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Chemotypes in *Achillea collina* based on sesquiterpene lactone profile

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Abstract

The lactone profile of six origins of *Achillea collina* growing in Bulgaria was studied and significant variability was observed. The reasons for the differences in the lactone composition are discussed. Twenty-five components in total were isolated and identified, while the presence of ten lactones was proved by intensive TLC analysis in comparison with reference compounds. The structures of the components **17**, **20**, **25**–**30** were established by spectroscopic methods. The structure of **7**, a cyclization product of **6**, was also discussed. The anti-inflammatory activity of some extracts, fractions and individual compounds was tested in vitro by determining the inhibitory effects on induced human neutrophils.

Keywords: *Achillea collina*; Guaianolides; Germacranolides; Bisabolanes; Chemotaxonomy; Anti-inflammatory activity

1. Introduction

Achillea millefolium complex is represented by six species in Bulgaria – *A. millefolium*, *A. distans*, *A. setacea*, *A. pannonica*, *A. asplenifolia* and *A. collina* (Saukel et al., 2003). The study of *A. millefolium* group in Bulgaria showed that the tetraploid *A. collina* J. Becker ex Reichenb is the most distributed species in the country, from the see level up to 1800 m altitude (Vitkova et al., 2005). Often two or more species have been observed in the same habitat. Probably, this makes the hybridization easier which phenotypic manifestation are the morphological spectra of various transitional forms. Information about hybridization between the species in *A. millefolium* group as well as between *A. collina* and *A. clypeolata*, *A. crithmifolia* has been reported (Nedelcheva et al., 1998; Nejati, 2002, Nejati et al., 2001; Saukel et al., 2003). A certain phytochemical diversity could be also expected.

Previous investigations of sesquiterpene lactones in *A. collina* showed that some European populations have similar azulenogenic profile (Kubelka et al., 1999; Trendafilova et al., 2006). Only one taxon originating from Bulgaria was published to contain eudesmanolides and germacranolides, and no azulenogenic guaianolide (Mustakerova et al., 2002). It should be emphasized that proazulenes, responsible for anti-inflammatory activity, are required components of Millefolii herba according to the European Phamacopoeia.

In continuation of our study on terpenoids in the species of *A. millefolium* group, growing in Bulgaria we describe now intraspecific variability of *A. collina*. On the basis of comparative study of the lactone profile of six samples were found taxa rich of azulenogenic lactones, free of proazulenes as well as free of sesquiterpene lactones. Anti-inflammatory activity of some extracts, fractions and individual components was tested.

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2. Results and discussion

Flower heads of *A. collina* from six populations **A1**–**A6** were extracted and worked up as described in Experimental part to give crude lactone fractions **L1**–**L6**, respectively. Fractions **L1**–**L4** were purified by column chromatography and preparative TLC to give individual compounds.

Thus, **L1** afforded four eudesmanolides: 11α,13-dihydrosantamarin (**1**) (Sanz et al., 1990), 11-*epi*-artesin (**2**) (Marco, 1989), 11α,13-dihydroreynosin (**3**) (Sanz et al., 1990), 1β-hydroxy-11-*epi*-colartrin (**4**) (Sanz et al., 1990) and two germacranolides: artabin (**5**) (Akhmedov et al., 1970) and 1β-hydroxy-6βH,7αH,11αH-germacra-4(5), 10(15)-dien-6,12-olide (11-*epi*-gallicin, **6**) (Gordon et al., 1981). Surprisingly, new well-defined signals in ^{1}H NMR spectrum of **6** were observed after 3 days keeping the sample in $CDCl_3$. The initial compound **6** was completely converted after 10 days into a new component (**7**), the NMR data (Tables 1 and 3) of which correlated well with an eudesmanolide structure. Compound **7** did not possess a hydroxyl group although its MS exhibited molecular formula ($C_{15}H_{22}O_3$) containing three oxygen atoms, two of which belonging to the lactone ring. The third oxygen formed an epoxide group, as it was shown by ^{1}H NMR spectrum. Thus, the signal for carbinolic proton at δ 4.59 in **6** was replaced by doublet at δ 3.97 in **7**. Further, the COSY experiment, vicinal coupling constants and comparison of NMR data of **7** with those of its C-11 epimer confirmed the structure (Marco et al., 1995).

Table 1
^{1}H NMR (400 MHz) data of **7**, **17**, **19** and **20** in $CDCl_3$

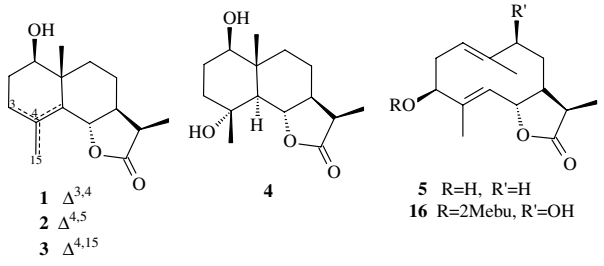
H	7	17	19	20
1	3.97 *d* (5.7)			
2	1.95–1.75	5.61 *d* (3.7)	4.77 *brt* (6.0)	4.84 *m*
3	1.65–1.45	3.55 *d* (12.5)	(α) 1.99 *dd* (6.0, 13.7)	(α) 2.40 *dd* (7.1, 13.6)
3′	1.45–1.35	4.01 *d* (12.5)	(β) 2.38 *dd* (5.1, 13.7)	(β) 1.96 *dd* (4.8, 13.6)
5	1.65–1.45			
6	4.26 *dd* (11.6, 9.5)	6.12 *d* (10.6)	5.30 *dd* (1.8, 10.4)	5.09 *dd* (2.4, 10.6)
7	1.90[a]	2.24 *q* (10.6)	2.19 *q* (10.4)	2.65 *q* (10.6)
8	1.80–1.78	5.62 *ddd* (4.5, 10.6, 10.9)	5.50 *ddd* (5.0, 5.0, 10.4)	5.49 *ddd* (4.1, 5.1, 10.6)
9	1.65–1.45	1.81 *dd* (10.9, 13.6)	1.93 *dd* (5.0, 14.5)	1.92 *dd* (4.1, 15.0)
9′	1.65–1.45	2.12 *dd* (4.5, 13.6)	2.30 *dd* (5.0, 14.5)	2.22 *dd* (5.1, 15.0)
11	2.60 *quint* (7.2)	2.59 *dq* (6.8, 10.6)	2.60 *dq* (6.9, 10.5)	2.58 *dq* (6.8, 10.6)
13	1.19 *d* (7.2)	1.25 *d* (6.8)	1.24 *d* (6.9)	1.29 *d* (6.8)
14	1.12 *s*	1.33 *s*	1.57 *s*	1.72 *s*
15	1.53 *s*	1.66 *s*	1.48 *s*	1.55 *s*
R		6.15 *qq* (1.2, 7.0) 2.02 *dq* (1.2, 7.0) 1.89 *dq* (1.2, 1.2)	6.14 *qq* (1.2, 7.1) 2.01 *dq* (1.2, 7.1) 1.89 *dq* (1.2, 1.2)	6.14 *qq* (1.2, 7.1) 2.01 *dq* (1.2, 7.1) 1.89 *dq* (1.2, 1.2)
OMe		3.32 *s*		
OH		2.68 *d* (3.7)		

