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Biologically active secondary metabolites from Asteraceae and Polygonaceae species

Ph.D. Thesis

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LIST OF PUBLICATIONS RELATED TO THE THESIS

I. **Lajter I**, Zupkó I, Molnár J, Jakab G, Balogh L, Vasas A, Hohmann J.
Antiproliferative activity of Polygonaceae species from the Carpathian Basin against human cancer cell lines
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II. **Lajter I**, Vasas A, Orvos P, Bánsághi S, Tálosi L, Jakab G, Béni Z, Háda V, Forgo P, Hohmann J.
Inhibition of G protein-activated inwardly rectifying K^+ channels by extracts of *Polygonum persicaria* and isolation of new flavonoids from the chloroform extract of the herb
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III. **Lajter I**, Vasas A, Béni Z, Forgo P, Binder M, Bochkov V, Zupkó I, Krupitza G, Frisch R, Kopp B, Hohmann J.
Sesquiterpenes from *Neurolaena lobata* and their antiproliferative and anti-inflammatory activities
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Pharmacological insight into the anti-inflammatory activity of sesquiterpene lactones from *Neurolaena lobata* (L.) R.Br. ex Cass.
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V. **Lajter I**, Pan SP, Nikles S, Ortmann S, Vasas A, Csupor-Löffler B, Forgó P, Hohmann J, Bauer R.
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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ABBREVIATIONS AND SYMBOLS

1D	one-dimensional
2D	two-dimensional
APCI	atmospheric pressure chemical ionization
COSY	correlated spectroscopy
EIMS	electron ionization mass spectrometry
ESIMS	electron spray ionization mass spectrometry
GF	gel filtration
GIRK	G protein-activated inwardly rectifying K^+ channel
HMBC	heteronuclear multiple-bond correlation spectroscopy
HPLC	high-performance liquid chromatography
HRE(S)IMS	high-resolution electron (spray) ionization mass spectrometry
HRMS	high-resolution mass spectrometry
HSQC	heteronuclear single-quantum coherence spectroscopy
JMOD	J-modulated spin-echo experiment
MPLC	medium-pressure liquid chromatography
MS	mass spectrometry
NF-κB	nuclear factor-κB
NMR	nuclear magnetic resonance
NO	nitric oxide
NOE	nuclear Overhauser effect
NOESY	nuclear Overhauser enhancement spectroscopy
NP	normal-phase
NSAID	non-steroidal anti-inflammatory drug
OCC	open-column chromatography
PLC	preparative-layer chromatography
RP	reversed-phase
RPC	rotation planar chromatography
SL	sesquiterpene lactone
TLC	thin-layer chromatography
TMS	tetramethylsilane
UV	ultraviolet
VLC	vacuum-liquid chromatography
δ	chemical shift

1. INTRODUCTION

Cancer and cardiovascular diseases are the leading causes of death in the western world.[1] Cancer and certain cardiovascular diseases, such as atherosclerosis, are often associated with inflammation, and it has been demonstrated that chronic inflammation may be the common factor in many diseases.[2] The clinically used anti-inflammatory drugs (steroids) are effective, but their long-term use may require increasing doses and cause unwanted side-effects, such as hypertension, oedema, ulcer, weight gain and insulin resistance. There are other types of drugs that are non-steroidal anti-inflammatory agents (NSAIDs) and have lower toxicity. The treatment of cardiovascular diseases includes a series of indications, such as cardiotonic and antiarrhythmic agents, lowering the level of low-density lipoprotein cholesterol, decreasing the blood pressure, preventing blood clots, relieving fluid buildup and managing stress hormones. Great efforts are ongoing worldwide in the search for new compounds that can selectively influence these diseases.

Plants have a long history of use in the treatment of the above diseases. Over 60% of the currently used anticancer agents are derived from natural sources, including plants, marine organisms and micro-organisms. The agents include vinblastine, vincristine, the camptothecin derivatives, etoposide and paclitaxel. Trabectedin (Yondelis®), isolated from the sea squirt, *Ecteinascidia turbinata*, provided the first marine anticancer drug to be approved in Europe.[3] Ingenol-3-angelate (Picato®), a metabolite found in *Euphorbia peplus*, has attracted considerable interest in the past few years since its approval by the EMA in 2013 for the treatment of actinic keratosis, a precancerous skin condition. Much time has passed since a natural product without structural modification was introduced into clinical practice.[4] Several plant-derived agents are currently undergoing clinical development, among them flavopiridol, combretastatins and roscovitine.

The best-known example of a natural anti-inflammatory agent is salicylic acid, isolated from *Salix* species, and its derivative acetylsalicylic acid which has more favorable side-effects and a wide range of applications.[5-7] Cannabidiol, a secondary metabolite in *Cannabis sativa* has also been approved for the treatment of inflammation in many countries (e.g. Germany, the UK, Canada and New Zealand) in 2005. In contrast to NSAIDs that inhibit the enzyme cyclooxygenase, the mode of action underlying the anti-inflammatory effects of natural products, such as sesquiterpene lactones (SLs) has been explained by mechanisms involving the inhibition of nuclear factor-kB (NF-κB) and the production of inflammatory cytokines.[8,9] This has raised interest in SLs as prospective therapeutics for the treatment of inflammation.

A number of bioactive compounds generally obtained from terrestrial plants such as isoflavones, resveratrol, quercetin, catechin, sulforaphane, tocotrienols and carotenoids have been proven to

promote cardioprotection and to reduce the risk of cardiovascular diseases. The cardioprotective effects of the various phytochemicals may be due to their antioxidative, antihypercholesterolaemic, antiangiogenic, anti-ischaemic, platelet aggregation-inhibitory and anti-inflammatory activities that reduce the risk of cardiovascular disorders.[10]

Over the past few years, there has been a rapid escalation in the discovery of molecular targets that may be applied to the discovery of novel tools for the diagnosis, prevention and treatment of human diseases (e.g. diabetes, bacterial and viral infections and cancer).[11] Natural products possess a broad diversity of structures and functions, and have traditionally provided substantial inspiration for drug development programs. Modern isolation and screening technologies have enhanced the search for new lead molecules and increased interest in folk-medicinal plant extracts.[12]

A number of traditionally used plants exhibit pharmacological properties of great potential in therapeutic applications. The process that leads from a plant to the discovery of a bioactive compound includes the selection of the plant for investigation. Plants can be selected for screening on the basis of ethnopharmacological information or chemotaxonomic relationships to medicinal plants with pharmacological properties.

Secondary metabolites of the Asteraceae and Polygonaceae species can be regarded as promising starting materials for pharmaceutical discoveries, in consequence of their pharmacological potential, and in particular their noteworthy antitumour and anti-inflammatory effects, which provides a rationale for screening for new active constituents from these families for the treatment of cancer and cardiovascular disorders.

This thesis summarizes our phytochemical and pharmacological investigations on Asteraceae (*Onopordum acanthium* and *Neurolaena lobata*) and Polygonaceae (*Polygonum persicaria*) species with the aim of finding new natural compounds of promise against cancer, inflammation or cardiovascular diseases.

2. AIMS OF THE STUDY

A few years ago, the research group of the Department of Pharmacognosy at the University of Szeged started a screening programme to investigate the antiproliferative activity of the species of the Asteraceae family and to identify the bioactive compounds in the selected plants.[13] The aim of the present work as part of this project was the chemical investigation and detailed pharmacological analysis of two species belonging to the Asteraceae family. In the course of the work, pharmacological screening was extended to plants of the Polygonaceae family.

In order to achieve the aims, the main tasks were:

- A review of the literature on the Asteraceae and Polygonaceae families, from aspect of the chemistry and pharmacological properties of the plants.
- Extraction of plant materials of Polygonaceae species with various solvents for the screening, and investigation of the tumour cell proliferation-inhibitory and GIRK channels modifying activities of the extracts.
- Identification of the bioactive secondary metabolites of *Neurolaena lobata*: isolation, structure elucidation and *in vitro* and *in vivo* evaluation of antiproliferative and anti-inflammatory potential of the extracts and isolated compounds.
- Phytochemical and pharmacological analysis of *Onopordum acanthium*: isolation, structure determination of the compounds and *in vitro* anti-inflammatory evaluation of the extracts and compounds (including compounds previously isolated from the roots of *O. acanthium*).
- Isolation and structure determination of biologically active compounds from *Polygonum persicaria*, and liquid chromatographic−mass spectroscopic (LC-MS) investigation of samples of various origins from different vegetation stages.

3. LITERATURE OVERVIEW

3.1. BOTANY

3.1.1. Botany of the family Asteraceae and the investigated species

The Asteraceae (formerly Compositae; sunflower) family comprise the largest family of flowering plants, with over 1700 genera and ca. 24 000 species. Members of the Asteraceae are distributed throughout the world and occupy a wide range of habitat. There is great diversity in growth form, ranging from annual and perennial herbs to shrubs, vines or trees.[14,15]

Neurolaena lobata (L.) R. Br. ex Cass. (gavilana, capitana, tres puntas, jackass bitters), belonging in the tribe *Neurolaeneae* (Asteraceae), is a herb that grows from 1 to 4 m tall; it has only a few main stems, with numerous branches and yellow blooming florescence. The leaves are alternate, trilobed and extremely bitter-tasting. This plant occurs in the rainforest, in clearings, roadsides, fields and pastures. It is distributed widely in north-western parts of South America and Central America, and can be found also throughout the Caribbean islands.[16,17]

Onopordum acanthium L. (Scotch thistle, cotton thistle), a member of the tribe *Cardueae* (Asteraceae), is a biennial, herbaceous plant with a height of 2.5 m. In the first year it grows very spiny leaves in a large rosette, and develops its typical phenotype properties in the second year. The stems are yellowish, hairy, with spiny wings, and branched in the upper part. The leaves, measuring up to 35 × 20 cm, are oblong-ovate to broadly lanceolate or ovate, sessile, sinuate-dentate or with 6−8 pairs of broadly triangular teeth. Their lobes end in very sharp yellow, green or white spines. The shoots and leaves are covered by hairs, giving the plant a distinct bluish-green appearance. The vibrant purple flowers grow at the end of leafy stalks, as a single flower or a cluster of flowers, produced in the summer. It is native to Europe and Asia, and naturalized in various parts of the world, including Hungary.[18-20]

3.1.2. Botany of the family Polygonaceae and the investigated species

The family Polygonaceae (buckwheat, knotweed or smartweed) comprises approximately 1200 species, in some 48 genera. The family was divided into 2 subfamilies, Eriogonoideae and Polygonoideae by BRANDBYGE (1993).[21] The largest genera are *Polygonum, Rumex*, *Rheum*, *Coccoloba*, *Persicaria* and *Calligonum*. The family is most diverse in the Northern Temperate Zone. Some species of the genera *Fallopia*, *Persicaria*, *Rumex* and *Polygonum* are among the most troublesome invasive species in Europe and North America. There are 37 species that are native to or naturalized in Hungary, and 45 in the Carpathian Basin.[22,23]

Most Polygonaceae species are annual or perennial herbs; some are small shrubs, but vines and rarely trees are also present. A characteristic feature of the family is the ocreae, a nodal sheath

variously interpreted as an outgrowth of the sheathing base of the petiole, as connate stipules, or as an expanded axillary stipule. The leaves of buckwheat are nearly always alternate and penninerved, seldom opposite or whorled. In many genera the stem is characteristically swollen at the nodes. The flowers are usually perfect and actinomorphic with 2 to 6 uniform petaloid petals, often in 2 whorls of 3 or one whorl of 5, persistent in the fruit, the ovule is mostly orthotropus, the fruit is a trigonous or lenticular nut, and the seeds have copious endosperm.[21,22,24]

Polygonum persicaria L. (syn. *Persicaria maculosa* Gray, lady's thumb, redshank) belongs in the genus *Polygonum* (Polygonaceae). It is an annual herb with coarse, erect or ascending stems that grow 30 to 91 cm tall. The leaves are alternate, petiolate to subsessile, lanceolate to elliptic, 2 to 15 cm long, and up to 4 cm wide. They often have a purplish triangular spot near the centre. The flowers are born in terminal or sometimes axillary, cylindrical racemes, and the perianth is bright or pale pink. The petals are fused for one-third of their length and have 4 or 5 lobes. The seeds are lens-shaped to triangular, black, smooth and shiny. It is native to Europe and is widely distributed as a weed throughout temperate and tropical North and South America, Asia, North Africa and Australia.[22,24,25]

3.2. CHEMISTRY

3.2.1. Chemistry of the family Asteraceae and the investigated species

The Asteraceae are among the chemically most diverse groups of flowering plants. SLs, triterpene monools and diols, acetylenic compounds, methylated flavonols and flavones, inulin-type fructans, the cyclitols L-inositol and scyllitol and fatty oils in the seeds are common constituents of many species; they probably occur in all tribes and form the chemical make-up of the family. Essential oils and diterpenoids are also widely distributed. Alkaloids, cyanogenic glycosides, amides, coumarins and several types of phenolic constituents exhibit much more limited distribution.[26]

Neurolaena lobata

Chemical investigations of *N. lobata* have revealed the presence of SLs. In 1978, MANCHAD and BLOUNT isolated 2 germacranolide SLs from leaves of the plant collected in Trinidad and named them neurolenins A and B.[27] A few years later, the presence of a new germecranolide, lobatin A, and the furanoheliangolide lobatin B were reported from the plant.[28] PASSREITER *et al.* investigated the above-ground organs of *N. lobata* obtained from Guatemala. From the CH_2Cl_2 extract of the plant 7 further SLs, the germacralonide neurolenins C–F and the furanoheliangolide lobatin C, 8β-isovaleryloxy-9α-hydroxy-calyculatolide and 8β-isovaleryloxy-9α-acetoxy-calyculatolide were isolated.[16] Besides SLs, *N. lobata* contains pyrrolizidine alkaloids (tussilagine, isotussilagine and 2-pyrrolidineacetic acid methyl ester),[29] 6-hydroxy- and 6-methoxy-flavonoids (6-hydroxykaempferol 3,7-dimethyl ether,

6-hydroxyluteolin 3'-methyl ether, 6-hydroxykaempferol 7-glucoside, 6-hydroxykaempferol 7-glucoside, quercetagetin, 6-hydroxyluteolin 7-glucoside, 6-hydroxykaempferol 3-methyl ether 7-sulfate, 6-hydroxykaempferol 3-methyl ether 7-glucoside, quercetagetin 3-methyl ether 7-sulfate, quercetagetin 3,7-dimethyl ether, quercetagetin 3,6-dimethyl ether, quercetagetin 3-methyl ether 7-glucoside and quercetagetin 7-glucoside)[30] and thymol derivatives.[31]

Onopordum acanthium

In 1931, DRAGENDORFF and PRANTL described the presence of inulin in the petals of the plant. According to the investigations of BORNEMANN and the data of the Atlas of Medicinal Plants of USSR, the seeds contain 30-35% of fatty oils and 0.1% of alkaloids. An SL, named arctiopicrin, was also detected in the leaves.[32] The extensive phytochemical analysis of the aerial parts by BOGS *et al.* resulted in the identification of luteolin 7-*O*-glucoside, apigenin, quercetin, isorhamnetin, aesculin, choline, stachydrine, caffeic acid, chlorogenic acid and quinic acid.[32,33] BOHLMANN *et al.* isolated 4 polyines from the roots.[18] Later, an SL, onopordopicrin, was identified from the fresh leaves by DROZDZ *et al.*[18] In 1976, KARL *et al.* isolated luteolin, chrysoeriol, eriodictyol, isoquercetin, isorhoifolin, apigenin 7-*O*-glucuronide, cyaniding 3,5-diglucoside, saccharides and amino acids from the fresh flowers of *O. acanthium.*[18] In 1979, triterpene alcohols, lupeol and amyrin and their acetates were detected in the seeds.[34] NOLASCO *et al.* reported the presence of 6 steroids (Δ_5-avenasterol, campesterol, stigmasterol, β-sitosterol, brassicasterol and cholesterol) in the seeds.[35] Russian researchers studied various parts of *O. acanthium* and identified triterpenoids (taraxasterol, lupeol and α- and β-amyrin) and their acetates (taraxasteryl acetate, lupeol acetate and α- and β-amyrin acetate) from the flowers, leaves and stems, and a new phenylpropanoid glycoside, aconiside, from the seeds.[34-37] (*E*)-1-Oxo-3,4-dihydro-1*H*-isochromen-7-yl-3-(3,4-dihydroxyphenyl)acrylate was also isolated from the seeds of *O. acanthium* purchased at a local herbal store in Tehran.[38] In the course of work at the Department of Pharmacognosy University of Szeged, three SLs (4β,15-dihydro-3-dehydro-zaluzanin C, zaluzanin C and 4β,15,11β,13-tetrahydrozaluzanin C), a neolignan (nitidanin-diisovalerianate), an oxylipin (13-oxo-9*Z*,11*E*-octadecadienoic acid), 24-methylenecholesterol (for their structures see **Annex I**), α-linolenic acid and linolenic acid were previously identified from the roots of the plant.[39]

3.2.2. Chemistry of the family Polygonaceae and the investigated species

The most characteristic compounds identified from species belonging in the Polygonaceae family are flavonoids (particularly flavonols or their *O*/*C*-glycosides), polysaccharides and phenylpropanoids (caffeic acid and its glycosides, sinapic acid and chlorogenic acid).[40-42] Further important phenolic compounds are anthraquinones (the best-known are emodin, chrysophanol and physcion)[43-45] and stilbenes (frequently *trans*-resveratrol and its glycoside, piceid).[40,41,46,47] Characteristic components

of some *Polygonum* species are drimane-type sesqui- and norsesquiterpenoids and sulfated flavonoids.[48-50] Interestingly, *Rumex* species contain 24-norursane-type triterpenoids.[51]

The majority of the species occurring in the Carpathian Basin have only been poorly analysed, with the exception of *Fallopia* species. Flavonoids, anthraquinones, stilbenes and polysaccharides have been isolated from their roots, stems, flowers and leaves.[41,52,53] Phytochemical investigations of *Rumex scutatus*, *R. conglomeratus*, *R. stenophyllus*, *R. thyrsiflorus*, *R. obtusifolius* subsp. *alpinus*, *R. obtusifolius* subsp. *obtusifolius*, *Polygonum arenarium* and *P. rurivagum* have not been reported previously.

Polygonum persicaria

In 1968, MUKHAMED'YAROVA isolated flavonoids (quercetin, isoquercetin and hyperoside) from *P. persicaria* for the first time.[54] In the 1970s, the presence of isoquercitrin, avicularin, kaempferol, kaempferol 3-galactoside and quercetin 3-galactoside in the seeds was demonstrated by Russian and Italian researchers.[55,56] SMOLARZ investigated the flavonoid composition of the aerial parts of *P. persicaria* by HPLC. Flavonoid glycosides [rutin, quercitrin, miquelianin, quercetin-3-*O*-β-(6"galloyl) glucoside, quercetin-3-*O*-β-(6"-galloyl)galactoside, spiraeoside, kaempferol 3-*O*-glucoside and orientin], and flavonoid aglycons (taxifolin, luteolin, quercetin 3-methylether and rhamnetin) were detected.[55,57] From the CH_2Cl_2 extract of the leaves of the plant collected in Argentina, 3 flavonoids (pinostrobin, flavokawin B and cardamonin) were isolated by DERITA and ZACCHINO.[58] In 2013, KURNIKA *et al*. reported 2 further known flavonoids, polygochalcone and astragalin, and a new natural compound (persicochalcone).[59] Additionally, a new carboxystilbene (persilben),[60] drimane-type sesquiterpenes (polygodial, isopolygodial, drimenol and confertifolin),[58,61] a 5,7-dihydroxychromone, sitosterol[56] and phenolic acids (protocatechuic, genistic, *p*-hydroxybenzoic, vanillic, caffeic, syringic, *p*-coumaric, ferulic and synaptic acid)[62] have been identified in *P. persicaria*.

3.3. FOLK-MEDICINAL USES OF THE INVESTIGATED PLANT SPECIES

N. lobata is a frequently used medicinal plant. In the Central American and Caribbean regions decoctions are utilized for the treatment of cancer, hepatic ailments, anaemia, hypertension, diabetes, stomach pains and skin diseases, and as a tonic and antipyretic.[28,63-65] Among the Yucatec Maya, the crushed leaf is used to treat itchy inflamed skin.[64] In Guatemala and Belize, the leaves of *N. lobata* are widely used to cure or prevent a variety of diseases, particularly fevers, malaria, diarrhoea, ulcers and diabetes, and also amoebiasis, fungal infections, ringworm and intestinal parasites.[17,66,67] More recently, it is also used as oncolytic home remedy. In some areas of Guatemala and Costa Rica, it is additionally known as an insect repellent.[68]

O. acanthium has been used traditionally for its antibacterial, cardiotonic, hemostatic and hypotensive properties.[36,37] Moreover, the juice of this plant is used as a folk remedy against

cancerous ulcers, carcinoma of the face and other tumours.[65] A decoction or powder (mixed with honey) from the seeds is applied in some Turkish regions against hepatic diseases, hemorrhoids, gastric disorders and gynacological diseases and as an antipyretic.[69,70] On the other hand, the inflorescences, roots, seeds and late-developing leaves of Scotch thistle are used internally or externally in traditional medicine in Central Asia for the treatment of different types of inflammation, common colds, purulent wounds, ulcers, furuncles and skin diseases.[71]

P. persicaria is applied as an astringent, rubefacient and vermifuge.[72] Decoctions and infusions of the plant have been used in Russian traditional medicine for the treatment of hemorrhoidal bleeding and as a laxative and diuretic. The infusion of *P. persicaria* is recommended for the treatment of patients with atonic constipation.[73] In Argentina, this plant has been applied against fungal infections, such as skin ailments and vaginal diseases.[74] Additionally, the leaves of *P. persicaria* have been used widely against various cancers, tumours, swellings and ulcers.[75]

3.4. PHARMACOLOGY

3.4.1. Pharmacology of the family Asteraceae and the investigated species

The biological and chemical profiles of the plants belonging in the Asteraceae, with nearly 1700 genera and about 24 000 species, are characterized by the complexity and diversity.[15,26] According to ZDERO and BOHLMANN, around 7000 different compounds were isolated and identified from 5000 species up to the 1990s, including numerous SLs, di- and triterpenoids, flavonoids, polyacetylenes, alkaloids, benzofurans, benzopyrans and phenylpropanes.[76,77] This extraordinary diversity is accompanied by extensive bioactivity. The plants and their secondary metabolites have been demonstrated to possess multiple pharmacological activities, such as antioxidant, antiprotozoal, antimicrobial, cytotoxic, anti-inflammatory, antidiabetic, hepatoprotective and antipasmodic effects, activities on the central nervous and cardiovascular systems, etc. Some groups of compounds found in Asteraceae species, e.g. pyrrolizidine alkaloids, are toxic and are sometimes implicated in human and veterinary poisonings.[78]

Neurolaena lobata

The widespread use of *N. lobata* by different Maya groups and indigenous healers throughout the Caribbean region inspired researchers to investigate the chemical and pharmacological properties of this traditional medicinal plant. Various biological activities have been reported for the crude leaf extract of the plant, and for some of the isolated SLs. Examples are summarized in **Table 1**.

