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Cytotoxic sesquiterpene lactones from the aerial parts of *Inula aucheriana*

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ABSTRACT

Inula aucheriana DC is a member of the family Asteraceae which is known to produce cytotoxic secondary metabolites noted as sesquiterpene lactones. In the present study, sesquiterpene lactones inuchinenolide B, 6-deoxychamissonolide (stevin) and 14-acetoxy-1β,5α,7αH-4β-hydroxy-guai-9(10),11(13)-dien-12,8α-olide were isolated from *I. aucheriana*. Inuchinenolide B and 14-acetoxy-1β,5α,7αH-4β-hydroxy-guai-9(10),11(13)-dien-12,8α-olide were further evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay to demonstrate cytotoxic activity with IC_{50} values of (56.6, 19.0), (39.0, 11.8), and (55.7, 15.3) μg/mL against HepG-2, MCF-7 and A-549 cells, respectively. The cytotoxic activity of the two evaluated sesquiterpene lactones partly explains the cytotoxic activity that was previously observed for the extracts of *Inula aucheriana*. The isolated compounds could be further investigated in cancer research studies.

Key words: cytotoxicity, *Inula aucheriana*, MTT assay, sesquiterpene lactones.

INTRODUCTION

Inula aucheriana DC. a perennial species of Asteraceae grows in the West Azerbaijan province, Iran. It is a member of the *Inula* genus which has about 100 species of mainly perennial and subshrubs, distributed across warm and temperate parts of Europe, Asia and Africa (Bown 2002). Some species of this genus have been used in traditional medicine by Greek, Roman and Chinese healers for their expectorants, antitussives, diaphoretics, antiemetics, and antibacterial properties (Seca et al. 2014). Compounds like monoterpenes, sesquiterpenes, diterpenes, flavonoids, and glycolipids have been isolated from *Inula* while several biological effects like antiproliferative, antibacterial and hepatoprotective activity have been reported from this genus (Zhao

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et al. 2006). Some phytochemical studies about different species of *Inula* have introduced several cytotoxic and apoptotic compounds, most of these isolated materials are sesquiterpene lactones of different structure types. There has been limited research about the cytotoxicity of *Inula aucheriana*; however, our previous study demonstrated that the extracts from the aerial parts of this species exhibit cytotoxicity to several cancerous cell lines (Hamzeloo-Moghadam et al. 2012); thus the present study was focused on the isolation of compounds from the aerial parts of this Asteraceae species as well as on the evaluation of their cytotoxic activity to find the compound/ compounds that are at least partially responsible for the previously observed cytotoxic results.

EXPERIMENTAL

General Experimental Procedures

IR (Infrared) spectra were recorded on a Nicolet Maga 550, and NMR spectra on a Bruker instrument, operating at 500 MHz for ^{1}H and 125 MHz for ^{13}C. TMS (Tetramethylsilane) was used as internal standard. HRMS (High Resolution Mass Spectroscopy) were obtained with an Orbitrap LTQ XL (Thermo Fisher Scientific, San Jose Ca, USA) ion trap mass spectrometer using a nanospray (nanoelectrospray) ionization source to generate ions from methanol solutions.

Chemicals and Reagents

RPMI 1640 medium, penicillin-streptomycin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) from Sigma, DMEM medium and FBS (Gibco). Organic solvents (analytical grade) were purchased from Merck.

Plant Material

Inula aucheriana was collected from the West Azerbaijan province, Iran (July 2010) and was authenticated by botanists of the Traditional Medicine and Materia Medica Research Center (TMRC), Shahid Beheshti University of Medical Sciences, Tehran, Iran. A voucher specimen was deposited at TMRC Herbarium for future reference (voucher no. TMRC 3173). The aerial parts of the species were dried in shade and ground for extraction.