[a] Overlapped signals.

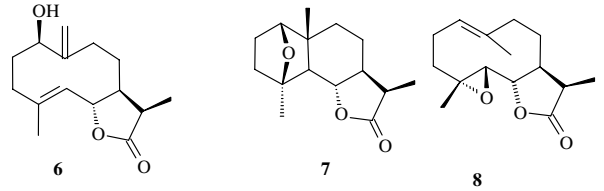
Compound **7**, a product of cyclization of **6** confirmed once again the position of hydroxyl group in **6** at C-1, and rejected the other possibility – at C-9. On the other hand, ^{1}H NMR data of **6** coincided with those published for herbolide H (Segal et al., 1985), which contain a hydroxyl group at C-9 and α-oriented methyl group at C-11. The present study gave evidence to correct the structure of herbolide H to 11-*epi*-gallicin.

From fraction **L2** were isolated 11α,13-dihydroparthenolide (**8**) (Galal et al., 1999), matricarin (**9**) (Ahmed et al., 2003), desacetylmatricarin (**10**) (Ahmed et al., 2003) and 11,13-dehydrodesacetylmatricarin (**11**) (Ohno et al., 1980) in addition to the compounds found in **L1**. Compounds **9**–**11** have been recently described for the proazulene containing taxon of *A. collina* (Trendafilova et al., 2006).

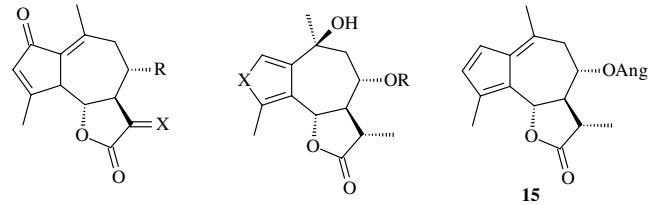
Fraction **L3** was found to contain mainly achillin (**12**) (White and Winter, 1963), 8α-hydroxyachillin (**13**) (White and Winter, 1963) and one germacranolide – artabin (**5**) (Ahmed et al., 2003), found also in **L1**. It is noteworthy that **L3** contained guaianolides of achillin series, while these found in **L2** were of matricarin type.



1 $\Delta^{3,4}$
2 $\Delta^{4,5}$
3 $\Delta^{4,15}$
4
5 R=H, R'=H
16 R=2Mebu, R'=OH



6 **7** **8**



9 R=OAc, X=H,αCH$_3$
10 R=OH, X=H,αCH$_3$
11 R=OH, X=CH$_2$
12 R=OAc, X=H,βCH$_3$
13 R=OH, X=H,βCH$_3$
31 R=H, X=CH$_2$

14 R=Ang, X=CH$_2$
32 R=Tig, X=CH$_2$
33 R=Ac, X=CH$_2$
34 R=Ang, X=O
35 R=Tig, X=O
36 R=Ac, X=O

15

Fraction **L4** gave the known 8α-angeloyloxyartabsin (**14**) (Verzár-Petri et al., 1980; Schroeder et al., 1994), 8α-angeloyloxytannunolide (**15**) (Todorova et al., 2006),

3β-[2-methylbutyroyloxy]-9β-hydroxy-germacra-1(10),4-dienolide (**16**) (Yang et al., 2005), 8α-angeloxy-1β,2β,4β,5β-diepoxy-10β-hydroxy-6βH,7αH,11βH-12,6α-guaianolide (**21**) (Glasl et al., 2001), and the new sesquiterpenoids described below.