Table 1. Selected pharmacological studies of *N. lobata* [compounds (NA = neurolenin A, NB = neurolenin B, NC = neurolenin C, ND = neurolenin D, LA = lobatin A, LB = lobatin B, 8β-iV-9α-H-C = 8β-isovaleryloxy-9α-hydroxy-calyculatolide)]

Herbal preparation/ compounds tested	Model used	Dosage	Results	Ref.
ANTIMALARIAL ACTIVITY				
CH_2Cl_2, MeOH extract of leaves, NA, NB, NC/D (1:3), NC/D (3:2), LA, LB, 8β-iV-9α-H-C	*in vitro*, *Plasmodium falciparum* (NF54), human erythrocytes	0.012-50 μg/mL	The activity is inversely related to the solvent polarity. IC_{50} = 8.7 μg/mL (CH_2Cl_2); 13.8 μg/mL (MeOH); 0.2−0.3 μg/mL (neurolenins); 6−8 μg/mL (lobatins, 8β-iV-9α-H-C)	[79]
70% EtOH extract of dried, fresh flowers and young leaves	*in vitro*, *P. berghei*, mice erythrocytes	5-100 μg/mL	The fresh extract showed higher antimalarial activity. IC_{50} = 4.3 μg/mL, 8.9 μg/mL (dried extract); 4.1 μg/mL (extract of fresh young leaves)	[80]
Freeze-dried CH_2Cl_2 extract of leaves	*in vitro*, *P. falciparum* (K1; NF54) human erythrocytes	250 mg/mL	IC_{50} of freeze-dried CH_2Cl_2 extract: 8.6 μg/mL, 10.6 μg/mL for *P. falciparum* NF54 and K1 (P = 0.9)	[81]
Freeze-dried H_2O, MeOH extract of leaves	*in vivo*, *P. berghei* (NK 65), mice	750 mg/kg	Parasitaemia was 9.8% and 6.5% after 7 days of treatment with aqueous and MeOH extracts (P = 0.0002; P < 0.0001), respectively	
ANTI-INFLAMMATORY ACTIVITY				
80% EtOH extract of leaves NB, NC/D, LB, 8β-iV-9α-H-C	*in vitro,* TNF-α reduction in LPS-stimulated THP-1 cells	10, 100 μg/mL 0.4−40 μM	The extract of leaves administered at 100 μg/mL reduced LPS-stimulated TNF-α production in THP-1 cells by 72%. IC_{50} for compounds: 0.2−2.3 μM (P ≤ 0.05)	[82]
EtOH extract of leaves	*in vivo,* carrageenan-induced paw oedema in mice	1.25 g/kg	The extract reduced swelling by 19.5%.	[83]
ANTIULCEROGENIC ACTIVITY				
Hexane, $CHCl_3$, aqueous fractions of hydroalcoholic extract of aerial parts	*in vivo*, EtOH/HCl-induced gastric mucosal lesions, mice	50, 100, 200, 1000 mg/kg	Hydroalcoholic extract (1000 mg/kg) and hexane, $CHCl_3$ fractions (100 mg/kg) significantly reduced the ulcers induced by EtOH/HCl solution, by 77, 86 and 83% (P < 0.001)	[84]
	in vivo, hypothermic restraint stress-induced lesion, mice	100, 1000 mg/kg	In a stress-induced gastric model, the hydroalcoholic extract (1000 mg/kg) and hexane, $CHCl_3$ fractions (100 mg/kg) produced a significant reduction of gastric lesion formation, by 48%, 70% and 52% (P < 0.05; P < 0.001)	
	in vivo, NSAID-induced gastric ulcers in cholinomimetic-treated mice	100, 1000 mg/kg	Hydroalcoholic extract (1000 mg/kg) and hexane, $CHCl_3$ fractions (100 mg/kg) also reduced (41%, 57% and 51%) the gastric lesions induced by the combination of indomethacin and bethanechol (P < 0.05; P < 0.001)	
ANTIPROLIFERATIVE ACTIVITY				
NA, NB, NC/D (1:1), LA, LB, 8β-iV-9α-H-C	*in vitro*, MTT assay, GLC_4 and COLO 320 cells	-	The most active compounds against both cell lines were LB and NB (IC_{50} = 0.6 μM and 1.1 μM on GLC_4; 1.1 μM and 1.2 μM on COLO 320) (P < 0.05)	[79]

Table 1. *continued*

PLATELET AGGREGATION-INHIBITORY ACTIVITY				
Crude aqueous extract of whole plant	*in vitro*, human platelet aggregation induced by thrombin (0.075 U/mL)	-	The aggregation of human platelets induced by thrombin was inhibited by the extract (IC_{50} = 0.7 mg/mL)	[85]

Moreover, the leaf extract of *N. lobata* possesses antiparasitic activities, including antitrypanosomal, antitrichomonas and antileishmaniasis activity both *in vitro* and *in vivo*.[66,86,87] The analgesic,[88] antibacterial and antifungal[89,90] activities of *N. lobata* leaf extracts have also been reported. The hypoglycemic activity of the EtOH extract was demonstrated *in vivo*, and in another study the inhibitory effect of *N. lobata* extracts on the transfer of HIV from dendritic cells to lymphocytes was reported *in vitro*.[91,92]

Onopordum acanthium

Several research groups have investigated the antioxidant activity and total phenolic contents of various parts of *O. acanthium*.[93-95] Its anticancer, angiotensin converting enzyme (ACE) inhibitory and cytotoxic properties have also been investigated. Pharmacological investigations have mainly been performed in South-west Asia and South-east Europe.

ABUHARFEIL *et al.* studied the stimulatory activities of aqueous and EtOH extracts prepared from 13 Jordanian plants (including *O. acanthium*) on murine natural killer (NK) cells in generating cytotoxicity against YAC tumour cells *in vitro.*[96] These plants are currently utilized in traditional medicine to treat different type of cancers. The fresh aqueous extracts of *O. acanthium* at concentrations of 5 and 0.1 mg/mL induced an intermediate level (30% and 38.3%) of NK cytotoxicity. Furthermore, the dried aqueous and EtOH extracts of *O. acanthium* showed lower increases in NK cell activity in comparison with the fresh aqueous plant extract.

Our research group earlier evaluated the antiproliferative activities of extracts prepared from roots and aerial parts with solvents of different polarity on 3 human tumour cell lines (HeLa, MCF7 and A431). The $CHCl_3$-soluble leaf and root extracts at 10 µg/mL markedly inhibited the proliferation (70.5–85.1% and 53.1–87.1%).[97] Later, chemical investigation of the roots resulted in the isolation of SLs, a neolignane, steroids and fatty acids. It was found that 4β,15-dihydro-3-dehydrozaluzanin C (**Annex I**), the most active antiproliferative compound in the extract, exerted noteworthy tumour cell growth-inhibitory activity (IC_{50} 2.68–15.06 µM).[39]

In 2013, SHARIFI *et al.* isolated a new compound, (*E*)-1-oxo-3,4-dihydro-1*H*-isochromen-7-yl-3-(3,4-dihydroxyphenyl)acrylate, with ACE-inhibitory activity (83 ± 1% at 330 µg/mL) from *O. acanthium* seeds. This effect was in accordance with the hypotensive property of *O*. *acanthium*. In addition, the DPPH radical scavenging assay revealed the higher antioxidant activity of the isolated compound (IC_{50} = 2.6 ± 0.04 µg/mL) than those of the positive control, BHT (IC_{50} = 10.3 ± 0.15 µg/mL) and Trolox

(IC_{50} = 3.2 ± 0.06 μg/mL). The enzyme inhibition and ACE-C or -N domain specificity of the compound were further evaluated through molecular modeling and docking studies. Molecular docking predicted competitive-type enzyme inhibition and approximately similar affinities of the isolated compound for the ACE-C and -N domains.[38]

3.4.2. Pharmacology of the family Polygonaceae and the investigated species

Many species of Polygonaceae are rich sources of bioactive constituents which contribute to a wide range of medicinal properties. Antioxidant, anti-inflammatory, antimicrobial, antitumour, antiulcerogenic and antileukaemic effects and aldose reductase-, α-glycosidase-, lipid peroxidation- and platelet aggregation-inhibitory activities were reported earlier.

The polysaccharides have been found to display significant radical-scavenging activities (in the DPPH free radical assay), indicating their potential application as novel natural antioxidants.[52] Stilbene derivatives (e.g. resveratrol and piceid) with antibacterial and antifungal activities have also been isolated from many Polygonaceae species.[98] Flavonoids and chalcones possess various biological activities. Their strong antioxidant effects in particular play important roles against radical oxidative stress-causing pathological processes, such as arteriosclerosis or cancer.[99] Quercetin, isolated from many plants of this family, is able to induce apoptosis in human leukaemic cells.[100]

A quercetin derivative, quercetin-3-*O*-β-D-glucuronopyranoside was isolated in large amount from *R. aquaticus* and has been investigated in a number of experimental models. It proved to inhibit neutrophil infiltration into the gastric mucosa, pro-inflammatory cytokine (TNF-α and IL-1β) production,[101] the production of intracellular ROS and ERK ½ activation,[102] and it decreased the area injuries of gastric lesion sizes, acid output and the gastric pH.[103] Furthermore, its antioxidative and anti-inflammatory effects were evaluated on cultured feline oesophageal epithelial cells.[104]

Sesquiterpenes isolated from *Polygonum hydropiper* exhibited interesting biological activities, such as lens aldose reductase-inhibitory, antifungal and antitumour-promoting effects,[50,105] and this plant also has tyrosinase-inhibitory, oestrogenic, antinociceptive and antimutagenic effects. [106-108]

Rheum palmatum, among the best-known members of the Polygonaceae family, produces anthranoids as the most characteristic compounds. Their pharmacological activities have been investigated in many assays. Aloe emodin induces the apoptosis of human nasopharyngeal carcinoma cells.[109] Emodin exerts antidiabetic and antitumour activity.[110-112] Furthermore, emodin has been investigated for its lipid-lowering and neuroprotective effects in rat cortical neurons.[113,114]

Polygonum persicaria

Only a limited number of pharmacological studies have been reported on *P. persicaria*. Most of them deal with the biological activity of extracts with different polarity prepared from the aerial parts.

In 1999, SMOLARZ and SKWAREK found that the EtOH extract of the herb did not influence interferon induction in the cell cultures prepared from monkey kidney.[115] FARRUKH *et al.* investigated the antibacterial, antifungal and insecticidal activities of the crude extract of *P. persicaria,* which displayed significant activity in *in vitro* assays, and concluded that this plant is a potential source of natural antifungal, antibacterial and insecticidal agents.[116] DERITA and ZACCHINO later confirmed the antifungal properties of CH_2Cl_2 extract of the aerial parts *in vitro* by microbroth dilution assay.[117]

YANO *et al*. tested the anti-inflammatory effect of a hydroalcoholic extract of the aerial parts in *in vivo* studies. It was concluded that the extract has anti-inflammatory activity and decreases locomotion after intraperitoneal administration to rats.[118]

As concerns the chemical constituents responsible for the observed activities, persilben, a naturally occurring *E*-stilbene, attracted great interest because of its antimicrobial, antifungal and antioxidant activities and its good penetration through biological membranes in consequence of its high lipophilicity.[60,119]

4. MATERIALS AND METHODS

4.1. PLANT MATERIAL

Plants for screening for antiproliferative and GIRK channel activities

27 species of the Polygonaceae family belonging in the *Fallopia*, *Oxyria*, *Persicaria*, *Polygonum* and *Rumex* genera were collected in the flowering period between June and September 2010, in several regions of the Carpathian Basin (Croatia, Hungary and Romania). Botanical identifications were performed by Gusztáv Jakab (Institute of Environmental Sciences, Faculty of Water and Environmental Management, Szent István University, Szarvas, Hungary) and Lajos Balogh (Natural History Collection, Savaria Museum, Szombathely, Hungary). The plants were separated into different parts. The air-dried plant organs were comminuted and stored at room temperature until processing.

Neurolaena lobata

Plant material was collected 0.5 km north–north-west of San José in the area of the Chakmamantokrock formation (16° 59' 16" N, 89° 53' 45" W), near the north-western shore of Lago Petén Itzá, Departamento Petén, Guatemala, and within the botanical garden of the Institute for Ethnobiology, San José, Guatemala in February 2011.[120] The fresh plant material (aerial plant parts, leaves, caulis and florescence) of *N. lobata* was air-dried (3.0 kg) in Guatemala, sent to Austria, and frozen and stored at –70 °C until preparation.

Onopordum acanthium

Aerial parts of the plant were collected in Kiskundorozsma (Hungary) in May 2008 and authenticated by Dr. Tamás Rédei (Institute of Ecology and Botany, Centre for Ecological Research, Hungarian Academy of Sciences, Vácrátót, Hungary). The air-dried plant material was stored at room temperature until processing.

Polygonum persicaria

Aerial parts were collected in the flowering period in Szarvas-Cserebökény (Hungary) in June 2010. Further *P. persicaria* samples for the LC-MS investigation were collected in Bélbor, Romania (flowering plant, collected in July 2012), in Szarvas-Furugy, Hungary (before the flowering period, June 2012), and in Homoródalmás, Romania (before the flowering period, in July 2012). Botanical identifications were performed by Dr. Gusztáv Jakab. These plant materials were stored at room temperature until processing.

4.2. EXTRACTION

4.2.1. Preparation of extracts for pharmacological screening

All extracts were prepared from 10 g of air-dried, powdered plant material with 100 mL of MeOH with the use of a VWR ultrasonic bed (type USC500TH) at room temperature. After filtration, the solutions were evaporated to dryness with a Büchi Rotavapor R-210 (40 °C, 337 mbar). The residues were dissolved in 50 mL of 50% aqueous MeOH and were subjected to solvent–solvent partition between *n*-hexane (3 × 50 mL) (extracts A) and $CHCl_3$ (3 × 50 mL) (extracts B) and the residue gave extracts C. After extraction with MeOH, the residual plant materials were dried and extracted with 30 mL of boiling H_2O for 15 min in a multiple water bath (type 1041, GFL). The filtered extracts were freeze-dried, using a Hetosicc lyophilizator (type CD 52, Heto Lab Equipment), affording extracts D.

4.2.2. Extraction of the plant materials for preparative phytochemical work

Neurolaena lobata

The air-dried aerial parts of the plant (3.00 kg), which was stored at –70 °C before processing, were ground with a Retsch (type GM 2000) grinder and percolated with MeOH (50 L) at room temperature. The extract was concentrated by using a Rotavapor R-210 (40 °C, 337 mbar). H_2O (1 L) was added to the extract after concentration (1 L) and solvent–solvent partition was performed with petroleum ether (5 × 1 L), then with CH_2Cl_2 (5 × 1 L) and finally with EtOAc (5 × 1 L).

Onopordum acanthium

The air-dried aerial parts (4.4 kg) were ground with a Retsch (type SM 100) grinder and extracted with MeOH (61 L) at room temperature. The extract was concentrated *in vacuo* (1500 mL) and then diluted with 1500 mL H_2O, and liquid−liquid partition was performed with *n*-hexane (9 × 3 L), followed by $CHCl_3$ (10 × 3 L), and the residue gave the aqueous MeOH extract.

Polygonum persicaria

The air-dried aerial parts (300 g) were ground with a Retsch (type SM 100) grinder.The raw material was extracted by percolation with MeOH (3 L) at room temperature. The extract was concentrated to 100 mL, diluted with 100 mL H_2O, and subjected to solvent–solvent partition between *n*-hexane (3 × 500 mL) and $CHCl_3$ (3 × 500 mL).

4.3. PURIFICATION AND ISOLATION OF COMPOUNDS

4.3.1. Open-column chromatography (OCC)

OCC was carried out on polyamide for column chromatography (ICN). Sorbent: OCC 1: 287 g; OCC 2: 30 g. Mobile phases (compositions reported as volumetric ratios):

OCC 1: MeOH–H_2O [1:4, 2:3, 3:2, 4:1 (3000 mL each)]; volume of collected fractions: 250 mL.

OCC 2: MeOH–H_2O [1:4, 2:3, 3:2, 4:1 (1500 mL each)]; volume of collected fractions: 500 mL.

4.3.2. Vacuum-liquid chromatography (VLC)

VLC was performed on SiO_2 NP-VLC: silica gel (SiO_2) 60 GF254, 15 µm, Merck 11678; RP-VLC: LiChroprep RP-18, 40-63 µm, Merck 13900 and LiChrospher RP-18, 15 µm, Merck 11022). Sorbent: NP-VLC 1: 281 g; NP-VLC 2: 69 g; NP-VLC 3: 259 g, NP-VLC 4: 254 g; NP-VLC 5: 14 g, NP-VLC 6: 200 g, NP-VLC 7: 60 g, RP-VLC 1: 15 g. Mobile phases:

NP-VLC 1: cyclohexane–EtOAc–EtOH [30:10:0, 30:15:0, 30:20:0, 30:30:0, 30:30:1, 30:30:2 and 30:30:6 (520 mL, 440 mL, 520 mL, 600 mL, 640 mL and 360 mL)]; volume of collected fractions: 40 mL.

NP-VLC 2: CH_2Cl_2–acetone [100:0 (150 mL), 99:1, 49:1, 97:3, 19:1, 9:1 and 8:2 (100 mL each); volume of collected fractions: 10 mL.

NP-VLC 3: CH_2Cl_2–acetone [100:0 (1250 mL), 99:1, 97:3, 19:1, 9:1 and 4:1 (500 mL each)]; volume of collected fractions: 50 mL.

NP-VLC 4: CH_2Cl_2–acetone [99:1, 97:3, 19:1, 9:1, 17:3 and 4:1 (1000 mL, 500 mL, 500 mL, 500 mL, 500 mL and 250 mL, respectively); and MeOH (400 mL)] volume of collected fractions: 50 mL.

NP-VLC 5: cyclohexane–EtOAc–EtOH [30:10:0 (100 mL), 30:20:0, 30:20:0.5, 30:30:0.5 and 30:30:1 (60 mL each)]; volume of collected fractions: 4 mL.

NP-VLC 6: *n*-hexane–$CHCl_3$–MeOH [7:3:0, 7:3:0.5, 5:5:0.5, 1:9:0.5, 0:10:0.5, 0:12:1, 0:10:1, 0:9:1.5, 0:8:2, 0:6:4 and 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 MeOH (1500 mL)]; volume of collected fractions: 200 mL and 100 mL.

NP-VLC 7: cyclohexane–EtOAc–MeOH [8:2:0, 7:3:0, 1:1:0, 4:6:0, 2:8:0, 0:1:0 and 0:8:2 (260 mL, 200 mL, 300 mL, 200 mL, 300 mL, 200 mL and 200 mL, respectively) and MeOH (300 mL)]; volume of collected fractions: 20 mL.

RP-VLC: MeOH–H_2O [3:7, 1:1, 7:3, 4:1 and 9:1 (70 mL each) and MeOH (70 mL)]; volume of collected fractions: 10 mL.

4.3.3. Rotation planar chromatography (RPC)

RPC was carried out on manually coated SiO_2 (silica gel 60 GF254, Merck 7730; RPC 1–RPC 4 and RPC 6–RPC 8) or Al_2O_3 (aluminium oxide G, type E, Merck 1090; RPC 5) plates of 1 (RPC 1, RPC 2, RPC 5), 2 (RPC 3, RPC 4, RPC 7) or 4 (RPC 6, RPC 8) mm thickness, at a flow rate of 3, 4 or 10 mL/min on a Harrison Model 8924 Chromatotron instrument (Harrison Research). Mobile phases:

RPC 1: CH_2Cl_2–acetone [100:0, 99:1, 97:3, 19:1 and 9:1 (50 mL each)]; volume of collected fractions: 2 mL.

RPC 2: CH_2Cl_2–acetone [100:0, 99:1, 49:1, 19:1, 9:1 and 7:3 (40 mL each); volume of collected fractions: 2 mL.

