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Cytotoxic constituents of *Achillea clavennae* from Montenegro

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Abstract

Examination of the aerial parts of *Achillea clavennae* afforded eight guaianolides (**1**–**8**), three bisabolenes (**9**–**11**), four flavonols (**12**–**15**), sesamin (lignan) and isofraxidin (coumarin). The structures of the new compounds (**2**, **4**, **5**, **7** and **10**) were determined by spectroscopic methods. The antiproliferative action of **2**, **8**, **9** and **12** were tested to HeLa, K562 and Fem-X human cancer cell lines. Guaianolides **2** (9α-acetoxyartecanin) and **8** (apressin) showed significant cytotoxic effects to all tested lines and inducumenone (**9**) exhibited a moderate activity. The most active was flavonol centaureidin (**12**), already known as cytotoxic compound.

Keywords: *Achillea clavennae*; Asteraceae; Guaianolides; Bisabolenes; Flavonoids; Cytotoxicity

1. Introduction

The genus *Achillea* L. (family Asteraceae, tribe Anthemidae) comprises about 100 species that commonly occur in temperate regions throughout the Old World, especially on higher mountains of the Mediterranean (Gajić, 1975). Due to numerous medicinal properties, aerial parts of the members of the genus are used widely in traditional medicine. *Achillea clavennae* L. (silvery yarrow), the examination of which is reported, is found on elevated carbonate rocks of eastern and southern Alps as well as on mountains in the west part of Balkan peninsula at the altitude between 1500 and 2500 m. One of the first reports on the medicinal properties of *A. clavennae*, such as cholagogue, stomachic and antihelminthic activities, originated in the beginning of the seventeenth century (Chivenna, 1609). In a recent report on antibacterial activity of *A. clavennae* essential oil from Croatia (Skočibušić et al., 2004) the use of the species in traditional medicine to treat various disorders is also quoted.

Previous phytochemical investigations of *A. clavennae* involved polyacetylenes in the roots (Bohlmann and Jastrow, 1962) and flavonoids in the leaves (Franzén, 1988; Valant-Vetschera and Wollenweber, 2001). Recently, Stojanović et al. (2005b) identified in the aerial parts of *A. clavennae* from Macedonia *n*-alkanes, fatty acids, various monoterpenes, guaianolides (rupicolin A and B, 1-deoxy-1α-peroxy-rupicolin A and B) and flavonoids (apigenin and centaureidin). This paper also reports high inhibitory effects of the extract against medically important pathogens, *Staphylococcus aureus*, *Escherichia coli*, *Candida albicans* and *Aspergillus niger*. In addition, the essential oil composition of the aerial parts of *A. clavennae* from different localities and examination of their antimicrobial activities in some cases are reported elsewhere (Chalchat et al., 2000; Bezić et al., 2003; Skočibušić et al., 2004; Stojanović et al., 2005a). We now report the examination of the aerial parts of *A. clavennae* L. from the Komovi mountain (Montenegro).

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2. Results and discussion

Repeated silica gel column chromatography and preparative TLC of the crude extract of the air-dried aerial parts of *A. clavennae*, obtained following the usual procedure for isolation of sesquiterpene lactones (Bohlmann et al., 1984), yielded eight guaianolides (**1**–**8**), three bisabolenes (**9**–**11**), flavonols, centaureidin (**12**) (Long et al., 2003), penduletin (**13**) (Sy and Brown, 1998), casticin (**14**) (Timmermann et al., 1979) and artemetin (**15**) (Sy and Brown, 1998), the lignan sesamin (**16**) (Meselhy, 2003) and the coumarin isofraxidin (**17**) (Banthorpe and Brown, 1989). Among the guaianolides, artecanin (**1**) (Hewllet et al., 1996), epoxychlorohydrin **3** (Wagner et al., 1988), anadalucin (**6**) (Aguilar et al., 1988) and apressin (**8**) (Tsankova et al., 1981) were known. Lactone **3** has also been reported recently, together with its 8α-hydroxy analogue, in the aerial parts of *A. depressa* (Trifunović et al., 2005). The bisabolenes indicumenone (**9**) (Mladenova et al., 1987) and chrysetunone (**11**) (Mladenova et al., 1988) were found previously in *Chrysanthemum indicum* from Bulgaria. The isolation of **12** and **13** is not unexpected since these flavonols were detected in the majority of studied populations of *A. clavennae* from alpine central Europe, Croatia, Kosovo and Macedonia (Franzén, 1988; Valant-Vetschera and Wollenweber, 2001; Stojanović et al., 2005b). However, **14** and **15** have not been previously isolated from this species.

