Targeted isolation of secondary plant metabolites to discover new pharmacologically active natural compounds

OTKA K 109846 project final report

The aim of the present project was the discovery of new bioactive plant secondary metabolites from Hungarian plants using rational, efficient selection of plant extracts and target compounds. Accordingly this, after bioactivity and chemical screening and literature survey, previously uninvestigated or poorly studied plant species were analysed in detail, most of them the member of the Hungarian flora. In some cases plants from other countries were also involved in the analysis, usually such studies were supported by international cooperations.[1] Relevant ethnomedicinal knowledges and reported phytochemical and pharmacological data were also considered for selection of the plants. Species of plant families Asteraceae, Elaeagnaceae, Euphorbiaceae, Fabaceae, Juncaceae, Lamiaceae, Papaveraceae, Polygonaceae, Ranunculaceae, Rutaceae, Scrophulariaceae and Solanaceae families were in the focus of the research, together with some macrofungus belonging to Basidiomycetes and Ascomycetes classes.

Collection of plant materials

Small amounts (gram scale) of plants were gathered for screen investigations and kg scale plant materials were collected for preparative work with the help of well-experienced botanists (Gyula Pinke, Gusztáv Jakab, Gergely Király) or by us. In all cases voucher specimens were deposited in the Herbarium of Department of Pharmacognosy, University of Szeged.

Screening for bioactivities

Extracts of different polarity were prepared from the samples after separation of the plant parts (root, leaf, stem, fruit, flower, etc.).[2] In most cases *n*-hexane, dichloromethane (or chloroform), aqueous methanol and aqueous extracts were screened for different activities. The same tests were used for bioactivity guided isolation, and for the evaluation of the purified compounds.

Electrophysiological assays were carried out on cardiovascular K^+ ion channels. The effects of the samples on the G protein-activated inwardly rectifying K^+ (GIRK) channel and on the human Ether-à-go-go-Related Gene (hERG) channel were investigated. Sodium ion channel activities were studied on CHO cells stably expressing human $Na_v1.2$ sodium channels by the whole-cell patch clamp technique, using the QPatch-16 automated patch clamp system.

Xanthine oxidase (XO) isolated from bovine milk (lyophilized powder) and xanthine powder purchased from Sigma-Aldrich were applied for XO inhibitory assay. The protocol used was adapted

[1] Investigation of the Guatemalan plant *Neuroleana lobata* was suggested by G. Krupitza. The African plant *Cyclopia genistoides* was studied in cooperation with Prof. Kobus Elof (University of Pretoria). Saharan plants, namely *Anthyllis henoniana*, *Centropodia forskalii*, *Cornulaca monacantha*, *Ephedra alata* var. *alenda*, *Euphorbia guyoniana*, *Helianthemum confertum*, *Henophyton deserti*, *Moltkiopsis ciliata* and *Spartidium saharae* were sent us by Prof. M. Chaieb (Department of Biology and Ecology of Arid Land, University of Sfax, Tunisia).

[2] The biological evaluations were made partly in our department (XO-screening, DPPH), partly by our cooperative partners, such as Department of Medical Microbiology and Immunobiology, Department of Pharmacodynamics and Biopharmacy of University of Szeged, Institute of Biochemistry, Biological Research Centre of the Hungarian Academy of Sciences, Rytmion Ltd. and Karl Franzens University of Graz.

from that recommended by Sigma, performing the measurements in 96-well plate, with the plate reader FluoSTAR.

Antimicrobial screening was carried out on Gram-positive and negative strains of microbes. The inhibition zones produced by plant extracts were determined by disc-diffusion method. Minimum inhibitory concentrations (MIC) of the highly effective samples were also determined.

Anti-inflammatory activities: samples were evaluated for their inhibitory effects on cyclooxygenase-2 and nuclear factor kappa B1 gene expression, inducible nitric oxide synthase, 5-lipoxygenase, and cyclooxygenase-1 and cyclooxygenase-2 enzymes in *in vitro* assays. The *in vivo* anti-inflammatory effect was investigated in carrageenan- or 48/80-induced inflammatory paw oedema model in Male Sprague-Dawley.