RPC 3: CH_2Cl_2–acetone [100:0, 99:1, 97:3, 19:1, 9:1 and 4:1 (50 mL each); volume of collected fractions: 2 mL.

RPC 4: cyclohexane–EtOAc–EtOH [60:20:0.5, 60:30:0.5, 60:40:0.5, 60:60:0.5 and 60:60:1 (100 mL each)]; volume of collected fractions: 5 mL.

RPC 5: cyclohexane–CH_2Cl_2–MeOH [5:15:1 (100 mL)]; volume of collected fractions: 2 mL.

RPC 6: *n*-hexane–CH_2Cl_2–MeOH [2:7:1 (250 mL) and 2:8:1.5 (100 mL)] and MeOH (150 mL); volume of collected fractions: 25 mL.

RPC 7: CH_2Cl_2–MeOH [9:1 (500 mL)]; volume of collected fractions: 30 mL.

RPC 8: cyclohexane–CH_2Cl_2–MeOH [2:9:0.5, 1:10:1 and 0:10:1 (80 mL, 360 mL and 120 mL, respectively)] and MeOH (120 mL); volume of collected fractions: 40 mL.

4.3.4. Medium-pressure liquid chromatography (MPLC)

MPLC was performed at a flow rate of 100 mL/min on a Büchi apparatus (Büchi Labortechnik AG, Flawil, Switzerland) with an RP18ec column (40-63 µm, 40 × 150 mm, Büchi). Mobile phase:

RP-MPLC: MeOH–H_2O [1:1, 3:2, 7:3, 4:1 and 9:1 (100 mL each)] and MeOH (150 mL); volume of collected fractions: 10 mL.

4.3.5. Preparative layer chromatography (PLC)

PLC was performed on SiO_2 plates (NP-PLC: 20 × 20 cm or 20 × 10 cm, silica gel 60 F254, Merck 5715; RP-PLC: 20 × 20 cm, silica gel 60 RP-18 F254S, Merck 5559). Separation was monitored in UV light at 254 and 366 nm (NP-PLC 1, 3–10; RP-PLC) or by spraying the border of the plates with concentrated H_2SO_4 (NP-PLC 2). Compounds were eluted from the scraped adsorbent with $CHCl_3$. Mobile phases:

NP-PLC 1: cyclohexane–EtOAc–EtOH 30:20:0.5

NP-PLC 2–4: cyclohexane–EtOAc–EtOH 60:60:1

NP-PLC 5: cyclohexane–EtOAc–EtOH 60:40:1

NP-PLC 6,8: cyclohexane–EtOAc–EtOH 30:30:1

NP-PLC 7: CH_2Cl_2–MeOH 18:1

NP-PLC 9–10: cyclohexane–EtOAc–EtOH 15:15:2

RP-PLC: MeOH–H_2O 7:3

4.3.6. Gel filtration (GF)

GF was performed on Sephadex LH-20 (25–100 µm, Pharmacia Fine Chemicals). Sorbent: 10 g. Mobile phase: MeOH (70 mL); volume of collected fractions: first fraction: 10 mL, further fractions: 2 mL each.

4.3.7. High-performance liquid chromatography (HPLC)

HPLC was performed on a LiChrospher RP-18 (5 µm, 250 × 4 mm, Merck) column, using Waters Controller 600 instrument with a 2487dual λ absorbance detector. Mobile phase containing MeCN–H_2O 3:2 was applied at a flow rate of 0.3 mL/min; the chromatographic separation was monitored at 254 nm and 280 nm.

4.4. STRUCTURE DETERMINATION OF THE ISOLATED COMPOUNDS

Optical rotations were determined in $CHCl_3$ at room temperature with a Perkin-Elmer 341 polarimeter.

NMR spectra were recorded on a Bruker Avance DRX 500 spectrometer at 500 MHz (^{1}H) or 125 MHz (^{13}C) and on a Varian 800 MHz NMR spectrometer equipped with a ^{1}H{^{13}C/^{15}N} triple resonance ^{13}C enhanced salt-tolerant cold probe operating at 799.9 MHz and 201 MHz for ^{1}H and ^{13}C nuclei, respectively. Chemical shifts were referenced to tetramethylsilane (TMS) (^{1}H) or to residual solvent resonances. Two-dimensional data (^{1}H–^{1}H COSY, NOESY, HSQC and HMBC) were acquired and processed with standard Bruker and VNMRJ-3.2 software.

HRMS analyses were performed on a Thermo LTQ FT Ultra system (Thermo Fisher Scientific, Bremen, Germany). The ionization method was electron spray ionization (ESI) and the system was operated in positive ion mode. For collision-induced dissociation (CID) experiments, helium was used as the collision gas. The capillary temperature was 280 °C.

APCIMS measurements were performed on an API 2000 Triple Quad mass spectrometer (AB SCIEX, Framingham, MA, USA) with an atmospheric pressure chemical ionization (APCI) interface, using positive and negative polarity. The source temperature was 350 °C and the samples were dissolved in MeCN.

EIMS measurements were carried out on a Finnigan MAT 95XP mass spectrometer (Finnigan MAT, San Jose, CA) equipped with a Finnigan electrospray ion source. The source temperature was 220 °C and samples were dissolved in MeOH.

UV spectra were recorded in MeOH on a Shimadzu spectrometer.

4.5. LC-MS INVESTIGATION OF *P. PERSICARIA* SAMPLES

Chromatographic separations were performed on a Shimadzu LC system [2 pumps (LC-20AD); UV-Vis detector (SPD-20A); autosampler (SIL-20A); column thermostat (CTO-20AC)] equipped with a LiChrospher 100 RP-18e (4 × 250 mm, 5 µm) column (Merck). Mobile phase A was MeCN and mobile phase B was H_2O. Isocratic elution was applied with 60% A + 40% B as mobile phase. The flow rate was 0.4 mL/min. The components were monitored at 254 nm. The HPLC was coupled to an API 2000 quadrupole MS/MS with an APCI interface. The source temperature was 450 °C. The measurements

were carried out in positive ionization mode and the qualification was accomplished by using multiple reaction monitoring (MRM). Data acquisition and evaluation were performed by using Analyst 1.5.1 software.

4.6. PHARMACOLOGICAL TESTS

Pharmacological investigations were performed in cooperation with the Department of Pharmacodynamics and Biopharmacy, University of Szeged; Rytmion Ltd; the Clinical Institute of Pathology, Department of Vascular Biology and Thrombosis Research, Center of Physiology and Pharmacology, Medical University of Vienna; the Department of Pharmacognosy, University of Vienna; and the Institute of Pharmaceutical Sciences, Department of Pharmacognosy, Karl-Franzens University Graz.

4.6.1. *In vitro* antiproliferative investigations

MTT assay

Antiproliferative effects were measured *in vitro* on four human cell lines (A2780, A431, HeLa and MCF7, isolated from ovarian cancer, skin epidermoid carcinoma, and cervical and breast cancers, respectively) by means of an MTT ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]) assay, as described previously.[121] Doxorubicin and cisplatin were used as positive controls. The reduced MTT was assayed at 545 nm, using a microplate reader and the IC_{50} values were calculated by means of GraphPad Prism 4.0.

XTT viability assay

The XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide) viability assay was performed as reported previously and in accordance with the manufacturer's protocol (Roche Diagnostics, Mannheim, Germany).[128] The absorbance was determined with a microplate reader (TACEN Rainbow) at a wavelength of 490 nm, and the reference wavelength was 650 nm. Vinblastine served as positive control.

4.6.2. GIRK channel-inhibitory assay

Experiments were performed on HEK293 (human embryonic kidney) cells stably expressing the G protein-activated inwardly rectifying K^+ channels, GIRK1/4 (Kir3.1/3.4). Propafenone was used as a positive control. The GIRK channel current was measured by using planar patch-clamp technology in the whole-cell configuration with a 4-channel semi-high-throughput automated patch clamp system (Nanion Technologies GmbH). For the detailed protocol of the GIRK assay, see ref.[122]

4.6.3. *In vitro* and *in vivo* anti-inflammatory investigations

Interleukin-8 (IL-8) and E-selectin expression-inhibitory assays

The inhibition of LPS- and TNF-α-induced IL-8 and E-selectin production was determined in HUVECtert or THP-1 cells. BAY (Sigma−Aldrich, St. Louis, MO, USA) was used as positive control. IL-8 release and E-selectin expression were measured by ELISA. The statistical significance of differences was calculated by using ANOVA. For the details of this assay, see ref.[123]

Interleukin-8 (IL-8) and E-selectin mRNA expression assays

IL-8 and E-selectin mRNA expression assays were performed as described previously.[123] The inhibition of mRNA expression was determined by real-time PCR (ABI 7300 Real-Time PCR System). BAY (Sigma−Aldrich, St. Louis, MO, USA) was used as positive control. The statistical significance of differences was calculated by using ANOVA test.

NF-κB1/COX-2 gene expression assay

For the detailed protocols of the NF-κB1 and COX-2 gene expression assays see refs.[124,125]. The inhibitory effects were measured *in vitro* on human monocytic cell line THP-1 cells. Quercetin for NF-κB1 and dexamethasone for COX-2 were used as positive controls. Total RNA (GenElute™ Mammalian Total RNA Miniprep Kit) and reverse transcription (High capacity cDNA Reverse Transcription Kit) were carried out according to the manufacturer´s manual. mRNA expression was quantified by real-time PCR (ABI 7300 Real-Time PCR System).

Nitric oxide (NO) assay

The inhibition of NO production *in vitro* in LPS/IFN-γ-induced RAW264.7 cells was determined by the Griess assay method, as described by BLUNDER *et al*.[126] The NO synthase inhibitor N^G-monomethyl-L-arginine (L-NMMA) was used as positive control. The absorbance was determined with a microplate reader (Perkin Elmer Wallac Victor 1420 Multilabel Counter) at 540 nm.

Leukotriene biosynthesis (5-LOX) inhibition assay

The LOX inhibition assay was performed as described earlier,[127] with slight modifications.[128] Human neutrophilic granulocytes with 5-LOX activity were isolated from venous human blood, on the basis of sedimentation rates and lysis tolerance. The concentration of LTB_4 formed during incubation was determined by means of a competitive LTB_4 EIA Kit (Cayman Chemical Company, Ann Arbor, MI, USA). The positive control for this test system was zileuton (Sequoia Oxford, UK).

COX-1 and COX-2 inhibition assays

The assays were carried out as reported elsewhere,[129,130] with the use of purified PGHS-1 from ram seminal vesicles (Cayman Chemical Company, Ann Arbor, MI, USA) and human recombinant *N*-

terminal hexahistidine-tagged PGHS-2 isolated from a Baculovirus overexpression system in S*f*21 cells. PGE_2 was determined with a competitive PGE_2 EIA kit (Enzo Life Sciences, Farmingdale, NY, USA). Indomethacin and NS-398 (Cayman Chemical Company, Ann Arbor, MI, USA) were used as positive controls.

Carrageenan-induced paw oedema model in rats

The anti-inflammatory action was investigated *in vivo* by means of a rat paw oedema test described in ref.[131] Mature male Sprague-Dawley rats (9 rats/group, 175-200 g) were treated intraperitoneally with 20 or 60 mg/kg extract dissolved in physiological saline containing 20% dimethylsulfoxide. The control group was treated with the vehicle, while dexamethasone (0.5 mg/kg) was used as a reference anti-inflammatory agent. The local inflammatory response was elicited 60 min later by a subplantar injection of 0.5 mg carrageenan dissolved in 0.1 mL isotonic saline. The contralateral foot was injected with physiological saline. The volume of the paws was determined 5 hours later with a plethysmometer (Hugo Sachs Elektronik, March-Hugstetten, Germany).

5. RESULTS

5.1. SCREENING OF POLYGONACEAE SPECIES FOR ANTIPROLIFERATIVE AND GIRK CHANNEL INHIBITORY ACTIVITIES

As part of our screening program for antiproliferative compounds in plants occurring in the Carpathian Basin, 27 species of the Polygonaceae family were investigated for their antiproliferative effects. The extracts prepared with *n*-hexane (A), $CHCl_3$ (B), aqueous MeOH (C) or H_2O (D) from selected plant organs (altogether 196 extracts) were evaluated at concentrations of 10 µg/mL and 30 µg/mL against the cell lines HeLa, A431 and MCF7 through use of the MTT assay (see sections **4.2.1** and **4.6.2**). The results of the antiproliferative assays are listed in **Table 1.** in **Appendix I**. (Antiproliferative effects of < 10% are not presented.)

A total of 6 extracts demonstrated substantial cell growth-inhibitory activity (≥ 50% inhibition of cell proliferation) against one or more cell lines at 10 µg/mL or 30 µg/mL, as presented in **Table 2**. These active fractions mostly originated from the roots of the plants. Additionally, 36 samples inhibited cell proliferation moderately (25–49.99%), while 154 extracts did not display inhibitory potency on the investigated cells.

Fractions B (containing $CHCl_3$-soluble lipophilic constituents) and fractions A (*n*-hexane extracts) proved to be active. The residual aqueous and aqueous MeOH extracts (fractions C and D, respectively) did not demonstrate pronounced antiproliferative effects (>50% inhibition) against any cell line.

Table 2. Antiproliferative effects of the most active extracts (>50% inhibition) against the used cancer cell lines.

			Growth inhibition (% ±SEM)					
			HeLa		**A431**		**MCF7**	
Spec.[a]	**P.p.[b]**	**S.[c]**	**10 µg/mL**	**30 µg/mL**	**10 µg/mL**	**30 µg/mL**	**10 µg/mL**	**30 µg/mL**
P.h.	R	A	38.23 ± 1.10	**54.75 ± 1.04**	26.72 ± 2.21	46.58 ± 1.02	20.69 ± 1.66	48.23 ± 2.20
R.a.	H	B	**77.67 ± 0.31**	**97.02 ± 0.26**	-	14.92 ± 1.64	22.56 ± 0.97	**55.13 ± 1.89**
R.al.	R	A	-	42.60 ± 1.61	12.27 ± 1.90	**58.32 ± 0.63**	14.68 ± 2.50	**58.81 ± 1.51**
R.aq.	R	A	21.00 ± 1.07	**60.89 ± 0.86**	15.81 ± 2.55	32.57 ± 0.97	-	**69.25 ± 2.58**
R.s.	W	A	47.09 ± 1.02	**51.19± 1.46**	28.92 ± 1.41	38.66 ± 1.33	49.34 ± 1.12	**56.15 ± 0.78**
R.t.	R	B	-	-	11.51 ± 1.94	**96.20 ± 0.20**	-	**88.55 ± 0.30**

[a] Spec.: species (*P.h.* = *Polygonum hydropiper*, *R.a.* = *Rumex acetosa*, *R.al.* = *Rumex alpinus*, *R. aq.* = *Rumex aquaticus*, *R.s.* = *Rumex scutatus*, *R.t.* = *Rumex thyrsiflorus*)
[b] P.p.: plant parts (H = herbs, R = roots, W = whole plant); [c] S.: solvent (A = *n*-hexane, B = chloroform)

In the genus *Rumex*, noteworthy antiproliferative activities were recorded for *R. acetosa, R. alpinus, R. aquaticus, R. scutatus* and *R. thyrsiflorus.* The $CHCl_3$ extract of the herb of *R. acetosa* was the most potent of the whole screen against HeLa cells at both concentrations (77.67% and 97.02%) and also exhibited a considerable antiproliferative effect on MCF7 cells at higher concentration. The *n*-hexane extract of the roots of *R. alpinus* was active on A431 and MCF7 cells. Moreover, high activities were detected for the *n*-hexane extract of *R. aquaticus* at 30 μg/mL on HeLa and MCF7 cells. Similarly, in the case of *R. scutatus*, the *n*-hexane extract displayed considerable antiproliferative effects on both cell lines, and also demonstrated moderate activity at 10 μg/mL on all three cell lines. The $CHCl_3$ extract prepared from the roots of *R. thyrsiflorus* was the most effective on A431 and MCF7 cells (96.20% and 88.55%). Phytochemical and pharmacological investigations of *R. aquaticus* and *R. thyrsiflorus* are in progress.

As regards the *Polygonum* species, *P. hydropiper* proved to have marked efficacy against HeLa cells, while it was found to be moderately active on the other cell lines. For *Fallopia*, *Oxyria* and *Persicaria* species, moderate (25–49.99%) cell growth inhibition was detected.

In the course of GIRK channel inhibitory activity investigation 51 extracts [*n*-hexane (A), $CHCl_3$ (B) and aqueous MeOH (C)] of 11 species (*F. bohemica*, *F. japonica*, *F. sachalinensis*, *P. amphibia* f. *terrestris*, *P. aviculare*, *P. persicaria*, *R. crispus*, *R. hydrolapathum*, *R. obtusifolius*, *R. patientia* and *R. stenophyllus*) were tested at 0.01 and 0.1 mg/mL concentrations. Among them mainly the $CHCl_3$ (B) extracts proved to be the most active ones; *P. aviculare* (75 ± 5%), *P. amphibia* (70 ± 12%), *P. persicaria* (76 ± 8%), *R. stenophyllus* (72 ± 3%), *R. patientia* (74 ± 2%) and *R. crispus* (72 ± 2%) showed higher than 70% inhibitory activity at 0.1 mg/mL on GIRK channels (unpublished data).

5.2. INVESTIGATION OF *N. LOBATA*, *O. ACANTHIUM* AND *P. PERSICARIA* EXTRACTS FOR BIOACTIVITY

Extracts of different polarity (aqueous and organic) prepared from the aerial parts of *N. lobata* have been tested previously by KRUPITZA et al. (our cooperative partner) on human promyelocytic leukaemia cells (HL-60) with analyses of the inhibition of cell proliferation and apoptosis induction. The most active extract was further investigated against anaplastic large cell lymphoma (ALCL) cell lines of human and mouse origin. The CH_2Cl_2 extract inhibited the proliferation of HL-60, and human and mouse ALCL cells with an IC_{50} of ~2.5, 3.7 and 2.4 μg/mL, respectively, and arrested cells in the G2/M phase.[120]

The extracts of *O. acanthium* (see section **4.2.1**.) were evaluated for their inhibitory activity on COX-2 and NF-κB1 gene expression, inducible nitric oxide synthase (iNOS), 5-LOX, and COX-1 and COX-2 enzymes at 10 or 50 μg/mL in *in vitro* assays (**Table 1** in **Appendix V**). In most cases, the $CHCl_3$ extract exerted strong inhibitory effects [inhibition of iNOS (76.7 ± 7.0%), 5-LOX (62.6 ± 6.8%), and inhibition of COX-2 enzyme (61.8 ± 9.0%)]. Additionally, the effect of different *P. persicaria* extracts

(see section **4.2.1**.) on the GIRK channel was investigated by using an automated patch clamp method (**Table 1** in **Appendix II**). The $CHCl_3$ extract at 0.1 mg/mL exhibited significant GIRK channel-inhibitory activity (76 ± 8%).

On the basis of the results of the preliminary screening, the lipophilic extracts ($CHCl_3$ and CH_2Cl_2) of *N. lobata, O. acanthium and P. persicaria* were chosen for more detailed phytochemical studies, with the aim of the identification of their bioactive constituents.

5.3. ISOLATION OF COMPOUNDS FROM *NEUROLAENA LOBATA*

Dried and ground aerial parts of the plant were percolated with MeOH. The crude extract was concentrated and subjected to solvent–solvent partitioning with petroleum ether, then with CH_2Cl_2 and finally with EtOAc (see section **4.2.2**). The concentrated CH_2Cl_2 phase (95.4 g) was chromatographed on a polyamide column (**OCC 1**) with mixtures of MeOH and H_2O as eluents. The **OCC-1** fractions were combined into 7 fractions (BI–BVII) according to the TLC monitoring (**Figure 1**).

Fraction BII, obtained with MeOH–H_2O 1:4, was subjected to VLC (**NP-VLC 1**), using a gradient system of cyclohexane–EtOAc–EtOH. The **NP-VLC 1** fractions were combined after TLC monitoring into 9 fractions (BII/1-BII/9).

Fraction BII/4 obtained with cyclohexane–EtOAc 3:2, was chromatographed by RPC on silica gel in 2 steps, first with a gradient system of CH_2Cl_2–acetone (**RPC-1**). The subfraction eluted with CH_2Cl_2–acetone 97:3 was next separated by RPC with mixtures of CH_2Cl_2–acetone (**RPC 2**), and then further purified by preparative layer chromatography (**NP-PLC 1**) on silica gel with the mobile phase cyclohexane–EtOAc–EtOH, to afford **LOB-2** (3.5 mg) and **LOB-3** (15.6 mg).

Fraction BII/5, obtained with cyclohexane–EtOAc 1:1, was separated on VLC (**NP-VLC 2**) with a CH_2Cl_2–acetone gradient system. The subfraction eluted with CH_2Cl_2–acetone 49:1 was further fractionated with RPC (**RPC 3**), using a mobile phase of CH_2Cl_2–acetone, and finally separated by **RP-PLC** to yield **LOB-5** (4.4 mg) and **LOB-6** (5.6 mg).

Fraction BII/6, eluted with cyclohexane–EtOAc 1:1, was separated by VLC (**NP-VLC 3**), using a gradient system of CH_2Cl_2–acetone. The subfraction obtained with CH_2Cl_2–acetone 97:3, was subjected to PLC (**NP-PLC 2**), using a system of cyclohexane–EtOAc–EtOH, to give **LOB-9** (1.6 mg) and **LOB-10** (5.1 mg). The subfraction eluted with CH_2Cl_2–acetone 95:5, was further purified by PLC (**NP-PLC 3**) on silica gel with cyclohexane–EtOAc–EtOH, leading to the isolation of **LOB-11** (1.1 mg). Finally, the subfraction eluted with MeOH was purified first by **RP-PLC 2**, using a mixture of MeOH–H_2O, and then by PLC (**NP-PLC 4**), using cyclohexane–EtOAc–EtOH mixtures, to yield **LOB-13** (5.9 mg).

