SUPPLEMENTARY MATERIAL

Lignans and indole alkaloids from the seeds of *Centaurea vlachorum* Hartvig (Asteraceae), growing wild in Albania and their biological activity.

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Abstract:

The present phytochemical investigation of the seeds of *Centaurea vlachorum* led to the isolation and characterization of four compounds including two indole alkaloids N-(p-coumaroyl) serotonin (**1**) and moschamine (**2**) and two dibenzylbutyrolactone lignans matairesinol (**3**) and arctiin (**4**). This is the first report on the isolation of non-volatile secondary metabolites from *C. vlachorum*. The chemataxonomic significance of these compounds was summarized. Moreover, the isolated compounds were tested for their free radical scavenging activity using the following in vitro assays: i) interaction with the free stable radical of DPPH (1,1-diphenyl-2-picrylhydrazyl), ii) inhibition of linoleic acid peroxidation with the dihydrochloric acid of 2,2-Azabis-2-aminepropane (AAPH). Finally, their inhibitory activity toward soybean lipoxygenase was evaluated, using linoleic acid as substrate.

Key words: *Centaurea vlachorum*, Asteraceae, indole alkaloids, lignans

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1. Experimental

1.1. Chemicals

1,1-Diphenyl 2-picryl hydrazyl (DPPH), Lipoxygenase (1.13.11.12) type I-B (Soybean) and linoleic acid (sodium salt), 99% purity, were purchased from Sigma (St Louis, MO, USA). Nordihydroguaiaretic acid (NDGA) and trolox were purchased from Merck. All other chemicals were of analytical grade. A Perkin Elmer Lambda 20 UV-Vis spectrophotometer has been used for the radical scavenging activity experiments.

1.2. Inhibition of linoleic acid lipid peroxidation

Production of conjugated diene hydroperoxide by oxidation of sodium linoleate in an aqueous solution is monitored at 234 nm in the presence of 2,2'-Azobis(2-amidinopropane) dihydrochloride (AAPH) of 50 μ l of 40 mM AAPH solution as a free radical initiator in 0.05 M phosphate buffer, pH 7.4. Oxidation was carried out in the presence of the tested samples (100 μ M). The rate of oxidation at room temperature was monitored by recording the increase in absorption at 234 nm caused by conjugated diene hydroperoxides. Trolox was used as a reference drug (Peperidou et al., 2014).

1.3. Soybean lipoxygenase inhibition study in vitro

The tested compounds dissolved in DMSO (10mM stock solution) were incubated (final concentration 100 μ M) at room temperature with sodium linoleate (0.1 mM) and 0.2 ml of enzyme solution ($1/9 \times 10^{-4}$ w/v in saline) in tris buffer pH 9. The conversion of sodium linoleate to 13-hydroperoxylinoleic acid was recorded at 234 nm compared with the appropriate standard inhibitor Nordihydroguaiaretic acid (NDGA) (Peperidou et al., 2014).

1.4. Interaction with DPPH

To a solution of DPPH (0.1mM in methanol) the tested samples (stock solution 10mM) dissolved in DMSO were added (100 μ M). After 20/60 min the antioxidant activity is recorded at 517nm and the percentage of reducing activity (RA) was calculated and compared to the reference compound NDGA (nordihydroguaiaretic acid) (Peperidou et al., 2014).

1.5. Plant material

The aerial parts and seeds of *C. vlachorum* were collected on early July 2011, the plant was taxonomically identified by Prof. Shuka L. (Faculty of Natural Sciences, University of Tirana); a voucher specimen was deposit in the Herbarium of Faculty of Natural Sciences, Tirana under the code *Shuka* 2017-2020 (TIR).

1.6. Extraction and isolation of compounds

The air-dried seeds of *C. vlachorum* (52.0 g) were finely ground and successively extracted at room temperature with petroleum ether, CH₂Cl₂, MeOH. This procedure yielded 4.1 g, 3.0 g and 4.2 g of PE-, CH₂Cl₂- and MeOH-soluble fractions, respectively.