Antiproliferative effects of the extracts were evaluated on a panel of human adherent cell lines (HeLa, A431, A2780, MCF7, T47D and MDA-MB-231) using the MTT assay.

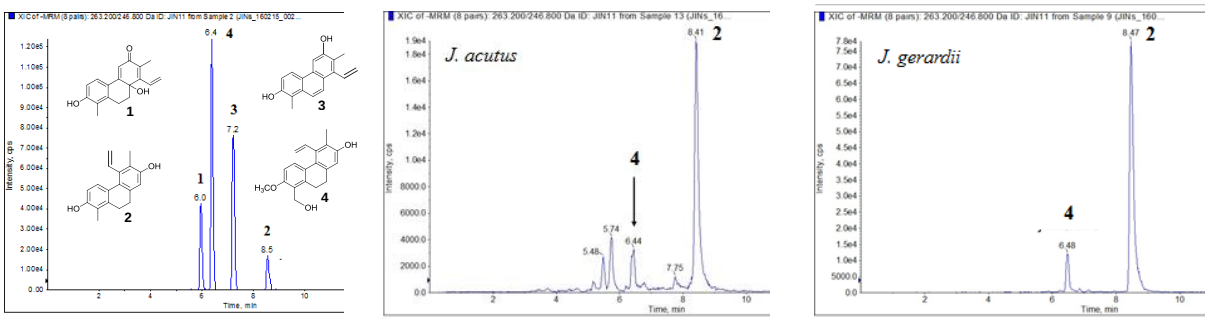
MDR modulating effects of the samples were determined on L5178 mouse T-lymphoma cell line using rhodamine 123 accumulation assay. The fluorescence activity ratios (FAR) between the MDR1-transfected cell populations over-expressing P-glycoprotein and its parental counterparts were measured at two concentrations by flow cytometry.

Antioxidant activities were determined by the spectrophotometric DPPH (1,1-diphenyl-2-picrylhydrazyl) and ferric reducing antioxidant power (FRAP) methods.

TRPV1 antagonist activity: a functional TRPV1 specific Ca^{2+}-uptake assay was made. CB_1 and CB_2 receptor binding was determined in a radioligand displacement assay.

Dereplication study

Literature data were collected for all selected species and the active extracts were evaluated by LC-MS-DAD, in order to recognise compounds, which have already been identified. As example for the dereplication study, LC-MS of *Juncus* species is presented on Figure 1.



A B C

Figure 1. MRM chromatogram of four phenanthrenes (**1–4**) from *Juncus inflexus* with anti-MRSA activity in the reference mixture (A), and in extract of *J. acutus* (B) and *J. gerardii* (C).

Isolation of the compounds

Isolation of the compounds was carried out using modern extraction and separation techniques. CC, VLC, CPC, RPC, PLC, NP- and RP-HPLC were used for purification of the secondary metabolites of the active extracts. In the whole project altogether ***187 compounds, including 54 (!) new natural products*** were obtained as the results of the preparative phytochemical works. The isolated

compounds were identified by 1D- and 2D-NMR (^{1}H, ^{13}C, JMOD, ^{1}H-^{1}H COSY, HSQC, HMBC and NOESY) and mass spectroscopy (HRMS, APCI-MS, ESI-MS). In some cases literature data were considered, or authentical standards were used for chromatographic identification of the isolated compounds.