Extraction and Isolation

Dried aerial parts of *I. aucheriana* (500 g) were successively extracted with *n*-hexane and chloroform (each solvent for 3 days), every day the solvent was replaced with fresh solvent to yield *n*-hexane (9.4 g) and chloroform (8.9 g) extracts. The chloroform extract (5 g) was subjected to Vacuum Liquid Chromatography [(VLC) silica gel, 40-63 Nm] using sequential washings with EtOAc, EtOAc –MeOH (2:1)-(1:1) and MeOH as eluent to give two fractions (F1-F2). Fraction F1 (1600 mg) was fractionated by silica gel SPE (7.5 × 2.5 cm, 40-63 μm) with CH_2Cl_2, CH_2Cl_2- EtOAc (10:1)- (1:1), EtOAc , EtOAc- MeOH (3:1)-(1:1) and MeOH respectively, to afford 17 fractions (Fr1-Fr17). Fraction Fr8 (139 mg) was further subjected to semi preparative HPLC (Shimadzu, SPD 20A :ODS, PDA detector) with water-acetonitrile (75:25) as the mobile phase and flow rate of 8 mL/ min, to finally afford compound A (8 mg, 0.0016%), B (26 mg, 0.0052%) and C (5 mg, 0.0010%).

Preparation for MTT Assay

The compounds were dissolved in DMSO (5 mg/500 μL). The final concentrations (100 μg/mL, 50 μg/mL, 25 μg/mL, 12.5 μg/mL, 6.25 μg/mL and 3.125 μg/mL) were provided from the above solution (final DMSO concentration was 1%).

Cell Lines

The human tumor cell lines were obtained from the Pasteur Institute, Tehran, Iran: HepG-2 (human hepatocellular carcinoma), MCF-7 (human breast adenocarcinoma), and A-549 (Non-small cell lung carcinoma). MCF-7 cells were cultured in DMEM

medium with 5% FBS, while the other two cell lines were maintained in RPMI 1640 medium with 10% FBS to obtain the desired growth. All cell lines were treated with 1% penicillin-streptomycin, in a humidified incubator at 37°C in an atmosphere of 5% CO_2.

MTT Assay

The assessment of the viability of the cells was carried out in a micro culture tetrazolium/formazan assay (MTT assay) (Mosaddegh et al. 2006, 2010). Ninety six-well plates were used and HepG-2 cells 15×10^3, MCF-7 cells 8×10^3 and A-549 cells 8×10^3 were seeded in each well. They were then incubated at 37°C. After 24h the medium was replaced with fresh medium containing different concentrations of the compounds to be tested. After 72h exposure of the cells at 37°C to each sample, the medium was replaced with fresh medium containing MTT, with a final concentration of 0.5 mg/mL. The cells were incubated for another 4h in a humidified atmosphere at 37°C, then the medium containing MTT was removed and the remaining formazan crystals dissolved in DMSO. The absorbance was recorded at 570 nm with an ELISA reader (TECAN). Tamoxifen was used as positive control. The relative cell viability (%) related to control wells containing cells, cell culture medium and DMSO 1%, was calculated by [A]samples /[A]control × 100 where [A]samples was the absorbance of test sample and [A]control was the absorbance of wells containing cells + medium+DMSO1%. To calculate IC_{50}, viability (%) versus log concentrations was graphed by Microsoft Excel program, and the concentration at 50% viability was determined.

RESULTS AND DISCUSSION

Compounds A, B and C, reported for the first time from *Inula aucheriana* were identified as inuchinenolide B **1**, 14-acetoxy-1β,5α,7αH-4β-hydroxy-guai-9(10),11(13)-dien-12,8α-olide **2** (here named aucherinolide) and 6-deoxychamissonolide **3** (Fig. 1), respectively.

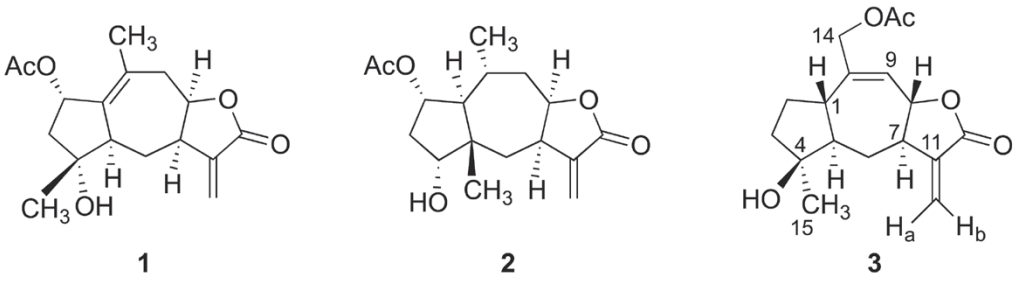

Figure 1 - Chemical structures of inuchinenolide B **1**, aucherinolide **2** and 6-deoxychamissonolide **3.**