Compound **17** was isolated as colourless oil. Its molecular formula $C_{21}H_{30}O_8$ was deduced from HRMS (ESI) – *m/z* 474.1964 $[C_{21}H_{30}O_8+CH_3CN+Na]^+$. IR absorption bands at 1730 and 1760 cm^{-1} together with the signals for two carbonyl groups at δ 166.6 and δ 178.0 in ^{13}C NMR spectrum (Table 3) showed the presence of an esterified sesquiterpene lactone. The MS fragments at *m/z* 310 $[M-100]^+$, 292 $[310-18]^+$ and 274 $[292-18]^+$ indicated the loss of an unsaturated $C_5H_8O_2$ acid and two molecules of water. The acid residue was identified as angeloyl on the basis of the characteristic ^1H NMR signals. The combined use of ^1H NMR, ^{13}C NMR, ^1H–^1H COSY and HMQC allowed to distinguish C_6–C_9 spin system, including C_{11}–C_{13} moiety giving the structural fragment **B** (Fig. 1). Furthermore, the carbon resonances at δ 127.4 (*s*), δ 140.0 (*d*), δ 138.6 (*s*) and δ 139.2 (*s*) showed the presence of a tetrasubstituted double bond, besides a trisubstituted one from the angeloyl ester. In addition, ^1H NMR spectrum (Table 1) displayed two signals at δ 2.68 *d* ($J = 3.7$ Hz) and δ 5.61 *d* ($J = 3.7$ Hz) ascribed to OH and a carbinolic proton, respectively, because the former doublet disappeared while the latter was transformed into a singlet after D_2O exchange. The ^1H–^1H COSY experiment showed interaction only between these two protons, which means that there was no neighbouring proton. Further, the methylene carbon at δ 66.9 (C-3) and two doublets at δ 3.55 and δ 4.01 with $J_{gem} = 12.5$ Hz revealed an isolated methylene group, whereas the three-proton singlets at δ 1.33 (H-14) and δ 1.66 (H-15) indicated the presence of two methyls, all of them attached to oxygen containing groups. The connectivity of the described structural units through quaternary carbons in partial structure **A** was achieved by the observed heteronuclear correlations in HMBC spectrum. Thus, the signal for sp^2 carbon atom at δ 139.2 (*s*) exhibited a long-range interaction with H-2 and H-14, while the carbon singlet at δ 138.6 correlated with H-3 and H-15. In this way the pointed signals were attributed to C-1 and C-5. The C-4 carbon signal at δ 70.6 was proved by the observed cross-peaks with H-3 and H-15. The correlation of C-4 with the three-proton singlet at δ 3.32 indicated the location of methoxy group at C-3. The coupling of structural fragments **A** and **B** followed from HMBC cross-peaks between C-5/H-7, C-1/H-9 and C-4/H-6. The signal at δ 71.8 was assigned to C-10 because of the interaction with H-9 and H-15. The only logical connectivity between C-2 and C-4 was by ether-type linkage because one more oxygen has to be included in the structure, according to the molecular formula. An additional argument for this assumption was the chemical shift of C-2 (δ 88.8) and C-4 (δ 70.6). The relative stereochemistry at C-2, C-4 and C-10 followed from the NOEs between H-15/H-7, H-14/H-7, H-3/H-6 and H-2/H-14. Thus, the proposed structure of the lactone **17** was determined as a hemiacetal of 2,3-secoguaianolide.

The ^1H NMR spectra (Table 1) of compounds **19** and **20** were very similar. Significant differences were observed in the chemical shifts of H-3α, H-3β, H-6, H-7, and H-14, while the multiplicity of the corresponding signals was nearly identical. The relative stereochemistry of the discussed compounds was deduced from 2D NOESY spectra. Thus, the observed interaction between H-14 and H-7 confirmed their *sin*-α-disposition, while the cross-peaks between H-6/H-8/H-11 indicated their *sin*-β-disposition in both lactones **19** and **20**, i.e. they share a common configuration at the chiral centres in the seven-membered ring. The discussed structures differed in the stereochemistry at C-2 and C-4. Thus, the NOEs between H-15/H-2, H-15/H-3α, H-14/H-2 and H-2/H-3α in **19** were in agreement with their orientation to the α-face of the molecule. Further, NOEs between H-6/H-15, H-15/H-3β, H-15/H-2, H-2/H-3β were an evidence for their *sin*-β-disposition in **20**. Thus, the paramagnetic shift of H-3β in **19** and H-3α in **20** was caused by *sin*–oriented hydroxy groups at C-2 and C-4. These facts demonstrated that lactones **19** and **20** were C-2, C-4-diastereomers. On the other hand, it worth to be noted that ^1H NMR data reported for 8α-angeloxy-2α,4α,10β-trihydroxy-6βH,7αH,11βH-1(5)-guaien-12,6α-olide (Glasl et al., 2001) were almost identical with those of **19**, i.e. the configuration at C-2 and C-4 in the isolated previously compound has to be corrected.

In addition to the described above sesquiterpene lactones, six compounds (**25**–**30**) with bisabolane carbon

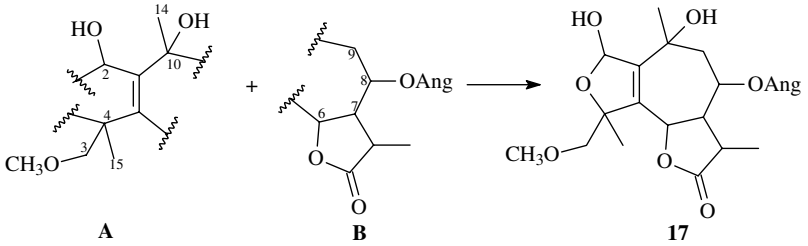


Fig. 1. Structural fragments of compound **17**.