Altogether ***24 plant species*** was subjected to preparative phytochemical investigation in order to isolate their bioactive constituents. The isolated compounds represent different chemical classes of secondary plant metabolites (Figure 2). The compounds belong to the following main classes:

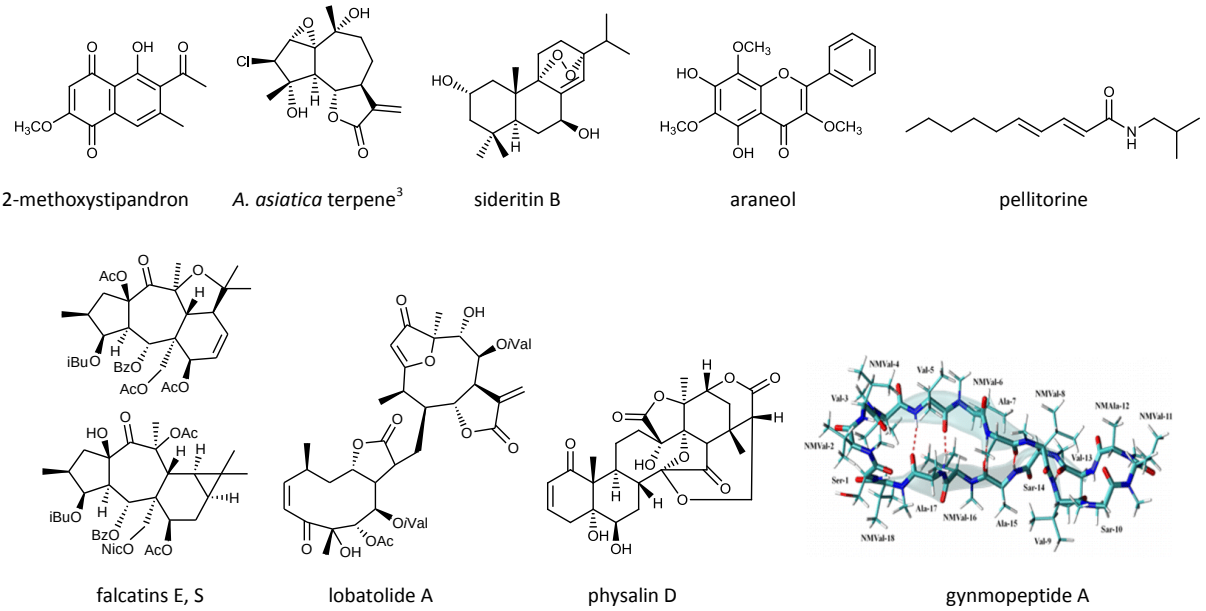


Figure 2. Examples of the isolated compounds

N-containing compounds

- Alkaloids: isolated from *Aconitum moldavicum* and *Chelidonium majus*
- Alkylamides: obtained from *Helianthus helianthoides* var. *scabra* and *Tetradium daniellii*
- Peptides were found in the macrofungus *Gymnopus fusipes*

Terpenes

- Monoterpenes: were obtained from *Artemisia asiatica, Melampyrum barbatum, M. bihariense*
- Sesquiterpenes: were found in *Artemisia asiatica, Centaurea virgata, Neurolaena lobata* and *Onopordum acanthium*
- Diterpenes: were isolated from *Euphorbia falcata, E. exigua, E. guyoniana, E. taurinensis* and *Sideritis montana*
- Triterpenes: were obtained from *Hippophae rhamnoides, Melampyrum bihariense* and *Physalis alkekengi, Onopordum acanthium*

Phenolic compounds

- Flavonoids (including flavones, flavonols, isoflavones, flavanones, pterocarpans) were isolated from *Artemisia asiatica, Centaurea virgata, Cyclopia genistoides, Euphorbia davidii,*

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

Filago vulgaris, Luzula luzuloides, Melampyrum barbatum, M. bihariense, Onopordum acanthium, Rumex aquaticus and *Sideritis montana*

- Lignans were isolated from *Filago vulgaris, Hippophae rhamnoides, Onopordum acanthium* and *Sideritis montana*
- Benzophenone in glycosidic form were obtained from *Cyclopia genistoides*
- Furocoumarines: were found in *Tetradium daniellii*
- Phenanthrenes: were isolated from *Juncus inflexus* and *Luzula luzuloides*
- Naphtalenes, anthraquinones and stilbenes: were isolated from *Rumex aquaticus*