Although they were known compounds (Ito and Iida 1981, Cheng et al. 2012, Willuhn et al. 1981), their 2D NMR data were not available in the literature, and for 6-deoxychamissonolide **2**, the previously reported NMR spectra was obtained in acetone-D6, while those in this report were obtained in CDCl3. The 1D and 2D NMR data for inuchinenolide B and 6-deoxychamissonolide are reported in Table I. The molecular formulae of $C_{17}H_{22}O_5$ for inuchinenolide B (m/z: 329.1357 $[M+Na]^+$) and $C_{17}H_{24}O_5$ for 6-deoxychamissonolide (m/z: 331.1515 $[M+Na]^+$) were revealed by high-resolution QTOF mass spectrometric analysis.

Compound B, which we name aucherinolide, was unusual. The molecular formula of $C_{17}H_{22}O_5$ (m/z: 345.1310 $[M+K]^+$) was obtained by high-resolution QTOF mass spectrometry. The ^{1}H- and ^{13}C-NMR spectra and the key HMBC correlations

TABLE I
^{1}H and ^{13}C NMR chemical shifts (δ, ppm), ^{1}H multiplicities (mult.) and coupling constants (J, Hz), ^{1}H-^{13}C correlations in HMBC spectra and ^{1}H-^{1}H correlations for compounds A and C in CDCl3.

No.	A				C			
	δ_H (mult, *J*/Hz)	δ_C	COSY	HMBC	δ_H (mult, *J*/Hz)	δ_C	COSY	HMBC
1	-	135.8	-	-	1.86 (m)	52.8	H-2	C-2, 15
2	5.46 (t, 7.6)	72	H-3a, b	C-1, 10, 17	4.93 (tw, 7.8)	74.9	H-1	C-4, 5, 17
3a	1.72 (dd, 7.8, e12.6)	46.9	H-2	C-2, 4, 14	1.84	38.4	H-2, 4	C-4, 7, 9
3b	2.48 (dd, 7.6, 12.6)		H-2	C-1, 4, 5, 14	2.06		H-2, 4	C-2, 4,
4	-	77	-	-	4.04	80.6	H-3	C-6, 15
5	2.70 (brd, 11.5)	52.2	H-6	-	-	27.1	-	-
6a	1.48 (m)	25	H-5, 7	C-1, 5, 7	1.41	38.0	H-7	C-1, 15
6b	2.26 (m)		H-7	C-5, 7	2.33		H-7	C-7, 8, 15
7	3.3 (m)	41.9	H-8	-	2.82	45.1	H-8, 13	-
8	4.88 (m)	78.7	H-9a, b	-	4.24	81.4	H-7, 9	-
9a	2.26 (m)	36.6	H-8	C-1, 7, 8, 10, 15	1.40	44.0	H-8	C-7, 8, 9, 11, 14
9b	2.56 (m)		H-8	-	2.37		H-8	-
10	-	131.8	-	-	1.97	29.1	H-1, 9, 14	-
11	-	138.3	-	-	-	140.4	-	-
12	-	169.9	-	-	-	169.5	-	-
13a	5.64 (d, 3.0)	122	H-7	C-7, 12	5.46	119.8	H-7	C-7, 12
13b	6.32 (d, 3.0)		H-7	C-7, 11, 12	6.18		H-7	C-7, 11, 12
14	1.05 (s)	22.7	-	C-3, 4, 5	0.97	19.9	-	C-1, 9, 10
15	1.66 (s)	21.6	-	C-1, 9, 10	0.93	18.7	-	C-1, 4, 6, 7
16	-	170.7	-	-	-	170.5	-	-
17	2.06 (s)	20.9	C-16	-	2.04	21.3	-	C-17

(Fig. 2) proved it to be a 12,8-guaianolide sesquiterpene lactone with an acetate group at C-14. The structure was concluded as being 14-acetoxy-1β,5α,7αH-4β-hydroxy-guai-9(10),11(13)-dien- 12,8α-olide **2** (Fig. 1) based on this information and after comparison of the spectroscopic data with those of a new substance introduced by Cheng et al. (2012) for *Inula hookeri*. In particular, relative stereochemistry was able to be assigned by Cheng et al. (2012) through NOESY experiments. The NMR data are given in Table II and agree very closely with those of Cheng et al. (2012). The only exception is for the assigned proton chemical shift for H_a2, for which we see clear correlation evidence for assignment at δ1.78, overlapped with the signal for H_b3, and not at δ1.40, as reported.