The similarity of most of the NMR data of **2** ($C_{17}H_{20}O_7$) (Table 1) to those of the co-occurring artecanin (**1**, Hewllet et al., 1996) indicated a close relationship. Lactone **2** exhibited an acetoxy group ($\nu_{OAc} = 1742\ cm^{-1}$; δ 2.18 *s*, 3H) attached to C-9, as evidenced by a low-field *dd* of H-9β (δ 4.85, $J_1 = 2.0$, $J_2 = 5.8$ Hz), coupled to H_2-8. A 9α-position of the acetoxy group was indicated by the NOE between H-9 and H_3-14, as well as the observed downfield shift of H-7 in **2** (δ 3.52) compared with **1** (δ 3.30), typical for the anisotropic effect of *syn*-γ-positioned OAc group. Thus, compound **2** was assigned as 9α-acetoxyartecanin.

According to $[MH]^+$ and $[MH + 2]^+$ ions, *m/z* 315 and 317 (3:1), observed in CIMS, lactone **4** exhibited the same molecular formula ($C_{15}H_{19}O_5Cl$) as the known chlorohydrin **3**. The overall appearance of the ^1H NMR spectrum (Table 1), very similar to that of **3**, as well as HMBC correlations (Table 2), indicated the same gross structure and a diastereomeric relationship between **3** and **4**. A strong NOE between H_3-15 and H-5 and a much weaker one between H_3-15 and H-3 revealed *cis*-H_3-15/H-5 and *trans*-H_3-15/H-3 relationships, corresponding to 4α-Me (4β-OH) and 3α-Cl configuration. Unfortunately, an additional cross-peak between H-3 and H-2 did not shed light on the relative configuration of the 1,2-epoxy group. Inspection of molecular models for both 1α,2α- and 1β,2β-epoxides indicated that in both instances the distances between H-2 and H-3β were about the same. A proton of **4** vicinal to the

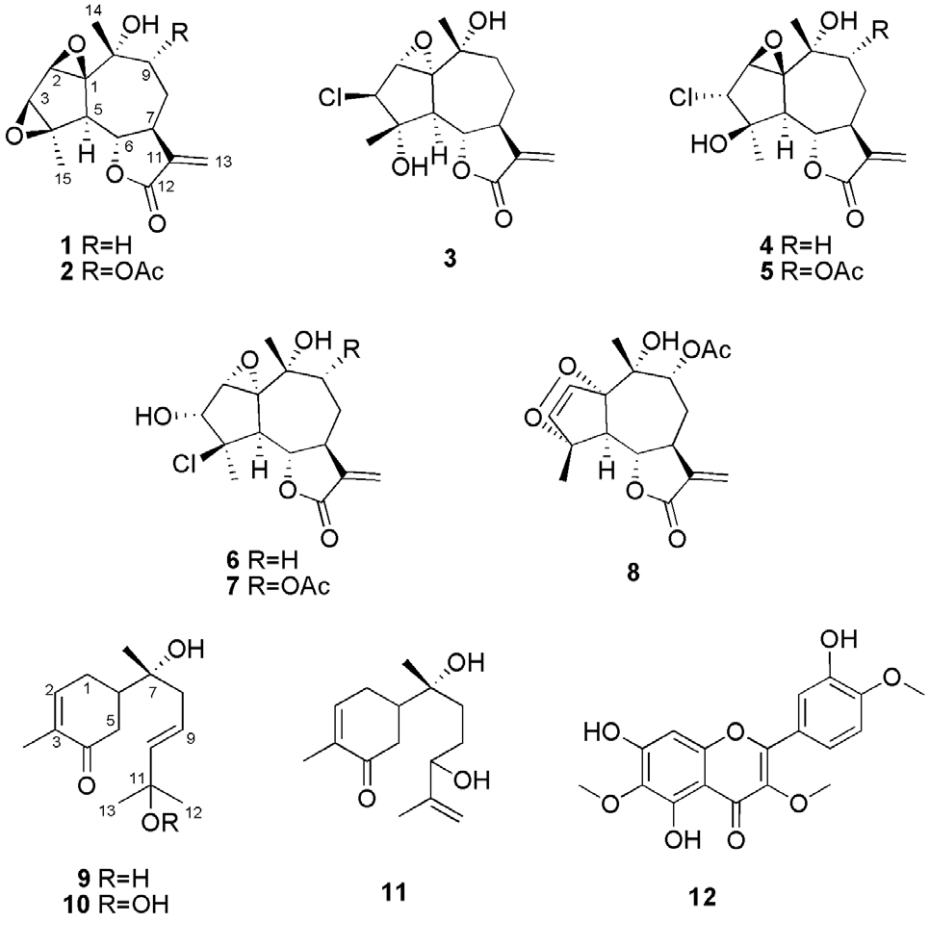

Table 1
^{1}H NMR data of compounds **1**, **2**, **4**, **5** and **7** (200 MHz, $CDCl_3$, TMS as internal standard)[a]