Table 2
^{1}H NMR (400 MHz) data of compounds **25**–**30** in $CDCl_3$

H	25	26	27	28	29	30
1	–	3.99 *brs*	–	–	–	–
2	5.84 *q* (1.4)	5.56 *brs*	5.83 *d* (1.4)	5.82 *d* (1.3)	5.8 *q* (1.7)	5.81 *q* (1.5)
4	4.34 *brd* (3.0)	3.97 *brd* (3.9)	4.32 *brs*	4.30 *t* (3.9)	4.32 *t* (3.8)	4.30 *t* (3.4)
5	2.10–1.90	1.45 *ddd* (13.5, 13.5, 3.9)	2.10–2.00	2.08–1.98	2.05[a]	1.95 *m*
5′	2.10–1.90	1.71 *ddd* (13.5, 2.0, 2.0)	2.10–2.00	2.08–1.98	2.05[a]	2.05 *m*
6	2.58 *ddd* (9.9, 5.5, 4.5)	1.66 *m*	2.60 *ddd* (10.2, 5.1, 5.1)	2.60 *ddd* (9.2, 7.2, 4.1)	2.58 *ddd* (9.9, 4.8, 4.5)	2.76 *ddd* (12.3, 3.7, 3.7)
7	2.30 *m*	2.00 *m*	2.35 *m*	2.32 *m*	2.26 *m*	2.50 *m*
8	2.10–2.00	2.10–1.90	2.10–1.90	1.95–2.15	2.12–2.02	2.05 *ddd* (13.5, 5.1, 2.7)
8′	2.10–2.00	2.10–1.90	2.10–1.90	1.95–2.15	2.12–2.02	1.90 *dt* (13.5, 3.7)
9	2.15–2.10	2.10–2.05	2.15–1.90	2.08–1.98	1.45–1.65	5.58 *m*
10	5.11 *tq* (7.2, 1.4)	5.12 *tq* (7.2, 1.4)	6.48 *tq* (7.2, 1.4)	5.43 *tq* (6.5, 1.6)	4.29 *t* (6.8)	5.58 *m*
12	1.61 *q* (1.4)		1.74 *s*	1.65 *s*	1.72 *t* (1.3)	1.35 *s*
13	1.69 *q* (1.4)	1.67 *s*	9.38 *s*	3.97 *s* (2H)	5.00 *s* (2H)	1.25 *s*
14	2.06 *d* (1.4)	1.83 *dd* (1.3)	2.04 *brs*	2.02 *d* (1.3)	2.03 *brs*	2.00 (1.5)
15	0.83 *d* (6.8)	0.84 *d* (6.8)	0.87 *d* (6.8)	0.80 (6.8)	0.82 *d* (6.8)	0.82 *d* (6.8)
OOH						8.70 *s*

[a] Overlapped signals.

skeleton were isolated and identified. The spectral data of **25** (Tables 2 and 3) were identical with those published for 4α-hydroxy-bisabol-1-one, isolated from *Chrysothamnus nauseusus* (Bohlmann et al., 1979). It should be noted that the two compounds had different specific rotation. 4α-Hydroxy-bisabol-1-one exhibited a positive value of optical rotation, while this parameter showed negative value for compound **25** due to the presence of two stereoisomers. Unfortunately, the spectral data are insufficient evidence for determination of stereochemistry in this type of sesquiterpenoids. According to ^{1}H and ^{13}C NMR spectra of the isolated components (Tables 2 and 3), compound **26** was 1-hydroxy derivative of **25**, while **27**–**30** differed from **25** in the substituents in the side chain (C-9/C-13). Thus, one proton singlet at δ 9.38 in the ^{1}H NMR of **27** and a broad singlet for two protons at δ 3.97 in the ^{1}H NMR of **28** were evidences for the replacement of C-12 methyl (in **25**) with aldehyde (in **27**) and hydroxymethyl (in **28**) group, respectively. The comparison of the ^{1}H NMR spectrum of **29** with that of **25** showed one proton triplet at δ 4.29 instead of signals for the C-10 olefinic proton. In addition, a singlet at δ 5.00 for two protons and the absence of signals for vinylic methyl group confirmed the presence of C-11

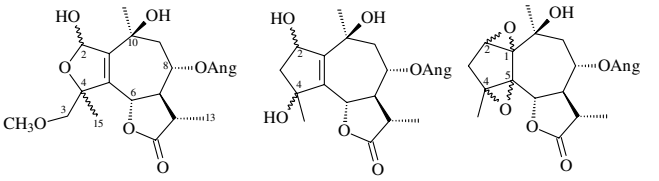
Table 3
^{13}C NMR (100 MHz) data of compounds **7**, **17**, **20** and **25**–**30** in $CDCl_3$

C	7	17	20	25	26	27	28	29	30
1	84.0	139.2	147.2	200.8	67.9	200.1	200.8	200.6	200.6
2	23.5	88.8	74.4	127.6	129.9	127.6	127.1	127.7	128.1
3	42.3	66.9	48.7	158.3	136.7	158.2	158.2	158.4	156.4
4	82.9	70.6	81.9	67.3	69.1	67.2	67.3	67.3	67.8
5	58.3	138.6	140.7	31.1	29.7	31.3	30.7	30.0	29.1
6	78.8	76.0	75.7	45.1	40.6	45.1	44.4	45.0	42.2
7	52.4	54.0	53.1	30.4	30.5	30.5	30.6	30.6	29.7
8	23.3	73.3	71.3	34.4	35.2	33.0	33.8	32.3	37.4
9	30.6	45.3	45.8	25.8	26.0	26.9	25.6	28.2	130.0
10	52.2	71.8	71.6	124.3	124.6	124.3	126.1	89.6	135.7
11	38.3	40.5	41.1	131.5	131.4	139.4	134.7	143.7	81.7
12	180.2	178.0	177.9	17.6	17.7	9.2	68.8	17.41	22.2
13	9.6	15.7	14.6	25.7	25.7	195.3	13.7	114.3	25.3
14	21.2	21.6	30.1	21.3	20.5	21.3	21.4	21.3	21.4
15	17.4	30.1	27.6	15.9	14.4	15.8	16.0	16.0	15.9
R		166.6	166.6						
		127.4	127.2						
		140.0	140.1						
		20.5	20.8						
		16.0	15.9						
OMe		52.4							

exomethylene function. The oxygen-containing function, which deshielded H-10 was identified as OOH group on the basis of MS peak at m/z 234 $[M-H_2O_2]^+$. Compound **30** also contained a double bond in the side chain. The ^1H NMR signal for two olefinic protons at δ 5.58 as well as carbon doublets at δ 130.0 and 135.7 exhibited a disubstituted double bond which position was proved by COSY experiment. A quaternary carbon at δ 81.7 and methyl singlets at δ 1.25 and 1.35 showed an oxygen-containing group attached at C-11. The singlet at δ 8.70 and MS fragment at m/z 234 $[M-H_2O_2]^+$ indicated the presence of an OOH group. The proposed structures for compounds **25**–**30** were deduced by 2D COSY, and HMQC experiments, HRMS and mass fragmentation.

