Inhibition of COX-2 and NF-κB1 Gene Expression, NO Production, 5-LOX, and COX-1 and COX-2 Enzymes by Extracts and Constituents of *Onopordum acanthium*

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Abstract

The present study focused on the investigation of the inhibition of cyclooxygenase-2 and nuclear factor kappa B1 gene expression, nitric oxide production, leukotriene biosynthesis (5-lipoxygenase), and cyclooxygenase-1 and cyclooxygenase-2 enzymes of *Onopordum acanthium*, and the isolation and identification of its active compounds. From the chloroform soluble part of the MeOH extract prepared from aerial parts, lignans [pinoresinol (**1**), syringaresinol (**2**), and medioresinol (**3**)] and flavonoids [hispidulin (**4**), nepetin (**5**), apigenin (**6**), and luteolin (**7**)] were isolated by a combination of different chromatographic methods. The structures of the compounds were determined by means of mass spectrometry and 1D- and 2D-nuclear magnetic resonance spectroscopy, and by comparison of the spectral data with literature values. Extracts of different polarity and the isolated compounds obtained from the aerial parts, together with those previously isolated from the roots of the plant [4*β*,15-dihydro-3-dehydrozaluzanin C (**8**), zaluzanin C (**9**), 4*β*,15,11*β*,13-tetrahydrozaluzanin C (**10**), nitidanin diisovalerianate (**11**), 24-methylenecholesterol (**12**), and 13-oxo-9Z,11E-octadecadienoic acid (**13**)], were evaluated for their inhibitory effects on cyclooxygenase-2 and nuclear factor kappa B1 gene expression, inducible nitric oxide synthase, 5-lipoxygenase, and cyclooxygenase-1 and cyclooxygenase-2 enzymes in *in vitro* assays. It was found that *O. acanthium* extracts exert strong inhibitory activities *in vitro* and some lignans, flavonoids, and sesquiterpenes may play a role in these activities. 4*β*,15-Dihydro-3-dehydrozaluzanin C and zaluzanin C at 20 µM were the most active constituents tested against lipopolysaccharide/interferon-*γ*-induced nitric oxide production (100.4 ± 0.5% and 99.4 ± 0.8%) in the inhibition of cyclooxygenase-2 (98.6 ± 0.2% and 97.0 ± 1.1%) and nuclear factor kappa B1 gene expression (76.7 ± 7.3% and 69.9 ± 3.4%). Furthermore, it was shown that these inhibitory effects are not due to cytotoxicity of the compounds.

Introduction

Onopordum is a large genus in the tribe Cardueae (Asteraceae), which contains about 50 biennial species. These plants are distributed in the Mediterranean area of Europe, northern Africa, the Canary Islands, the Caucasus, and southwest and central Asia [1,2]. Characteristic metabolites of the genus *Onopordum* are flavonoids, acetylenic compounds, steroids, triterpenes, lipids, and nitrogen-containing compounds. Moreover, different germacrane-, elemane-, eudesmane-, and guaiane-type sesquiterpenoids were identified in the species, which possess a number of biological activities (antibacterial, antifungal, antioxidant, anti-inflammatory, and cytotoxic activities) [1].

Onopordum acanthium L., commonly named Scotch thistle, is a medicinal plant belonging to the genus *Onopordum* that is naturalized in various parts of Europe and Asia. The plant has been used traditionally for its antibacterial, cardiotonic, hemostatic, hypotensive, and anticancer properties [3,4]. On the other hand, the inflorescences, roots, seeds, and late developing leaves of *O. acanthium* are used internally in the traditional medicine of Central Asia for the treatment of inflammation of the bladder and the respiratory and urinary systems [5]. Phytochemical studies revealed the presence of sesquiterpenes, flavonoids, triterpenes, sterols, lipids, nitrogen-containing compounds, phenolic acids, and coumarins in this species [1]. Pharmacological investigations revealed its antioxidant, angiotensin-con-

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Table 1 Inhibition of COX-2 and NF-κB1 gene expression, NO production, 5-LOX, and COX-1 and COX-2 enzymes by extracts from herbs of *O. acanthium*. N = two experiments in duplicate.

Extract	Inhibition % ± SD					
	COX-2* (10 µg/mL)	NF-κB1 (10 µg/mL)	iNOS (10 µg/mL)	5-LOX (50 µg/mL)	COX-1 (50 µg/mL)	COX-2** (50 µg/mL)
n-Hexane extract of the aerial parts (**A**)	18.5 ± 17.9	< 10	16.9 ± 13.8	57.2 ± 2.8	40.5 ± 9.1	82.8 ± 8.4
Chloroform extract of the aerial parts (**B**)	41.8 ± 8.3	21.8 ± 6.7	76.7 ± 7.0	62.6 ± 6.9	< 10	61.8 ± 9.0
Aqueous-methanol extract of aerial parts (**C**)	< 10	< 10	10.2 ± 6.0	31.2 ± 7.6	< 10	< 10
Dexamethasone***	47.6 ± 4.2	ND	ND	ND	ND	ND
Quercetin***	ND	46.0 ± 8.4	ND	ND	ND	ND
L-NMMA***	ND	ND	52.2 ± 4.9	ND	ND	ND
Zileuton***	ND	ND	ND	63.0 ± 3.8	ND	ND
Indomethacin***	ND	ND	ND	ND	32.3 ± 4.8	ND
NS-398***	ND	ND	ND	ND	ND	30.5 ± 3.1