H	1	2	4	5	7
2	3.56 *d* (1.0)	3.60 *d* (1.2)	3.86 *d* (0.8)	3.92 *d* (0.8)	3.91 *d* (3.4)
3	3.30 *d* (1.0)[b]	3.34 *d* (1.2)	4.10 *d* (0.8)	4.11 *d* (0.8)	4.18 *dd* (3.4, 10.2)
5	2.87 *d* (11.0)	2.94 *d* (10.8)	2.81 *d* (11.0)	2.87 *d* (10.9)	2.59 *d* (10.9)
6	4.10 *dd* (10.6, 11.0)	4.04 *dd* (10.4, 10.8)	4.38 *dd* (9.6, 11.0)	4.35 *dd* (10.2, 10.9)	4.59 *dd* (10.0, 10.9)
7	3.30 *m*[b]	3.52 *m*	3.58 *m*	3.75 *m*	3.07 *m*
8α	2.05 *m*	~2.15 *m*[b]	~2.32 *m*	~2.22 *m*[b]	2.51 *m*
8β	1.67 *m*	2.08 *m*	1.62 *m*	~2.22 *m*[b]	1.80 *m*
9α	1.75 *m*		~1.91 *m*[b]		
9β	1.90 *m*	4.85 *dd* (2.0, 5.8)	~1.91 *m*[b]	4.97 *t* (7.3)	5.11 *dd* (1.6, 5.2)
13a	6.20 *d* (3.4)	6.22 *d* (3.4)	6.19 *d* (3.6)	6.22 *d* (3.6)	6.28 *d* (3.6)
13b	5.44 *d* (3.2)	5.41 *d* (3.0)	5.46 *d* (3.4)	5.46 *d* (3.3)	5.54 *d* (3.2)
14	1.14 *s*	1.08 *s*	1.23 *s*	1.19 *s*	1.42 *d* (1.2)
15	1.56 *s*	1.57 *s*	1.57 *s*	1.56 *d* (1.1)	1.79 *s*
OAc		2.18 *s*[b]		2.15 *s*	2.18 *s*
10-OH	[c]	2.65 *s*	[c]	[c]	3.07 *d* (1.2)
4-OH			3.22 *br s*	3.06 *d* (1.1)	
3-OH					2.49 *d* (10.2)

[a] Chemical shifts, multiplicity and coupling constants (*J*, Hz) were assigned by means of characteristic chemical shifts, coupling patterns and 2D NMR techniques.
[b] Overlapped signals.
[c] Obscured signal.

Table 2
^{13}C NMR chemical shifts of lactones **2**, **4**, **5** and **7** in $CDCl_3$

C	2[a]		4[b]		5[a]	7[b]	
	δ_C	HMBC	δ_C	HMBC	δ_C	δ_C	HMBC
1	75.7	H-3,14	73.0	H-3,9α,14	74.2[c]	74.4	H-5,14
2	56.5	H-3	63.5	H-3	62.9	62.2	
3	57.2	H-2,15	64.0		63.4	77.8	H-2,15
4	71.1	H-2,15	80.0	H-2,3,15	78.2	86.7	H-2,15
5	42.3		50.0	H-3,15	49.2	58.0	H-15
6	82.1	H-5,7,8α,8β	78.5	H-5	79.6	79.4	H-5
7	40.7	H-9,13a,13b	43.5	H-8β,9α,13a,13b	41.0	41.1	H-13a
8	31.0		22.5		30.8	28.0	
9	73.8	H-14,8α	33.5	H-14	73.6[c]	78.6	H-14
10	72.4	H-14	72.0	H-14	71.4[c]	71.7	H-14
11	138.5	H-7,13a	140.5	H-13a	139.1	[d]	
12	169.5[c]	H-13a,13b	170.5	H-13a,13b	169.5[e]	169.2	H-13a,13b
13	119.1		119.0		119.6	120.6	
14	24.6		28.0		22.8[f]	23.0	
15	19.4		24.0		23.9[f]	24.7	
OAc	170.0[c]	C*H*$_3$(OAc)			169.9[e]	170.9	C*H*$_3$(OAc)
	21.2				21.0[f]	21.1	