The dichloromethane extract was subjected to CC (column chromatography) on silica gel with CH₂Cl₂-MeOH to give eighteen fractions (A-S). Fraction O (CH₂Cl₂-MeOH 90:10), was identified as compound (**4**, 606.8 mg). Fraction P (95.2 mg), eluted with CH₂Cl₂-MeOH (85:15), was refractionated over silica gel with CH₂Cl₂-MeOH to give seven fractions (PA-PG). Fraction PE (CH₂Cl₂-MeOH 85:15) was identified as compounds (**4**, 62.1 mg).

The MeOH extract was concentrated and the residue redissolved in boiling water. The water-soluble fraction was filtered and extracted successively with Et₂O, EtOAc and n-BuOH to yield 0.4 g, 1.1 g and 1.6 g of Et₂O, EtOAc and n-BuOH-soluble fractions, respectively.

The EtOAc residue (1.1 g) was subjected to CC on silica gel with CH₂Cl₂-MeOH to give eleven fractions (A-Q). Fractions E (CH₂Cl₂-MeOH 98:2), I (CH₂Cl₂-MeOH 95:5) and N (CH₂Cl₂-MeOH 90:10) were identified as compounds (**3**, 21.7 mg), (**2**, 64.3 mg) and (**4**, 324.0 mg), respectively. Fraction K (72.6 mg), eluted with CH₂Cl₂-MeOH (94:6), was refractionated over silica gel with CH₂Cl₂-MeOH to give eight fractions (KA-KH). Fraction KF (CH₂Cl₂-MeOH 94:6) was identified as compound (**1**, 14.5 mg). The BUOH residue (1.6 g) was subjected to CC on silica gel with CH₂Cl₂-MeOH to give twenty two fractions (A-Y). Fraction Q (CH₂Cl₂-MeOH 90:10) was identified as compound (**4**, 155.7 mg). Fraction O (52.0 mg), eluted with CH₂Cl₂-MeOH (92:8), was refractionated over silica gel with CH₂Cl₂-MeOH to give nine fractions (OA-TI). Fraction OG (CH₂Cl₂-MeOH 94:6) was identified as compounds (**1**, 16.1 mg). Fraction T (95.2 mg), eluted with CH₂Cl₂-MeOH (85:15), was refractionated over silica gel with CH₂Cl₂-MeOH to give twelve fractions (TA-TM). Fraction TC (CH₂Cl₂-MeOH 92:8) was identified as compounds (**4**, 39.4 mg). Fraction V (97.8 mg), eluted with CH₂Cl₂-MeOH (80:20), was refractionated over silica gel with CH₂Cl₂-MeOH to give fourteen fractions (VA-VO). Fraction VE (CH₂Cl₂-MeOH 90:10) was identified as compounds (**4**, 5.2 mg).

Column chromatography (CC) was carried out on silica gel 60 (Merck Art. 9385), gradient elution with the solvents mixtures indicated in each case. TLC: silica gel (Kieselgel F₂₅₄, Merck, Art. 5554); Detection on TLC plates: UV light (absorbance: 254 and 366 nm), vanillin-H₂SO₄ spray reagent on silica gel.

All isolated compounds were analyzed by spectroscopic methods (1D and 2D NMR) and their data were compared with those of samples from our collection and/or by a comparison with reported data in literature.

Spectroscopic data. NMR: The ^1H NMR spectra (500 MHz) and ^{13}C NMR spectra (125.0 MHz) were recorded in CD_3OD using AGILENT DD2 500 spectrometer. Chemical shifts are reported in δ (ppm) values relative to TMS. COSY, HSQC, HMBC were performed using standard Agilent microprograms.