* COX-2 gene expression inhibition; ** COX-2 enzyme inhibition; *** positive control; ND = not determined

verting enzyme (ACE) inhibitory activities and the ability to activate natural killer cells *in vitro* against tumor cells [6, 7]. Formerly, the antiproliferative activities of extracts prepared from roots and aerial parts with solvents of different polarity were evaluated on three human tumor cell lines (HeLa, MCF7, and A431), and the chemical investigation of the roots resulted in the isolation of sesquiterpene lactones, a neolignane, steroids, and fatty acids. Some of the isolated compounds were responsible for the antiproliferative activity of *O. acanthium* [8, 9].
As a continuation of phytochemical and pharmacological analyses of *O. acanthium*, the isolation, identification of bioactive compounds, and evaluation of *in vitro* inhibitory activity of the extracts and compounds from the aerial parts of *O. acanthium* on cyclooxygenase-2 (COX-2) and nuclear factor kappa B1 (NF-κB1) gene expression, inducible nitric oxide synthase (iNOS), 5-lipoxygenase (5-LOX), and cyclooxygenase-1 (COX-1) and COX-2 enzymes assays are reported in the present paper. The *in vitro* assays carried out on *O. acanthium* have not been performed previously. In addition, the cytotoxic activity of the compounds possessing COX-2 and NF-κB1 gene expression inhibitory effects was also evaluated by the XTT assay at different time points in various concentrations.

Results and Discussion

Dried and ground aerial parts of *O. acanthium* were extracted with MeOH. After concentration, the extracts were dissolved in 50% aqueous MeOH, and a solvent-solvent partition was performed first with *n*-hexane (A) and then with $CHCl_3$ (B), and the residue gave the aqueous-MeOH extract (C). In preliminary testings, COX-2 and NF-κB1 gene expression, iNOS, 5-LOX, and COX-1 and COX-2 inhibitory activities of these extracts were analyzed. Extract B, which showed marked inhibitory activity in three test systems (**Table 1**), was fractionated by vacuum liquid chromatography (VLC) on silica gel, resulting in six main fractions (BI–VI). These fractions were further evaluated for their inhibitory effects. Fractions BI, BIV, and BV demonstrated significant or moderate activity against lipopolysaccharide (LPS)/interferon-γ (IFN-γ)-induced nitric oxide (NO) production as well as the inhibition of COX-2 gene expression and the COX-2 enzyme [inhibition of iNOS (62.5 ± 16.5%, 102.0 ± 0.3%, and 79.9 ± 6.2%, respectively), inhibition of COX-2 gene expression (45.5 ± 8.3%, 31.5 ± 11.1%, and 12.6 ± 5.7%, respectively), and inhibition of the COX-2 enzyme (63.8 ± 9.8%, 19.9 ± 8.4%, and 44.9 ± 8.8%, respectively)].
Fraction BI was separated by column chromatography (CC) on polyamide to give seven subfractions (BI/1-I/7), and some of them were found to be moderately or highly active on investigated assays. The most active subfractions BI/2 (inhibition of COX-2: 73.3 ± 3.7%; and NF-κB1 gene expression: 56.4 ± 2.1%; inhibition of iNOS: 103.1 ± 2.4%), BI/6 (inhibition of iNOS: 66.2 ± 12.2%) and BI/7 (inhibition of iNOS: 73.4 ± 4.4%) were then subjected to multiple chromatographic separations, including VLC, rotation planar chromatography (RPC), medium-pressure liquid chromatography (MPLC), gel filtration on Sephadex LH-20, and preparative thin-layer chromatography TLC. This purification process afforded the isolation of seven compounds (**1**–**7**) in pure form.
The structure elucidation was carried out by extensive spectroscopic analysis using one- and two-dimensional NMR (^1H-^1H COSY, HSQC, HMBC) spectroscopy, atmospheric pressure chemical ionization-mass spectrometry (APCI-MS) experiments, and comparison of the spectral data with literature data. Three lignans, pinoresinol (**1**), medioresinol (**3**) [10], and (±)-syringaresinol (**2**) [11], and four flavonoids, hispidulin (**4**) [12], nepetin (**5**) [13], apigenin (**6**), and luteolin (**7**), were identified from the aerial parts of *O. acanthium* (**Fig. 1**). The compounds, excluding apigenin (**6**) and luteolin (**7**), were isolated for the first time from this species. Furthermore, pinoresinol (**1**), syringaresinol (**2**), hispidulin (**4**), and nepetin (**5**) were previously described from other *Onopordum* species [1] and medioresinol (**3**) was first detected in the genus.
The compounds isolated from aerial parts were tested for inhibitory effects on COX-2 and NFκB1 gene expression, iNOS, 5-LOX, and COX-1 and COX-2 enzymes in *in vitro* assays at 20 µM concentration (**Table 2**). Among the flavonoids, noteworthy inhibitory activities (> 50% inhibition) were recorded for luteolin (**7**), nepetin (**5**), and hispidulin (**4**). Luteolin (**7**) was the most potent in the inhibition of 5-LOX (74.6 ± 8.8%) in accordance with previously reported studies [14]. Moreover, luteolin (7) was moderately active on the COX-2 and NF-κB1 gene expression and iNOS assays.
Only moderate activities were observed for the lignans, but pinoresinol (**1**) proved to be active against LPS/IFN-γ-induced NO production. These results were in agreement with data published by Jung et al., who investigated the inhibition of inflammatory responses by pinoresinol (**1**) from the fruits of *Forsythia koreana*

