	56 ± 4
9	>100[c]	>100[c]	>100[c]
12	>100[c]	>100[c]	>100[c]
14	>100[c]	>100[c]	>100[c]
Etoposide	16 ± 2	10 ± 2	4.3 ± 0.4

[a] Each data represents mean ± SD from two different experiments performed in triplicate.
[b] Significantly different from compound (**4**), P <0.05, RM ANOVA on ranks and post hoc Student–Newman–Keuls method.
[c] Compounds having IC_{50} higher than 100 μM were considered inactive.

Table 3
Brine shrimp (*Artemia salina*) lethality bioassay

Doses (μg/mL)	Number of shrimps	Number of survivors	%Deaths at doses	LD_{50} (μg/mL)	Standard drug	LD_{50} (μg/mL)
1000	30	0	100	13.077	Etoposide	7.463
100	30	6	80			
10	30	16	47			

crude chloroform extract (850 g) was further subjected to open chromatographic column using silica gel as adsorbent. Elution was performed with *n*-hexane, *n*-hexane/$CHCl_3$/EtOAc and $CHCl_3$/EtOAc/MeOH in increasing polarity and by using air pressure. It is important to insure that the mixture $CHCl_3$/EtOAc was always in a 1:1 proportion. Thus, sub-fractions (86 × 800 mL) were collected and grouped into eight fractions (A–H) on the basis of TLC. Fractions A (5% *n*-hexane/$CHCl_3$/EtOAc), B (25% *n*-hexane/$CHCl_3$/EtOAc) and C (50% *n*-hexane/$CHCl_3$/EtOAc) were further purified by silica gel column chromatography using *n*-hexane, *n*-hexane/$CHCl_3$/EtOAc and $CHCl_3$/EtOAc/MeOH as eluent to yield **1** (1.8 g), **2** (800 mg), **3** (11 g), **5** (43 mg) and **9** (16 g). From the MeOH extract (141 g), compounds **4–9** were obtained by silica gel column chromatography and preparative TLC on silica gel plates using 5% $CHCl_3$/MeOH as eluent. Concerning compounds **10–16**, they were obtained from the roots extract by the identical procedure described above.

Helebelicine A (**2**): Red crystal; mp 122–123 °C; UV (log ε) 256 (2.92), 312 (2.39), 410 (1.82) nm; ^{1}H (300 MHz, $CHCl_3$) and ^{13}C NMR (75 MHz, $CHCl_3$) data (see Table 1); HRESIMS: *m/z* 286.1078 $[M+H]^+$ (calcd for $C_{16}H_{16}NO_4$, 286.1074).

Helebelicine B (**3**): Yellow amorphous powder; UV (log ε) 259 (2.92), 401 (2.24) nm; ^{1}H (300 MHz, $CHCl_3$) and ^{13}C NMR (75 MHz, $CHCl_3$) data (see Table 1); HRESIMS: *m/z* 286. 1077 $[M+H]^+$ (calcd for $C_{16}H_{16}NO_4$, 286.1074).

10-O-Demethyl-17-O-methylarnottianamide (**10**): Brown amorphous powder; IR (KBr) υ_{max} in cm^{-1}: 3341, 1650, 1621, 1510, 1281 and 1133; UV ($CHCl_3$) λ_{max} nm (log ε): 232 (4.63), 290 (3.99), 329 (3.82); ^{1}H (300 MHz, $CHCl_3$) and ^{13}C NMR (75 MHz, $CHCl_3$) data (see Table 1); HRESIMS: *m/z* 382.1285 $[M+H]^+$ (calcd for $C_{21}H_{20}O_6N$, 382.1246).

3.3. Brine shrimp lethality bioassay

It is an excellent and simple preliminary method to determine the cytotoxicity of crude plant extract and pure natural compounds. In this method, artificial 'sea water' was prepared by dissolving 3.8 g sea salt per liter of double distilled water and filtered.[21] 'Sea water' was placed in a small tank, added brine-shrimp eggs (1 mg) (*Artemia salina*) and was darkened by covering with aluminum foil. It was allowed to stand for 24 h at 25 °C which provided a large number of larvae. Twenty milligrams of the concentrated sample was dissolved in 2 mL $CHCl_3$ (20 mg/2 mL) and transferred to 500, 50 and 5 μL vials corresponding to 1000, 100 and 10 μg per mL, respectively. Then three replicates were prepared for each concentration making a total of nine vials. The vials containing material was concentrated, dissolved in DMSO (50 μL) and 5 mL 'sea water' added to each. Then, 30 shrimps were added per vial, the preparation was allowed to stand for 24 h after which time the shrimps were counted and the number of surviving shrimps were recorded. The data were analyzed with a Finney computer program to determine the LD_{50} values.

3.4. Cell culture

The human lung carcinoma A549 (#CCL-185), colorectal adenocarcinoma DLD-1 (#CCl-221) and skin fibroblast WS1 (#CRL-1502) cell lines were all obtained from the American Type Culture Collection (ATCC, Manassas, USA). Cells lines were grown in minimum essential medium containing Earle's salts (Mediatech Cellgro®, Herndon, USA), supplemented with 10% fetal calf serum (Hyclone, Logan, USA), 1× solution of vitamins, 1× sodium pyruvate, 1× non-essential amino acids, 100 IU of penicillin and 100 μg mL^{-1} of streptomycin (Mediatech Cellgro®). Cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2.

3.5. Cytotoxicity assay

Exponentially growing cells were plated at a density of 5×10^3 cells per well in 96-well microplates (BD Falcon) in 100 μL of culture medium and were allowed to adhere for 16 h before treatment. Then, the cells were incubated for 48 h in the presence or absence of 100 μL of increasing concentrations of extract, fraction or pure compounds dissolved in culture medium and DMSO. The final concentration of DMSO in the culture medium was maintained at 0.25% (v/v) to avoid toxicity. Cytotoxicity was assessed using the resazurin reduction test.[22] Fluorescence was measured on an automated 96-well Fluoroskan Ascent Fl™ plate reader (Labsystems) using an excitation wavelength of 530 nm and an emission wavelength of 590 nm. Cytotoxicity was expressed as the concentration of drug inhibiting cell growth by 50% (IC_{50}).

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