Significance, utilization of the results of the project

Discovery of biologically active natural products

A new alkaloid, 1-*O*-demethylswatinine and the known cammaconine, columbianine, swatinine, gigactonine, delcosine, lycoctonine and ajacine were isolated from the root of *Aconitum moldavicum*. The effects of the isolated compounds, together with eighteen other Aconitum diterpene and norditerpene alkaloids were studied on $Na_v1.2$ channels by the whole-cell patch clamp technique, using the QPatch-16 automated patch clamp system. Pyroaconitine, ajacine, septentriodine, and delectinine demonstrated significant $Na_v1.2$ channel inhibition (57-42%) at 10 µM concentration; several other compounds (acovulparine, acotoxicine, hetisinone, 14-benzoylaconine-8-*O*-palmitate, aconitine, and lycoctonine) exerted moderate inhibitory activity (30–22%).

A series of fatty acid substituted aconitine derivatives, among them 4 new compounds, were prepared by semisynthesis and, together with 28 genuine natural Aconitin alkaloids, subjected to electrophysiological assay on cardiac K^+ ion channel. The activities of these compounds were measured *in vitro* on GIRK and hERG channels using whole-cell patch clamp technique. Considering the GIRK/hERG selectivity, as an indicator of perspective therapeutic applicability and cardiac risk ratio, it can be stated that lipo-alkaloids are significantly more selective than genuine diterpene-alkaloids. 14-Benzoyl-8-O-eicosa-8*Z*,11*Z*,14*Z*-trienoate and 14-benzoyl-8-O-eicosa-11*Z*,14*Z*,17*Z*-trienoate are strong and selective inhibitors of GIRK channels, thus they are promising antiarrhythmic lead compounds.

Thirteen new and three known sesquiterpenes were isolated from the aerial parts of *Neurolaena lobata*. Some compounds were shown to have noteworthy antiproliferative activities against human tumor cell lines (A2780, A431, HeLa and MCF7). The anti-inflammatory effects of five sesquiterpenes were evaluated in vitro using LPS- and TNF-α-induced IL-8 expression inhibitory assays, which revealed that all these compounds strongly down-regulated the LPS-induced production of IL-8 protein, with neurolobatin B and 3-epi-desacetylisovaleroylheliangine being the most effective.

Sesquiterpene lactones of the guaianolide, seco-guianolide, germacranolide and eudesmanolide-type were isolated from *Artemisia asiatica* and investigated for antiproliferative activity on a panel of four adherent cancer cell lines. The IC_{50} values were in 4-30 µM range. In addition, two new 2,2-dimethyl-chromene derivatives and the new pentaisovaleryl sucrose were isolated from the roots of *A. asiatica*.

Rarely occurring sesquiterpenes were isolated from *Onopordum acanthium* root. Their antiproliferative activities were assessed on HeLa, MCF7 and A431 cells and found that 4β,14-dihydro-3-dehydrozaluzanin C exerted remarkable tumour cell growth inhibitory activity (IC50 2.68–15.06 µM). From the aerial part of the plant lignans (pinoresinol, syringaresinol, medioresinol), and flavonoids (hispidulin, nepetin, apigenin, luteolin) were isolated. Compounds obtained both from

root and herb were evaluated for their inhibitory effects on COX-2 and NF-κB1 gene expression, inducible nitric oxide synthase (iNOS), 5-LOX, and COX-1 and COX-2 enzymes in *in vitro* assays. It was concluded that some lignans, flavonoids and sesquiterpenes may play a role in the anti-inflammatory processes.

Juncus inflexus phenanthrenes [jinflexin B (new!), juncusol, juncuenin D, and dehydrojuncuenin B] showed significant activity (MIC value range 12.5-100 μg/mL) against methicillin-resistant *Staphylococcus aureus* (MRSA) strains.

Four diterpenes (3 new!) were isolated from *E. taurinensis* and three jatrophane (2 new!) from *E. exigua* which exerted substantial MDR-reversing activity on the T-lymphoma cells with FAR values between 34 and 59 at 20 μM (*E. taurinensis*) and between 26 and 36 at 80 μg/mL (*E. exigua*).