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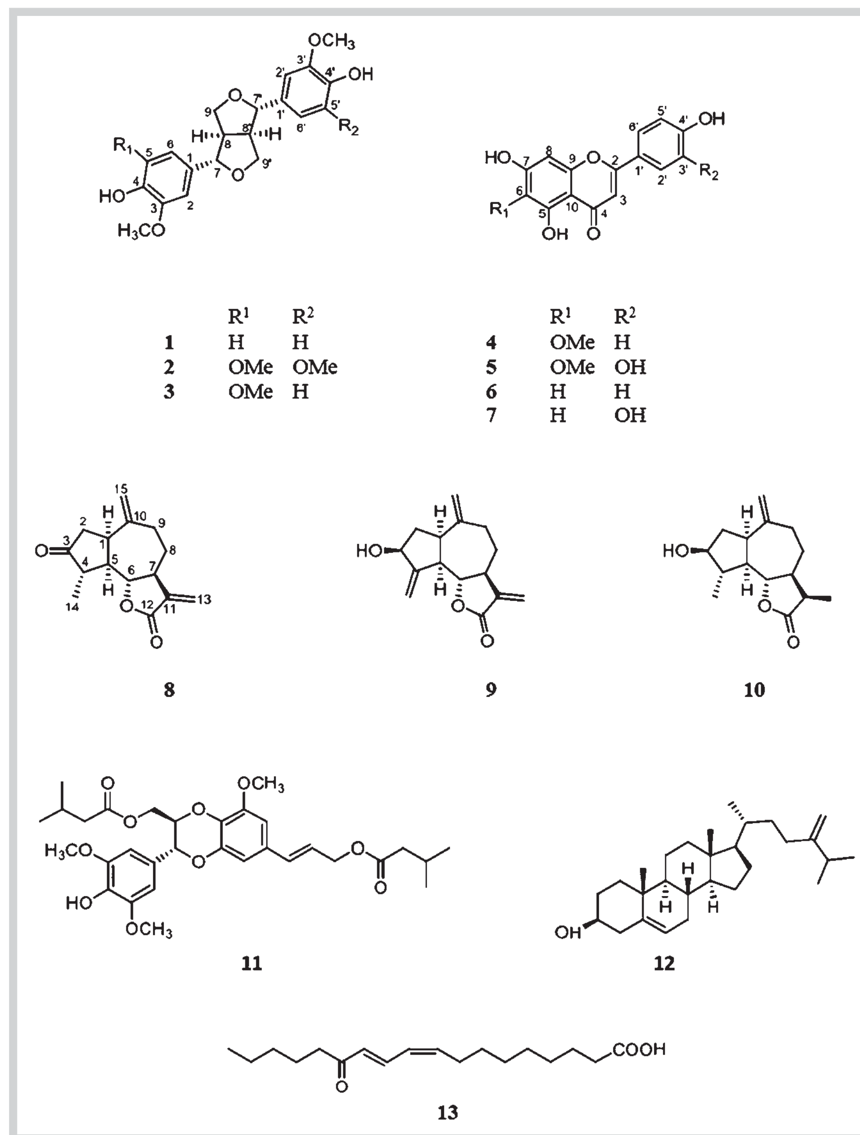

Fig. 1 Structures of compounds **1–13**.

Table 2 Inhibition of COX-2 and NF-*κ*B1 gene expression, NO production, 5-LOX, and COX-1 and COX-2 enzymes by compounds isolated from herbs of *O. acanthium*. N = two experiments in duplicate.

