Bioactive compounds of invasive goldenrod plants

Full flowering goldenrod plants *Solidago virgaurea*, *S. gigantea*, *S. canadensis* and *S. graminifolia* (*Euthamia graminifolia*) were collected in Hungary from the summer of 2018. *S. graminifolia* was found only in Eger-Almár as previously reported, thus samples were obtained also from botanical gardens (National Botanical Garden, Vácrátót, Hungary and Botanical Garden of Masaryk University, Brno, Czech Republic). From samples used for isolation procedure voucher plant specimens were deposited at the Herbarium of the Hungarian Natural History Museum, Budapest, Hungary. *S. rugosa* (it is an invader only in Switzerland and France) was purchased from Lichtnelke Pflanzenversand, Hamburg, Germany. Plant extracts were prepared using various organs (root, stem, leaf, flower) and solvents (*n*-hexane, ethyl acetate, ethanol and 50% ethanol) by maceration for screening bioactive components to accomplish our goal the valorisation of goldenrod plants.

During the project we have developed and improved several separation and detection methods, and detection, isolation and identification of antimicrobial goldenrod compounds have been achieved, as well as action mechanism of two giant goldenrod component has been studied.

Improvements of separation and detection methods

- The crude extracts were tested against Gram positive soil bacterium *Bacillus subtilis*, luminescent marine *Aliivibrio fischeri*, luminescence gene-tagged Arabidopsis pathogen *Pseudomonas syringae* pv. *maculicola* and Gram negative pepper pathogen *Xanthomonas euvesicatoria* bacterial strains and cereal pathogen *Fusarium avenaceum* and the barley and wheat pathogen *Bipolaris sorokiniana* fungal strains in 96-well microplates. The active ones were investigated also by (high-performance) thin-layer chromatography ((HP)TLC)-bioassays to search for the separated active components of the extracts. For this reason, in one hand HPTLC methods were developed for the crude extracts and on the other hand new HPTLC-antifungal assays for both test organisms were introduced in the lab ((HP)TLC-antibacterial assays were developed previously) (NÖVÉNYVÉDELEM 2021, 82 [N. S. 57]: 12.).

- The HPTLC method developed for the separation of compounds of root extracts allowed the rapid and easy-to-perform discrimination between the investigated five species. The chemotaxonomic distinction was based on the chemical profile obtained by HPTLC-vanillin sulphuric acid reagent method (Journal of Chromatography A, 10.1016/j.chroma.2019.460602) that was confirmed by principal component analysis. Similarly, the HPTLC–*Aliivibrio fischeri* bioautograms of 13 goldenrod root extracts combined with chemometrics allowed us to distinguish the four Solidago species present in Europe. Principal component analysis (PCA) and HPTLC–HRMS revealed the main biomarkers of the species responsible for their distinction, such as Z,Z-matricaria ester from *S. virgaurea*, solidagenone from *S. canadensis*, solidagoic acid A, and a dialdehyde clerodane diterpene from *S. gigantea*, and Z-dehydromatricaria ester from *S. graminifolia* (JPC – Journal of Planar Chromatography – Modern TLC, 10.1007/s00764-022-00159-3). Thus,

based on both above methods the identification of these species has become possible also in wintertime due to their persistent rhizomes.

- We introduced the bilateral band compression (BBC) to increase the sensitivity of the direct bioautographic assays. BBC is a process where a solvent flow perpendicular to the direction of development squeezes bands into a smaller area. When we compressed a 10 mm wide HPTLC band, more than 6 times increase in peak height and peak area of *Canadian goldenrod* labdane diterpenes solidagenone and presolidagenone was observed. Simultaneously, the signal-to-noise ratio increased only up to 2.2–2.4 times due to the higher noise level. BBC increased the relative standard deviations (RSDs) of peak height and area reached three to four times of the original as well. However, what is more important, the use of BBC before direct bioautography resulted in higher sensitivity in the bio-detection. Thus HPTLC separation combined with BBC and bioassay, enabled the detection of the antimicrobial minor components, which were not detectable by a conventional process (Journal of Liquid Chromatography and Related Technologies, 10.1080/10826076.2020.1725553).