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Table S1. ^1H and ^{13}C NMR of compound **1** (CD_3OD , 500MHz)

Position	δ_{C}	C	δ_{H}	Number H	Multiplicity J (Hz)
1	122.85	CH	7.02	1	s
2	110.97	C	-	-	-
3	129.20	C	-	-	-
4	102.11	CH	6.96	1	br s
5	149.69	C	-	-	-
6	111.10	CH	6.66	1	br d (J=10.0)
7	111.25	CH_2	7.15	1	d (J=9.0)
8	131.70	C	-	-	-
9	40.05	CH_2	3.56	2	t (J=7.0)
10	25.05	CH_2	2.92	2	t (J=7.0)
1'	126.35	C	-	-	-
2'	129.13	CH	7.50	1	d (J=7.5)
3'	115.27	CH	6.78	1	d (J=7.0)
4'	159.02	C	-	-	-
5'	115.27	CH	6.78	1	d (J=7.0)
6'	128.01	CH	7.50	1	d (J=7.5)
7'	140.29	CH	7.44	1	d (J=15.5)
8'	117.16	CH	6.38	1	d (J=15.5)
9'	167.85	C	-	-	-

Figure S1. ^1H NMR of compound **1** (CD_3OD , 500MHz)

Figure S2. ^{13}C NMR of compound **1** (CD_3OD , 125MHz)

Figure S3. gDQCOSY of compound **1** (CD_3OD , 500MHz)

Figure S4. gHSQCAD of compound **1** (CD₃OD, 500MHz)

Figure S5. gHMBCAD of compound **1** (CD₃OD, 500MHz)

Table S2: ^1H and ^{13}C NMR of compound **2** (CD_3OD , 500MHz)

Position	δ_{C}	C	δ_{H}	Number H	Multiplicity J (Hz)
1	124.34	CH	7.02	1	s
2	112.49	C	-	-	-
3	129.43	C	-	-	-
4	103.56	CH	7.08	1	br d (J=2.0)
5	151.09	C	-	-	-
6	112.40	CH	6.68	1	dd (J=9.0, 2.5)
7	112.75	CH	7.16	1	d (J=8.0)
8	133.09	C	-	-	-
9	26.45	CH_2	2.92	2	m
10	41.49	CH_2	3.56	2	m
1'	128.28	C	-	-	-
2'	111.48	CH	6.97	1	o.s
3'	149.21	C	-	-	-
4'	149.71	C	-	-	-
5'	116.44	CH	6.78	1	d (J=8.0)
6'	123.22	CH	6.99	1	dd (J=8.2, 1.9)
7'	142.03	CH	7.44	1	d (J=16.0)
8'	118.83	CH	6.41	1	d (J=16.0)
9'	169.23	C	-	-	-
$-\text{OCH}_3$	56.34	$-\text{OCH}_3$	3.83		s

Figure S6. ^1H NMR of compound **2** (CD_3OD , 500MHz)

Figure S7. ^{13}C NMR of compound **2** (CD_3OD , 125MHz)

Figure S8. gDQCOSY of compound **2** (CD₃OD, 500MHz)

Figure S9. gHSQCAD of compound **2** (CD₃OD, 500MHz)

Figure S10. gHMBCAD of compound **2** (CD₃OD, 500MHz)

Table S3: ^1H and ^{13}C NMR of compound **3** (CD_3OD , 500MHz)

Position	δ_{C}	C	δ_{H}	Number H	Multiplicity J (Hz)
1	131.49	C	-	-	-
2	113.89	CH	6.66	1	o.s
3	149.05	C	-	-	-
4	146.18	C	-	-	-
5	116.11	CH	7.00	1	o.s
6	123.07	CH	6.58	1	o.s
7	35.38	CH_2	2.84	2	m
8	47.77	CH	2.66	1	m
9	181.89	C	-	-	-
1'	130.80	C	-	-	-
2'	113.24	CH	6.55		o.s
3'	149.01	C	-	-	-
4'	146.37	C	-	-	-
5'	116.19	CH	7.01	1	o.s
6'	122.25	CH	6.50	1	o.s
7'	38.92	CH_2	2.49	2	m
8'	42.57	CH	2.48	1	m
9a'	72.97	CH_2	3.90	1	dd (9.0, 8.0)
9b'			4.14	1	dd (9.0, 7.5)
-OCH ₃ -3'	56.36	OCH ₃	3.78	3	s
-OCH ₃ -3	56.31	OCH ₃	3.77	3	s