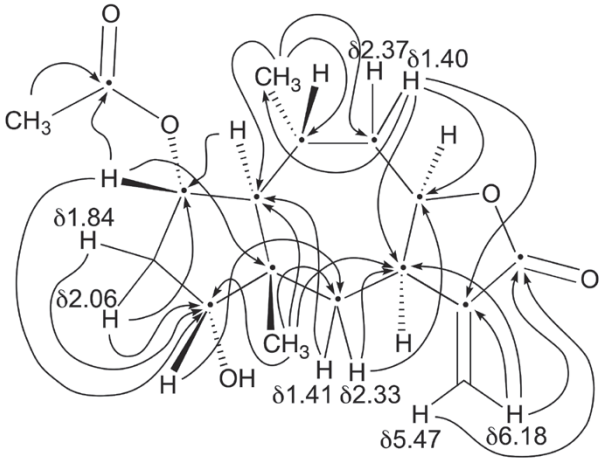

Figure 2 - Key HMBC correlations for aucherinolide.

There are several reports of cytotoxic and apoptotic sesquiterpene lactones from the genus *Inula*. A brief report follows. 1,6-O,O-diacetylbritannilactone from *Inula britannica* L. demonstrated strong cytotoxic activity and induction of apoptosis in HL-60 cells through the activation of caspase-8, -9, and -3, phosphorylation of Bcl-2 and Bid, and by augmenting the

TABLE II
^{1}H and ^{13}C NMR chemical shifts (δ, ppm), ^{1}H multiplicities (mult.) and coupling constants (J, Hz), ^{1}H-^{13}C correlations in HMBC spectra and ^{1}H-^{1}H correlations for compounds A and C in CDCl3.

No.	B			
	δ_H (mult, J/Hz)	δ_C	COSY	HMBC
1	2.58* (m)	45.0	H-5	C-5, 9, 10/11
2a	1.78 (m)	23.7	H-1, 3	C-1/7, 4, 5
2b	1.88 (m)		H-1, 3	C1/7, 3, 4, 10
3a	1.67 (m)	41.5	H-2	C-1, 2, 4, 5
3b	1.80 (m)		Ha-3	C-1, 2, 4
4	-	79.5	-	-
5	1.63 (m)	52.5	H-1, 6	C-1/7, 4, 10
6a	1.43 (m)	31.0	H-5,7	C-5, 1/7, 8
6b	2.50 (m)		Ha-6	C-1/7
7	2.58* (m)	45.2	H-8, Hb-13	C-5, 9, 10/11
8	4.76 (d, 9.3)	81.7	H-9	C-1/7, 6,9, 10/11
9	6.15 (brs)	129.9	H-8, 14	C-1/7, 14
10	-	138.6	-	-
11	-	139.0	-	-
12	-	169.9	-	-
13a	5.53 (d, 2.8)	119.7	H-7	C1/7, 12
13b	6.21 (d, 2.8)		H-7	C-1/7, 11, 12
14	4.63 (s)	65.5	H-9	C-1/7, 9, 10, 16
15	1.24 (s)	31.4	-	C-3, 4, 5
16	-	170.5	-	-
17	2.10 (s)	20.9	-	C-14, 16

release of cytochrome c from mitochondria (Pan et al. 2007). Acetylbritannilactone, also from *Inula britannica,* has enforced apoptosis in VSMC cells as shown by induction of a greater ratio of Bax/Bcl-2, activation of caspase-9, caspase-3, and the breakdown of Poly (ADP-ribose) polymerase (Liu et al. 2011). It has also hindered the growth *via* impelling cell cycle arrest in G0/G1 phase of HT-29 cancer cells (Fang et al. 2011). Eupatolide, another sesquiterpene from the same species has been shown to induce apoptosis in human breast cancer cells through tumor necrosis factorrelated apoptosis-inducing ligand (Lee et al. 2010). Ergolide has also been isolated from *Inula britannica*. Studies about the potential of this cytotoxic sesquiterpene lactone have proposed the mechanism for its inducing apoptosis through down regulation of cell survival signal molecules which occur as a consequence of inhibiting the NFkappaB signaling pathway (Song et al. 2005).