- The stability assessment is essential for the evaluation of not only pharmaceuticals but also agrochemicals. For this reason, two-dimensional (2D)-HPTLC-MS method was introduced using the various length of break (0-24 h) between the two orthogonal development runs with mobile phase of the same composition (original compounds are along the diagonal). After the 2nd development run, derivatives of the compounds of interest are detectable below and above of their chromatographic zones. Furthermore, the purity of the separated zones and the quality of the present compounds can be evaluated by MS. 2D-HPTLC-MS was shown in the paper (Journal of Chromatography A, 10.1016/j.chroma.2020.461230) as a suitable and easy-to-perform approach for stability assessment of the analytes of interest.

- In our bioassay-guided analyses, a fast non-targeted screening (HPTLC-bioassay) is followed by a highly targeted characterization (HPTLC-UV/Vis/FLD/MS) and isolation of bioactive compounds. An orthogonal hyphenation of two different liquid chromatographic techniques (planar and column chromatography) was introduced to characterize the compounds coeluted in an HPTLC zone. The heart-cutting HPTLC-UV/Vis/FLD-HPLC-DAD-MS system including HPTLC-densitometry and HPTLC-TLC-MS Interface-HPLC-DAD-MS enabled the separation and identification of two closely related constitutional isomers, which were not distinguishable by high-resolution MS (HRMS) (Talanta, 10.1016/j.talanta.2020.121306).

- Microdilution antimicrobial assays were developed and improved for the determination of IC50, MIC and MBC in the course of the test of the natural compounds isolated from methanol extract of *Inonotus nidus-pici* poroid fungus. Among the examined four compounds, a phenolic derivative and a triterpene steroid were found to be moderate antibacterials and the former had strong antioxidant effect as well (Molecules, 10.3390/molecules26185453).

- For the quantitative evaluation of eight bioactive clerodane diterpenes of *Solidago gigantea* Ait. (giant goldenrod) root extract, previously isolated and identified during this project, high-performance thin-layer chromatography (HPTLC) combined with two hyphenated methods:

one with vanillin sulphuric acid derivatization and densitometry, and another with acetylcholinesterase (AChE) inhibition assay and video densitometry, were developed. Both methods gave figures of merit for quantification including 5.8-33.9 ng and 175.5-448.7 ng LOQs and 2.7-6.9 RSD% and 8.8-13.9 RSD% inter-day precisions, respectively. Excepting one diterpene (with the lowest retardation factor), the quantitative results for the richest sample obtained by the two methods were in harmony. The difference could be due to a matrix effect. Based on the diterpenes' content of 14 root samples collected over a year from the same plant population, most probably, the contents in the persistent root are influenced also by such environmental factors, which had no well-marked tendency with the change of the seasons. Comparing the changes with the alteration of the daily mean temperature recorded at Szeged (located also in the Great Hungarian Plain, 90 km far from the collection area), no relationship was found. However, the cumulative content of these diterpenes decreased during late spring growth but increased during the strong vegetative growth and inflorescence development, and remained relatively high during flowering and also winter dormancy. Based on this result and the fact that dry mass peaks in the flowering stage, we suggest the end of summer as the optimal time to collect the root as a source of bioactive compounds (Virág Lapat's master thesis and Journal of Chromatography B, 10.1016/j.jchromb.2021.123004).

Isolation and identification of bioactive goldenrod components

HPTLC-based techniques and methods enabled the non-target screening for antimicrobial compounds and their subsequent highly targeted characterization by means of e.g. chemical reagents and mass spectrometry (HPTLC-TLC-MS Interface-ESI-MS). The chromatographic behaviour and the mass signals of several promising goldenrod compounds were determined that helped the development of further preparative scale chromatographic separation methods, thus the isolation of compounds of interest. Sample collection in bigger amount and the preparative work resulted in the isolation and identification of the following bioactive compounds:

- Three antibacterial compounds of *S. canadensis* root were isolated by preparative normal phase TLC fractionation and semi-preparative RP-HPLC purification. These compounds were identified by NMR as solidagenone, (13*R*)-presolidagenone and (13*S*)-presolidagenone. Based on HPTLC-effect-directed analysis results, apart from the anti-cholinesterase and anti-hyperglycaemic effects, these labdane diterpenes also inhibited *B. subtilis*, *X. euvesicatoria* and *A. fischeri* bacterial strains (Journal of Chromatography A, 10.1016/j.chroma.2019.460602 and Acta Pharm. Hung., 10.33892/aph.2021.91.276-277).