The above discussed lactones **15**, **17** and **19**–**21** isolated from fraction **L4** could be regarded as artefacts formed from 8α-angeloxyartabsin (**14**) by photo-oxygenation as it has been suggested previously (Beauhair and Fourrey, 1982). This process will result in the unstable 2β,4β-and 2α,4α-endoperoxides **23** and **24** which could be expected to be precursors of 2,4-*syn*-α- and 2,4-*syn*-β-disubstituted stereoisomeric couples **17**/**18**, **19**/**20** and **21**/**22**. Compounds **18** and **22** were not found in the studied plant but the co-occurrence of diastereomers **19** and **20** supported this hypothesis. It should be noted that the lactones **15**, **17** and **19**–**21** as well as their precursor **14** were present in very low concentrations. The germacranolide **16** was a major lactone in **L4**. The other important feature of the studied taxon was the presence of bisabolanes (**25**–**30**). This type of sesquiterpenes is described now for the first time in the species of *A. millefolium* group. To the best of our knowledge, bisabolane compounds have been isolated up to now from three *Achillea* species (Barrero et al., 1990; Bruno et al., 1996; Trifunović et al., 2006).

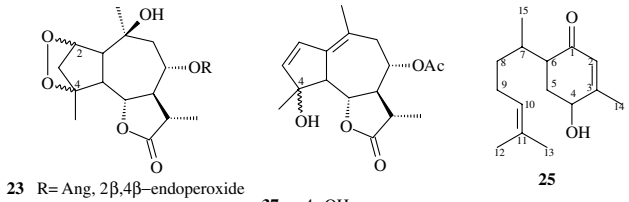
Fractions **L5** gave positive reaction with modified acetic acid-phosphoric acid reagent, specific for azulenogenic lactones (Stahl, 1967). The lactone pattern of **L5** on TLC corresponded to that of *A. collina*, described previously (Trendafilova et al., 2006). Further, TLC comparison of **L5** with authentic samples as references using different mobile phases showed the presence of matricarin (**9**), desacetylmatricarin (**10**), dehydroleucodin (**31**), 8α-angeloxyartabsin (**14**), 8α-tigloxyartabsin (**32**), achillicin (**33**), 8α-angeloxy-3-oxa-artabsin (**34**), 8α-tigloxy-3-oxa-artabsin (**35**), 3-oxa-achillicin (**36**), matricin (**37**), 4-*epi*-matricin (**38**), 8α-tigloyloxy-11(βH),13-dihydro-10-*epi*-tanaparthin-α-peroxide (**39**), 8α-isobutyryloxy-11(βH),13-dihydro-10-*epi*-tanaparthin-α-peroxide (**40**). The 2-oxo-guaianolides **9**, **10** and **31** were detected by intensive fluorescence quenching under UV 254 nm. Further, spraying with modified acetic acid-phosphoric acid reagent resulted in different coloured stains. Thus, the proazulenes **14**, **32**, **33**, **37** and **38** gave blue-green, 3-oxaguainolides **34**–**36** – pink, 2,4-endoepreoxides **39** and **40** – yellowish colour, while 2-oxoguainolides **9**, **10** and **31** could not be detected under these conditions.



17 2β-OH, 4α–CH_3
18 2α-OH, 4β–CH_3

19 2β, 4β–diOH
20 2α, 4α–diOH

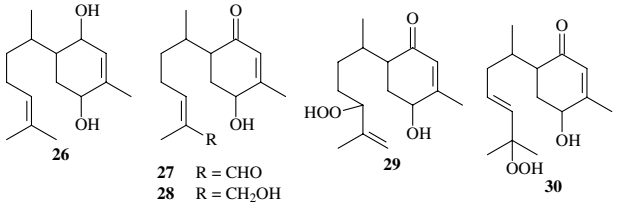
21 1β,2β,4β,5β-diepoxy
22 1α,2α,4α,5α-diepoxy



23 R= Ang, 2β,4β–endoperoxide
24 R= Ang, 2α,4α–endoperoxide
39 R= Tig, 2α,4α–endoperoxide
40 R= iBu, 2α,4α–endoperoxide

37 4αOH
38 4βOH

25



26

27 R = CHO
28 R = CH_2OH

29

30

Finally, the IR spectrum of fraction **L6** did not exhibit absorption band at 1770–1750 cm^{-1}. Obviously, the corresponding taxon (**A6**) did not contain lactones.

The anti-inflammatory effects of the tested samples were assessed in vitro using the modified cell-based assay of Tan and Berridge (Tan and Berridge, 2000) based on reduction of the highly water-soluble tetrazolium salt WST-1 in the presence of activated neutrophils. The activity is presented as percentage inhibitory effect of the extracts, fractions and individual components against the superoxide produced by Zymozan-activated neutrophils. The analysed samples showed the following activity: total extracts **A1** – 90.61%, **A4** – 75.60%, lactone fractions **L1** – 75.67%, **L4** – 78.82%, and the pure compounds **4** – 76.74%, **16** – 98.27% and **25** – 55.27%. Among the tested samples only the lactone **3** was inactive. With exception of **25** (55.27% activity) the effect of all the tested samples exceeded the effect of indomethacine (72.2%), a well-known non-steroidal anti-inflammatory agent. Obviously, the eudesmanolide **4** and the germacranolide **16** contribute for the significant activity of the total extracts and the crude lactone fractions.

3. Concluding remarks

The obtained results indicated a significant intraspecific variability (Table 4). The analysed samples differed in the lactone composition as follow: **L1** and **L2** contained the same eudesmanolides **1**–**4** and germacranolides **5** and **6**.