Compound	Inhibition % ± SD					
	COX-2* (20 µM)	NF-*κ*B1 (20 µM)	iNOS (20 µM)	5-LOX (20 µM)	COX-1 (20 µM)	COX-2** (20 µM)
Pinoresinol (**1**)	< 10	< 10	49.13 ± 4.10	37.5 ± 12.2	12.7 ± 6.1	12.3 ± 9.5
Syringarsinol (**2**)	< 10	16.1 ± 11.5	17.44 ± 8.28	28.5 ± 7.1	< 10	< 10
Medioresinol (**3**)	< 10	11.2 ± 7.7	< 10	11.4 ± 12.0	16.2 ± 8.1	< 10
Hispidulin (**4**)	< 10	10.3 ± 1.9	< 10	51.6 ± 11.0	10.9 ± 2.5	< 10
Nepetin (**5**)	21.1 ± 23.4	10.9 ± 5.7	< 10	62.4 ± 7.7	< 10	10.1 ± 7.2
Apigenin (**6**)	30.4 ± 12.6	28.6 ± 8.2	21.6 ± 7.7	41.3 ± 10.2	< 10	24.3 ± 10.3
Luteolin (**7**)	37.2 ± 25.1	30.9 ± 1.0	38.9 ± 10.1	74.6 ± 8.8	10.2 ± 9.4	39.1 ± 10.8
Dexamethasone***	47.6 ± 4.2	ND	ND	ND	ND	ND
Quercetin***	ND	46.0 ± 8.4	ND	ND	ND	ND
L-NMMA***	ND	ND	52.5 ± 4.9	ND	ND	ND
Zileuton***	ND	ND	ND	63.0 ± 3.8	ND	ND
Indomethacin***	ND	ND	ND	ND	32.3 ± 4.8	ND
NS-398***	ND	ND	ND	ND	ND	30.5 ± 3.1

* COX-2 gene expression inhibition; ** COX-2 enzyme inhibition; *** positive control; ND = not determined

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Table 3 Inhibition of COX-2 and NF-*κ*B1 gene expression, NO production, 5-LOX, and COX-1 and COX-2 enzymes by extracts from roots of *O. acanthium*. N = two experiments in duplicate.

Extract	Inhibition % ± SD					
	COX-2* (10 µg/mL)	NF-*κ*B1 (10 µg/mL)	iNOS (10 µg/mL)	5-LOX (50 µg/mL)	COX-1 (50 µg/mL)	COX-2** (50 µg/mL)
n-Hexane extract of the roots (**A**)	60.6 ± 7.9	17.5 ± 7.6	83.2 ± 1.1	66.9 ± 10.0	60.3 ± 8.4	86.5 ± 8.9
Chloroform extract of the roots (**B**)	53.7 ± 8.5	21.0 ± 6.8	51.8 ± 1.7	56.7 ± 8.1	< 10	31.8 ± 10.5
Aqueous-methanol extract of the roots (**C**)	3.2 ± 6.2	10.0 ± 3.6	< 10	59.7 ± 7.7	< 10	28.5 ± 10.3
Dexamethasone***	47.6 ± 4.2	ND	ND	ND	ND	ND
Quercetin***	ND	46.0 ± 8.4	ND	ND	ND	ND
L-NMMA***	ND	ND	52.5 ± 4.9	ND	ND	ND
Zileuton***	ND	ND	ND	63.0 ± 3.8	ND	ND
Indomethacin***	ND	ND	ND	ND	32.3 ± 4.8	ND
NS-398***	ND	ND	ND	ND	ND	30.5 ± 3.1

* COX-2 gene expression inhibition; ** COX-2 enzyme inhibition; *** positive control; ND = not determined

Table 4 Inhibition of COX-2 and NF-*κ*B1 gene expression, NO production, 5-LOX, and COX-1 and COX-2 enzymes by compounds isolated from roots of *O. acanthium*. N = two experiments in duplicate.

Compound	Inhibition % ± SD					
	COX-2* (20 µM)	NF-κB1 (20 µM)	iNOS (20 µM)	5-LOX (20 µM)	COX-1 (20 µM)	COX-2** (20 µM)
4*β*,15-Dihydro-3-dehydrozaluzanin C (**8**)	98.6 ± 0.2	78.7 ± 7.3	100.4 ± 0.5	26.4 ± 12.9	< 10	10.5 ± 10.3
Zaluzanin C (**9**)	97.0 ± 1.1	69.9 ± 3.4	99.4 ± 0.8	< 10	< 10	< 10
4*β*,15,11*β*,13-Tetrahydrozaluzanin C (**10**)	< 10	< 10	61.4 ± 17.3	< 10	< 10	29.5 ± 9.6
Nitidanin diisovalerianate (**11**)	< 10	13.1 ± 14.6	< 10	16.1 ± 11.0	< 10	< 10
24-Methylenecolesterol (**12**)	< 10	11.7 ± 10.6	< 10	< 10	10.1 ± 6.3	36.4 ± 9.5
13-Oxo-9Z,11E-octadecadienoic acid (**13**)	< 10	18.6 ± 6.6	< 10	20.4 ± 11.5	16.4 ± 5.5	16.6 ± 8.8
Dexamethasone***	47.6 ± 4.2	ND	ND	ND	ND	ND
Quercetin***	ND	46.0 ± 8.4	ND	ND	ND	ND
L-NMMA***	ND	ND	52.5 ± 4.9	ND	ND	ND
Zileuton***	ND	ND	ND	63.0 ± 3.8	ND	ND
Indomethacin***	ND	ND	ND	ND	32.3 ± 4.8	ND
NS-398***	ND	ND	ND	ND	ND	30.5 ± 3.1

* COX-2 gene expression inhibition, ** COX-2 enzyme inhibition, *** positive control, ND = not determined

(Rehder) Nakai (Oleaceae). Pinoresinol (**1**) inhibited the production of NO, prostaglandin-E2 (PGE_2), tumor necrosis factor-*α*, and interleukin-6 in LPS-activated microglia. Furthermore, pinoresinol (**1**) attenuated mRNA and protein levels of iNOS, COX-2, and proinflammatory cytokines in LPS activation [15].