There has also been an effort to synthesize derivatives of *Inula britannica* sesquiterpene lactones through a study by Liu et al. (2005); derivatives of 1-O-acetylbritannilactone, (2- Oalkyloxime-3-phenyl)-propionyl-1-O-acetylbritannilactone esters which are sesquiterpene lactones of the above mentioned species, have been synthesized and four of them have exhibited antiproliferative effects on HL-60 and Bel-7402 cell lines (Liu et al. 2005). Dimeric sesquiterpene lactones japonicones A-D have been previously isolated from *Inula japonica* Thunb., among which japonicone A has displayed the most potent cytotoxic activity on A-549, LOVO, CEM and MDA-MB-435 cells (Qin et al. 2009). Germacranolide inulacappolide from *Inula cappa* DC, has been evaluated for cytotoxicity and has shown strong activity against HeLa, K-562 and KB cell lines (Xie et al. 2007) and bigelovin isolated from *Inula helianthus-aquatica* has been cytotoxic to U937 cells and it has also instigated apoptosis and arrested the cell cycle at G(0)/G(1) phase (Zeng et al. 2009). Pseudoguaianolides and guaianolides from *Inula hookeri* C. B. Clarke have been likewise investigated for antiproliferative activity on HepG-2, HeLa, PC-3, and MGC-803 cell lines and have demonstrated cytotoxic results (Cheng et al. 2012). The sesquiterpene lactone isocostunolide from the roots of *Inula helenium* has strongly enforced cytotoxic activity in A-2058, HT-29, and HepG-2 cell lines. It has also been reported that isocostunolide could induce apoptosis in A-2058 cells via a mitochondria-dependent pathway (Chen et al. 2007) and finally, gaillardin a sesquiterpene lactone isolated from the aerial parts of *Inula oculus-christi* has demonstrated cytotoxic activity and apoptotic induction in MCF-7 cells (Hamzeloo-Moghadam et al. 2013).

We have previously reported the apoptotic sesquiterpene lactone britannin from *Inula aucheriana* and in the present study three other

sesquiterpene lactones have been isolated from the species. The amount of 6-deoxychamissonolide was not enough for biologic assay so the cytotoxic activities of inuchinenolide B and aucherinolide were evaluated and the results are shown in Figures 3-5 and Table III. Qin and colleagues have examined the cytotoxicity of inuchinenolide B in MCF-7 cells and have reported the IC_{50} value as 15.5 µM (Qin et al. 2013); however, we have determined the IC_{50} value to be 39.0 µg/mL (118 µM) in MCF-7 cells. The values were higher in HepG-2 and A-549 cell lines but still revealed the cytotoxicity of the compound to these cells (56.6 µg/mL equal to 172 µM) and (55.7 µg/mL equal to 169 µM) in HepG-2 and A-549 cells, respectively. Aucherinolide demonstrated to be more potent in the above mentioned cells with IC_{50} values of 19.0 µg/mL (55 µM), 15.3 µg/mL (44 µM) and 11.8 µg/mL (34 µM) in HepG-2, A-549 and MCF-7 cells, respectively. The results could explain the previously reported cytotoxic results of *Inula aucheriana* extracts.