Table 4
Sesquiterpene distribution in the investigated samples of *A. collina*

Type[a]	L1	L2	L3	L4	L5	L6
Eu	**1–4**	**1–4**	–	–	–	–
Ger	**5**, **6**	**5**, **6**, **8**	**5**	**16**	–	–
Gu						
Mat	–	**9–11**	–	–	**9**, **10**, **31**	–
Ach	–	–	**12**, **13**	–	–	–
Azu	–	–	–	**14**, **15**, **17**, **19–21**	**14**, **32–40**	–
Bis	–	–	–	**25–30**	–	–

[a] Eu, eudesmanolides; Ger, germacranolides; Gu, guaianolide; Mat, matricarin derivatives; Ach, achillin derivatives; Azu, azulenogenic derivatives; Bis, bisabolanes.

Besides these lactones, germacranolide **8**, matricarin derivatives **9–11** presented in **L2**, which were not found even in traces in **L1**. Artabin (**5**) was the only germacranolide isolated from **L3**. The other two lactones were of achillin series (**12** and **13**). Achillin (**13**) was the major constituent in **L3**. The germacranolide **16** was the principal component of **L4** but no achillin or matricarin derivatives were detected. Instead, 8α-angeloxyartabsin (**14**), products of its degradation (**15**, **17**, **19–21**) and six bisabolanes (**25–30**) were identified. Two main terpenoids **16** and **25** characterized this taxon. Fraction **L5** differed in the content of azulenogenic guaianolides, while **L6** was a chemotype free of sesquiterpene lactones.

This study showed that population **A5** was rich in azulenogenic lactones and should be assigned to the group of typical *A. collina*. The second group includes the populations **A1–A4**, that have no or a small quantity of proazulenes. It was proved that 42% of the Bulgarian 4× – *A. collina*, which were cultivated or collected in the field, have no or only traces of proazulenes (Saukel et al., 2003). The same authors announce that *A. collina* from Bulgaria is closer to *A. setacea* than to *A. collina* from Central Europe. A population **A6**, free of sesquiterpene lactones was included in the third group. This taxon may originate from sympatric *A. collina* and *A. clypeolata* in the investigated habitat. Deviation of the pattern from *A. collina* s.lat. in Bulgaria from the pattern of typical *A. collina* s.str. could be explained by regional differentiation and/or by hybrid influences from *A. clypeolata*, *A. setacea*, or other taxa.

4. Experimental

4.1. General

Column chromatography (CC): Sephadex LH-20; Silica gel 60 (Merck); Analytical and preparative thin layer chromatography (TLC): Silica gel 60 GF_{254} (Merck); Spot detection: UV light at 254 nm, spraying with sulfuric acid, modified acetic acid-phosphoric acid reagent (EP-reagent) (Stahl, 1967); NMR spectra: Bruker DRX-250 (^1H 250 MHz/^{13}C 62.8 MHz) and Bruker DRX-400 (^1H 400 MHz/^{13}C 100 MHz), solvent $CDCl_3$; TMS as internal standard; Mass spectra (EI and HRMS): VG Autospec and Micromass LCT, respectively; IR spectra (film): Specord IR-75.

4.2. Plant material

Six samples **A1–A6** of *A. collina* originated from natural populations of four floristic regions of Bulgaria: **A1** and **A3** – from Sredna Gora Mt. (western), **A2** – from Black sea coast (northern), **A4** – from Stara Planina Mt. (central), **A5** and **A6** – from Sofia region, were planted in the experimental field of the Institute of Botany, BAS, near Sofia. The flower heads used for the present study were collected in July 2004. Voucher specimens of the corresponding taxa **A1–A6** (Co 1102; 502; 2602; 1099, 3802; 992) were deposited in the Herbarium of the Institute of Botany, Bulgarian Academy of Sciences. The identification of the species was based on morphological and caryological data. The chromosome numbers of the all studied populations ($2n = 36$) were investigated in the Institute of Botany, Sofia.

4.3. Extraction, isolation and identification

Air dried plant material of each sample [**A1** (150 g), **A2** (20 g), **A3** (20 g), **A4** (35 g), **A5** (25 g) and **A6** (20 g)] was extracted with $CHCl_3$ at room temperature. After evaporation of the solvent under vacuum the residue (9.0 g, 0.75 g, 0.78 g, 1.7 g, 0.77 g and 0.75 g, respectively) was worked up using a standard procedure (Todorova et al., 2006) to obtain purified lactone fractions **L1–L6**.

Fraction **L1** (770 mg) was subjected on Silica gel CC using $CHCl_3/Me_2CO$ mixtures with increasing polarity to give subfractions L1/1–L1/15. Subsequent preparative TLC ($CHCl_3/Me_2CO$, 30:1) of selected subfractions afforded **1** (5 mg) from L1/2, **2** (11 mg) from L1/3, **3** (40 mg) from L1/7, **4** (18 mg) and **5** (12 mg) from L1/9, and **6** (7 mg) from L1/13.

Fraction **L2** (196 mg) was separated into 13 subfractions (L2/1–L2/13) by CC ($CHCl_3/Me_2CO$, 30:1 with increasing polarity). Preparative TLC of L2/3 yielded **7** (6 mg) and **8** (2 mg). The presence of compounds **1–5** in L2/6 and **6** in L2/13 was proven by analytical TLC of L2/6, L2/13 and authentic samples. Compounds **9** (8.5 mg) and **11** (3 mg) were isolated by preparative TLC (Et_2O/hexane, 1:1) from L2/7 and L2/9, respectively.

Fraction **L3** (120 mg) gave five subfractions by CC ($CHCl_3$/Me_2CO, 15:1). L3/1 (70 mg) afforded **12** (57 mg) after recrystallization. Compounds **5** (4 mg) and **13** (8 mg) were isolated from L3/3 and L3/4 by preparative TLC ($CHCl_3$/Et_2O, 1:1).