Besides the extracts and compounds of the aerial parts of *O. acanthium*, extracts of different polarity prepared from the roots were also tested for their inhibitory effect on all test models (**Table 3**). The *n*-hexane and $CHCl_3$ extracts of the roots showed a considerable inhibiting effect on more tests, therefore, the compounds **8**–**13** isolated earlier from the lipophilic extract [9] were also included in the assays. The most active compounds were 4*β*,15-dihydro-3-dehydrozaluzanin C (**8**) and zaluzanin C (**9**), which exhibited inhibitory activities on the gene expression of COX-2 and NF-*κ*B1, and decreased the NO production at different concentrations (**Tables 4** and **5**). These results confirm the previously reported anti-inflammatory activities of compounds **8** and **9** [1]. 4*β*,15,11*β*,13-Tetrahydrozaluzanin C (**10**) also demonstrated activity against LPS/IFN-*γ*-induced NO production. Other tested compounds were found to be moderately active or inactive in the used assays. As far as we know, this is the first report on inhibitory activity of compounds **8** and **9** against COX-2 and NF-*κ*B1 gene expression (mRNA level) in THP-1 cells.

Table 5 Inhibition of COX-2 and NF-*κ*B1 gene expression in THP-1 cells by isolated compounds (**8**, **9**) from roots at various concentrations.

Compound	Inhibition % ± SD			
		COX-2 gene expression		NF-*κ*B1 gene expression
8	1 µM	41.6 ± 7.6	1 µM	19.3 ± 2.7
	5 µM	79.7 ± 14.8	10 µM	54.3 ± 8.3
	10 µM	96.0 ± 2.1	40 µM	91.2 ± 0.1
9	1 µM	19.4 ± 22.6	1 µM	13.5 ± 5.2
	5 µM	51.9 ± 17.6	10 µM	44.6 ± 4.7
	10 µM	83.7 ± 0.8	40 µM	87.9 ± 0.0
References*		47.6 ± 4.2		ND
		ND		46.0 ± 8.4

* Dexamethasone at 2.5 nM (COX-2 gene expression); quercetin at 25 µM (NF-*κ*B1 gene expression); ND = not determined

In order to determine whether the gene expression inhibitory effects were due to cytotoxicity, the compounds were investigated by the XTT assay at different time points (4 h, 24 h, 48 h, 72 h) and at different concentrations. It was found that the active compounds had no or low effects on cell viability at the tested concentrations (**Fig. 2**).

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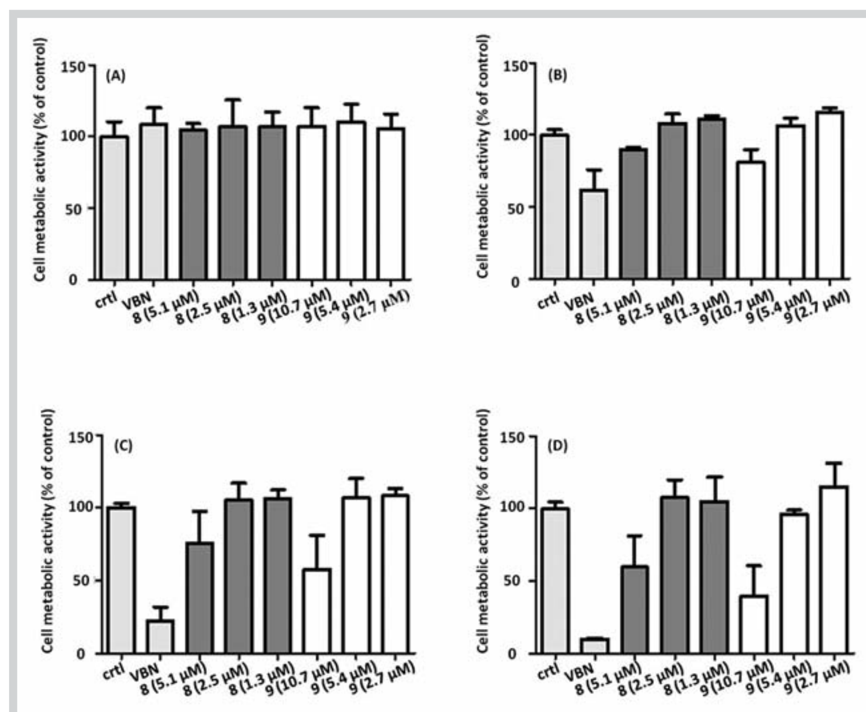

Fig. 2 Effects of 4*β*,15-dihydro-3-dehydrozaluzanin C (**8**) and zaluzanin C (**9**) in different concentrations on human leukemia cell lines (THP-1). Effect after 4 h, 24 h, 48 h, and 72 h using the XTT assay (n = 6, mean ± SD). Vehicle-treated cells (crtl, 0.1% DMSO) served as a control; vinblastin as a positive control (0.1 µg/mL).