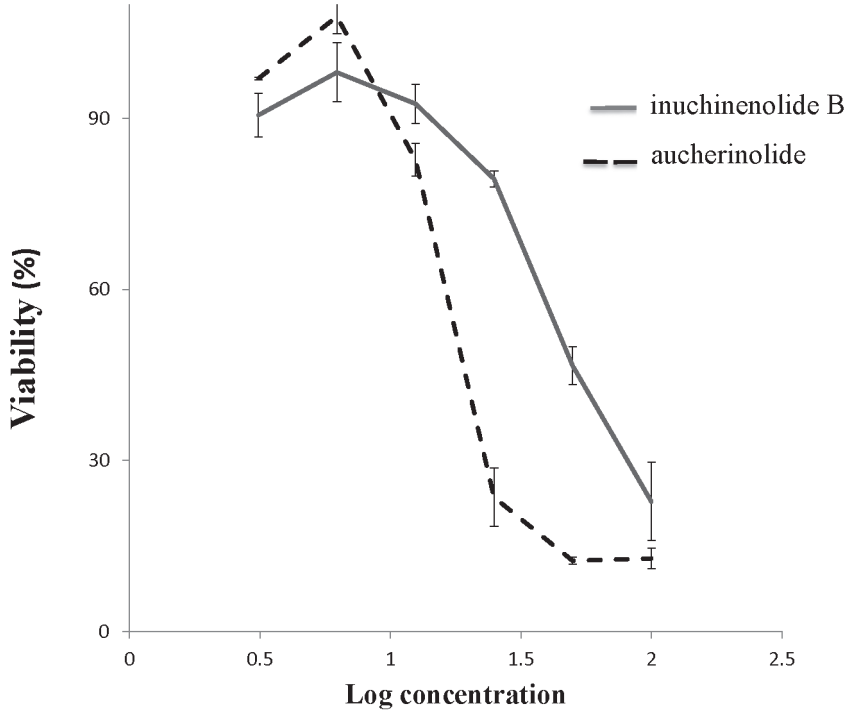

Figure 3 - Viability of inuchinenolide B and aucherinolide in HepG-2 cells. Values represent the mean ±SD of 3 separate experiments.

Surveying the literature about the cytotoxicity of various sesquiterpene lactones which have been evaluated through MTT assay, reveals different ranges of IC_{50} for these compounds. Examples include deoxyelephantopin, a sesquiterpene lactone from *Elephantopus scaber* which has been evaluated in A-549 cell line to show cytotoxicity with IC_{50} 12.287 µg/mL (Kabeer et al. 2013). In another study, IC_{50} values of 6.37, 6.20 and 4.76 µg/mL have been reported for sesquiterpene lactone gaillardin from the aerial parts of *Inula oculus-christi* in MCF-7, HepG-2 and A-549 cell lines, repectively (Hamzeloo-Moghadam et al. 2013), while sesquiterpene lactones from *Laserpitium* species have exhibited cytotoxicity with IC_{50} values of 4.32-97.54 µM against MCF 7/6 cell line (Popović et al. 2013). Moreover, Rosselli et al. (2012) have demonstrated IC_{50} values of

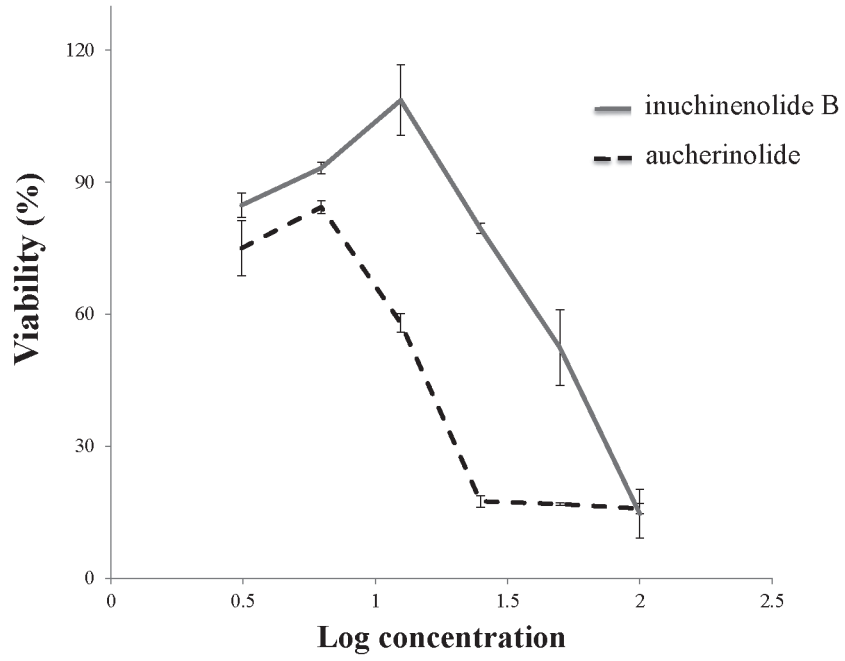

Figure 4 - Viability of inuchinenolide B and aucherinolide in A-549 cells. Values represent the mean ±SD of 3 separate experiments.