Phytochemical investigation of *Sideritis montana* afforded two new abietane diterpenes (sideritins A and B) and six known compounds (pomiferin E, 9α,13α-epi-dioxyabiet-8(14)-en-18-ol, paulownin, 6-methoxysakuranetin, 3-oxo-α-ionol and 4-allyl-2,6-dimethoxyphenol glucoside). The antiproliferative effect of the isolated compounds was investigated on human cancer cell lines (HeLa, SiHa and C33A). The results demonstrated that pomiferin E and 6-methoxysakuranetin displayed considerable activity.

5,7-dihydroxyflavones isolated from *Centaurea virgata,* apigenin, hispidulin, luteolin and diosmetin from *Cyclopia genistoides*, and eupatilin, hispidulin, jaceosidin, cirsilineol, 5,7,4',5'-tetrahydroxy-6,3'-dimethoxyflavone, 6-methoxytricin and chrysosplenetin from *Artemisia asiatica* significantly inhibited the xanthine oxidase enzyme *in vitro*, while hesperetin and 5,7,3',5'-tetrahydroxyflavone exerted weak activity. The degree of XO inhibition suggests the importance of the free OH group on C-7.

Information for plant taxonomy and biosynthesis of compounds

Phytochemical analysis of *Filago vulgaris* demonstrated that lignans, flavonoids and coumarins are the representatives of phenolic compounds of the *Filago* genus, and methoxylated flavonols lacking substitution on ring B, can be regarded as a taxonomic marker of the tribe Inuleae. Moreover, identification of lignans in *Filago* genus has taxonomical value for evaluation of relationships in the tribe Inulae.

The chemical characterization of *Luzula luzuloides*, and the presence of vinylated phenanthrenes in the plant confirmed the close botanical relationship of the genus *Luzula* and *Juncus*. Moreover, three vinyl substituted phenanthrenes can be considered as chemotaxonomic markers for Juncaceae family, since such specifically substituted phenanthrenes were only reported from *Juncus* and *Luzula* species.

Information for evaluation the medicinal uses of plants

Members of the *Heliopsis* genus, including *H. helianthoides*, have been used by the North American Indians as medicinal plants, primarily as pain remedy. Four *N*-alkylamides, octadeca-2*E*,4*E*,8*E*,10*E*,14*Z*-pentaene-12-ynoic acid isobutylamide, octadeca-2*E*,4*E*,8*E*,10*E*,14*E*-pentaene-12-ynoic acid 2'-methylbutylamide, hexadeca-2*E*,4*E*,9*E*-triene-12,14-diynoic acid isobutylamide and hexadeca-2*E*,4*E*,9,12-tetraenoic acid 2'-methylbutylamide were identified from *Heliopsis helianthoides* var. *scabra.* The latter compound showed submicromolar and selective binding affinities to the cannabinoid CB_1 receptor (Ki value = 0.31 μM), suggesting by this that the analgesic effect can be attributed to the alkylamide content of the plant.

Melampyrum barbatum showed anti-inflammatory activity using *in vivo* carrageenan-induced rat paw oedema test after intraperitoneal (i.p.) administration. Mussaenoside, aucubine, 8-epiloganin, loganic acid, apigenin and luteolin were obtained from the active extract. These data validate the ethnomedicinal use of *M. barbatum* for the treatment of inflammatory diseases and reveal that iridoids and flavonoids could be responsible for the anti-inflammatory effect of this species. In case of *M. bihariense* antioxidant activity was proved by DPPH and FRAP methods, and aucubin, mussaenoside, 8-epi-loganin, apigenin, luteolin, ursolic acid and oleanolic acid were isolated.

Tetradium daniellii has been used empirically to block or lessen nociceptive pain signalling in folk medicine for hundreds of years. In *in vitro* assay TRPV1 antagonist activity of the fresh fruits was noted and then pellitorine was isolated which was found to be a competitive inhibitor of the TRPV1 (it blocks capsaicin-evoked Ca^{2+} uptake through TRPV1 with an IC_{50} of 154 µg/ml).