- Eight bioactive compounds isolated from *Solidago gigantea* Ait. (giant goldenrod) root extract showed antibacterial (against *Bacillus subtilis* and *Aliivibrio fischeri*) and/or antifungal (against the cereal pathogen *Fusarium avenaceum*) effect. These compounds were identified as furan-containing clerodane diterpenes by NMR: a glycol (Sg1, known also as kingidiol, white solid), an epoxy-hemiacetal (Sg2, oil), a dialdehyde (Sg3a, white solid), the clerodane lactone (Sg3b, oil, also known as hautriwaic lactone), an alcohol (Sg3c, oil), a hemiacetal (Sg4, oil), the solidagoic acid A (Sg5, white solid) and the solidagoic acid B (Sg6, white

solid). The newly developed high-performance thin-layer chromatography (HPTLC)-*F. avenaceum* assay using mycelium suspension and the antifungal, antibacterial and enzyme inhibiting activity of the eight clerodane diterpenoids were reported first time (Journal of Chromatography A, 10.1016/j.chroma.2020.461727).

- Four of the five antibacterial isolates from roots of *S. virgaurea*, *S. graminifolia* (*Euthamia graminifolia*) and *S. speciosa* were found to be active also against the barley and wheat pathogen *Bipolaris sorokiniana*. The bioactive compounds were identified by NMR. The MIC and/or IC50 values of the antimicrobial isolates were determined in liquid phase in 96-well microplates, thus their antibacterial (against *Bacillus subtilis*, *Aliivibrio fischeri* and *Pseudomonas syringae* pv. *maculicola*) and/or antifungal (against crop pathogenic fungi *Fusarium avenaceum* and *Bipolaris sorokiniana*) activities were confirmed. The five identified compounds were as follows: 2Z,8Z- and 2E,8Z-matricaria esters from European goldenrod (*Solidago virgaurea*) and E- and Z-dehydromatricaria esters from grass-leaved goldenrod (*S. graminifolia*) and firstly from showy goldenrod (*S. speciosa*) as well as benzyl 2-hydroxy-6-methoxybenzoate for the first time found in showy goldenrod root. The latest one showed the strongest antifungal effect against both fungal strains (with IC50 of 25.1 µg/mL against *B. sorokiniana* and 26 µg/mL against *F. avenaceum*). The newly developed HPTLC- *Bipolaris sorokiniana* assay as well as the antifungal activity of the isolates were published (J. Agric. Food Chem., https://doi.org/10.1021/acs.jafc.1c03676).

- Four antibacterial cis-clerodane diterpene compounds (solidagoic acid H (1), solidagoic acid E (2), solidagoic acid I (3), and solidagoic acid F (4)) were isolated for the first time from *S. gigantea* leaf extract and tested against several bacterial and fungal strains and a new HPTLC-bioassay method with Gram-positive *Rhodococcus fascians* bacterial plant pathogen was introduced. Compounds 1 and 3 exhibited moderate antibacterial activity against the Gram-positive *B. subtilis* subsp. *spizizenii* and *R. fascians* bacterial strains in microdilution assays with half-maximal inhibitory concentration (IC50) values in the range of 32.3–64.4 µg/mL. The mass spectrometric fragmentation of the isolated compounds was interpreted and their previously published NMR assignments lacking certain resonances were completed (Márton Baglyas' master thesis and Journal of Chromatography A, 10.1016/j.chroma.2022.463308). Meanwhile, further 5 compounds were isolated from *S. gigantea* leaves that are under structure elucidation and biological assessment.