Fraction BII/7, obtained with cyclohexane–EtOAc 1:1**,** was separated by VLC (**NP-VLC 4**) with a CH_2Cl_2–acetone gradient system. The subfraction eluted with CH_2Cl_2–acetone 19:1, was subjected to PLC (**NP-PLC 5**), using a solvent system of cyclohexane–EtOAc–EtOH, to give **LOB-14** (2.8 mg) and

LOB-15 (3.2 mg). The subfraction obtained with CH_2Cl_2–acetone 4:1, was separated by RPC (**RPC 4**) with a gradient system of cyclohexane–EtOAc–EtOH and then further purified by **NP-PLC 6** and **RP-PLC** on silica gel with the mobile phases cyclohexane–EtOAc–EtOH and MeOH–H_2O, affording **LOB-20** (1.8 mg). The subfraction eluted with MeOH was further fractionated by VLC (**NP-VLC 5**), using a mobile phase of cyclohexane–EtOAc–EtOH, and finally subjected to PLC (**RP-PLC** and **NP-PLC 7**), using MeOH–H_2O and CH_2Cl_2–MeOH, to yield **LOB-26** (3.5 mg). Finally, a subsequent subfraction obtained with MeOH, was purified by PLC, first on reversed phase with MeOH–H_2O (**RP-PLC**), and then on normal phase with cyclohexane–EtOAc–EtOH (**NP-PLC 8**) as developing system on silica gel, resulting in the isolation of **LOB-18** (7.8 mg).

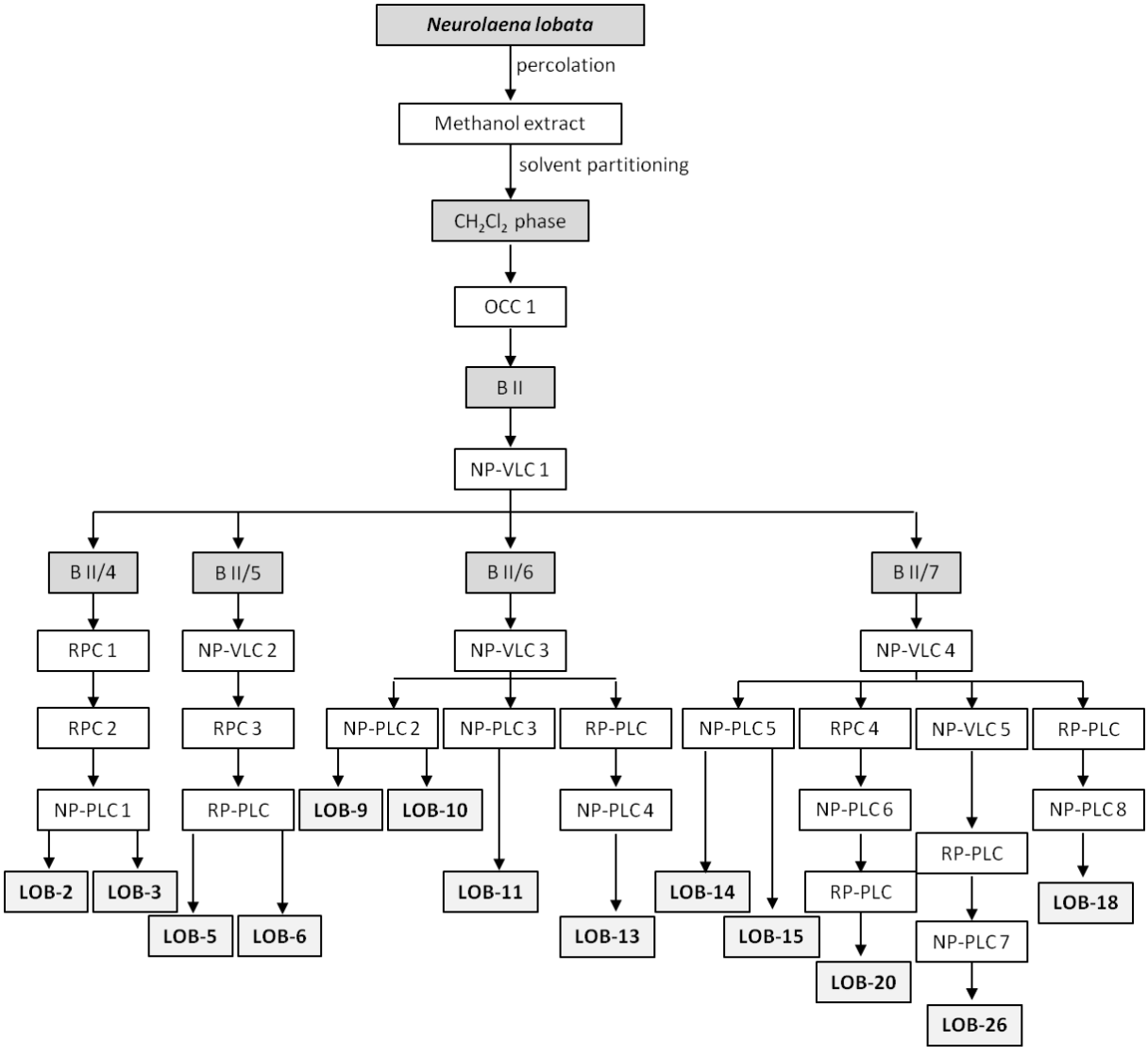


Figure 1. Isolation of compounds from *Neurolaena lobata*

5.4. ISOLATION OF COMPOUNDS FROM *ONOPORDUM ACANTHIUM*

After percolation of the air-dried and ground aerial parts with MeOH, the extract was evaporated *in vacuo* and subjected to solvent–solvent partitioning, first with *n*-hexane and then with $CHCl_3$, and the residue gave the aqueous-MeOH extract (see section **4.2.2**). The concentrated $CHCl_3$ phase (66 g) was fractionated by VLC on silica gel (**NP-VLC 6**), with a gradient system of *n*-hexane–$CHCl_3$–MeOH. The **NP-VLC 6** fractions were combined into 6 major fractions (BI-VI) according to the TLC monitoring (**Figure 2**).

Fraction BI eluted with *n*-hexane–$CHCl_3$–MeOH 1:9:0.5, was chromatographed on a polyamide column (**OCC 2**), using a gradient system of MeOH–H_2O. The combination of fractions of similar composition furnished 7 fractions (BI/1-I/7).

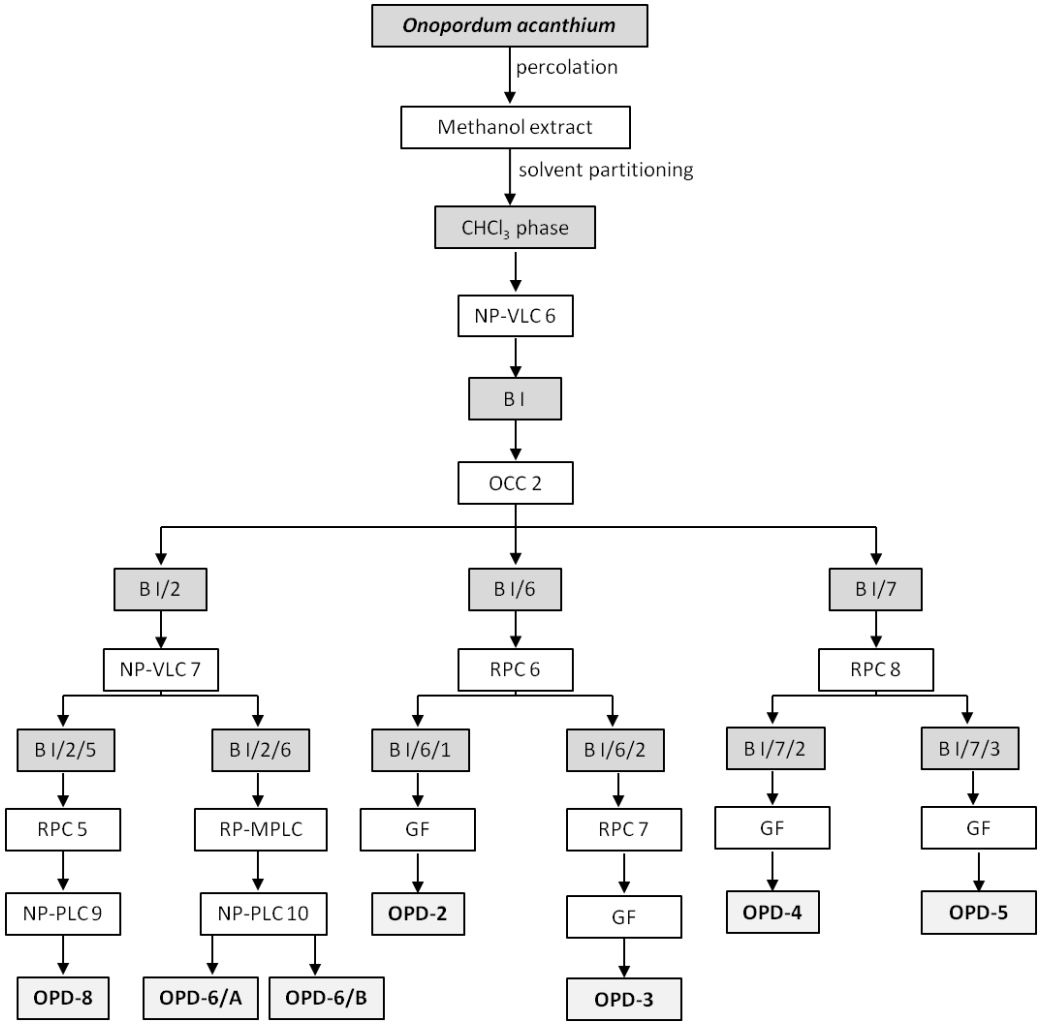


Figure 2. Isolation of compounds from *Onopordum acanthium*

Fraction BI/2, obtained from **OCC 2** with MeOH–H_2O 1:4, was chromatographed by VLC (**NP-VLC 7**), using a solvent system of cyclohexane–EtOAc–MeOH with increasing polarity, to yield 11 subfractions (BI/2/1-BI/2/11). Subfraction BI/2/5 was further purified by RPC on Al_2O_3 (**RPC 5**) with

mixtures of cyclohexane–CH_2Cl_2–MeOH, and finally subjected to **NP-PLC 9** using a mobile phase of cyclohexane–EtOAc–EtOH, to afford **OPD-8** (5.5 mg). Subfraction BI/2/6 eluted with cyclohexane–EtOAc 1:4, was separated by **RP-MPLC** with gradient system of MeOH–H_2O. Further PLC (**NP-PLC 10**; cyclohexane–EtOAc–EtOH 15:15:2) led to the isolation of **OPD-6/A** (9.4 mg) and **OPD-6/B** (3.4 mg).

For the separation of fraction BI/6 (eluted previously with MeOH–H_2O 4:1), RPC was carried out (**RPC 6**), with the application of gradient system *n*-hexane–CH_2Cl_2–MeOH. Subfraction BI/6/1, eluted with *n*-hexane-CH_2Cl_2–MeOH 2:7:1, was purified on Sephadex LH-20 column (**GF**) with MeOH as eluent to afford **OPD-2** (3.5 mg). Subfraction BI/6/2, eluted with *n*-hexane-CH_2Cl_2–MeOH 2:8:1.5, was separated by RPC (**RPC 7**) with the isocratic solvent system CH_2Cl_2–MeOH (9:1), and was finally purified on a Sephadex LH-20 column (**GF**), to yield **OPD-3** (8 mg).

Fraction BI/7 obtained with MeOH–H_2O 4:1, was also subjected to RPC (**RPC 8**), using a gradient system of cyclohexane–CH_2Cl_2–MeOH. The subfractions (BI/7/2 and BI/7/3) obtained with cyclohexane–CH_2Cl_2–MeOH 1:10:1 and 0:10:1, were further chromatographed on a Sephadex LH-20 column (**GF**) to afforded **OPD-4** (4.5 mg) and **OPD-5** (4.5 mg).

5.5. ISOLATION OF COMPOUNDS FROM *POLYGONUM PERSICARIA*

The powdered and dried aerial parts of the plant were extracted with MeOH. After concentration under vacuum, the crude extract was subjected to solvent–solvent partitioning to yield an *n*-hexane-soluble phase and a $CHCl_3$-soluble phase (see section **4.2.2**). Evaporation of the $CHCl_3$ phase resulted in a greenish-brown residue (3.88 g), which was fractionated by **RP-VLC** on silica gel with mixtures of MeOH–H_2O. The fractions were combined into 6 subfractions B/1–B/6 according to the TLC monitoring (**Figure 3**).

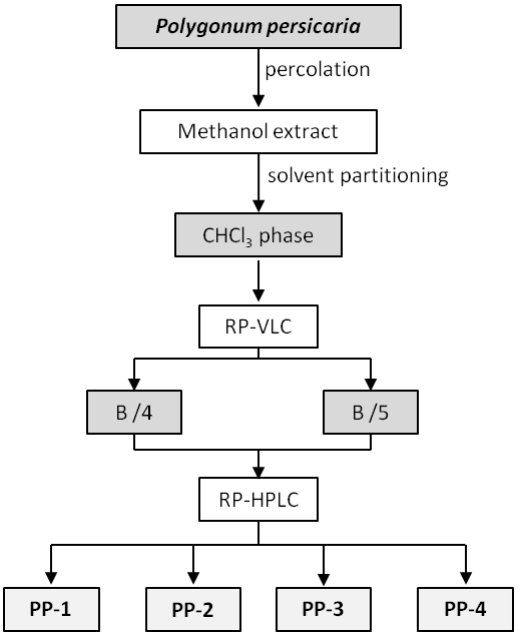


Figure 3. Isolation of compounds from *Polygonum persicaria*

Fractions B/4 and B/5, eluted with MeOH–H_2O 4:1 and 9:1 (**RP-VLC**), were further separated by **RP-HPLC**, using the mobile phase MeCN−H_2O 3:2, to yield **PP-1** (2.0 mg), **PP-2** (2.4 mg), **PP-3** (3.0 mg) and **PP-4** (1.8 mg) and the HPLC eluates containing the minor compounds.

5.6. CHARACTERIZATION AND STRUCTURE DETERMINATION OF THE ISOLATED COMPOUNDS

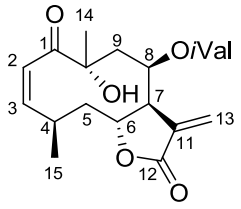
The structure elucidation was performed by means of NMR and MS measurements. Information from 1D (^{1}H-NMR and JMOD) and 2D (^{1}H-^{1}H COSY, NOESY, HSQC and HMBC) NMR experiments proved valuable for the chemical structure determination. HRESIMS, ESIMS, APCIMS and EIMS measurements revealed the molecular masses and molecular compositions of the compounds (**Table 3**).

Table 3. MS data on the isolated new natural compounds

Compound	M.W.	MS	MS fragments (*m/z*)
LOB-15 (**9**)	394	HRESIMS	449.1784 $[M + MeOH + Na]^+$ (calcd for $C_{21}H_{30}O_9Na$, 449.1788); 417.1525 $[M + Na]^+$ (calcd for $C_{20}H_{26}O_8Na$, 417.1525);
		APCIMS	395 $[M + H]^+$, 377 $[M + H - H_2O]^+$, 293 $[M + H - 102]^+$.
LOB-14 (**10**)	394	HRESIMS	449.1788 $[M + MeOH + Na]^+$ (calcd for $C_{21}H_{30}O_9Na$, 449.1788); 417.1530 $[M + Na]^+$ (calcd for $C_{20}H_{26}O_8Na$, 417.1525)
		APCIMS	395 $[M + H]^+$, 377 $[M + H - H_2O]^+$, 293 $[M + H - 102]^+$
LOB-18 (**11**)	394	HRESIMS	395.1701 $[M + H]^+$ (calcd for $C_{20}H_{27}O_8$, 395.1700)
		ESIMS-MS	395.1703 $[M + H]^+$; 377 $[M + H - H_2O]^+$, 359 $[M + H - 2 \times H_2O]^+$, 311 $[M + H - C_5H_8O]^+$, 293 $[M + H - C_5H_8O - H_2O]^+$, 275 $[M + H - C_5H_8O - 2 \times H_2O]^+$, 257 $[M + H - C_5H_8O - 3 \times H_2O]^+$, 231 $[C_{14}H_{15}O_3]^+$, 213 $[C_{14}H_{13}O_2]^+$, 203 $[C_{13}H_{15}O_2]^+$, 185 $[C_{13}H_{13}O]^+$
LOB-20 (**12**)	364	HRESIMS	365.1961 $[M + H]^+$ (calcd for $C_{20}H_{29}O_6$; 365.1959)
		ESIMS-MS	365.2035 $[M + H]^+$; 347 $[M + H - H_2O]^+$, 295 $[C_{15}H_{19}O_6]^+$, 277 $[C_{15}H_{17}O_5]^+$, 263 $[C_{15}H_{19}O_4]^+$, 261 $[C_{15}H_{17}O_4]^+$, 245 $[C_{15}H_{17}O_3]^+$, 227 $[C_{15}H_{15}O_2]^+$, 219 $[C_{13}H_{15}O_3]^+$, 217 $[C_{14}H_{17}O_2]^+$, 201 $[C_{13}H_{13}O_2]^+$, 199 $[C_{14}H_{15}O]^+$, 179 $[C_{10}H_{11}O_3]^+$, 163 $[C_{10}H_{11}O_2]^+$
LOB-26 (**13**)	406	HRESIMS	407.2065 $[M + H]^+$(calcd for $C_{22}H_{31}O_7$; 407.2064)
		ESIMS-MS	407.2135 $[M + H]^+$; 329 $[M + H - H_2O - CH_3COOH]^+$, 305 $[M + H - C_5H_{10}O_2]^+$, 245 $[M + H - C_5H_{10}O_2 - CH_3COOH]$, 227 $[M + H - C_5H_{10}O_2 - CH_3COOH - H_2O]^+$, 217 $[C_{14}H_{17}O_2]^+$, 209 $[M + H - C_5H_{10}O_2 - CH_3COOH - H_2O - H_2O]^+$, 199 $[C_{14}H_{15}Og]$, 181 $[C_{14}H_{13}]$, 171 $[C_{13}H_{15}]$
PP-1 (**21**)	398	HRESIMS	421.0897 $[M + Na]^+$ (calcd for $C_{21}H_{18}O_8Na$; 421.0894)
		EIMS	398 $[M]^+$, 316 $[M-(CH_3)_2C=C=CO]^+$, 83 $[(CH_3)_2C=CHCO]^+$, 55 $[(CH_3)_2C=CH-]^+$
PP-2 (**22**)	398	HRESIMS	421.0903 $[M + Na]^+$ (calcd, for $C_{21}H_{18}O_8Na$; 421.0894)
		EIMS	398 $[M]^+$, 316 $[M-(CH_3)_2C=C=CO]^+$, 83 $[(CH_3)_2C=CHCO]^+$, 55 $[(CH_3)_2C=CH-]^+$
PP-3 (**23**)	386	HRESIMS	387.1078 $[M + H]^+$ (calcd. for $C_{20}H_{19}O_8$; 387.1074)
		ESIMS-MS	387 $[M + H]^+$, 372, 339, 310, 283, 282, 255
		EIMS	386 $[M]^+$
PP-4 (**24**)	416	HRESIMS	417.1184 $[M + H]^+$ (calcd. for $C_{21}H_{21}O_9$; 417.1180)
		ESIMS-MS	417 $[M + H]^+$, 402, 387, 374, 359, 355, 341, 299
		EIMS	416 $[M]^+$

Compounds from Neurolaena lobata

From the CH_2Cl_2 extract of *N. lobata*, neurolenin A [**LOB-2** (**1**)], neurolenin B [**LOB-3** (**2**)], neurolenin D [**LOB-5** (**3**)], neurolenin C [**LOB-6** (**4**)], 8β-isovaleryloxy-9α-acetoxy-calyculatolide [**LOB-9** (**5**)], lobatin A [**LOB-10** (**6**)], lobatin B [**LOB-11** (**7**)] and 8β-isovaleryloxy-9α-hydroxy-calyculatolide [**LOB-13** (**8**)] were isolated and identified by comparison of their APCI-MS and ^{1}H- and ^{13}C-NMR data with those published in the literature.[16,132,133]



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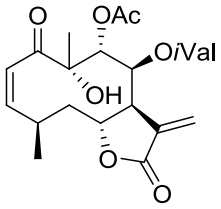

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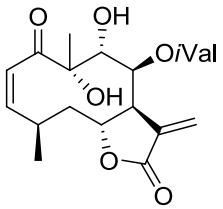

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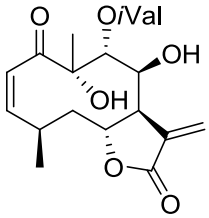

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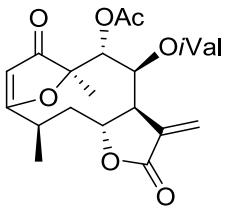

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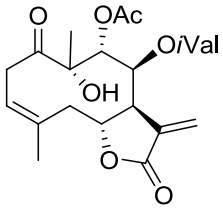

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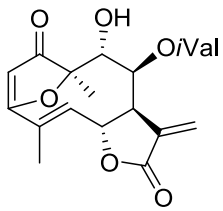

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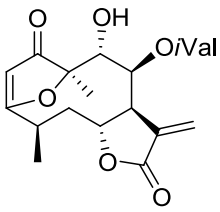

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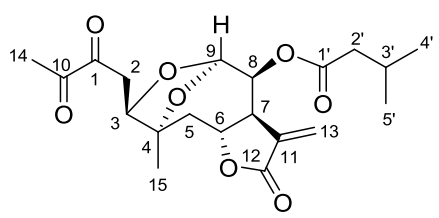
LOB-15 (**9**) and **LOB-14** (**10**) were isolated as amorphous solids with $[\alpha]^{27}_{D}$ −89 (*c* 0.2, $CHCl_3$) and $[\alpha]^{27}_{D}$ −32 (*c* 0.2, $CHCl_3$), respectively; HRESIMS and APCIMS revealed the molecular formula of $C_{20}H_{26}O_8$ (**Table 2**) for both compounds. Analysis of the HRESIMS and ^{1}H- and ^{13}C-NMR spectra demonstrated that the two compounds are stereoisomers with closely comparable spectroscopic features. Additionally, the ^{1}H- and ^{13}C-NMR spectra indicated the presence of an isovaleroyl group in the molecules (**9** and **10**). The informative signals for **LOB-15**/**LOB-14** at δ_H 4.91/4.71 (H-6), 3.45/3.23 (H-7), 5.38/5.47 (H-13a), and 6.26/6.25 (H-13b) and δ_C 73.7/74.1 (C-6), 46.3/46.2 (C-7), 134.1/134.7 (C-11), 169.2 (C-12) and 121.3 (C-13) identified the presence of a *trans*-fused α-methylene-γ-lactone ring at H-6 in the β position.[16,134] With this partial structure as starting point, analysis of the ^{1}H- and ^{13}C-NMR spectra and 2D homo- (^{1}H-^{1}H COSY) and heteronuclear correlation data (HSQC and HMBC) resulted in complete ^{1}H- and ^{13}C-NMR assignments (**Annex IV** and **V**). In the HMBC spectra, no

correlations were detected between H-9 and C-10 or between H-15 and C-9, confirming fission of the C-9–C-10 bond in both compounds. The HMBC correlation observed between H-9 and C-4 suggested that the bond fission was followed by ring closure between C-4 and C-9. In view of the molecular composition, the chemical shift of C-9 (98.8 in **LOB-15** and 102.0 in **LOB-14**) was indicative of the presence of a bicyclic acetal moiety in both compounds. The HMBC cross-peak of the carbonyl carbon signal (isovaleroyl CO) with the H-8 signal demonstrated the presence of the ester group at C-8.