[a] Measured at 50 MHz.
[b] Detected via HSQC and HMBC (at 500 MHz for ^{1}H).
[c,e,f] The assignments can be interchanged.
[d] Not observed.

epoxide (H-5) exhibited (δ 2.81) a downfield shift compared with the co-occurring 1α,2α-epoxy-3α-hydroxy-4β-chloroguaianolides andalucin (**6**, δ 2.52) and 9α-acetoxyanadalucin (**7**, δ 2.59) (vide infra), whereas the C-5 resonance of **4** occurred at higher field (δ 50.0) than in **7** (δ 58.0). A similar trend of the ^{1}H and ^{13}C chemical shifts of C(5)H moiety has been observed previously in the diastereomeric 1α,2α:3α,4α and 1β,2β:3β,4β-diepoxyguaianolides canin and artecanin (**1**) (Hewllet et al., 1996). This indicated that **4** had the opposite (1β,2β) epoxy configuration, which was also supported by different couplings H-2/H-3 in **4** ($J_{2,3} = 0.8$ Hz) and in **6** and **7** ($J_{2,3} = 3.4$ Hz).

In the co-occurring lactone **5** ($C_{17}H_{21}O_7Cl$) the chemical shifts of H-5 and C-5 (δ_H 2.87/δ_C 49.2) were similar to those of **4**. Apart from this, signals of the majority of the remaining protons (i.e., H-2, H-3, H-6, H_3-14 and H_3-15) in **5** were rather similar to those of **4** (Table 1), thus indicating the same constitutional and stereochemical arrangement, i.e., 1β,2β-epoxy-3α-chloro-4β,10α-dihydroxy. Lactone **5** exhibited an acetoxy group (δ_H 2.15 *s*, 3H; δ_C 169.5–169.9),

attached to C-9, as evidenced by one-proton triplet of H-9β (δ 4.97, $J = 7.3$ Hz), coupled to H_2-8. The 9α-OAc position was also supported by a downfield shift ($\Delta\delta = 0.17$ ppm) of H-7 in **5** in comparison to that in **4**, effected by the paramagnetic effect of *syn*-γ-positioned OAc.

Chlorohydrin **7** ($C_{17}H_{21}O_7Cl$) exhibited the same basic structural and stereochemical features as the co-occurring guaianolide andalucin (**6**) (Aguilar et al., 1988). The ^{1}H NMR spectral data (Table 1; Aguilar et al., 1988), the downfield shift of H_3-15 ($\Delta\delta$ ca. 0.22 ppm), also observed in **6**, compared with 4-hydroxy lactones **3**–**5** and a strong vicinal coupling between 3-OH (δ 2.49 *d*, $J = 10.2$ Hz) and H-3 (δ 4.18 *dd*, $J_{3,2} = 3.4$, $J_{3,OH} = 10.2$ Hz) were in accordance with a 3-hydroxy, 4-chloro substitution in **7**. As in **4**, H_3-15 exhibited a strong NOE to H-5 and a weaker one to H-3 in accordance with a 3α-OH,4β-Cl pattern. The ^{1}H and ^{13}C chemical shifts of C(5)H moiety, mentioned above, are also indicative of the five-membered ring stereochemistry. Lactone **7** contained an acetoxy group (δ 2.18 *s*, 3H) at the 9α-position, as evidenced by one-proton *dd* (δ 5.11, $J_1 = 1.6$, $J_2 = 5.2$ Hz), assigned as H-9β according to scalar couplings with H_2-8 (observed in COSY) and NOE between H-9 and H_3-14. The downfield shift of H-7 ($\Delta\delta$ 0.32 ppm) compared with H-7 of **6**, due to the *syn*-γ-positioned 9α-OAc group, with the HMBC correlations of **7** (see Table 2) are in accordance with the proposed structure.