Previously, the phytochemical investigation of another *Onopordum* species (*O. laconicum* Heldr. & Sart. ex Rouy) also resulted in the isolation of 4*β*,15-dihydro-3-dehydrozaluzanin C (**8**) and zaluzanin C (**9**) [16], which were the most effective compounds in our assays. The presence of these compounds is limited to these species in the *Onopordum* genus. Furthermore other metabolites, e.g., flavonoids [apigenin (**6**), luteolin (**7**), hispidulin (**4**), and eriodictyol], were also found in both plant species [1]. Similarities in the phytochemical profile confirm the close relationship of these two species. The anti-inflammatory activity of *O. laconicum* has not been investigated yet, but it can be promising for pharmacological examination from this point of view.

In summary, the present study reveals the *in vitro* COX-2 and NF-*κ*B1 gene expression, iNOS, 5-LOX, and COX-1 and COX-2 enzymes inhibitory effects of *O. acanthium*. The inhibitory activities of the isolated compounds were demonstrated in these assays. The results confirm that the inhibitory activities of the extracts may be attributed mainly to flavonoids, lignans. and sesquiterpenoids, and other compounds are exerting additional effects. The traditional uses of *O. acanthium* in ethnomedicine against inflammatory diseases seems to be supported by our data.

Materials and Methods

General experimental procedures

VLC was carried out on silica gel G (15 µm, Merck); CC on polyamide (ICN) and Sephadex LH-20 (25–100 µm, Pharmacia Fine Chemicals); preparative TLC on silica gel 60 F_{254} plates (Merck); and RPC on silica gel 60 GF_{254} and Al_2O_3 60 G (Merck) using a Chromatotron instrument (Model 8924, Harrison Research). MPLC was performed by a Büchi apparatus (Büchi Labortechnik AG) using a 40 × 150 mm RP18ec column (40–63 µm, Büchi).

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) and 125 MHz (^{13}C). The signals of the deuterated solvents were taken as a reference. Two-dimensional (2D) experiments were performed with standard Bruker software. In the COSY, HSQC, and HMBC experiments, gradient-enhanced versions were used. MS spectra were recorded on an API 2000 Triple Quad mass spectrometer with an APCI ion source using positive and negative polarities.

Plant material

The roots and aerial parts of *O. acanthium* were collected in 2008 from the Southern Great Plain, Hungary, and identified by Dr. Tamás Rédei (Institute of Ecology and Botany, Centre for Ecological Research, Hungarian Academy of Sciences, Vácrátót, Hungary). A voucher specimen (No. 814) has been deposited at the Department of Pharmacognosy, University of Szeged.

Extraction and isolation

The air-dried and ground aerial parts of *O. acanthium* (4.4 kg) were extracted with MeOH (61 L) at room temperature in a percolator. The crude extract (1550 g) was evaporated *in vacuo* and subjected to solvent-solvent partitioning first with 30 L of *n*-hexane (A) and then with 29 L of $CHCl_3$ (B). The residue gave the aqueous-MeOH exract (C). After concentration, the $CHCl_3$ extract (66 g) was fractionated by VLC on silica gel (85 mm × 210 mm), which was eluted with a gradient system of *n*-hexane-$CHCl_3$-MeOH [from 7 : 3 : 0 to 0 : 4 : 6 (600 mL, 400 mL, 500 mL, 500 mL, 2000 mL, 1400 mL, 1400 mL, 1000 mL 1000 mL, 500 mL, and 500 mL, respectively), and finally MeOH (1500 mL); the volumes of collected fractions were 200 mL and 100 mL to yield six major fractions (BI-VI).

Fraction BI (12 g) was chromatographed on a polyamide column (60 mm × 200 mm) with mixtures of MeOH and H_2O [1 : 4, 2 : 3, 3 : 2, 4 : 1, 9 : 1 and 1 : 0 (1500 mL each); the volume of collected fractions was 500 mL] as eluents to give seven fractions (BI/1–7).

Fraction BI/2 (1.26 g) was separated by VLC (45 mm × 200 mm) with a gradient system of cyclohexane-EtOAc-MeOH [from 8 : 2 : 0 to 0 : 8 : 2 (260 mL, 200 mL, 300 mL, 200 mL, 300 mL, 200 mL, and 200 mL, respectively) and MeOH (300 mL; the vol-

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ume of collected fractions was 20 mL] to yield eleven subfractions (BI/2/1–11). The BI/2/5 subfraction (79.5 mg) was further purified by RPC on Al_2O_3 (plates of 1 mm thickness) with mixtures of cyclohexane-CH_2Cl_2-MeOH [5 : 15 : 1 (100 mL); the volume of collected fractions was 2 mL], and finally subjected to preparative NP-TLC using the mobile phase of cyclohexane-EtOAc-EtOH (15 : 15 : 2) to yield compound **1** (5.5 mg). Moreover, subfraction B/I/2/6 (244.4 mg) was separated by RP-MPLC with a gradient system of MeOH-H_2O [from 1 : 1 to 9 : 1 (100 mL each) and MeOH (150 mL); the volume of collected fractions was 10 mL] and then by preparative TLC, using cyclohexane-EtOAc-EtOH (15 : 15 : 2), to give compounds **2** (9.4 mg) and **3** (3.4 mg).