The stereochemistry of **LOB-15** and **LOB-14** was assessed by analysing the coupling constants and nuclear *Overhauser* effects (NOEs) were detected in a NOESY experiment (**Annex IV** and **V**). The relative configurations of the lactone ring and C-8 were similar to those determined in sesquiterpenes isolated earlier from *N. lobata*.[16,134] Thus, H-6 was in a β orientation, while H-7 and H-8 were in α positions in compounds **LOB-15** and **LOB-14**. The coupling constants observed between H-8 and H-9 suggested that these protons are in equatorial positions in both compounds. The most significant difference in the NOE correlations was observed for H-6. In the case of **LOB-15**, a strong NOE correlation was detected between H-6 and the two H-2 protons, while a strong correlation between H-6 and the H-15 methyl protons was detected instead of this in **LOB-14**. These data indicated a seco-germacranolide structure of **LOB-15** and **LOB-14** for the two stereoisomers, named neurolobatin A (**9**) and neurolobatin B (**10**), respectively. The oxygen in the eight-membered ring in **LOB-15** and in the seven-membered ring in **LOB-14** is in the β position. It was assumed that the two isomers have a common origin: they may be formed from germacranolide-type sesquiterpenes after ring opening between C-9 and C-10, followed by formation of the bicyclic acetal structure.



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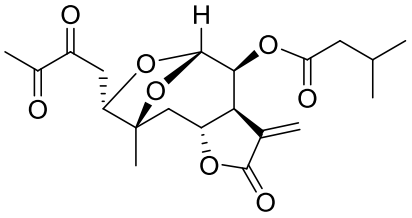

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LOB-18 (**11**) was obtained as an amorphous solid with $[\alpha]^{27}_{D}$ +53 (*c* 0.2, $CHCl_3$). Its molecular formula was assigned via HRESIMS and NMR analyses as $C_{20}H_{26}O_8$ (*m/z* 395.1701 $[M + H]^+$). Its ^{1}H- and ^{13}C-NMR spectra confirmed the presence of an isovaleroyl group. Additionally, the 1D and 2D NMR spectra contained resonances for 1 carbonyl group, 1 α-methylene-γ-lactone ring, 1 quaternary carbon, 1 trisubstituted olefin, 4 methines and 2 methyl groups (**Annex VI**).

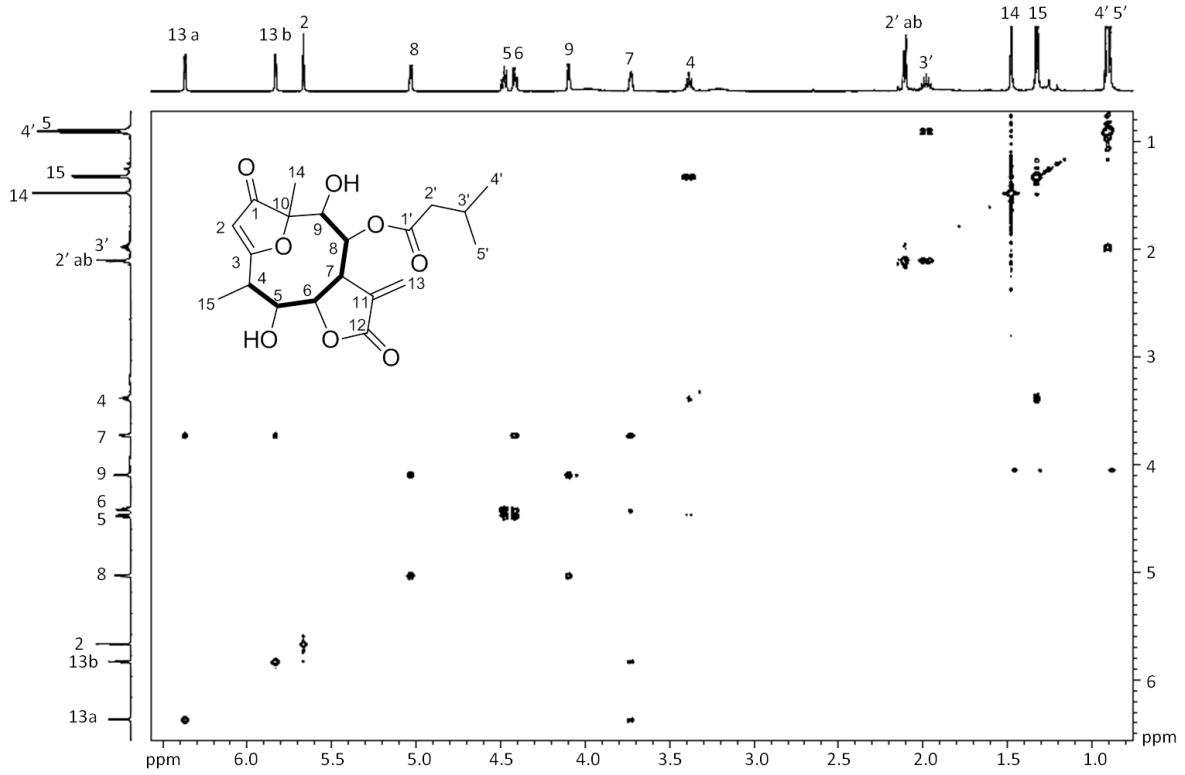


Figure 4. ^{1}H-^{1}H COSY spectrum and structural fragment (**—**) of **11**

The ^{1}H-^{1}H COSY spectrum (**Figure 4**) revealed one structural fragment with correlated protons: δ_H 3.39 dq, 4.49 t, 4.41 dd, 3.74 dt, 5.03 d and 4.09 d [–CHR-CHR-CHR-CHR-CHR-CHR–] (C-4–C-9). Further, the chemical shifts and coupling constants of **LOB-18** were closely related to those of the 1-keto-furanoheliangolide derivative 8β-isovaleroyloxy-9α-hydroxycalyculatolide (**LOB-13**, **8**),[16] the only difference being the absence of the signals of a methylene and the appearance of the signal of a methine group (δ_H 4.49 t, δ_C 73.7).

This methine was assigned as C-5 with regard to the HMBC correlations between C-5 and H-6, C-5 and H-7, and C-5 and H-15 (**Figure 5**). The relative configuration of **LOB-18**, studied by means of a NOESY experiment (**Figure 6**), was deduced by starting from the β orientation of H-6 and the α orientation of H-7, indicated by the coupling constant $J_{6,7}$ = 4.9 Hz, and found in all sesquiterpenes isolated previously from *N. lobata*. The cross-peaks between H-6 and H-15 proved the β orientation of the 15-methyl group, while the NOE effects observed between H-7 and H-5, H-5 and H-4, H-7 and H-13b, and H-13b and H-8 dictated the α orientation of these protons. All of the above evidence supported the structure of **11** for this compound.

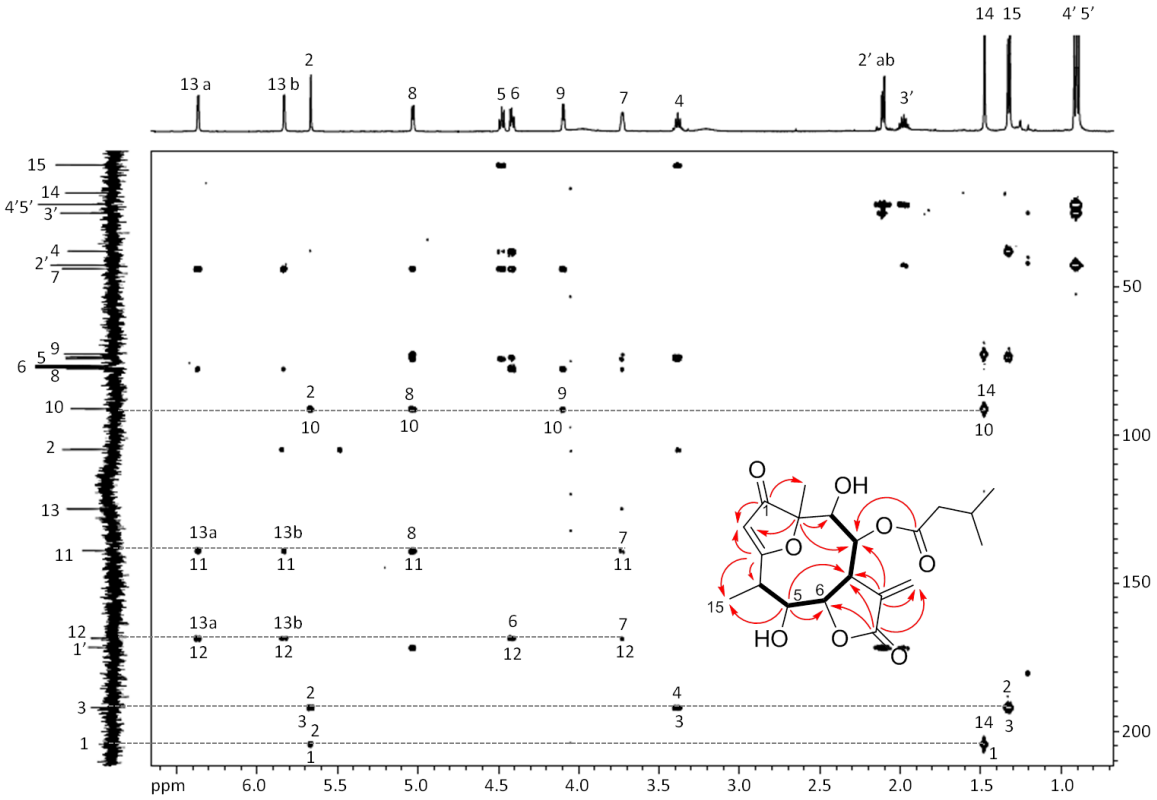


Figure 5. HMBC spectrum and ^{1}H-^{1}H COSY (▬) and HMBC (C→H) correlations of **11.**

Figure 6. Diagnostic NOESY correlations of **LOB-18** (**11**). **11**

LOB-20 (**12**) was obtained as a colourless gum. From the molecular ion peak at *m/z* 365.1961 $[M + H]^{+}$, in the positive-ion HRESIMS, its molecular formula was determined to be $C_{20}H_{28}O_6$. The ^{1}H-NMR spectrum showed the presence of signals due to an isovaleroyl side-chain (**Annex VI**). Furthermore, the JMOD spectrum suggested that the skeleton consists of 15 carbons, including 2 methyls, 3 methylenes, 6 methines and 4 quaternary carbons. The ^{1}H-^{1}H COSY spectrum was used to define two structural fragments with correlated protons: –CHR-CH_2-CH– (A) (δ_H 2.57, 2.36, 1.68 and 4.96) (C-1–C-3) and =CH-CHR-CHR-CHR-CH_2– (B) (δ_H 5.28, 5.53, 2.88, 5.21, 2.71 and 1.29) (C-5–C-9). These structural parts and the tertiary methyls and quaternary carbons were connected by inspection of the long-range C–H correlations observed in the HMBC spectrum. The two- and three-bond correlations between the quaternary carbon C-4 and H-6, H-15 and H_2-2, and between the

quaternary C-10 and H-8, H_2-2, H-14 and H_2-9 signals revealed that structural fragment A together with C-10, C-4 and the 14- and 15-methyl groups forms a germacrane skeleton. The lactone ring connected to the macrocycle in position C-6, C-7 was evident from the HMBC cross-peaks between C-12 and H_2-13, C-12 and H-6, C-11 and H_2-13, and C-11 and H-7. The position of the ester group was proved by the long-range correlation between the ester carbonyl group (δ_C 172.0) and H-8 (δ_H 5.21 t). The remaining epoxy and hydroxy groups, which were elucidated from the molecular composition, were placed at C-10–C-1 and C-3, respectively, with regard to the ^{13}C-NMR chemical shifts ($\delta_{C\text{-}10}$ 57.1, $\delta_{C\text{-}1}$ 59.7 and $\delta_{C\text{-}3}$ 66.8) and literature data for similar epoxy germacranolides.[135]

The relative configuration of the chiral centers was studied by NOESY measurements (**Figure 7**). Diagnostic NOE correlations were detected between H-6 and H-3, and H-6 and H-14, demonstrating the β orientation of these protons. Furthermore, NOESY cross-peaks were observed between H-1 and H-2a, H-2b and H-3, H-14 and H-9a, and H-7 and H-8, indicating the α-oriented H-1, H-2a, H-7, H-8 and H-9b and the β orientation of H-9a and H-2b. All of the above evidence was used to propose the structure of this compound as depicted in structural formula **12**. **LOB-20** (**12**) is the 3-epimer of desacetylisovaleroylheliangine, reported earlier from *Calea megacephala* (Asteraceae).[135]

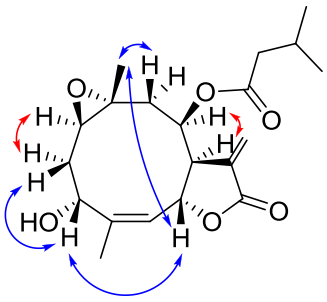
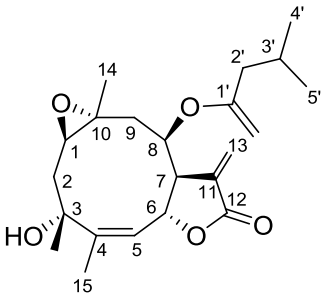


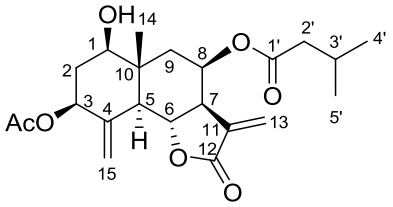
Figure 7. Diagnostic NOESY correlations of **LOB-20** (**12**).



12

LOB-26 (**13**) was isolated as a colourless oil with $[\alpha]^{27}_D$ +5 (*c* 0.1, $CHCl_3$). It was shown by HRESIMS to have the molecular formula $C_{22}H_{30}O_7$ (*m/z* 407.2065 $[M + H]^+$). The ^{1}H- and ^{13}C-NMR spectra of **LOB-26** revealed the presence of 1 acetyl and 1 isovaleroyl group. Additionally, the ^{13}C and JMOD spectra suggested a sesquiterpene skeleton consisting of 1 methyl, 4 methylenes, 6 methines and 4 quaternary carbons (**Annex VI**). The ^{1}H-^{1}H COSY spectra indicated two structural elements: –CHR-CH_2-CHR– (A δ_H 3.61, 2.20, 1.63 and 5.18) (C-1–C-3) and –CHR-CHR-CHR-CHR-CH_2– (B δ_H 2.22, 4.56, 2.83, 5.75, 2.32 and 1.58) (C-5–C-9). Their connectivity was determined from the HMBC spectrum observed between the quaternary carbons and protons of the structural fragments A and B. The positions of the ester groups were also established via the HMBC experiment on the basis of the $^3J_{C,H}$ couplings of the acetyl and isovaleroyl *C*O (δ_C 170.2 and 172.5) and skeletal protons H-3 (δ_H 5.18) and H-8 (δ_H 5.75), respectively.

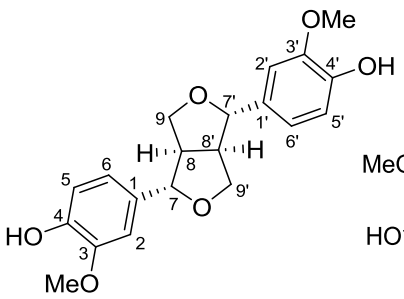
The relative configuration of **LOB-26** was studied by means of a NOESY experiment. Starting from the α orientation of H-5, characteristic cross-peaks between H-5 and H-7, H-7 and H-8, H-9b and H-1, and H-1 and H-3 confirmed the β orientation of these protons, while the NOE effects of H-6 and H-14, H-14 and H-2b, and H-14 and H-9a revealed the α orientation of H-6 and the 14-methyl group. All of the above evidence proved the eudesmanolide structure 3β-acetoxy-8β-isovaleroyloxyreynosin (**13**). **LOB-26** (**13**) is the first eudesmanolide isolated from the genus *Neurolaena*.



13

Compounds from Onopordum acanthium

OPD-8 (**14**) was obtained as an amorphous powder with $[\alpha]_D^{24}$ +53 (*c* 0.1, $CHCl_3$). Its molecular formula was $C_{20}H_{22}O_6$, established by APCI-MS, which showed a molecular ion peak at *m/z* 357 $[M - H]^-$. The ^{1}H- and ^{13}C-NMR investigations suggested that the compound contained a lignan structure. On the basis of its spectral data, **OPD-8** was identified as (+)-pinoresinol (**14**), isolated earlier from *O. acaulon, O. caricum* and *O. macracanthum.*[136-138] Complete ^{1}H- and ^{13}C-NMR shift assignments were achieved for the compound in $CDCl_3$ (in **Appendix V**).