Fraction **L4** (400 mg) was separated into 15 subfractions by CC using $CHCl_3$/Me_2CO mixtures with increasing polarity. Selected fractions (TLC screening) were used for isolation of individual compounds. Preparative TLC ($CHCl_3$/Me_2CO, 25:1) of L4/2 (38 mg) afforded compounds **25** (17 mg), **29** (3 mg) **30** (4 mg) and **15** (2.5 mg). Preparative TLC ($CHCl_3$/Et_2O, 2:1, ×2) of L4/3 allowed the isolation of **21** (3 mg), and **27** (1.0 mg). Preparative TLC ($CHCl_3$/MeOH, 30:1, ×2) of L4/7 (22 mg) afforded **16** (16 mg) and **26** (3 mg). Preparative TLC ($CHCl_3$/MeOH, 30:1, ×2) of L4/9 (40 mg) gave **17** (2.5 mg). Preparative TLC ($CHCl_3$/Me_2CO, 1:1) of L4/12 (15 mg) afforded **19** (3 mg), while **20** (4 mg) was purified from L4/14 under the same conditions. Known compounds were identified by comparison of their spectral data with those reported in the literature and by comparison with authentic samples.

4.3.1. Compounds

*4.3.1.1. 1β,4β-Epoxy-6β,7α,11α-selinan-6,12-olide (**7**).* Colourless oil; IR (film) ν_{max}: 1770, 1465, 1380, 1240, 1130 cm^{-1}; HREIMS: 250.1564 $[M]^+$ (calc. for $C_{15}H_{22}O_3$ 250.1569); EIMS: (70 eV) *m/z* (rel. int.): 250 $[M]^+$ (25), 235 $[M-15]^+$ (58), 221 (50), 206 (30), 193 (60), 165 (70), 133 (56), 119 (80) 55 (100); ^1H NMR: see Table 1; ^{13}C NMR: see Table 3.

*4.3.1.2. 8α-Angeloxy-2β,10β-dihydroxy-4β-methoxymethyl-2,4-epoxy-6βH,7αH,11β-1(5)-guaien-12,6α-olide (**17**).* Colourless oil; IR (film) ν_{max}: 3450, 1760, 1730, 1670 cm^{-1}; HRESIMS: 474.1964 $[M+CH_3CN+Na]^+$ (calc. for $C_{23}H_{33}O_8$ 474.1970); EIMS (70 eV) *m/z* (rel. int.): 410 $[M]^+$ (1), 310 $[M-100]^+$ (1), 292 $[310-18]^+$ (47), 274 $[292-18]^+$ (18), 262 $[292-15-15]^+$ (67), 247 $[262-15]^+$(36), 203 (62), 175 (50), 83 (89), 55 (100); ^1H NMR: see Table 1; ^{13}C NMR: see Table 3.

*4.3.1.3. 8α-Angeloxy-2α,4α,10β-trihydroxy-6βH,7αH,11βH-1(5)-guaien-12,6α-olide (**20**).* Colourless oil, IR (film) ν_{max}: 3620, 1780, 1720 cm^{-1}; HRESIMS: 444.1981 $[M+CH_3CN+Na]^+$ (calc. for $C_{22}H_{31}O_7Na$ 444.1998); EIMS (70 eV) *m/z* (rel. int.): 380 $[M]^+$ (1), 365 $[M-15]^+$ (55), 362 $[M-18]^+$ (20), 262 $[362-100]^+$ (50), 244 $[362-18]^+$ (55), 219 (40), 201 (42), 191 (35), 173 (38), 147 (38), 111 (37), 91 (42), 83 (10), 69 (57), 55 (100); ^1H NMR: see Table 1; ^{13}C NMR: see Table 3.

*4.3.1.4. 4-Hydroxy-bisabol-1-one (**25**).* Yellowish oil, $[\alpha]_D^{23}$ − 28.6 ($CHCl_3$; *c* 0.81); IR (lit.): 3630, 1678, 1610 cm^{-1}; HREIMS: 236.0869 $[M]^+$ (calc. for $C_{15}H_{24}O_2$ 220.1828); EIMS (70 eV) *m/z* (rel. int.): 236 $[M]^+$ (80), 221 [M−15] (2), 218 $[M-18]^+$ (15), 153 $[M-C_6H_{11}]^+$ (75), 151 (88), 135 $[153-18]^+$ (40), 125 $[M-C_8H_{15}]^+$ (96), 109 (100), 95 (55), 83 (80), 69 (98), 55 (90). ^1H NMR: see Table 2; ^{13}C NMR: see Table 3.

*4.3.1.5. Bisabol-1,4-diol (**26**).* Yellowish oil, $[\alpha]_D^{23}$ − 15.0 ($CHCl_3$; *c* 0.13); IR (film) ν_{max}: 3630, 1680 cm^{-1}; HREIMS: 238.193268 $[M]^+$(calc. for $C_{15}H_{26}O_2$ 238.193280); EIMS (70 eV) *m/z* (rel. int.): 338 $[M]^+$ (12), 220 $[M-18]^+$ (25), 202 $[220-18]^+$ (13), 187 $[202-15]^+$ (10), 127 $[C_7H_{12}O_2]^+$ (20), 111 $[C_8H_{15}]^+$ (78) 109 (100), 95 (90), 83 (82), 69 (98); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3.

*4.3.1.6. 4-Hydroxy-1-oxo-bisabol-13-al (**27**).* Yellowish oil, IR (film) ν_{max}: 3630, 1680, 1670, 1610 cm^{-1}; HRESIMS: 273.1639 $[M+Na]^+$ (calc. for $C_{15}H_{22}O_3Na$ 273.1681); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3.