According to modern ethnobotanical records, the fruit of *Hippophae rhamnoides* is effective in the treatment of different allergic symptoms. Investigation of the fruit extract for anti-inflammatory activity using in vivo 48/80 induced rat paw oedema model afforded pharmacological evidence for this observation. The peel extract was effective in this assay, and the main compounds responsible for the activity were identified as ursolic acid and oleanolic acid.

Plants belonging to the genus *Rumex* are used in traditional medicine for the treatment of various bacteria-related dermatologic conditions, bacterial and fungal infections, e.g. dysentery or enteritis. Our antibacterial assay indicated the high activity of *R. aquaticus* extract, and demonstrated that naphthalenes (musizin, musizin-8-*O*-glucoside and 2-methoxystipandron) are the most potent antibacterial secondary metabolites, which can be responsible for the antibacterial activity of the plant.

In our study the inhibitory effect of *Chelidonium majus* herb extract and purified alkaloids on hERG potassium current as well as on cardiac action potential were investigated. Experimental data show that hydroalcoholic extract of the plant and its alkaloids, especially berberine, chelidonine and sanguinarine have significant hERG potassium channel blocking effect, and also prolong the cardiac action potential in dog ventricular muscle. Therefore these compounds may increase the risk of potentially fatal ventricular arrhythmias.

Main discoveries of the project

1. Isolation of gymnopeptides A and B, the largest cyclic peptides of mushroom origin, with antiproliferative acticity in nanomolar range (IC_{50} 14–88 nM) from *Gymnopus fusipes*. These cyclopeptides were now syntetised by a Chinese group in 2017.

2. Isolation of a series of new myrsinane, premyrsinane, cyclomyrsinane and jatrophane diterpenes from *Euphorbia falcata* and *E. guyoniana* and recognition of their high K^+ ion channel inhibitory activity.

3. Discovery of vanilloid type 1 receptor (TRPV1) activity of a simple alkylamide, pellitorine from *Tetradium daniellii*, which is structurally analogue to capsaicin.

4. Recognition of antibacterial activity of *Juncus* species against drug-resistant strains, and isolation of new phenanthrenes from *J. inflexus* responsible for this activity.

5. Structure determination of a structurally complex new dimeric sesquiterpene from *Neurolaena lobata* and dimeric phenanthrene from *Juncus inflexus*.

Changes related to the original workplan

Plans not to be accomplished

Investigation of Scrophulariaceae species (*Euphrasia, Odontities, Rhinanthus*)

Reason: Collection of plants was not possible. The only investigated Scrophulariaceae genus was *Melampyrum* (*M. barbatum, M. bihariense*).

Investigation of Lamiaceae species (*Stachys, Salvia, Lavandula, Nepeta*)

Reason: *Stachys, Salvia, Lavandula, Nepeta* species are well-studied, many papers were published recently about the phytochemical-pharmacological investigation of these species. *Sideritis montana* was the only studied Lamiaceae species.

Isolation of secondary metabolites from *Pulicaria, Cirsium, Grindelia, Thymelaea, Daphne* species

Reason: plants belonging to these genus were collected, but on chemical screening (TLC, HPLC) no any interesting compound worthy for isolation was observed.

Expansion of the project

Screening of Saharan plants, detailed investigation of *Euphorbia guyoniana*, *Cyclopia genistoides* and *Neurolaena lobata.*

Reason: our foreigner partners asked us to perform the studies

Screening and detailed investigation of Hungarian macrofungi and Juncaceae plants (Juncus, Luzula)

Reason: these are promising source of new bioactive natural products, and most of the Hungarian species are poorly studied.

Isolation of *Gymnopus fusipes, Juncus inflexus, Luzula luzuloides* metabolites

Reason: positive hits in the screening of extracts for bioactivity

All the above studies afforded new bioactive natural compounds using targeted isolation strategies!