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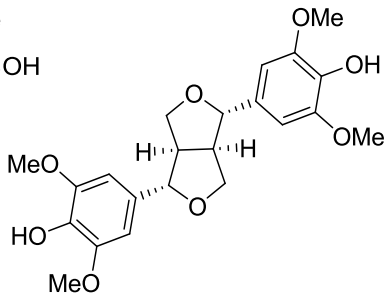

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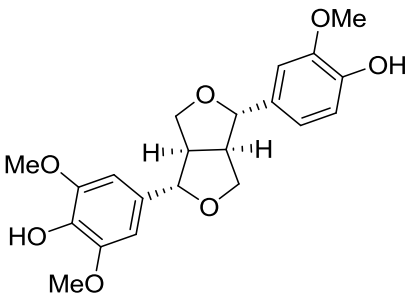
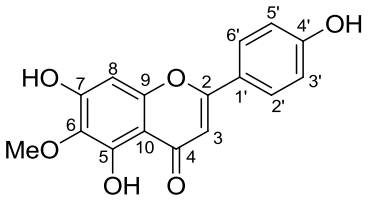


16

OPD-6/A (**15**) and **OPD-6/B** (**16**) were isolated as colourless oils. Spectral analysis led to the elucidation of their structures as lignans. The MS, ^{1}H- and ^{13}C-NMR data were in good agreement with the literature values. **OPD-6/A** and **OPD-6/B** were therefore identified as (+)-syringaresinol (**15**) and medioresinol (**16**).[139,140] Compound **15** was isolated earlier from *O. acaulon* by CARDONA *et al.* and **16** was first detected in the genus *Onopordum*.[35,136]



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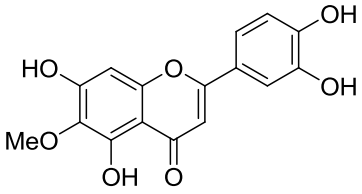

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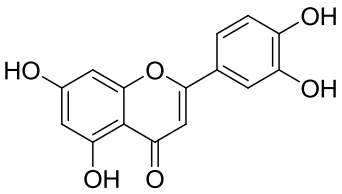

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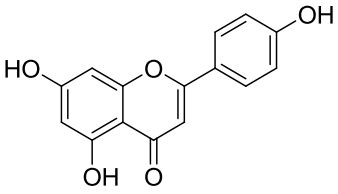


20

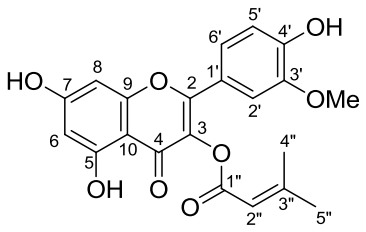
OPD-2 (**17**)**, OPD-3** (**18**)**, OPD-4** (**19**) **and OPD-5** (**20**) were obtained as yellow amorphous powders. As a result of MS, ^{1}H- and ^{13}C-NMR investigations, **OPD-2**–**OPD-5** were identified as hispidulin (**17**), nepetin (**18**), luteolin (**19**) and apigenin (**20**), respectively, by comparison of their spectral data with those published in the literature.[141-143] The flavonoids, excluding **19** and **20**, were isolated for the first time from *O. acanthium*, while **17** and **18** had been described previously from other *Onopordum* species.[35]

Compounds from Polygonum persicaria

PP-1 (**21**) was isolated as a pale-yellow oil. Its UV absorptions at 244, 270 sh and 337 nm indicated its nature as a flavonol derivative.[144] HRESIMS suggested that its molecular formula is $C_{21}H_{18}O_8$, with the base peak at *m/z* 421.08974 $[M + Na]^+$. From the ^{1}H- and ^{13}C-NMR spectra of **PP-1**, the presence of 1 methoxy and 1 senecioyl group was recognized (**Annex VII**). The ^{1}H-NMR spectrum indicated a 5,7-dihydroxylated flavonoid ring A (two *meta*-coupled doublets at δ_H 6.29 and 6.36, *J* = 1.6 Hz). The signals at δ_H 7.41 d (1.6 Hz), 7.02 d (8.3 Hz) and 7.48 dd (8.3, 1.6 Hz) allowed the identification of an ABX aromatic ring system, corresponding to 3',4'-substituted flavonoid ring B. All the ^{1}H- and ^{13}C-NMR signals were assigned by means of ^{1}H-^{1}H COSY, HSQC and HMBC experiments, and demonstrated an acylated isorhamnetin structure. The position of the senecioyl group was confirmed by the downfield-shifted ^{13}C-NMR signal of C-3 (δ_C 163.1) relative to that of isorhamnetin ($\delta_{C\text{-}3}$ 135.62).[145] The positions of the OH groups at C-5, C-7 and C-4' were indicated by the HMBC cross-peaks between the OH signals at δ_H 12.34, 6.00 and 5.97 and the carbon signals at δ_C 162.2 (C-5), 162.2 (C-7) and 148.6 (C-4'), while the location of the methoxy group was evident from the HMBC correlation between the δ_H 3.95 and δ_C 146.4 signals. All of the above evidence confirmed that **PP-1** was 3-*O*-senecioyl-isorhamnetin (**21**).

PP-2 (**22**) was obtained as a pale-yellow oil, with UV absorption similar to that of **PP-1**. The HRESIMS and NMR spectra indicated the same molecular mass as in the case of **PP-1**. The acyl group

was the only significant difference in the NMR spectra of **PP-2** (**Annex VII**) and **PP-1**. In **PP-2**, an angeloyl group was identified in consequence of the typical signals at δ_H 6.34 qq, 2.09 p (3H) and 2.08 dq (3H) and δ_C 164.6, 126.1, 142.7, 22.7 and 16.3 [CO-C(CH_3)=$CHCH_3$]. The 2D NMR experiments (^{1}H-^{1}H COSY, HSQC and HMBC) supported that this compound was 3-*O*-angeloyl-isorhamnetin (**22**).



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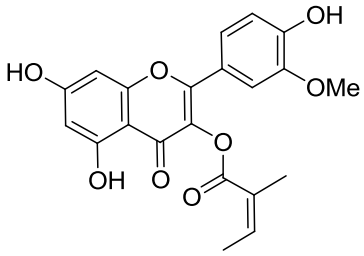

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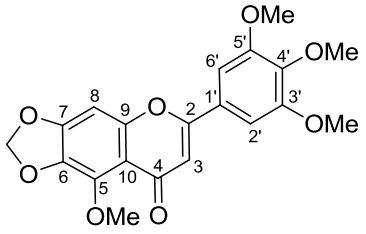

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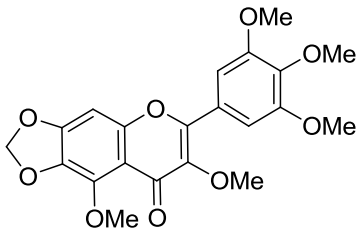

24

PP-3 (**23**) was isolated as a pale-yellow oil with UV absorption bands characteristic of a flavone derivative[144]. The molecular formula $C_{20}H_{18}O_8$ was established from the pseudomolecular ion peak at *m/z* 387.10780 $[M + H]^+$ in the HRESIMS. The ^{1}H-NMR and JMOD spectra exhibited typical signals for 1 methylenedioxy and 4 methoxy groups (**Annex VII**). The singlet signal at δ_H 6.60 in the ^{1}H-NMR spectrum was assigned to H-3 with regard to its HMBC correlation to C-2, C-4, C-10 and C-1'. The other singlet signal (δ_H 6.75) was assigned to H-8 with respect to its long-range correlation to C-9, thereby indicating a 5,6,7-substituted ring A. Moreover, the two-proton singlet signal at δ_H 7.06 s (H-2',6') suggested that ring B is symmetrically substituted, which means that the three methoxy groups are situated on ring B,[146] and one methoxy and the methylenedioxy group are on ring A. The methylenedioxy group must be in position 6,7 as indicated by the HMBC correlations between the OCH_2O (δ_H 6.07) protons and C-6 (δ_C 134.8) and C-7 (δ_C 153.0), and a methoxy group (δ_H 4.15) at C-5 (δ_C 153.0) with regard to their HMBC cross-peaks. These results indicated that **PP-3** is 5,3',4',5'-tetramethoxy-6,7-methylenedioxyflavone (**23**).

PP-4 (**24**) was proved by UV, ESIMS and 1D and 2D NMR spectra to be the 3-methoxy derivative of **PP-3**. This was earlier reported as a synthetic compound, but its NMR spectral data were not given.[147] The NMR assignments of all protons and carbons were determined (**Annex VII**). This is the first report of the isolation of 3,5,3',4',5'-pentamethoxy-6,7-methylenedioxyflavone (**24**) from a natural source.

6. DISCUSSION

6.1. SCREENING STUDIES

The aim of the study was to continue the screening program on the flora of the Carpathian Basin for plants and compounds with antiproliferative activity.[97,148] Moreover, the GIRK channel inhibitory activity of the extracts was also investigated. Although appreciable experimental evidence and ethnobotanical data have accumulated concerning the anticancer and other properties of Polygonaceae species, no comprehensive screening studies have yet been published on the plants of this family.[75]

In our work, the antiproliferative effects of 27 species belonging in the *Fallopia* (3), *Oxyria* (1), *Persicaria* (2), *Polygonum* (8) and *Rumex* (13) genera of the Polygonaceae family were tested *in vitro* against three human tumour cell lines, using the MTT assay.

The investigated Polygonaceae species were collected in several regions of Croatia, Hungary and Romania. Many of them were selected by virtue of their traditional application or their chemotaxonomical aspects, while some species originated from random collection. The plant samples were extracted with an amphipolar solvent (MeOH), which permitted the isolation of lipophilic and polar components. Solvent–solvent partitioning between *n*-hexane, $CHCl_3$ and H_2O afforded fractions (n = 196) differing in polarity. On the basis of the screening results, the *n*-hexane (A) and the $CHCl_3$-soluble fractions (B), and in particular those of the aerial plant parts or roots, were found to be the most effective. In contrast, only moderate activities were recorded for some fractions C (remaining MeOH) and some H_2O-soluble fractions (D). The characteristic compounds identified in the species of the Polygonaceae family are flavonoids, anthraquinones and other phenolic compounds, e.g. stilbenes, some of them having noteworthy activity. These compounds are present mainly in the *n*-hexane or $CHCl_3$ phases. It may be supposed that these compounds contribute to the antitumour effects of the active extracts.

Five species (*R. acetosa*, *R. alpinus*, *R. aquaticus*, *R. scutatus* and *R. thyrsiflorus*) of the *Rumex* genus and *P. hydropiper* proved to be the most active (**Table 2**), and 16 species of 27 investigated plants were found to be moderately active. A survey of the literature data on the investigated species did not reveal any earlier pharmacological and phytochemical studies on secondary metabolites of *R. thyrsiflorus* or *R. scutatus*.

A comparison of the measured activities with the ethnomedicinal uses of the plants reported by HARTWELL (1970) led to the conclusion that the present screening results for *P. hydropiper*, *R. acetosa*, *R. alpinus* and *R. aquaticus* are in accordance with the traditional uses of the plants against cancers.[75] Different preparations of *P. hydropiper* have been used since ancient times for the treatment of many kind of tumours, e.g. “hard, scirrhous, hydropic, oedematous, indurated and

mesenteric tumours", while *R. alpinus* has been applied against parotid tumours.[75] Especially the $CHCl_3$ extracts (B) of the herba of *R. acetosa*, and the root extracts (A) of *R. aquaticus* exhibited high activity against HeLa and MCF7 cells.

Interestingly, it emerged that some of the plants proved ineffective or exerted only a marginal effect on the tested cell lines, in spite of their traditional use in cancer treatment. Although HARTWELL's series reports the ethnomedicinal use of *O. digyna*, *Persicaria amphibia*, *P. maculosa*, *Polygonum aviculare*, *P. bistorta*, *R. crispus*, *R. hydrolapathum* and *R. obtusifolius* against cancers, warts and tumours,[75] only moderate activities (25–50% cell proliferation-inhibitory potency at 10 μg/mL and 30 μg/mL) were recorded for these species in our study. On the other hand, the extracts of *R. scutatus* and *R. thyrsiflorus* demonstrated a strong anticancer profile, although their ethnomedicinal use has not been described previously.

In case of GIRK channel inhibitory investigation 51 extracts of 11 Polygonaceae species were tested, among them the lipophilic $CHCl_3$ (B) fractions proved to be the most active. Especially the $CHCl_3$ extracts (B) of the aerial parts of *P. persicaria*, and the whole plant of *P. aviculare* exhibited high activity. To the best of our knowledge, this was the first application of the GIRK channel-inhibitory assay for the screening of plant extracts.

In conclusion, the antiproliferative and GIRK channel inhibitory screens has provided important preliminary data promoting the selection of Polygonaceae plant species (native to the Carpathian Basin). These selected species are promising for the discovery of new compounds with antitumour and GIRK channel inhibitory properties.

6.2. INVESTIGATION OF N. LOBATA, O. ACANTHIUM AND P. PERSICARIA

Chemical investigations of *N. lobata, O. acanthium* and *P. persicaria* resulted in the isolation of 24 compounds, including 9 new natural products (**Annex I** and **II**). The structures were identified by means of spectral methods as SLs, lignans and flavonoids. Pharmacological analysis confirmed that some of the isolated compounds possess biological activity.

6.2.1. Isolation of bioactive compounds

Our previous pharmacological investigations of the extracts with different polarity prepared from the aerial parts of *N. lobata*, *O. acanthium* and *P. persicaria* (see section **5.1** and **5.2**) and reviewing the literature data led to the conclusion that the lipophilic extracts ($CHCl_3$ or CH_2Cl_2) contain the bioactive secondary metabolites.

In the initial step of the phytochemical work, the dried plant materials were percolated with an amphipolar solvent (MeOH) at room temperature; solvent–solvent extraction was then applied, which resulted in the $CHCl_3$ or CH_2Cl_2 phases. All of them were subjected to a multistep

chromatographic procedure in order to isolate the compounds responsible for the pharmacological activities.

The purification of the CH_2Cl_2-soluble phase of *N. lobata* was carried out by OCC, affording 7 main fractions (BI-BVII); among them, fraction BII was the most interesting. Since this fraction contained several compounds and demonstrated a chemical complexity, more selective methods (VLC, RPC and PLC) were applied, with the use of silica gel and different solvent systems. Finally, NP- and RP-PLC proved the most effective and selective methods for the isolation of compounds (**1**–**13**).

The $CHCl_3$ phase of *O. acanthium* was separated by VLC to furnish 6 main fractions (BI-VI). These fractions were further evaluated for their inhibitory effects on COX-2 and NF-κB1 gene expression, iNOS, 5-LOX, and COX-1 and COX-2 enzymes in *in vitro* assays. Fractions BI, BIV and BV at 10 or 50 μg/mL exhibited significant or moderate activity in the inhibition of COX-2 gene expression (45.5 ± 8.3%, 31.5 ± 11.1% and 12.6 ± 5.7%), NO production (62.5 ± 16.5%, 102.0 ± 0.3% and 79.9 ± 6.2%), and COX-2 enzyme (63.8 ± 9.8%, 19.9 ± 8.4% and 44.9 ± 8.8%). Fraction BI was chromatographed by CC on polyamide to give 7 subfractions (BI/1-I/7). The most active subfractions BI/2 (inhibition of COX-2: 73.3 ± 3.7%; inhibition of 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, RPC, MPLC, gel filtration on Sephadex LH-20 and preparative TLC. This purification process led to the isolation of 7 compounds (**14**–**20**) in pure form.

In the case of *P. persicaria*, the $CHCl_3$ phase was fractionated by RP-VLC on silica gel. The fractions were combined into 6 main fractions (B/1-B/6) according to the TLC monitoring, and were tested for GIRK channel-inhibitory activity. Fractions B/4 and B/5 displayed considerable activity (60 ± 10% and 68 ± 11%), while the fractions B/1-3 and B/6 at 0.1 mg/mL were moderately active (13–25%). Two active fractions (B/4 and B/5) were further separated by RP-HPLC, and yielded 4 compounds (**21**–**24**) in pure form and a mixture containing the minor constituents.

The preparative work was completed with analytical TLC on silica gel with various solvent systems. The detection was carried out in UV light at 254 and 366 nm, followed by spraying with cc. H_2SO_4.

After extensive chromatographic purification, 13 SLs were isolated from *N. lobata* (**LOB-2**, **LOB-3**, **LOB-5**, **LOB-6**, **LOB-9**–**11**, **LOB-13**–**15**, **LOB-18**, **LOB-20** and **LOB-26**) (**1**–**13**), 3 lignans (**OPD-8**, **OPD-6/A** and **OPD-6/B**) (**14**–**16**) and 4 flavonoids from *O. acanthium* (**OPD-2**–**5**) (**17**–**20**), and 4 flavonoids from *P. persicaria* (**PP-1**–**4**) (**21**–**24**) (**Annex I** and **II**). The lignans and flavonoids were obtained as oils or amorphous powders, and the SLs were oils, gums, crystals or amorphous solids.

6.2.2. Structure elucidation

The chemical structures of the isolated compounds were determined by means of spectroscopic methods. The molecular masses and compositions were obtained from MS investigations; UV spectroscopic and optical rotation measurements provided further important information for characterization of the compounds. The most useful data concerning the structures were furnished by 1D and 2D NMR spectroscopy. The constitutions of the compounds were elucidated via ^{1}H-NMR, JMOD, ^{1}H-^{1}H COSY, HSQC and HMBC experiments, and the relative configurations were then characterized with the aid of NOESY spectra. As a result of the NMR studies, complete ^{1}H- and ^{13}C-assignments were made for the new compounds and also in the case of some known compounds, where previously published data were incomplete.

Thirteen SLs, esterified with isovaleric acid at C-8 or C-9, were isolated from *N. lobata*. Eight of them, the germacranolide type **LOB-2** (neurolenin A, **1**), **LOB-3** (neurolenin B, **2**), **LOB-5** (neurolenin D, **3**), **LOB-6** (neurolenin C, **4**), and **LOB-10** (lobatin A, **6**) and the furanoheliangolide-type **LOB-11** (lobatin B, **7**), **LOB-9** (8β-isovaleryloxy-9α-acetoxy-calyculatolide, **5**) and **LOB-13** (8β-isovaleryloxy-9α-hydroxy-calyculatolide, **8**), had already been isolated from this species. **LOB-15** (**9**) and **LOB-14** (**10**) are unusual isomeric seco-germacranolide sesquiterpenes with a bicyclic acetal moiety. **LOB-18** (**11**), is an 1-keto-furanoheliangolide derivative similarly to **LOB-13** (**8**), from which it differs only at the substitution of C-5. **LOB-20** (**12**), also an unsaturated epoxy-germacranolide ester, is the 3-epimer of desacetylisovaleroylheliangine, reported from *Calea magecephala* previously, differing only in the ^{1}H-NMR chemical shifts and the coupling constants of H-3. **LOB-26** (**13**) is an eudesmanolide-type SL. Eudesmanolides occur widely in the family Asteraceae,[149] but **LOB-26** (**13**) is the first isolated from the genus *Neurolaena*. In summary, **LOB-15** (neurolobatin A, **9**), **LOB-14** (neurolobatin B, **10**), **LOB-18** (5β-hydroxy-8β-isovaleroyloxy-9α-hydroxycalyculatolide, **11**), **LOB-20** (3-*epi*-desacetylisovaleroyl-heliangine, **12**) and **LOB-26** (3β-acetoxy-8β-isovaleroyloxyreynosin, **13**) were identified as new SLs.

The structure analysis of compounds isolated from *O. acanthium* led to the identification of 3 lignans and 4 flavonoids. All of them [**OPD-8** ((+)-pinoresinol, **14**), **OPD-6/A** ((±)-syringaresinol, **15**), **OPD-6/B** (medioresinol, **16**), **OPD-2** (hispidulin, **17**), **OPD-3** (nepetin, **18**), **OPD-4** (luteolin, **19**) and **OPD-5** (apigenin, **20**)] were identified on the basis of the comparison of the measured and literature MS and NMR data. In the case of **OPD-8** (**14**), the NMR chemical shifts recorded in $CDCl_3$ were determined for the first time. (+)-Pinoresinol (**14**), (±)-syringaresinol (**15**), hispidulin (**17**) and nepetin (**18**) had been described previously from other *Onopordum* species, while medioresinol (**16**) was identified for the first time in the genus.

Four flavonoids were isolated from *P. persicaria*, among them, **PP-1** (**21**) and **PP-2** (**22**) being esterified at C-3. In the case of **PP-1**, the unusual senecioyl group is present in the molecule, while **PP-2** contains an angeloyl group. Compounds containing a senecioyl group is very rare in the plant kingdom. Such compounds have to date been isolated only from *Elaeoselinum foetidum*, *Pogostemon auricularis* and *Azadirachta indica*.[150-152] **PP-3** (**23**) and **PP-4** (**24**) were identified as 6,7-methylenedioxyflavones containing 4 or 5 methoxy groups in the molecule. **PP-1** (3-*O*-senecioyl-isorhamnetin, **21**), **PP-2** (3-*O*-angeloyl-isorhamnetin, **22**), **PP-3** (5,3',4',5'-tetramethoxy-6,7-methylenedioxyflavone, **23**) and **PP-4** (3,5,3',4',5'-pentamethoxy-6,7-methylenedioxyflavone, **24**) are new natural products, though **PP-4** was reported earlier only as a synthetic compound.

LC-MS investigation of the occurrence of compounds **21**–**24** in *P. persicaria* samples of various origins in different vegetation stages revealed that only samples collected in the flowering period contained the isolated flavonoids **21**–**24** (**Figure 3** in **Appendix II**).

6.2.3. Biological activities

Neurolaena lobata

The Maya ethnopharmacological plant *N. lobata* has been widely applied for the treatment of different types of cancer, ulcers, inflammatory skin disorders and diabetes. The previous pharmacological study of the plant demonstrated that the CH_2Cl_2 extract down-regulated the expression of oncogenes, induced tumour suppressors, inhibited cell proliferation, and triggered the apoptosis of malignant cells (see section **5.2**). On the basis of these results and the data published recently, the CH_2Cl_2 extract of the aerial parts was selected for further investigations.

The new compounds (**9**–**13**) isolated from the extract (CH_2Cl_2) of *N. lobata* which showed the most potent cell proliferation-inhibitory activities were tested for antiproliferative activities against a set of human adherent cell lines (A2780, A431, HeLa and MCF7) (**Table 4**).

Table 4. Antiproliferative effects of the isolated compounds (**9**-**13**) on different human tumour cell lines

Compound	IC_{50} (μM) ± SEM			
	HeLa	**A431**	**A2780**	**MCF7**
LOB-15 (**9**)	>10	>10	9.8 ± 1.0	>10
LOB-14 (**10**)	>10	**6.8 ± 0.56**	5.4 ± 0.67	>10
LOB-18 (**11**)	>10	>10	>10	>10
LOB-20 (**12**)	>10	**7.2 ± 0.99**	4.7 ± 0.19	**7.2 ± 0.57**
LOB-26 (**13**)	>10	**5.3 ± 0.47**	7.2 ± 0.42	>10
cisplatin	5.7 ± 0.84	8.8 ± 0.97	0.86 ± 0.12	8.0 ± 1.1

All these compounds except **11** inhibited the proliferation of A431 and A2780 cells, and were less active against MCF7 and HeLa cells. The IC_{50} values of **10**, **12** and **13** against A431 and MCF7 cells were comparable to those of the reference agent cisplatin. Neurolobatin A (**LOB-15**, **9**) and 5β-hydroxy-8β-isovaleroyloxy-9α-hydroxycalyculatolide (**LOB-18**, **11**) exhibited no or relatively low potencies on all tested cell lines.