Fraction BI/6 (1.1 g), which was eluted with MeOH-H_2O (4 : 1), was separated by RPC on silica gel (plates of 4 mm thickness) with a gradient system of *n*-hexane-CH_2Cl_2-MeOH [2 : 7 : 1 (250 mL), 2 : 8 : 1.5 (100 mL) and MeOH (150 mL); the volume of collected fractions was 25 mL] to yield two subfractions (BI/6/1 and BI/6/2). Subfraction BI/6/1 (102.5 mg) was purified on Sephadex LH-20 column using MeOH as an eluent to afford compound **4** (3.5 mg). Furthermore, subfraction BI/6/2 (310 mg) was separated by RPC on silica gel (plates of 2 mm thickness) with the isocratic solvent system of CH_2Cl_2-MeOH [9 : 1 (500 mL); the volume of collected fractions was 30 mL], and it was finally purified by a Sephadex LH-20 column using the mobile phase MeOH [(70 mL); the volume of collected fractions was 2 mL] to yield compound **5** (8 mg).

Fraction BI/7 (637 mg) was subjected to RPC on silica gel (plates of 4 mm thickness) using a gradient system of cyclohexane-CH_2Cl_2-MeOH [from 2 : 9 : 0.5 to 0 : 10 : 1 (80 mL, 360 mL, and 120 mL, respectively) and MeOH (120 mL); the volume of collected fractions was 40 mL]. Two subfractions, BI/7/2 (111.5 mg) and BI/7/3 (90 mg), were chromatographed on a Sephadex LH-20 column using the mobile phase MeOH [(70 mL); the volume of collected fractions was 2 mL] to get compounds **6** (4.5 mg) and **7** (4.5 mg), which were identified by co-TLC with authentic samples and via ^{1}H- and ^{13}C-NMR spectroscopy as apigenin and luteolin, respectively.

Compounds **8**–**13** were previously published from the roots of *O. acanthium* [9]. The purity of all isolated compounds was determined by integration of proton resonances, and was shown to be **1**–**8** and **12**: > 98 %, **9**–**11** and **13**: > 90 %.

(+)-Pinoresinol (**1**): amorphous powder; $[\alpha]_D^{24}$ + 53 (*c* 0.1, $CHCl_3$); ^{1}H-NMR δ_H: ($CDCl_3$): 3.10 (2 H, m, H-8, H-8′), 3.88 (2 H, dd, *J* = 9.1, 3.4 Hz, H-9a, H-9′a), 3.90 (6 H, s, 2 × OCH_3), 4.25 (2 H, dd, *J* = 9.1, 6.8 Hz, H-9b, H-9′b), 4.74 (2 H, d, *J* = 4.1 Hz, H-7, H-7′), 6.81 (2 H, dd, *J* = 8.2, 1.6, H-6, H-6′), 6.89 (2 H, d, *J* = 8.2, H-5, H-5′), 6.90 (2 H, d, *J* = 1.6, H-2, H-2′). ^{13}C-NMR δ_C: 54.2 (C-8, C-8′), 55.9 (OCH_3), 71.7 (C-9, C-9′), 85.9 (C-7, C-7′), 108.6 (C-2, C-2′), 114.3 (C-5, C-5′), 120.7 (C-6, C-6′), 132.9 (C-1, C-1′), 145.3 (C-4, C-4′), 146.7 (C-3, C.-3′); APCI-MS (negative mode): *m/z* 357 $[M - H]^-$, 151, 136.

Pharmacological tests

Cell culture: The human monocytic cell line THP-1 was obtained from European Collection of Cell Cultures (Item No: 88 081 201) and was maintained in RPMI 1640 (Gibco®) supplemented with 2 mM L-glutamine, 10 % fetal bovine serum (Gibco®), 10 mM HEPES (Gibco®), 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco®) at 5 % CO_2 and a 37 °C humidified atmosphere.

NF-κB1/COX-2 gene expression assay: For the monocyte macrophage differentiation, 1 × 10^6/mL cells were seeded into a 24-well plate with medium containing 12 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 48 h [17, 18]. Subsequently, cells were incubated with testing samples in a selected concentration for 1 h and stimulated with 7.5 ng/mL LPS (Sigma-Aldrich) for an additional 3 h [17]. The final concentration of the tested compounds was 20 µM and the extracts were 10 µg/mL, which were dissolved in DMSO. IC_{50} determinations of compounds **8** and **11** were performed in at least five concentrations, each in at least three independent experiments run in duplicate. Positive controls were quercetin (25 µM, purity ≥ 98 %) (Sigma-Aldrich) for NF-*κ*B1 and dexamethasone (2.5 nM, purity ≥ 97 %) (Sigma-Aldrich) for COX-2. 0.1 % DMSO was used as a calibrator sample.