Figure S11. ^1H NMR of compound **3** (CD_3OD , 500MHz)

Figure S12. ^{13}C NMR of compound **3** (CD_3OD , 125MHz)

Figure S13. gDQCOSY of compound **3** (CD₃OD, 500MHz)

Figure S14. gHSQCAD of compound **3** (CD₃OD, 500MHz)

Table S4: ^1H and ^{13}C NMR of compound **4** (CD_3OD , 500MHz)

Position	δ_{C}	C	δ_{H}	Number H	Multiplicity J (Hz)
1	132.72	C	-	-	-
2	113.07	CH	6.59	1	br s
3	150.42	C	-	-	-
4	146.81	C	-	-	-
5	113.61	CH	6.82	1	d (8.5)
6	122.13	CH	6.60	1	o.s
7	35.41	CH_2	2.52	2	m
8	42.48	CH	2.66	1	m
9	181.47	C	-	-	-
1'	134.24	C	-	-	-
2'	114.80	CH	6.74		d (2.0)
3'	150.60	C	-	-	-
4'	149.13	C	-	-	-
5'	117.81	CH	7.05	1	d (8.0)
6'	123.02	CH	6.65	1	dd (8.5, 2.0)
7'	38.90	CH_2	2.53	2	m
8'	47.65	CH	2.47	1	m
9a'	72.96	CH_2	3.89	1	dd (9.0, 8.0)
9b'			4.15	1	dd (9.0, 7.5)
-OCH ₃ -3'	56.74	OCH ₃	3.80	3	s
-OCH ₃ -4	56.55	OCH ₃	3.79	3	s
-OCH ₃ -3	56.50	OCH ₃	3.75	3	s
1''	102.88	CH	4.82	1	o.s
2''	74.89	CH	3.47	1	o.s
3''	77.78	CH	3.46	1	o.s
4''	71.33	CH	3.39	1	o.s
5''	78.14	CH	3.39	1	o.s
6a''	62.50	CH_2	3.81	1	o.s
6b''			3.70	1	o.s

Figure S15. ^1H NMR of compound **4** (CD_3OD , 500MHz)

Figure S16. ^{13}C NMR of compound **4** (CD_3OD , 125MHz)

Figure S17. gDQCOSY of compound **4** (CD₃OD, 500MHz)

Figure S18. gHSQCAD of compound **4** (CD₃OD, 500MHz)

Figure S19. gHMBCAD of compound **4** (CD₃OD, 500MHz)