- Two antimicrobial diterpenes were detected and isolated from roots and leaves of *S. rugosa* utilizing a bioassay-guided process. These compounds identified as (–)-hardwickiic acid and (–)-abietic acid, exhibited notable antimicrobial activity against Gram-positive bacterial (*Bacillus spizizenii*, *Clavibacter michiganensis* subsp. *michiganensis*, *Curtobacterium flaccumfaciens* pv. *flaccumfaciens*) and fungal (*Fusarium avenaceum* and *Bipolaris sorokiniana*) strains, including plant pathogens. Both compounds were present in both tissues, but (–)-hardwickiic acid was dominant in roots and (–)-abietic acid in leaves. Their antibacterial effect, especially against Gram-positive bacterial strains with half maximal inhibitory concentration (IC_{50}) between 1 and 5.1 µg/mL (5-20 times higher than that of the positive control gentamicin). In the used concentrations, minimal bactericidal concentration (MBC) was reached only against the non-pathogen *B. spizizenii*. The highest antifungal

activity was observed for (–)-hardwickiic acid against *Bipolaris sorokiniana* with an IC_{50} of 3.8 μg/mL (Molecules, 10.3390/molecules28093790).

- Flash chromatographic fractionation and purification procedures using both normal and orthogonal RP18 phases provided the isolation of nine antibacterial compounds from a goldenrod. Based on LC-HRMS/MS and NMR analysis, all nine compounds found to be brand new (so far we have not found literature data about compounds with such structures). The performance of further spectroscopic analyses and biological assessment is planned before publication.

Mechanism of antibacterial effect

The effectiveness of the seven different clerodane diterpenes isolated from giant goldenrod root was tested against Gram positive *Bacillus subtilis*, *Bacillus subtilis ssp. spizizenii* and *Rhodococcus fascians* by measuring minimal bactericidal concentration (MBC), minimal inhibitory concentration (MIC), and half-maximal inhibitory concentration (IC_{50}). Two of them (Sg3a and Sg6) proved to be the most effective (IC50 between 1.6 and 4.5 μg/mL; MIC between 2.2 and 8.3 μg/mL; MBC between 2.2 and 10.4 μg/mL) and these two diterpenes were selected for further study. To study the effect of these diterpenes in more details on bacterial activity genome wide transcriptional analyses were carried out with RNA-seq method. Two different experimental setting were applied to test the effect of diterpenes on bacteria. In one case the diterpenes were added at lower concentration of bacterial cells (OD_{600}=0.2) and incubated longer (5 hours) in LB medium and in other case higher bacterial concentration (OD_{600}=0.5) were supplemented with diterpenes and incubated shorter period (1 hour). The two experimental setups allow the investigation of the effect of the diterpenes on bacteria from two different aspects. The lower bacterial concentration and longer incubation period shows the effect of molecules on bacterial multiplication and the higher bacterial concentration and shorter incubation period rather reflects the direct rapid effect of the diterpenes on bacterial processes. The sequencing results showed that about same number of transcripts could be detected after all treatments. In the OD_{600}=0.2 samples collected after 5 hours 4064 genes were common in control, Sg3a and Sg6 samples. While in the OD_{600}=0.5 samples collected one hour after treatments somewhat more 4196 common genes. Compared to common genes, only few treatment specific genes could be detected. The highest number of specific genes can be seen after OD_{600}=0.2 Sg6 treatment (84 genes). The Sg3a and Sg6 treated samples were compared to control samples (containing ethanol as solvent of the diterpenes at equal concentrations) to detect genes that differentially express (DEG) after diterpene treatments. The highest number of DEG's that are activated at least two times or repressed at least to half ($|\log_2(\text{FoldChange})| > 1$) were found in samples that incubated for longer period (OD_{600}=0.2 Sg6 =1450, OD_{600}=0.2 Sg3a=1190, OD_{600}=0.5 Sg3a=493 OD_{600}=0.5 Sg6=315 genes). The numbers of up- and down regulated genes in all cases were about equal. The data also suggest that the Sg6 diterpene exerts its effect more slowly, but is able to induce greater changes in the bacterium in the longer term. There were a limited number of common genes (18 genes) that were differentially regulated after all treatments. These common genes can be the core genes that may determine the most typical processes that can influence the response of the bacteria to these diterpenes. Most of these common