Chlorohydrins **3**–**7** were most probably formed by nucleophilic attack of chloride ion on C-3 (**3**–**5**) or C-4 (**6** and **7**) in the corresponding bis-1,2:3,4-epoxides. In the case of **4** and **5** this is also supported by the co-occurrence of their possible precursors, artecanin (**1**) and 9α-acetoxyartecanin (**2**), respectively. Chlorohydrin **7** might be formed from the co-occurring *endo*-peroxide **8** which, via thermally induced stereospecific cyclopentene *endo*-peroxide–bis-epoxide rearrangement, might form the corresponding 1α,2α:3α,4α-diepoxide (Hewllet et al., 1996) which, however, was not detected in our extract. Nucleophilic attack of the chloride ion on C-4 of this bis-α-epoxide might result in formation of **7**.

Such chlorohydrins might be artefacts, formed during the isolation procedure, where chlorinated solvents might serve as the source of Cl^- (Hamburger et al., 1993; Engvild, 1986). Since in our case the chlorinated solvents ($CHCl_3$ and CH_2Cl_2) were used for chromatographic separations, we repeated the isolation procedure in the absence of such solvents. Unfortunately, since the original sample (collection #1, 1998) was completely used up in the first experiment, the repeat examination was carried out on a new collection (#2, 1999) from the same locality. This experiment afforded only two guaianolides, i.e., chlorohydrin **4** and the endoperoxide apressin (**8**). Thus, it seems that at least chlorohydrin **4** is not an artefact. It should also be noted that related chlorohydrin guaianolides were isolated previously from only a few *Achillea* species, such as *A. biebersteinii* and *A. santolina* (Yusupov et al., 1979), *A. clusiana* (Todorova et al., 1999), *A. depressa* (Trifunović et al., 2005) as well as *A. ligustica* (Ahmed et al., 2003).

The CI mass spectrum of **10** did not show the molecular ion. However, ^{1}H and ^{13}C NMR data (Table 3), closely similar to those of the co-occurring indicumenone (**9**) (Mladenova et al., 1987), as well as 2D NMR methods, indicated the molecular formula $C_{15}H_{24}O_4$. The presence of a hydroperoxide was evident from the occurrence of the typical low-field one-proton signal (δ 7.74). The chemical shift of C-11 (δ_C 82.5), assigned by its HMBC correlations to H_3-12 and H_3-13 (see Table 3), was consistent with attachment of the hydroperoxide to this carbon (Sy and Brown, 1997).

Related bisabolene derivatives have been detected previously in the aerial parts of two members of the genus *Achillea*, *A. odorata* (Barrero et al., 1990) and *A. cretica* (Bruno et al., 1996).

The antiproliferative action of the compounds **2**, **8**, **9** and **12** which were isolated in sufficient quantities to allow bioassays, were tested against malignant cell lines (human cervix carcinoma HeLa cells, human myelogenous leukemia K562 and human melanoma Fem-X cells) and to normal human cells (PBMC) with cisplatin as a positive control (Table 4).

Lactones **2** and **8** exhibited significant cytotoxic effects against all tested human cancer cells, especially to Fem-X, confirming the importance of the α-methylene-γ-lactone moiety for the cytotoxicity of γ-lactones. The very pronounced cytotoxicity of **8** to K562 cells ($IC_{50} = 4.44$ μM) might be especially interesting for further study this endoperoxide as a potential human myelogenous leukemia antiproliferation agent. Indicumenone (**9**) showed a moderate cytotoxicity to all tested cell lines, with IC_{50} in the range from 43.50 to 52.53 μM. The extraordinary activity of cyto-

Table 3
NMR data of **9** and **10** in $CDCl_3$

C/H	**9**	**10**		
	δ_C[a]	δ_C[b]	δ_H[c]	HMBC
1	27.2	27.5	2.35 *brd* (10.5) ~2.24 *m*[e]	
2	144.9	145.0	6.75 *brd* (~5.5)	H-15
3	135.3	136.0		H-15
4	200.4	[d]		
5	38.8	39.0	2.66 *brd* (~16) ~2.24 *m*[e]	
6	44.1	43.0	~2.24 *m*[e]	H-2,5,14
7	72.8	73.0		H-14
8	42.3	45.0	~2.24 *m*[e] 2.13 *m*	H-14
9	121.0	126.5	5.71 *m*[f]	
10	143.0	138.5	5.71 *m*[f]	H-12,13
11	70.7	82.5		H-12,13
12	29.8	24.5	1.34 *s*	
13	29.7	24.5	1.34 *s*	
14	23.9	24.0	1.18 *s*	
15	15.6	15.9	1.78 *s*	
OOH			7.74 *br s*	

[a] Measured at 50 MHz.
[b] Detected via HSQC and HMBC (at 500 MHz for ^{1}H).
[c] Measured at 500 MHz.
[d] Not observed.
[e,f] Overlapped signals.