Besides the antiproliferative effect, the anti-inflammatory activities of the new compounds (**9**–**13**) were also studied *in vitro* by means of LPS- and TNF-α-induced IL-8 expression-inhibitory assays. All the compounds down-regulated the LPS-induced production of IL-8 protein, with neurolobatin B (**10**) and 3-*epi*-desacetylisovalerylheliangine (**12**) being the most effective (**Figure 3** in **Appendix III**). However, the tested compounds (**9**–**13**) did not significantly influence the production of IL-8 in TNF-α-treated endothelial cells. Moreover, in contrast to the positive control BAY, the isolated compounds were selective as they inhibited only the LPS-induced IL-8 production.

In addition, the effects of the CH_2Cl_2 extract and the isolated known compounds (**1**–**8**) on the generation of pro-inflammatory proteins (IL-8 and E-selectin) were assessed *in vitro* in endothelial (HUVECtert) and monocytic (THP-1) cells by enzyme-linked immunosorbent assay (ELISA). Treatment with the extract or any of the SLs (**1**–**8**) decreased the LPS-induced secretion of the cytokine IL-8 in a dose-dependent manner (**Figure 2A** in **Appendix IV**). At the highest tested concentration (10 μM), all of the tested compounds (**1**–**8**) strongly decreased the secretion of IL-8 in LPS-stimulated endothelial cells. The most active compounds, neurolenin B (**LOB-3**, **2**), lobatin B (**LOB-11**, **7**) and 8β-isovaleryloxy-9α-acetoxy-calyculatolide (**LOB-9**, **5**) also down-regulated the production of IL-8 protein in TNF-α-induced endothelial cells (**Figure 2B** in **Appendix IV**). The extract (5 μg/mL) and the 8 known compounds (5 μM) demonstrated significant effects on another inflammation marker, the adhesion molecule E-selectin. After stimulation with LPS and TNF-α, all tested SLs (**1**–**8**) down-regulated the expression of E-selectin on endothelial cells (**Figure 3A** and **B** in **Appendix IV**). The highest activity was indicated by **LOB-11** (**7**), and **LOB-3** (**2**), **LOB-9** (**5**) and **LOB-10** (**6**) were also effective. The anti-inflammatory activities of the compounds (**1**–**8**) were observed in THP-1 monocytes too, where they significantly reduced the production of IL-8 after stimulation with LPS (**Figure 4** in **Appendix IV**). **LOB-11** (**7**) proved to be the most effective, followed by **LOB-9** (**5**), **LOB-3** (**2**) and **LOB-10** (**6**).

Furthermore, the WST-1 viability assay revealed that concentrations up to 10 μM in the case of SLs (**1**–**8**) and 30 μg/mL in the case of the extract were not toxic to endothelial cells. Hence, the observed anti-inflammatory activity was not due to a direct toxic effect.

In order to test whether *N. lobata* components modulate the expression of inflammatory genes at the mRNA level, endothelial cells were treated with the three most active SLs, **LOB-3** (**2**), **LOB-9** (**5**) and **LOB-11** (**7**), followed by analysis of the LPS-induced expression of mRNA encoding for IL-8 and

E-selectin. The relative mRNA expression of the IL-8 and E-selectin genes in the endothelial cells was strongly inhibited by the SLs as compared with activation with LPS alone (**Figure 5** in **Appendix IV**). **LOB-3** (**2**) exerted a significant effect only on E-selectin.

On the basis of these data, a structure–activity relationship was established. The highest activity was exerted by lobatin B (**LOB-11**, **7**); this compound differs from 8β-isovaleryloxy-9α-hydroxy-calyculatolide (**LOB-13**, **8**) only in the double bond at C-4–C-5, indicating the importance of this olefin for the anti-inflammatory potency. Comparison of 8β-isovaleryloxy-9α-hydroxy-calyculatolide (**LOB-13**, **8**) and 8β-isovaleryloxy-9α-acetoxy-calyculatolide (**LOB-9**, **5**), differing solely in the C-9 substituent, demonstrates an increase in the anti-inflammatory effect with acetylation at this position. Neurolenin B (**LOB-3**, **2**) with a 9-acetoxy group exhibited higher activity than that of neurolenin D (**LOB-5**, **3**) with a 9-hydroxy group. Comparison of neurolenin B (**LOB-3**, **2**) and lobatin A (**LOB-10**, **6**), which differ in the position of the olefin group in the 10-membered ring, revealed that C-2–C-3 double bond is preferred regarding the anti-inflammatory effect. Furthermore, the higher efficacy of neurolenin D (**LOB-5**, **3**) relative to that of neurolenin C (**LOB-6**, **4**) demonstrated that the position of the isovaleryloxy group at C-8 is more favourable than C-9.

The *in vivo* anti-inflammatory activity of the CH_2Cl_2 extract was evaluated by using a carrageenan-induced paw oedema model in rats. Both applied doses of *N. lobata* extract (20 and 60 mg/kg) inhibited the development of acute inflammation in rats (**Figure 6** in **Appendix IV**). The suppression of local oedema formation by the higher dose was more that 50%. This result arouses further interest in the therapeutic potential of such compounds for the treatment of inflammatory diseases.

The most active compounds neurolenin B (**LOB-3**, **2**) and lobatin B (**LOB-11**, **7**) were further studied to reveal the mechanism of their anti-inflammatory action.[153,154]

Onopordum acanthium

An anti-inflammatory investigation of *O. acanthium* (Scotch thistle), a plant traditionally used for the treatment of different types of inflammation in Central Asia, appeared promising, since the $CHCl_3$ extract proved to exert marked activity in three different test systems (see section **5.2**). Our primary goal was therefore to isolate and identify the compounds responsible for this activity of the plant.

The compounds (**14–20**) isolated from the active $CHCl_3$ extract of the aerial parts of the plant were tested at 20 μM for their inhibitory effects on COX-2 and NF-κB1 gene expression, iNOS, 5-LOX, COX-1 and COX-2 enzymes in *in vitro* assays (**Table 5**). Among the flavonoids, luteolin (**OPD-4**, **19**) was the most potent compound, markedly inhibiting the biosynthesis of leukotriene (74.6 ± 8.8%) and exhibiting moderate activity on COX-2 and NF-κB1 gene expression, and in iNOS and COX-2 assays. These findings are in accordance with those of previously reported studies.[155] Moreover, noteworthy inhibitory activities (> 50% inhibition) were recorded for hispidulin (**OPD-2**, **17**) and

nepetin (**OPD-3**, **18**) on 5-LOX, similarly to as in previously reported experimental models.[156] Interestingly, apigenin (**OPD-5**, **20**) exerted only a moderate effect in our experiments.

Table 5. Anti-inflammatory effects of compounds isolated from aerial parts of *O. acanthium*

Compound	% Inhibition ± SD					
	COX-2[a] (20 µM)	NF-κB1 (20 µM)	iNOS (20 µM)	LOX-5 (20 µM)	COX-1 (20 µM)	COX-2[b] (20 µM)
OPD-8 (**14**)	<10	<10	**49.1 ± 4.1**	37.5 ± 12.2	12.7 ± 6.1	12.33 ± 9.54
OPD-6/A (**15**)	<10	16.1 ± 11.5	17.4 ± 8.3	28.5 ± 7.1	<10	<10
OPD-6/B (**16**)	<10	11.9 ± 7.7	<10	11.4 ± 12.0	16.2 ± 8.1	<10
OPD-2 (**17**)	<10	10.3 ± 1.9	<10	**51.6 ± 11.0**	10.9 ± 2.5	<10
OPD-3 (**18**)	12.1 ± 23.4	10.9 ± 5.7	<10	**62.4 ± 7.7**	<10	10.1 ± 7.2
OPD-4 (**19**)	37.2 ± 25.1	30.9 ± 1.0	38.9 ± 10.1	**74.6 ± 8.8**	10.2 ± 9.4	39.1 ± 10.8
OPD-5 (**20**)	30.4 ± 12.6	28.6 ± 8.2	21.6 ± 7.7	41.3 ± 10.2	<10	24.3 ± 10.3
DEX[c]	47.6 ± 4.2	ND	ND	ND	ND	ND
Q[c]	ND	46.0 ± 8.4	ND	ND	ND	ND
L-NMMA[c]	ND	ND	52.5 ±4.9	ND	ND	ND
ZYFLO[c]	ND	ND	ND	63.0 ± 3.8	ND	ND
INN[c]	ND	ND	ND	ND	32.3 ± 4.8	ND
NS398[c]	ND	ND	ND	ND	ND	30.5 ± 3.1

[a] COX-2 gene expression inhibition; [b] COX-2 enzyme inhibition; [c] Positive control (DEX = dexamethasone, Q = quercetin, L-NMMA = N-Monomethyl-L-arginin monoacetat, ZYFLO = zilueton, INN = indomethacin, NS398 = N-[2-cyclohexyloxy-4-nitrophenyl] methanesulfonamide)
n = 2 experiments in duplicate. ND not determined

As concerns the lignans, only moderate activities were observed for **14–16**, but pinoresinol (**OPD-8**, **14**) was found to be active against LPS/IFN-γ-induced NO production. These results were in agreement with data published earlier.[157]

The extracts differing in polarity that were prepared from the roots were also tested for their inhibitory effects in all test models (**Table 2** in **Appendix V**). The $CHCl_3$ extracts of the roots exhibited activity in 3 tests, and the compounds isolated earlier from the lipophilic extract [4β,15-dihydro-3-dehydrozaluzanin C (**25**), zaluzanin C (**26**), 4β,15,11β,13-tetrahydrozaluzanin C (**27**), nitidanin-diisovalerianate (**28**), 24-methylenecholesterol (**29**) and 13-oxo-9*Z*,11*E*-octadecadienoic acid (**30**)][39] (**Annex III**) were also assayed (**Table 6**). Strong inhibitory activities were detected for 4β,15-dihydro-3-dehydrozaluzanin C (**25**) and zaluzanin C (**26**) in COX-2 (98.6 ± 0.2% and 97.0 ± 1.1%) and NF-κB1 gene expression (78.7± 7.3% and 69.9 ± 3.4%) , and NO assays (100.4 ± 0.5% and 99.4 ± 0.8%). 4β,15,11β,13-tetrahydrozaluzanin C (**27**) also demonstrated activity against LPS/IFN-γ-induced NO production (61.4 ± 17.3%). Other compounds (**28**–**30**) exerted only marginal effects in the applied

bioassays. As far as we know, this is the first report on inhibitory activity of compounds **25** and **26** against COX-2 and NF-κB1 gene expression (mRNA level) in THP-1 cells.

Table 6. Anti-inflammatory effects of compounds isolated from roots of *O. acanthium*

Compound	% Inhibition ± SD					
	COX-2[a] **(20 µM)**	**NF-κB1 (20 µM)**	**iNOS (20 µM)**	**LOX-5 (20 µM)**	**COX-1 (20 µM)**	**COX-2**[b] **(20 µM)**
25[c]	**98.6 ± 0.2**	**78.7± 7.3**	**100.4 ± 0.5**	26.4 ± 12.9	<10	10.5 ± 10.3
26[c]	**97.0 ± 1.1**	**69.9 ± 3.4**	**99.4 ± 0.8**	<10	<10	<10
27[c]	<10	<10	**61.4 ± 17.3**	<10	<10	29.5 ± 9.6
28[c]	<10	13.14 ± 14.63	<10	16.1 ± 11.0	<10	<10
29[c]	<10	11.65 ± 10.57	<10	<10	10.1 ± 6.3	36.4 ± 9.5
30[c]	<10	18.58 ± 6.58	<10	20.4 ± 11.5	16.4 ± 5.5	16.6 ± 8.8
DEX[d]	47.6 ± 4.2	ND	ND	ND	ND	ND
Q[d]	ND	46.0 ± 8.4	ND	ND	ND	ND
L-NMMA[d]	ND	ND	52.5 ±4.9	ND	ND	ND
ZIFLO[d]	ND	ND	ND	63.0 ± 3.8	ND	ND
INN[d]	ND	ND	ND	ND	32.3 ± 4.8	ND
NS398[d]	ND	ND	ND	ND	ND	30.5 ± 3.1

[a] COX-2 gene expression inhibition; [b] COX-2 enzyme inhibition

[c] **25:** 4β,15-dihydro-3-dehydrozaluzanin C, **26**: zaluzanin C, **27**: 4β,15,11β,13-tetrahydrozaluzanin C, **28**: nitidanin-diisovalerianate, **29**: 24-methylenecholesterol, **30**: 13-oxo-9*Z*,11*E*-octadecadienoic acid

[d] Positive control(DEX = dexamethasone, Q = quercetin, L-NMMA = N-Monomethyl-L-arginin monoacetat, ZYFLO = zilueton, INN = indomethacin, NS398 = N-[2-cyclohexyloxy-4-nitrophenyl] methanesulfonamide)

n = 2 experiments in duplicate. 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, 24, 48 and 72 h) and at different concentrations. It was found, that the active compounds have no or low effects on cell viability at the tested concentrations (**Figure 3** in **Appendix V**).

Overall, the results confirm that the inhibitory activities of the extracts may be attributed mainly to flavonoids, lignans and sesquiterpenoids, while other compounds exerting additional effects.

Polygonum persicaria

The GIRK channel-inhibitory activity of *P. persicaria* extracts was investigated aiming of identifying natural compounds with promising ion channel-blocking effect. GIRK channels are selectively expressed in the atrium and are not present in the ventricle. Electrical remodelling of atrial heart muscle during chronic atrial fibrillation may result in a constitutively active form of the GIRK channel, which may lead to an important role of this channel in this disease. Selective blockade of the GIRK channel might be a useful tool in the treatment of atrial fibrillation.[158]

The $CHCl_3$ extract of *P. persicaria* exhibited significant GIRK channel-inhibitory activity, its effect proving comparable to that of propafenone (**Table 1** in **Appendix II**). The most effective fractions of the extract were B/4 and B/5, from which compounds **PP-1**–**4** (**21**–**24**) were isolated. Surprisingly, neither the individual, nor the combined application of the isolated compounds of the active fractions (**21**–**24**) exerted activity on the GIRK channel (**Table 1** in **Appendix II**). However, the remaining HPLC eluates of fractions B/4 and B/5, containing mixtures of minor compounds, proved to have inhibitory activities of 63 ± 9% and 62 ± 4% at 0.1 mg/mL. The attempted isolation and identification of the compounds present in fractions B/4 and B/5 have so far failed because of their low quantities.

Investigation of the flavonoids (**21**–**24**) and the HPLC eluates containing the minor compounds revealed that the identified compounds display only weak GIRK channel-inhibitory activity; the more active agents can be found among the unidentified minor compounds.

7. SUMMARY

The primary aim of the present work was an evaluation of the antitumour and GIRK channel modulatory effects of Polygonaceae species native to the Carpathian Basin, and the isolation, structure determination and pharmacological investigation of biologically active compounds from *Neurolaena lobata*, *Onopordum acanthium* and *Polygonum persicaria.*

Lipophilic and hydrophilic extracts of 27 Polygonaceae species were screened *in vitro* against HeLa, A-431 and MCF-7 cells, using the MTT assay. Six of the tested species (*R. acetosa*, *R. alpinus*, *R. aquaticus*, *R. scutatus*, *R. thyrsiflorus and P. hydropiper*) were found to exert significant (> 50%) cell growth-inhibitory potency. Another six species (*P. aviculare*, *P. amphibia*, *P. persicaria*, *R. stenophyllus*, *R. patientia* and *R. crispus*) showed high inhibitory activity (> 70%) on GIRK channels. Our preliminary screen has provided important data on the anticancer and GIRK channel inhibitory properties of numerous Polygonaceae species native to the Carpathian Basin, which promotes the selection of further species for future pharmacological and phytochemical work.

Previous pharmacological evaluations of the extracts with different polarity prepared from *N. lobata*, *O. acanthium* and *P. persicaria* led to the conclusion that the lipophilic extracts ($CHCl_3$ or CH_2Cl_2) contain the bioactive secondary metabolites. The isolation of the compounds from these fractions was carried out by a multistep separation procedure, including OCC, VLC, RPC, MPLC, PLC, GF and RP-HPLC. The structures of the isolated compounds were elucidated by means of spectroscopic methods (HR-MS, MS and NMR). In addition, complete ^{1}H- and ^{13}C-NMR assignments were made for the characterization of the compounds.

Five new SLs [neurolobatin A (**LOB-15**, **9**), neurolobatin B (**LOB-14**, **10**), 5β-hydroxy-8β-isovaleroyloxy-9α-hydroxycalyculatolide (**LOB-18**, **11**), 3-*epi*-desacetylisovaleroylheliangine (**LOB-20**, **12**), 3β-acetoxy-8β-isovaleroyloxyreynosin (**LOB-26**, **13**)], together with 8 known ones [neurolenin A (**LOB-2**, **1**), neurolenin B (**LOB-3**, **2**), neurolenin D (**LOB-5**, **3**), neurolenin C (**LOB-6**, **4**), lobatin A (**LOB-10**, **6**), lobatin B (**LOB-11**, **7**), 8β-isovaleryloxy-9α-acetoxy-calyculatolide (**LOB-9**, **5**) and 8β-isovaleryloxy-9α-hydroxy-calyculatolide (**LOB-13**, **8**)], were obtained from the CH_2Cl_2 extract of the aerial parts of *N. lobata*. Among the new compounds, **LOB-15** (**9**) and **LOB-14** (**10**) are unusual isomeric seco-germacranolide sesquiterpenes with a bicyclic acetal moiety, **LOB-18** (**11**) and **LOB-20** (**12**) are unsaturated epoxy-germacranolide esters, and **LOB-26** (**13**) is the first eudesmanolide isolated from the genus *Neurolaena*.

The new SLs (**9**–**13**) were shown to have noteworthy antiproliferative activities against human tumour cell lines (A2780, A431, HeLa and MCF7). The anti-inflammatory effects of **9**–**13** were evaluated *in vitro* using an LPS- and TNF-α-induced IL-8 expression inhibitory assays, and it was found that all these compounds strongly down-regulated the LPS-induced production of IL-8 protein,

with neurolobatin B (**LOB-14**, **10**) and 3-*epi*-desacetylisovaleroylheliangine (**LOB-20**, **12**) being the most effective. The effects of the extract and the known SLs (**1**–**8**) on the generation of pro-inflammatory proteins were also assessed *in vitro* in endothelial and monocytic cells, and their potential to modulate the expression of inflammatory genes at the mRNA level was studied. Lobatin B (**LOB-11**, **7**) showed the most potent anti-inflammatory effect. 8β-Isovaleryloxy-9α-acetoxy-calyculatolide (**LOB-9**, **5**), neurolenin B (**LOB-3**, **2**) and lobatin A (**LOB-10**, **6**) were less active. Moreover, the structure–activity analysis revealed the importance of the double bond at C-4–C-5 and C-2–C-3 and the acetyl group at C-9 for the anti-inflammatory activity. *In vivo* confirmation of the pharmacological effect raises further interest in the therapeutic potential of lobatin B (**LOB-11**, **7**) and related compounds.

From the $CHCl_3$ extract of the aerial parts of *O. acanthium*, which exert a noteworthy inhibitory effects on iNOS, 5-LOX and COX-2 enzymes in *in vitro* assays, 3 known lignans [(+)-pinoresinol (**OPD-8**, **14**), (±)-syringaresinol (**OPD-6/A**, **15**) and medioresinol (**OPD-6/B**, **16**)], and 4 known flavonoids [hispidulin (**17**), nepetin (**18**), luteolin (**19**) and apigenin (**20**)] were identified. These compounds, excluding luteolin (**19**) and apigenin (**20**), were isolated for the first time from this species; furthermore, medioresinol (**16**) was also detected for the first time in the genus.

The inhibitory activities of the isolated compounds (**14**–**20**), together with the substances of the root extract (**25**–**30**), were tested on COX-2 and NF-κB1 gene expression, iNOS, 5-LOX, and COX-1 and COX-2 in *in vitro* assays. Two SLs, 4β,15-dihydro-3-dehydrozaluzanin C (**25**) and zaluzanin C (**26**), exhibited strong effects in the bioassays applied. Moreover, some of the flavonoids and lignans may play a role in the activities of the extract. The traditional use of *O. acanthium* against inflammatory diseases seems to be supported by our data.

Four new natural flavonoids [3-*O*-senecioyl-isorhamnetin (**PP-1**, **21**), 3-*O*-angeloyl-isorhamnetin (**PP-2**, **22**), 5,3',4',5'-tetramethoxy-6,7-methylenedioxyflavone (**PP-3**, **23**) and 3,5,3',4',5'-pentamethoxy-6,7-methylenedioxyflavone (**PP-4**, **24**)] were isolated from the aerial parts of *P. persicaria*. **PP-1** (**21**) contains a rare senecioyl group, while **PP-2** (**22**) is an angeloyl ester flavonoid. **PP-4** (**24**) was reported earlier only as a synthetic compound; this is the first report on its isolation from a natural source. The extract of the plant possessed high GIRK channel-inhibitory activity but the isolated compounds (**21**–**24**) did not affect the function of these channels. The minor compounds of the HPLC eluates are responsible for the K^+ channel-modulatory activity of the plant extract.

Our results reveal that secondary metabolites of Asteraceae and Polygonaceae species can be regarded as promising starting materials in the search for new pharmaceutical discoveries, in consequence of their pharmacological potential, and in particular their noteworthy anti-inflammatory and antitumour effects.