Table S5. Distribution of compounds **1–4** within the genus *Centaurea*

Species	Origin of species	Compounds				References
		1	2	3	4	
<i>C. affinis</i> Friv.	Serbia (aerial parts)	-	-	+	-	Janackovic et al., 2004
<i>C. americana</i> Nutt.	Cultivated seeds, United Kindom	-	-	+	+	Cooper et al., 2012
<i>C. arenaria</i> M.B. ex Willd.	Hungary (aerial parts)	-	+	+	+	Csapi et al., 2010
<i>C. aspera</i> L.		-	-	+	-	Marco et al., 2005
<i>C. bornmuelleri</i> Hausskn. Ex. Bornm.	Turkey	-	-	+	+	Shoeb et al., 2007c
<i>C. calcitrapa</i> L.	-	-	-	+	-	Dawidar et al., 1989
<i>C. cyanus</i> L.	United Kindom (seeds)	-	+	-	-	Sarker et al., 2001
<i>C. dealbata</i> Willd.	Seeds-	-	-	-	+	Shoeb et al., 2006a
<i>C. deflexa</i> Wagenitz	Turkey (aerial parts)	-	-	-	+	Chicca et al., 2011
<i>C. glomerata</i> Valh.	Egypt	-	-	-	+	Zaghloul et al., 1990
<i>C. huber-morathii</i> Wagenitz	Turkey	-	-	+	+	Shoeb et al., 2007c
<i>C. isaurica</i> Hub. Mor.	Turkey (aerial parts)	-	-	-	+	Flamini et al., 2004
<i>C. macrocephala</i> Muss. Puschk. ex Willd.	France (seeds)	-	-	+	+	Shoeb et al., 2004
<i>C. melitensis</i> L.	-	-	-	-	+	Negrete et al., 1989
<i>C. montana</i> L.	France, seeds	+	+	-	-	Shoeb et al., 2006b
<i>C. moschata</i> L.	United Kindom (seeds)	-	+	-	-	Sarker et al., 1997
<i>C. mucronifera</i> DC.	Turkey (aerial parts)	-	-	-	+	Shoeb et al., 2007b
<i>C. nicolai</i> Bald.	Montenegro (aerial parts)	-	-	+	-	Vajs et al., 1999
<i>C. nigra</i> L.	France (seeds)	+	+	+	+	Middleton et al., 2003
<i>C. ornata</i> Willd. ssp. <i>ornata</i> Franco	-	-	-	+	+	Bastos et al., 1994

<i>C. pamphylica</i> Boiss. & Heldr.	Turkey (aerial parts)	-	-	+	+	Shoeb et al., 2007a
<i>C. pannonica</i> (Heuff.) Simonk.	Serbia (aerial parts)	-	-	+	+	Milošević-Ifantis et al., 2013
<i>C. persica</i> Boiss.	-	-	-	+	-	Sanz et al., 1990
<i>C. ptosimopappa</i> Hayek	Turkey	-	-	+	-	Celik et al., 2006
<i>C. raphanina</i> Sm. ssp. <i>mixta</i> (DC.) Runemark	Greece	-	-	+	-	Panagouleas et al., 2003
<i>C. scabiosa</i> L.	France (seeds)	-	-	+	-	Ferguson et al., 2003
<i>C. schischkinii</i> Tzvelev	Turkey (seeds)	-	-	+	+	Shoeb et al., 2005
<i>C. sclerolepis</i> Boiss.	Turkey (seeds)	-	-	-	+	Erdemgil et al., 2006
<i>C. scoparia</i> Sieb.	-	-	-	+	-	Youssef et al., 1995
<i>C. solstitialis</i> subs. <i>schouwii</i> (DC.) Dostal	Italy (aerial parts)	-	-	+	-	Bruno et al., 1991
<i>C. sphaerocephala</i> L. ssp. <i>polyacantha</i> Willd	-	-	-	-	+	Bastos et al., 1990
<i>C. tweediei</i> Hook. et Arn.	Argentina (aerial parts)	-	-	+	-	Fortuna et al., 2001
<i>C. urvillei</i> subs. <i>armata</i> Wagenitz	Turkey (aerial parts)	-	-	-	+	Shoeb et al., 2007b
<i>C. zuccariniana</i> DC.	Greece (aerial parts)	-	-	-	+	Ciric et al., 2012

Table S6. Percentage (%) interaction of compounds (**1-4**) with DPPH, their % soybean LOX inhibitory activity and their % Inhibition of lipid peroxidation

Essential Oil	% Interaction with the stable free radical of DPPH		% Inhibition of LOX	% Inhibition of lipid peroxidation
Time	20 min	60 min		
Concentration	100µM	100µM	100µM	100µM
1	78.6	71.8	-	82.3
2	72.6	82.8	81.3	-
3	88.7	93.8	80.4	72.2
4	1.2	-	-	81.6
NDGA	81	93	96	
TROLOX				73.0