Table 4
In vitro antiproliferative activity of compounds **2**, **8**, **9** and **12**

Compound	IC_{50} (μM)				
	HeLa ($n = 3$)	Fem-X ($n = 2$)	K562 ($n = 2$)	PBMC ($n = 2$)	PBMC + PHA ($n = 2$)
2	7.49 ± 1.32	5.28 ± 1.16	9.84 ± 2.52	16.96 ± 1.35	11.86 ± 1.25
8	7.36 ± 1.98	5.27 ± 1.06	4.44 ± 0.76	15.89 ± 6.52	8.78 ± 0.71
9	44.46 ± 10.70	43.50 ± 12.43	52.53 ± 8.43	>100	71.65
12	2.55 ± 1.77	3.56 ± 1.47	5.37 ± 0.80	40.82 ± 2.31	35.93 ± 2.28
Cisplatin[a]	2.62 ($n = 3$)	3.85 ($n = 3$)	5.00 ($n = 1$)	>37 ($n = 1$)	22.92 ± 9.37 ($n = 4$)

[a] Cisplatin was used as a positive control.

toxic flavonol **12**, observed previously in the NCI 60-cell line in vitro screening panel (Beutler et al., 1993), was confirmed in our study. Centaureidin exerts its cytotoxicity through interference with tubulin polymerization into microtubules and inhibits [^{3}H]colchicine binding to tubulin (Beutler et al., 1998). The cytotoxic activity of all compounds was less pronounced on normal human peripheral blood mononuclear cells (PBMC) and increased when PBMC cells were stimulated for proliferation (PBMC + PHA) indicating the selectivity in the action of all tested compounds and their influence on dividing cells, in general.

3. Experimental

3.1. General

Optical rotations were measured on a Rudolph Research Analytical automatic polarimeter, Autopol IV. IR spectra were measured in the form of transparent films on a Perkin–Elmer FT-IR spectrometer 1725X. ^{1}H (200 MHz) and ^{13}C (50 MHz) NMR spectra were recorded on a Varian Gemini 2000 and 2D NMR on a Bruker DMX 500 spectrometer. Mass spectra were obtained on a Finnigan MAT 8230, BE DCI (150 eV, *iso*-butane). CC was carried out on silica gel 60 (0.063–0.200 mm, Merck). Silica gel G and silica gel F-254 were used for analytical (0.25 mm) and preparative (0.75 mm) TLC. Spots were detected under UV_{254} and by spraying with 50% H_2SO_4. Elemental analysis was performed on Vario EL *III* C,H,N,S-O elemental analyzer (Elementar).

3.2. Plant material

Aerial parts of *A. clavennae* were collected at the flowering stage in July 1998 (collection #1) and 1999 (collection #2) in Montenegro at Kučki Kom (the Komovi mountain, altitude ca. 2000 m), close to the Albanian border. A voucher specimen (AC00071998) is deposited in the BEOU-Herbarium, Institute of Botany, Faculty of Biology, Belgrade, Serbia and Montenegro.