*4.3.1.7. 4,13-Dihydroxy-bisabol-1-one (**28**).* Yellowish oil, $[\alpha]_D^{23}$ − 9.4 ($CHCl_3$; *c* 0.80); IR (film) ν_{max}: 3630, 1680, 1615 cm^{-1}; HRESIMS: 275.1623 $[M+Na]^+$ (calc. for $C_{15}H_{24}O_3Na$ 275.1623); EIMS (70 eV) *m/z* (rel. int.): 252 $[M]^+$ (1), 234 $[M-18]^+$ (43), 216 $[234-18]^+$ (8), 221 $[M-CH_2OH]^+$ (5), 153 $[M-C_6H_{10}OH]^+$ (43), 126 (82), 125 $[M-C_8H_{14}OH]^+$ (60), 109 (100), 95 (62), 83 (44), 69 (78); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3.

*4.3.1.8. 4-Hydroxy-10-hydroperoxy-11-bisabolen-1-one (**29**).* Yellowish oil, $[\alpha]_D^{23}$ − 26.7 ($CHCl_3$; *c* 0.32), IR (film) ν_{max}: 3630, 3600, 1675, 1650, 1620 cm^{-1}; HRESIMS: 332.1847 $[M+CH_3CN+Na]^+$ (calc. for $C_{17}H_{27}O_4Na$ 332.1838); EIMS (70 eV) *m/z* (rel. int.): 268 $[M]^+$ (1), 250 $[M-18]^+$ (5), 235 $[250-15]^+$ (16), 234 $[M-H_2O_2]^+$ (16), 181 $[M-C_4H_6OOH]^+$ (11), 167 $[M-C_5H_8OOH]^+$ (37) 153 $[M-C_6H_{10}OOH]^+$ (45), 149 $[167-18]^+$ (52), 135 $[153-18]^+$ (62), 126 $[167-41]^+$ (81), 125 $[M-C_8H_{14}OOH]^+$ (79), 111 $[126-15]^+$ (78) 109 (100), 97 (68), 83 (57), 69 (92); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3.

*4.3.1.9. 4-Hydroxy-11-hydroperoxy-9-bisabolen-1-one (**30**).* Yellowish oil, $[\alpha]_D^{23}$ − 6.8 ($CHCl_3$; *c* 0.42); IR (film) ν_{max}: 3630, 3600, 1680, 1615 cm^{-1}; EIMS (70 eV) *m/z* (rel. int.): 268 $[M]^+$ (1.5), 235 $[M-O_2H]^+$ (43), 234 $[M-H_2O_2]^+$ (26), 167 $[M-C_5H_9O_2]^+$ (37), 153 $[M-C_6H_{11}O_2]^+$ (35), 149 $[167-18]^+$ (49), 135 $[153-18]^+$ (50), 125 $[M-C_8H_{15}O]^+$ (72), 109 (100), 97 (77), 83 (71), 69 (85); ^1H NMR: see Table 2; ^{13}C NMR: see Table 3.

4.3.2. TLC identification of the sesquiterpene lactones in fraction **L5**

Fraction **L5** (1 mg) was dissolved in $CHCl_3$ (1 ml) and analysed by TLC using as references the following sesquiterpene lactones **9**, **10**, **14** and **31**–**40** and different mobile phases (systems A–C). All compounds were isolated from *A. collina* (Trendafilova et al., 2006). System A ($CHCl_3$/Me_2CO, 9:1): R_f values 0.75 (**31**), 0.65 (**9**), 0.60 (**14**), 0.58 (**34**), 0.56 (**32**), 0.54 (**35**), 0.48 (**33**), 0.46 (**36**), 0.44 (**38**), 0.42 (**37**), 0.37 (**39**), 0.35 (**40**) and 0.25 (**10**); System B (Et_2O/hexane, 1:1, twice): R_f values 0.48 (**14**), 0.46 (**34**), 0.43 (**32**), 0.41

(**35**), 0.31 (**33**), 0.29 (**31**), 0.27 (**36**), 0.25 (**9**), 0.17 (**38**) and 0.15 (**37**); System C (Et_2O/hexane, 3:1, twice): R_f values 0.44 (**38**), 0.42 (**37**), 0.40 (**39**), 0.38 (**40**) and 0.30 (**10**).

4.4. Anti-inflammatory activity

The anti-inflammatory activity was tested by the modified assay of Tan and Berridge (Tan and Berridge, 2000): total volume of 250 μl modified Hank's Solution (MHS) with pH 7.4 containing $0.5–1.0 \times 10^4$ neutrophils/ml, 500 μM WST-1 (1,2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt) (Dojindo Laboratories, Kumamoto, Japan) and concentrations of the test samples 500 μg/ml. The control contained only buffer, neutrophils and WST-1. All samples were equilibrated at 37 °C and the reaction initiated by adding Zymosan-activated serum ZAS (Sigma Chemicals, St. Louis, USA) for 30 min. Indomethacine was used as positive control. Absorbance was measured at 450 nm using Spectra MAX 340-plus microplate reader (Molecular Devices, Sunnyvale, California, USA).

4.4.1. Isolation of human neutrophils

Human neutrophils were isolated by the modified method of Siddiqui et al. (1995). Briefly fresh heparinized blood collected from healthy volunteers was diluted with equal volume of Hanks balance salts solution (HBSS). After leaving 20 min at room temperature, the upper leukocyte layer was collected, layered over Ficoll paque (Pharmacia Biotech., Uppsala, Sweden) and centrifuged at 1500 rpm for 30 min. Cells were lysed with lysis buffer for 10 min, centrifuged and diluted with HBSS. After centrifuging the pellet was re-suspended in HBSS with $CaCl_2$, $MgSO_4 \cdot 2H_2O$ and $MgCl_2 \cdot 6H_2O$. Cell counts were done using the improved Neubaur chamber. The viability of cells determined by the Trypan Blue method was above 97%.

4.4.2. Statistical analysis

Data are presented as the means ± SEM statistical analysis was performed using EziFit 5.0 Windows based software after analysis of variance. *P*-values <0.05 were considered to be significant.

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