RNA isolation and reverse transcription: Total RNA was extracted with a GenElute™ Mammalian Total RNA Miniprep Kit (Sigma-Aldrich). Reverse transcription was performed with a high-capacity cDNA Revers Transcription Kit (Applied Biosystems®). Both were carried out according to the manufacturer's manual. The cycler condition for reverse transcription was set to 25 °C for 25 min, 37 °C for 120 min, and 85 °C for 5 s.

Real-time PCR: Primers and probes for NF-*κ*B1 (NM_003 998) and COX-2 (NM_000 963) were designed with Primer Express Software supplied by Applied Biosystems® (COX-2 primers: forward 5′-GAA-TCATTC-ACC-AGG-CAA-ATT-G-3′, reverse 5′-TCT-GTA-CTG-CGG-GTG-GAA-CA-3′ and COX-2 probe: 5′-FAM-TCC-TAC-CAC-CAG-CAA-CCC-TGC-CA-TAMRA-3′; NF-*κ*B1 primers: forward 5′-CCA-CAG-ATGTTC-ATA-GAC-AAT-TTG-C-3′, reverse 5′-TTC-ACT-AGT-TTC-CAA-GTC-AGA-TTT-CC-3′ and NF-*κ*B1 probe 5′-FAM-CAG-CCT-CTG-TGT-TTG-TCC-AGC-TTC-GG-TAMRA-3′).

mRNA expression was quantified with an ABI 7300 Real-Time PCR System (Applied Biosystems®) using a TaqMan probe against endogenous control GAPDH (Pre-developed TaqMan® Assay, Applied Biosystems®) via the ΔΔct-method. The cycling condition was set to 50 °C for 2 min, 95 °C for 10 min, followed by 40 PCR cycles of 95 °C for 15 s and 60 °C for 1 min.

NO assay: The inhibition of NO production *in vitro* in LPS/IFN-γ-stimulated RAW264.7 cells was determined by the Griess assay method, as described before [19]. The NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) (100 µM, purity > 99 %) (Alexis Biochemicals) was used as a positive control. The absorbance, which is inversely proportional to the inhibition of NO production, was determined by a microplate reader (Perkin Elmer Wallac Victor 1420 Multilabel Counter) at 540 nm.

Leukotriene biosynthesis (5-LOX) inhibition assay: The LOX inhibition assay was carried out in a 96-well plate format as described previously [20], with slight modifications. Thirty mL of venous human blood from healthy volunteer donors were collected by a physician (Institute of Hygiene, Microbiology and Environmental Medicine, Medical University of Graz) with a BD Vacutainer® system, 9NC 0.129 M (BD, Belliver Industrial Estate). Human neutrophile granulocytes with 5-LOX activity were isolated from the venous human blood based on sedimentation rates and lysis tolerance.

The cell suspension (4500 cells/mL) was incubated with the sample, $CaCl_2$, ETYA, Calcium Ionophore A23187, and arachidonic acid in a shaking water bath at 37 °C. After 10 min, the incubation was stopped by the addition of 10 % formic acid. After centrifugation, the samples were diluted and the concentration of LTB_4 formed during incubation was determined by means of a competitive LTB_4 EIA Kit (Cayman Chemical Company). Zileuton (5 µM, purity > 98.5 %) (Sequoia Research Products Ltd.) was used as a positive control.

COX-1 and COX-2 inhibition assays: The COX-1 and COX-2 assays were carried out in a 96-well plate format with purified PGHS-1

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isolated from ram seminal vesicles (Cayman Chemical Company) and human recombinant N-terminal hexahistidine-tagged PGHS-2 isolated from a *Baculovirus* overexpression system in Sf21 cells, as reported previously [21,22]. The concentration of PGE_2, the main arachidonic acid metabolite in this reaction, was determined by a competitive PGE_2 EIA kit (Enzo Life Sciences). Indomethacin (1.25 µM, purity ≥ 99%) and NS-398 (5 µM, purity ≥ 98%) (Cayman Chemical Company) were used as positive controls.

XTT viability assay: The cell proliferation kit II (XTT) (Cat. No. 11 465 015 001) was obtained from Roche Diagnostics. THP-1 cells were seeded at a density of 2×10^4 cells (100 µL) per well in a 96-well plate and treated in relevant concentrations of **8, 9**, and **10** for different time periods (4 h, 24 h, 48 h, 72 h) at 37 °C and 5% CO_2. After the incubation, the fresh XTT solution [5 mL of XTT soln. (sodium 3-[1(phenylaminocarbonyl)-3,4-tetrazolium]-bis (4-methoxy-6-nitro)benzene sulfonic acid hydrate), plus 100 mL of electron coupling reagent] was added and analyzed after another 4 h using a microplate reader (TECAN Rainbow) at a wavelength of 490 nm; the reference wavelength was 650 nm. Vinblastine (0.1 µg/mL, purity ≥ 96%) (Sigma-Aldrich) served as a positive control.

Statistical analysis

Data were analyzed with Graph Pad Prism 4.03 (GraphPad Software Inc.) and are given as mean ± standard deviation.

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Conflict of Interest

The authors declare no conflicts of interest.

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