3.3. Extraction and isolation

A crude extract (19.5 g) of air-dried aerial parts (520 g) was obtained by two successive extractions with freshly distilled solvents (4.5 l): petrol–Et_2O–MeOH (1:1:1) at room temperature, followed by a treatment with MeOH to remove long chain saturated hydrocarbons by the usual procedure (Bohlmann et al., 1984). The total crude extract was divided into 28 fractions (fractions 1–28) by silica gel CC, by starting the elution with petrol and gradually increasing the polarity, first by addition of Et_2O (up to 100%) and then MeOH (up to 30%). Lactones **1** (3.8 mg), **7** (4.1 mg), **8** (7.1 mg) and terpene **11** (4.7 mg) were isolated from combined 19 (petrol–Et_2O, 5:95) and 20 (Et_2O) by silica gel CC (toluene–EtOAc, 1:1) followed by preparative silica gel TLC (**1**: $CHCl_3$–MeOH, 95:5; **7**: CCl_4–MeOH, 92:8; **8**: CH_2Cl_2–MeOH, 97:3; **11**: CCl_4–Et_2O–MeOH, 50:45:5). Silica gel CC ($CHCl_3$–MeOH, 95:5) of 21 (Et_2O), followed by additional CC (C_6H_6–Et_2O–MeOH, 7:2:1) and preparative TLC with the same solvent system yielded lactone **2** (15.0 mg). Lactone **3** (4.0 mg) and **5** (2.8 mg) were isolated from the combined 14 (petrol–Et_2O, 3:7) and 15 (petrol–Et_2O, 25:75) after silica gel CC (toluene–EtOAc, 3:2). Lactone **4** (3.9 mg) was obtained from 12 (petrol–Et_2O, 35:65) by silica gel CC (C_6H_6–Et_2O–MeOH, 7:2:1), followed by preparative TLC with the same solvent system. Lactone **6** (1.9 mg) was isolated after CC (CH_2Cl_2–MeOH, 98:2) of 25 (Et_2O–MeOH, 8:2). Bisabolene **9** (7.4 mg), isolated from 26 (Et_2O–MeOH, 8:2), was purified by means of preparative TLC (CH_2Cl_2–MeOH, 99:1). Fraction 13 (petrol–Et_2O, 35:65) yielded bisabolene **10** (3.9 mg) and artemetin (**15**, 5.0 mg) after CC (CH_2Cl_2–MeOH, 97:3), followed by preparative TLC (CH_2Cl_2–MeOH, 99:1). Isofraxidin (**17**, 1.2 mg) and casticin (**14**, 0.6 mg) were obtained from 16 (petrol–Et_2O, 2:8) by CC (CH_2Cl_2–MeOH, 9:1). Sesamin (**16**, 0.9 mg) was isolated from 7 (petrol–Et_2O, 6:4) after preparative TLC (C_6H_6–EtOAc, 9:1). Penduletin (**13**, 4.0 mg) was isolated from 17 (petrol–Et_2O, 15:85) by silica gel CC (toluene–Et_2O–MeOH, 7:2:1) and further purified by preparative TLC (petrol–Et_2O–MeOH, 5:4:1). Centaureidin (**12**, 31.0 mg) was obtained from 18 (petrol–Et_2O, 1:9) by silica gel CC with toluene–Et_2O–MeOH (65:20:15).

3.3.1. 9α-Acetoxyartecanin (**2**)

Yellowish gum, $[\alpha]_D^{22}$ −10.8° (CH_2Cl_2; *c* 0.222); IR ν_{max}^{film} cm^{-1}: 3465 (OH), 1767 (γ-lactone), 1742 (OAc); ^{1}H and ^{13}C NMR (see Tables 1 and 2, respectively); CIMS (*iso*-butane, probe), 150 eV, *m/z* (rel. int.): 337 $[MH]^+$ (100); Elemental analysis: Found: C, 60.44; H, 6.28. $C_{17}H_{20}O_7$ requires: C, 60.71; H 5.99%.

*3.3.2. 3α-Chloro-4β,10α-dihydroxy-1β,2β-epoxy-5α,7αH-guai-11(13)-en-12,6α-olide (**4**)*

Colorless gum, $[\alpha]_D^{22}$ −24.8° (CH_2Cl_2–MeOH (95:5); *c* 0.109); IR ν_{max}^{film} cm^{-1}: 3487 (OH), 1752 (γ-lactone); ^1H and ^{13}C NMR (see Tables 1 and 2, respectively); CIMS (*iso*-butane, probe), 150 eV, *m/z* (rel. int.): 317 $[MH+2]^+$ (33), 315 $[MH]^+$ (100), 299 $[(MH+2)-H_2O]^+$ (~2.5), 297 $[MH-H_2O]^+$ (8); Elemental analysis: Found: C, 57.52; H, 6.19. $C_{15}H_{19}ClO_5$ requires: C, 57.24; H, 6.08%.

*3.3.3. 3α-Chloro-9α-acetoxy-4β,10α-dihydroxy-1β,2β-epoxy-5α,7αH-guai-11(13)-en-12,6α-olide (**5**)*

Colorless oil; IR ν_{max}^{film} cm^{-1}: 3453 (OH), 1767 (γ-lactone), 1747 (OAc); ^1H and ^{13}C NMR (see Tables 1 and 2, respectively); CIMS (*iso*-butane, probe), 150 eV, *m/z* (rel. int.): 375 $[MH+2]^+$ (33), 373 $[MH]^+$ (100). ^1H and ^{13}C NMR (see Tables 1 and 2, respectively). Unfortunately, lactone **5** decomposed in the NMR tube prior to accurate mass measurements and determination of the optical rotation.