genes associated to cell membrane. Some of them are transporters that may export harmful substances or import necessary ions/molecules. Up- and down-regulation of these core response genes suggest that the membrane and membrane transport processes of the *B. spizizenii* are key players in the diterpene-bacteria reactions. Enrichment analyses were performed to highlight those processes that significantly changed during the treatments in bacterial cells compared to control. The results of these analyses showed common and specific changes induced by the two diterpenes. The most general response is that the bacterial chemotaxis-related genes were significantly repressed in all sample types. The other typical response is the changes of gene transcriptions related to biosynthesis of secondary metabolites. The strongest significant responses of these genes could be detected in Sg6 samples. In OD_{600}=0.2 Sg3a samples the up-regulation of the teichoic acid biosynthesis reveals that bacterial cells respond to the diterpene treatments with cell wall modification. Other significant changes in OD_{600}=0.2 Sg3a samples the high number of up- and down regulation of the ABC transporters. Based on the RNA-seq results, the inhibitory effect of diterpenes on the growth of bacteria is complex and can be associated to the inhibition of bacterial protein and nucleotide synthesis, energy metabolism, signaling and transport processes (publication is in preparation).

Presentations at conferences

HPLC2019, 16-19 June, 2019, Milano – 1 lecture (Discovery of natural compounds with potential bioactivity using HPTLC hyphenations)

12th Balaton Symposium, September 11-13, 2019, Siófok – 1 poster (Characterization of antibacterial substances with direct bioautography from grass-leaved goldenrod (*Euthamia graminifolia*)) and 1 lecture (Advantages of HPTLC in Non-Target-Based Drug Discovery)

LI. KROMATOGRÁFIÁS TOVÁBBKÉPZŐ TANFOLYAM, Szeged, 2020. január 27-29. – 1 lecture (A rétegkromatográfia előnyei a nem célzott vizsgálatokban, Benefits of the thin-layer chromatography in the non-target analyses).

METT25, Egerszalók, 2021. október 17-21. – 1 lecture (Rétegkromatográfia és oszlopkromatográfia, mint egymás segítői) and 1 poster (A magas aranyvessző (*Solidago gigantea* Ait.) antibakteriális komponenseinek kimutatása és izolálása)

International Conference on Advances in Pharmaceutical Drug Development, Quality Control and Regulatory Sciences (DDRS 2021), Budapest, 15-17 November, 2021 – 1 poster (High-throughput screening for bioactive natural compounds from plant extracts by HPTLC hyphenations)

LII. KROMATOGRÁFIÁS TOVÁBBKÉPZŐ TANFOLYAM, Szeged, 2022. június 27-29. – 1 lecture (Kétdimenziós folyadékkromatográfia kicsit másképp (HPTLC-HPLC))

25th International Symposium for High-Performance Thin-Layer Chromatography, 28th June - 1st July, 2022, Ljubljana, Slovenia – 1 lecture (Improved sensitivity and separation

efficiency in layer chromatography-based effect-directed analysis) and 1 poster (Detection and isolation of four antibacterial cis-clerodane diterpenes from giant goldenrod (*Solidago gigantea* Ait.) via high-performance thin-layer chromatography)

33rd International Symposium on Chromatography (ISC2022), Budapest, 18-22 September 2022 – 1 lecture (Two-dimensional liquid chromatography in different ways: 2D-HPTLC and HPTLC-HPLC) and 1 poster (Effect-directed Isolation of Bioactive Compounds from Giant Goldenrod (Solidago gigantea Ait.) Leaf)

LIII. KROMATOGRÁFIÁS TOVÁBBKÉPZŐ TANFOLYAM, Szeged, 2023. június 26-28. – 1 lecture (Bioaktív természetes vegyületek kutatása folyadékkromatográfiához csatolt technikák és módszerek segítségével)

13th Balaton Symposium, September 4-6, 2023, Siófok – 1 lecture (HPTLC Hyphenations as a Key for Preparative Bioassay-guided Isolation) and 1 poster (Antimicrobial potential of two diterpenes isolated from rough goldenrod (*Solidago rugosa* Mill.) against plant pathogens)

27th International Symposium on Separation Sciences (ISSS2023), 24-27 September 2023, Cluj Napoca – 1 lecture (Bioassay-Guided Isolation of Antimicrobial Components Monitoring by High-Performance Thin-Layer Chromatography Based Methods)