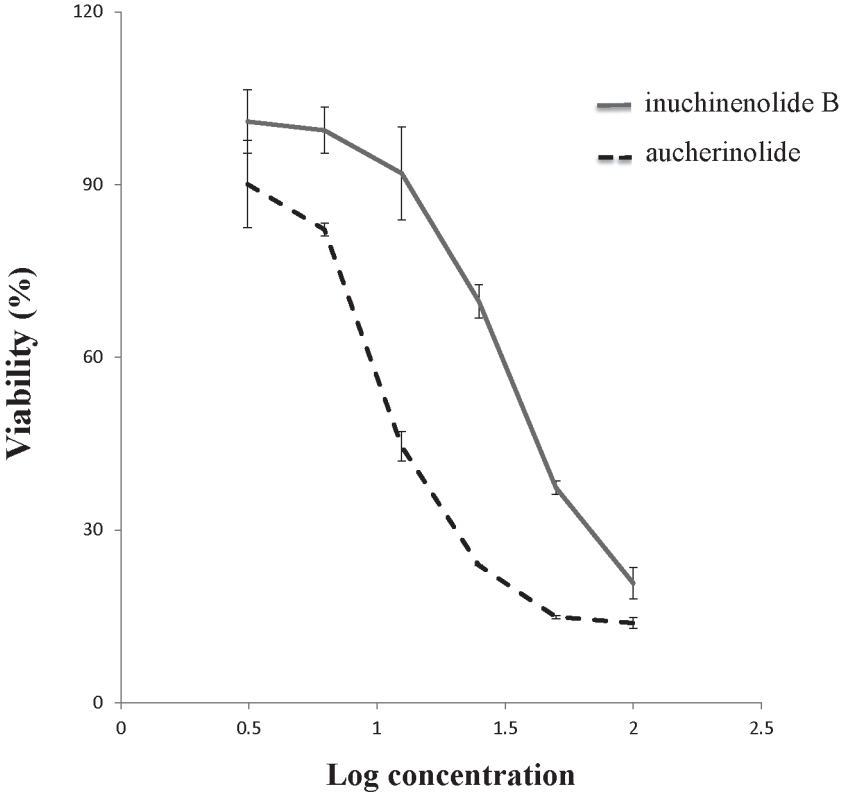

Figure 5 - Viability of inuchinenolide B and aucherinolide in MCF-7 cells. Values represent the mean ±SD of 3 separate experiments.

TABLE III
IC_{50} values for inuchinenolide B and aucherinolide in cell lines.

	IC_{50} µg/mL		
	HepG-2	A-549	MCF-7
inuchinenolide B	56.6	55.7	39.0
aucherinolide	19.0	15.3	11.8

five sesquiterpene lactones isolated from flowers of *Tanacetum vulgare* against A-549 cells to be 15.3-59.4 µM (Rosselli et al. 2012). Considering the outcomes of the present study, IC_{50} values of inuchinenolide B seemed rather high; however, the results of MTT assay for aucherinolide were comparable with some published reports about the *in vitro* toxcicity of sesquiterpene lactones mentioned above.

CONCLUSIONS

As mentioned earlier there are several reports about the cytotoxicity and apoptotic inducing properties of sesquiterpene lactones of the genus *Inula.* The sesquiterpene lactones of the present study, especially the two that have demonstrated cytotoxic activity somewhat explain the previously reported cytotoxicity demonstrated by extracts of *Inula aucheriana*. These compounds might execute their cytotoxic properties through apoptotic induction; however, this assumption needs further investigations about the molecular mechanisms of their cytotoxic activity.

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RESUMO

Inula aucheriana DC, uma espécie da família Asteraceae, é conhecida pela produção de lactonas sesquiterpênicas que são metabólitos secundários cititóxicos. No presente estudo, as seguintes lactonas sesquiterpênicas foram isoladas de *I. aucheriana*: inuchinenolida B, 6-desoxichamissonolida (estevina) e 14-acetoxi-1β,5α,7αH-4β-hidroxi-guai-9(10, 11(13)-dien-12,8α-olida. A atividade citotóxica da inuchinenolida B e da 14-acetoxi-1β,5α,7αH-4β-hidroxi-guai-9(10),11(13)-dien-12,8α-olida foi avaliada pelo ensaio do MTT (brometo de 3-(4,5 dimetiltiazol-2il)-2,5- difeniltetrazólio) em culturas de células HepG-2, MCF-7 e A-549 tendo sido determinados valores de IC50 de (56.6, 19.0 µg/mL), (39.0, 11.8 µg/mL), e (55.7, 15.3 µg/mL), respectivamente. A atividade citotóxica das duas lactonas sesquiterpênicas avaliadas explica a atividade citotóxica previamente observada para os extratos de *Inula aucheriana*. As substâncias isoladas merecem ser investigadas em estudos direcionados para pesquisas em câncer.

Palavras-chave: citotoxicidade, *Inula aucheriana*, ensaio do MTT, lactonas sesquiterpênicas.

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