*3.3.4. 9α-Acetoxyandalucin (**7**)*

Colorless gum, $[\alpha]_D^{22}$ −7.7° (CH_2Cl_2; *c* 0.129); IR ν_{max}^{film} cm^{-1}: 3457 (OH), 1769 (γ-lactone), 1739 (OAc); ^1H and ^{13}C NMR (see Tables 1 and 2, respectively); CIMS (*iso*-butane, probe), 150 eV, *m/z* (rel. int.): 375 $[MH+2]^+$ (33), 373 $[MH]^+$ (100), 357 $[(MH+2)-H_2O]^+$ (~4.5), 355 $[MH-H_2O]^+$ (13); Elemental analysis: Found: C, 54.45; H, 5.90%. $C_{17}H_{21}ClO_7$ requires: C, 54.77; H, 5.68%.

*3.3.5. (9E)-4-oxo-7-hydroxy-11-hydroperoxy-bisabola-2,9-diene (**10**)*

Colorless oil, $[\alpha]_D^{22}$ −6.5° (CH_2Cl_2; *c* 0.139); IR ν_{max}^{film} cm^{-1}: 3397 (OH), 1711 (C=O), 1657 (C=C); ^1H and ^{13}C NMR (see Table 3); CIMS (*iso*-butane, probe), 150 eV, *m/z* (rel. int.): 235 $[MH-H_2O_2]^+$ (100), 153 (62.5); Elemental analysis: Found: C, 67.29; H, 9.23. $C_{15}H_{24}O_4$ requires: C, 67.14; H, 9.01%.

3.4. Antiproliferative activity

Stock solutions of the compounds were prepared in DMSO at a concentration of 10 mM and afterwards diluted with a nutrient medium (RPMI 1640), supplemented with L-glutamine (3 mM), streptomycin (100 μg/ml) and penicillin (100 IU/ml), 10% heat inactivated (56 °C) fetal bovine serum (FBS) and 25 mM Hepes, adjusted to pH 7.2 by bicarbonate solution and applied to target cells to various final concentration ranging from 0 to 100 μM. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide) was dissolved (5 mg/ml) in phosphate buffer saline, pH 7.2, and filtered through Millipore filter, 0.22 μm, before use. RPMI 1640 cells culture medium, FBS and MTT were Sigma Chemicals products.

3.4.1. Cell culture

Human cervix carcinoma HeLa cells and human malignant melanoma Fem-X cells were cultured as a monolayers in the nutrient medium. Human myelogenous leukemia K562 cells were grown as a suspension culture in the same nutrient medium. The cells were grown at 37 °C in 5% CO_2 and humidified air atmosphere. Peripheral blood mononuclear cells (PBMC) were separated from whole heparinized blood of healthy volunteers by Lymphoprep™ gradient centrifugation. Interface cells, washed three times with Haemaccel (aqueous solution supplemented with 145 mM Na^+, 5.1 mM K^+, 6.2 mM Ca^{2+}, 145 mM Cl^- and 35 g/l gelatin polymers, pH 7.4) were counted and resuspended in the nutrient medium.

3.4.2. Treatment of HeLa, Fem-X, K562 and PBMC cells

HeLa or Fem-X cells were seeded (2000 cells per well) into 96-well microtiter plates and 20 h later, after the cell adherence, five different concentrations of investigated compounds were added to the wells. Leukemia K562 cells were seeded (3000 cells per well) in the nutrient medium. PBMC were seeded (150,000 cells per well) into the nutrient medium or in the nutrient medium enriched with (5 μg/ml) phytohaemaglutinin (PHA) in 96-well microtiter plates. Two hours later, investigated compounds were added to the wells, to five final concentrations. Only nutrient medium was added to the cells in the control wells with correspondent concentrations of DMSO. The nutrient medium with corresponding concentrations of compounds, but void of cells, was used as the blank.

3.4.3. Determination of cell survival

HeLa and Fem-X cell survival was determined indirectly by measuring total cellular protein by the Kenacid Blue R (KBR) dye binding method according to Clothier (1995). Inhibition of growth PBMC and K562 cell was determined by MTT test (Ohno and Abe, 1991). To get cell survival (%), *A* (at 570 nm) of a sample with cells grown in the presence of various concentrations of compounds was divided with control absorbance, A_c (the *A* of control cells grown only in nutrient medium), and multiplied by 100. It was implied that *A* of the blank was always subtracted from *A* of a corresponding sample with target cells. IC_{50} concentration was defined as the concentration of a drug which inhibits cell survival by 50%, compared with a vehicle-treated control.

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