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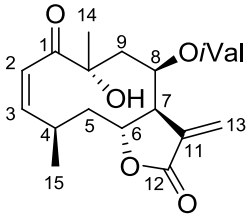
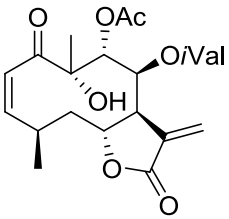
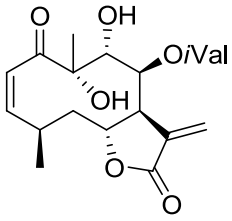
Annex I. Compounds isolated from the aerial parts of *N. lobata*



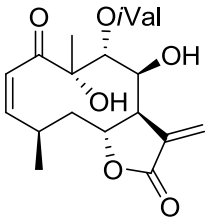
LOB-2 (**1**)
neurolenin A



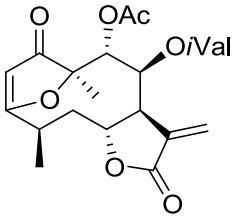
LOB-3 (**2**)
neurolenin B



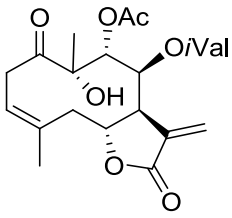
LOB-5 (**3**)
neurolenin D



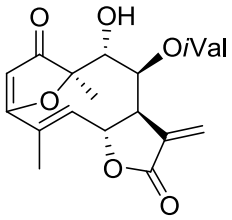
LOB-6 (**4**)
neurolenin C



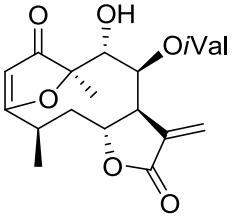
LOB-9 (**5**)
8β-isovaleroyloxy-9α-acetoxy-calyculatolide



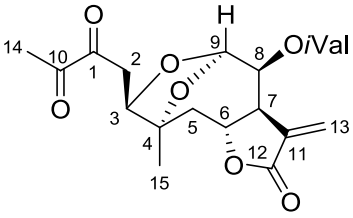
LOB-10 (**6**)
lobatin A



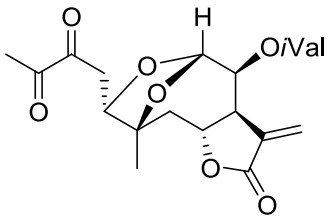
LOB-11 (**7**)
lobatin B



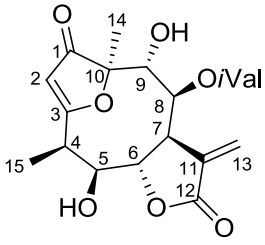
LOB-13 (**8**)
8β-isovaleroyloxy-9α-hidroxy-calyculatolide



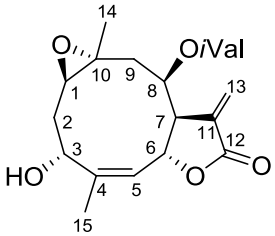
LOB-15 (**9**)
neurolobatin A



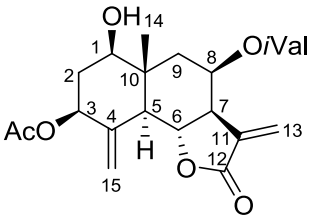
LOB-14 (**10**)
neurolobatin B



LOB-18 (**11**)
5β-hydroxy-8β-isovaleroyloxy-9α-hydroxycalyculatolide

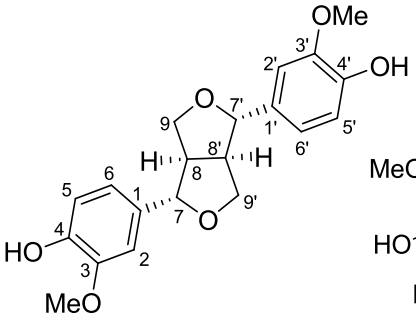


LOB-20 (**12**)
3-*epi*-desacetylisovaleroylheliangine

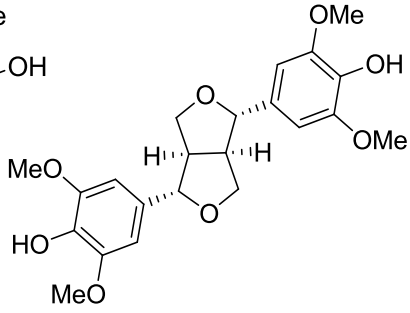


LOB-26 (**13**)
3β-acetoxy-8β-isovaleroyloxyreynosin

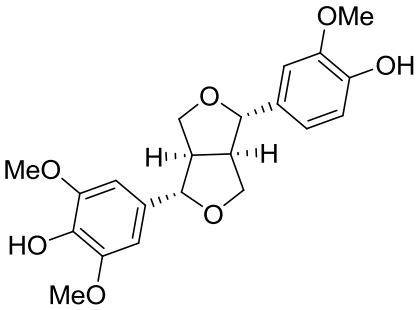
Annex II. Compounds isolated from the aerial parts of *O. acanthium*



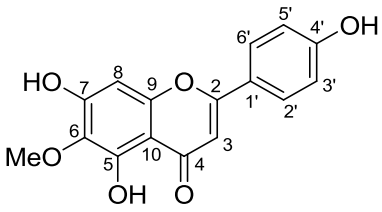
OPD-8 (**14**)
(+)-pinoresinol



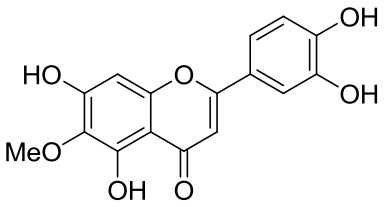
OPD-6/A (**15**)
(+)-syringaresinol



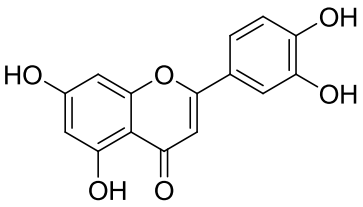
OPD-6/B (**16**)
medioresinol



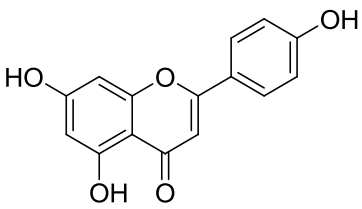
OPD-2 (**17**)
hispidulin



OPD-3 (**18**)
nepetin

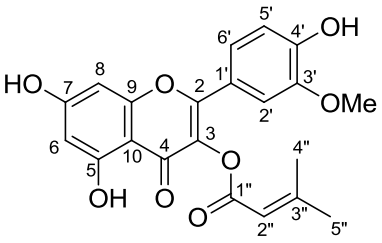


OPD-4 (**19**)
luteolin

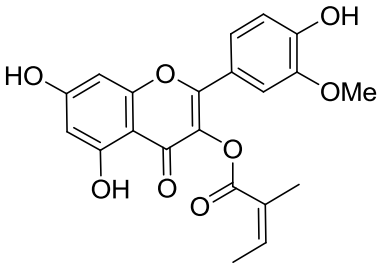


OPD-5 (**20**)
apigenin

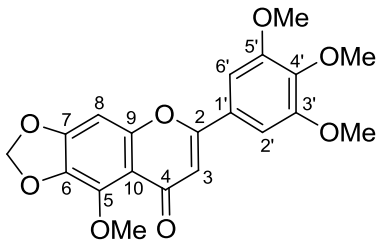
Compounds isolated from the aerial parts of *P. persicaria*



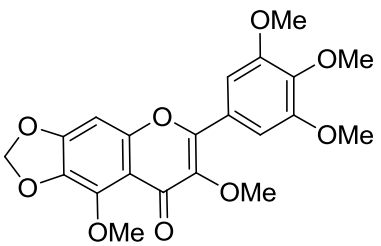
PP-1 (**21**)
3-*O*-senecioyl-isorhamnetin



PP-2 (**22**)
3-*O*-angeloyl-isorhamnetin

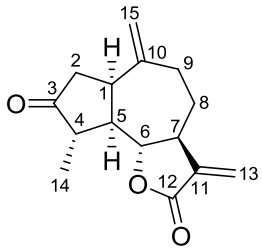


PP-3 (**23**)
5,3’,4’,5’-tetramethoxy-6,7-methylenedioxyflavone

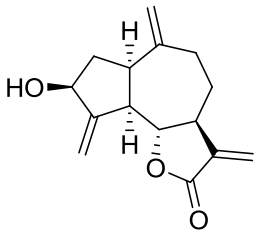


PP-4 (**24**)
3,5,3’,4’,5’-pentamethoxy-6,7-methylenedioxyflavone

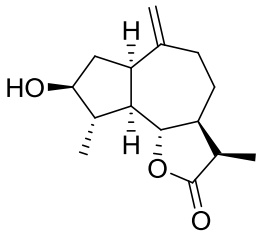
Annex III. Structures of compounds isolated previously from the roots of *O. acanthium* and tested in *in vitro* inhibition assays of COX-2 and NF-κB1 gene expression, NO production, 5-LOX and COX-1 and COX-2 enzymes.



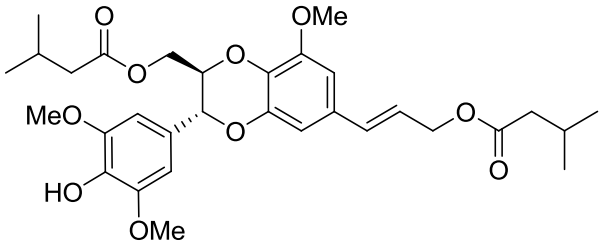
25
4β,15-dihydro-3-dehydrozaluzanin C



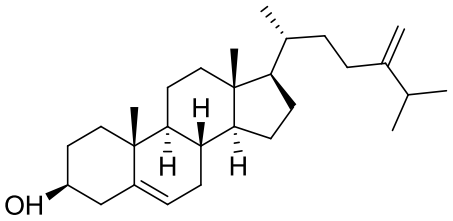
26
zaluzanin C



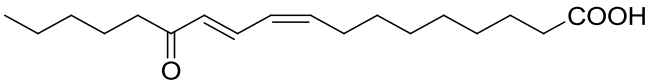
27
4β,15,11β,13-tetrahydrozaluzanin C



28
nitidanin-diisovalerianate



29
24-methylenecholesterol



30
13-oxo-9*Z*,11*E*-octadecadienoic acid

Annex IV. NMR data of **LOB-15** (**9**) isolated from *N. lobata* [$CDCl_3$, 800 MHz (^1H), 200 MHz (^{13}C), δ (ppm), J = Hz]

position	9			
	^1H	^{13}C	HMBC[a]	NOESY
1	-	194.8		
2a	2.77 dd (16.4, 3.2)	34.5	C-1, C-3	H-3, H-5a, H-15, H-6
2b	3.27 dd (16.4, 9.9)		C-1, C-3	H-3, H-5a, H-6
3	4.07 dd (9.7, 2.7)	79.0	C-1, C-4, C-5, C-15	H-2a, H-2b, H-9, H-14, H-15
4	-	81.9		
5a	2.40 dd (14, 4.1)	40.7	C-6, C-7	H-2a, H-2b, H-6, H-15
5b	1.84 dd (14.0, 11.8)		C-3, C-6, C-7, C-15	H-6, H-15
6	4.91 ddd (11.8, 9.4, 3.9)	73.7	C-7	H-2a, H-2b, H-5b
7	3.45 dddd (9.0, 4.1, 3.7, 3.1)	46.3	C-5, C-6, C-12	H-5a, H-8
8	5.40 t (4.1)	68.4	C-6, C-1′	H-9
9	5.62 d (4.1)	98.9	C-4, C-7	H-3, H-8, H-15
10	-	196.4		
11	-	134.1		
12	-	169.2		
13a	5.38 d (3.1)	121.3	C-7, C-11	
13b	6.26 d (3.7)		C-7, C-11, C-12	
14	2.37 s	23.5	C-10	H-3
15	1.40 s	23.0	C-3, C-4, C-5	H-2a, H-3, H-5a, H-5b, H-9
1′	-	172.5		
2′	2.16 m	42.9	C-3′, C-4′, C-5′	
3′	2.01 m	25.6	C-2′, C-4′, C-5′	
4′	0.92 d (6.6)	22.3	C-2′, C-3′	
5′	0.93 d (6.6)	22.3	C-2′, C-3′	

[a]HMBC correlations are from proton(s) stated to the indicated carbon.

Annex V. NMR data of **LOB-14** (**10**) isolated from *N. lobata* [$CDCl_3$, 800 MHz (^1H), 200 MHz (^{13}C), δ (ppm), J = Hz]

position	10			
	δH (J in Hz)	**δC**	**HMBC[a]**	**NOESY**
1	-	194.7		
2a	2.81 dd (16.9, 2.5)	35.9	C-1, C-3	H-3, H-9
2b	3.22 dd (16.9, 10.1)		C-1, C-3	H-3, H-9
3	4.14 dd (10.1, 2.6)	80.7	C-1, C-2, C-4, C-5, C-15	H-2a, H-2b, H-9, H-14, H-15
4	-	79.0		
5a	2.45 dd (13.8, 5.1)	38.5	C-3, C-4, C-6, C-7, C-15	H-6
5b	2.01 dd (13.8, 11.8)		C-6, C-7	
6	4.71 td (10.5, 5.1)	74.1	C-7	H-5a, H-9, H-15
7	3.23 dq (9.9, 3.1)	46.2	C-5, C-6, C-12	H-13a
8	5.43 t (2.5)	67.1	C-6, C-1′	
9	5.35 d (1.9)	102.0	C-4, C-7, C-8	H-2a, H-2b, H-3, H-6
10	-	196.4		
11	-	134.7		
12	-	169.2		
13a	5.47 d (3.4)	121.3	C-7, C-11	H-7
13b	6.25 d (3.4)		C-7, C-11, C-12	
14	2.40 s	23.5	C-10	H-3
15	1.48 s	26.0	C-3, C-4, C-5	H-3, H-6
1′	-	172.2		
2′	2.20 m	43.0	C-3′, C-4′, C-5′	
3′	2.04 m	25.6	C-2′, C-4′, C-5′	
4′	0.93 d (6.7)	22.3	C-2′, C-3′	
5′	0.92 d (6.7)	22.3	C-2′, C-3′	

[a]HMBC correlations are from proton(s) stated to the indicated carbon.

Annex VI. ^{1}H- and ^{13}C-NMR data of **LOB-18** (**11**), **LOB-20** (**12**) and **LOB-26** (**13**) isolated from *N. lobata* [$CDCl_3$, 500 MHz (^{1}H), 125 MHz (^{13}C), δ (ppm), J = Hz]

position	11[a]		12		13[b]	
	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C
1		210.2	2.57 dd (9.6, 5.0)	59.7	3.61 dd (11.5, 4.5)	76.4
2	5.67 d (0.7)	104.9	2.36 dt (14.8, 4.9)	34.5	2.20 m	36.8
			1.68 ddd (14.8, 11.9, 1.6)		1.63 m	
3	-	191.9	4.96 dd (11.9, 5.0)	66.8	5.18 dd (11.5, 5.5)	70.5
4	3.39 dq (6.8, 6.8)	38.0	-	141.9	-	139.9
5	4.49 t (8.0, 6.9)	73.7	5.28 d (11.1)	124.6	2.22 m	50.9
6	4.41 dd (8.1, 4.9)	74.3	5.53 dd (11.1, 2.2)	72.9	4.56 t (11.0)	74.6
7	3.74 dt (1.6, 4.9)	43.9	2.88 s	48.7	2.83 dd (11.0, 2.3)	52.2
8	5.03 d (4.9)	77.3	5.21 t (2.4)	75.5	5.75 dd (2.3, 4.0)	65.8
9	4.09 d (4.9)	72.7	2.71 dd (15.2, 2.4)	43.8	2.32 dd (15.5, 1.5)	40.6
			1.29 dd (15.2, 2.4)		1.58 dd (15.5, 3.0)	
10	-	91.2	-	57.1	-	42.9
11	-	139.1	-	137.1	-	134.5
12	-	168.6	-	169.1	-	169.9
13a	6.36 d (3.1)	125.1	6.40 d (2.0)	125.5	6.17 d (3.0)	120.1
13b	5.84 d (2.8)		5.79 d (2.0)		5.47 d (3.0)	
14	1.48 s	18.4	1.52 s	19.0	0.99 s	13.9
15	1.33 d (6.9)	9.0	1.88 s	17.3	5.20 s	108.6
					5.09 s	
1′	-	171.7	-	172.0	-	172.5
2′	2.11 m (2H)	42.8	2.18 m (2H)	43.7	2.18 d (7.5) (2H)	43.8
3′	1.98 sept (6.8)	25.2	2.04 m	25.8	2.06 m	25.9
4′	0.91 d (6.7)	22.3	0.90 d (6.6)	22.5	0.94 d (6.7)	22.7
5′	0.90 d (6.7)	22.3	0.91 d (6.6)	22.5	0.94 d (6.7)	22.7

[a]OH groups: δ 4.23 brs, 3.31 brs. [b]1-OH group: δ 2.04 brs, 3-*O*-acetate group: δ_H 2.14 s, δ_C 170.2, 21.4

Annex VII. ^{1}H- and ^{13}C-NMR data of **PP-1** (**21**), **PP-2** (**22**), **PP-3** (**23**) and **PP-4** (**24**) isolated from *P. persicaria* [$CDCl_3$, 500 MHz (^{1}H), 125 MHz (^{13}C), δ (ppm), J = Hz]

position	21		22		23		24	
	^{1}H [b, d]	^{13}C [b]	^{1}H [b,c]	^{13}C [b]	^{1}H [a]	^{13}C [a]	^{1}H [b]	^{13}C [b]
2		156.6	-	156.5	-	160.7	-	nd
3		163.1	-	163.1	6.60 s	108.5	-	141.2
4		176.1	-	176.1	-	177.4	-	nd
5		162.2	-	162.7	-	141.4	-	141.0
6	6.29 d (1.6)	99.3	6.29 d (1.6)	99.4	-	134.8	-	134.9
7		162.2	-	162.7	-	153.0	-	153.4
8	6.36 d (1.6)	94.2	6.44 d (1.6)	94.2	6.75 s	93.6	6.75 s	93.2
9		157.0	-	157.3	-	154.4	-	154.1
10		105.5	-	105.3	-	112.7	-	111.9
1’		121.6	-	121.7	-	126.4	-	125.7
2’	7.41 d (1.6)	110.5	7.36d (1.6)	110.5	7.06 s	103.5	7.34 s	105.8
3’		146.4	-	146.2	-	153.5	-	152.6
4’		148.6	-	149.1	-	140.9	-	140.0
5’	7.02 d (8.3)	114.9	7.03 d (8.3)	114.7	-	153.5	-	152.6
6’	7.48 dd (8.3, 1.6)	122.9	7.46 dd (8.3, 1.6)	123.0	7.06 s	103.5	7.34 s	105.8
-OCH_2O-	-	-	-	-	6.07 s	102.5	6.08 s	102.2
3-OCH_3	-	-	-	-	-	-	3.88 s	60.1
5-OCH_3	-	-	-	-	4.15 s	62.0	4.15 s	61.2
3’ -OCH_3	3.95 s	56.0	3.95 s	56.1	3.95 s	56.7	3.93 s	56.4
5’-OCH_3	-	-		-	3.95 s	56.7	3.93 s	56.4
4’-OCH_3	-	-		-	3.92 s	61.7	3.93s	61.1
1”	-	163.5	-	164.6				
2”	6.03 s	113.8	-	126.1				
3”	-	163.1	6.34 qq (7.5, 1.6)	142.7				
4”	2.23 s	27.8	2.09 p (1.6)	20.7				
5”	2.01 s	20.8	2.08 dq (7.5, 1.6)	16.3				

[a] 500/125 MHz; [b] 800/200 MHz; [c] 5-OH: 12.34 brs 7-OH: 5.60 brs, 4’-OH: 5.97 brs; [d] 5-OH: 12.34 brs, 7-OH: 6.00 brs, 4’-OH: 5.97 s. nd = not detected

APPENDIX

The thesis is based on the following publications:

I. **Lajter I**, Zupkó I, Molnár J, Jakab G, Balogh L, Vasas A, Hohmann J.
Antiproliferative activity of Polygonaceae species from the Carpathian Basin against human cancer cell lines.
Phytotherapy Research 2013; **27**: 77–85.

II. **Lajter I**, Vasas A, Orvos P, Bánsághi S, Tálosi L, Jakab G, Béni Z, Háda V, Forgo P, Hohmann J.
Inhibition of G protein-activated inwardly rectifying K^+ channels by extracts of *Polygonum persicaria* and isolation of new flavonoids from the chloroform extract of the herb.
Planta Medica 2013; **79**: 1736–1741.

III. **Lajter I**, Vasas A, Béni Z, Forgo P, Binder M, Bochkov V, Zupkó I, Krupitza G, Frisch R, Kopp B, Hohmann J.
Sesquiterpenes from *Neurolaena lobata* and their antiproliferative and anti-inflammatory activities.
Jorunal of Natural Products 2014; **77**: 576–582.

IV. McKinnon R, Binder M, Zupkó I, Afonyushkin T, **Lajter I**, Vasas A, de Martin R, Unger C, Dolznig H, Diaz R, Frisch R, Passreiter CM, Krupitza G, Hohmann J, Kopp B, Bochkov VN.
Pharmacological insight into the anti-inflammatory activity of sesquiterpene lactones from *Neurolaena lobata* (L.) R.Br. ex Cass.
Phytomedicine 2014; **21**: 1695–1701.

V. **Lajter I**, Pan SP, Nikles S, Ortmann S, Vasas A, Csupor-Löffler B, Forgó P, Hohmann J, Bauer R.
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*.
Planta Medica 2015; **81**: 1270-1276.