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Layer chromatography-bioassays directed screening and identification of antibacterial compounds from Scotch thistle

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Highlights:

- First direct transfer of OPLC mobile phase to flash chromatography
- Antibacterial profiling of Scotch thistle (*Onopordum acanthium* L.) leaf extract
- TLC/OPLC-DB using plant and human pathogen bacteria and a probiotic, human *gut bacterium L. plantarum*
- Infusion-transfusion OPLC method with on-line UV detection and fractionation
- preparative-scale bioassay-guided isolation
- Bioactive components are onopordopicrin, linoleic and linolenic acids

Abstract

The antibacterial profiling of *Onopordum acanthium L.* leaf extract and subsequent targeted identification of active compounds is demonstrated. Thin-layer chromatography (TLC) and off-

line overpressured layer chromatography (OPLC) coupled with direct bioautography were utilized for investigation of the extract against eight bacterial strains including two plant and three human pathogens and a soil, a marine and a probiotic human gut bacteria. Antibacterial fractions obtaining infusion-transfusion OPLC were transferred to HPLC-MS/MS analysis that resulted in the characterization of three active compounds and two of them were identified as, linoleic and linolenic acid. OPLC method was adopted to preparative-scale flash chromatography for the isolation of the third active compound, which was identified after a further semi-preparative HPLC purification as the germacranolide sesquiterpene lactone onopordopicrin. Pure onopordopicrin exhibited antibacterial activity that was specified as minimal inhibitory concentration in the liquid phase as well.

Keywords

Thin-layer chromatography-direct bioautography; overpressured layer chromatography-direct bioautography; overpressured layer chromatography-UV/fractionation; bioassay-guided isolation; *Lactobacillus plantarum*

1. Introduction

The introduction of the non-targeted, effect-directed processes provided a new chance to reduce the time and financial investment in the antibacterial drug discovery. Such analyses point out compounds with the desired activity in a complex matrix, irrespectively of their chemical nature and ensure a subsequent highly targeted procedure including characterization, bioactivity assay-guided isolation and identification of the components in question [1-3]. Planar layer chromatography coupled with antibacterial assay (direct bioautography, DB) [4,5] is a screening and biomonitoring tool that is high-throughput, rapid, easy to perform and suitable in such a workflow [6,7]. Compared to HPLC-antibacterial assay [1,2], the benefits of DB are: 1. while the compounds stuck on the stationary phase shorten the lifetime of a HPLC column, in layer chromatography the adsorbent is utilized once, thus sample preparation is not imperative, and no ingredient of a crude extract applied to the adsorbent layer is excluded from the test; 2. after separation, the elimination of the mobile phase and the setting of the appropriate conditions for bioassay can easily be done *in situ* in the adsorbent.

Overpressured layer chromatography (OPLC) [8-10] is a forced-flow technique, in which the planar adsorbent is closed and the mobile phase is accelerated through this stationary phase by a pump at a constant rate. In fully off-line mode, the sample application, separation, qualitative, quantitative and/or biological evaluation and isolation are performed as separate steps, like in conventional (high-performance) thin-layer chromatography ((HP)TLC). However, it has to be noted that due to the pressure, in OPLC the fronts caused by the demixation of the mobile phase components and the front of the total wetness are more characteristic. Nonetheless, OPLC with optimized separation conditions results in better separation, lower theoretical plate height, especially using longer development distances [8,9]. Moreover, the use of infusion mode (closed mobile phase outlet, the development stops automatically) and its combination with overrun (transfusion mode, opened mobile phase outlet before the automatic stop) provides longer development distance as well as on-line detection and fractionation [11].

Orally administered antibiotics can alter the very complex community of bacterial flora in the gastrointestinal tract, causing among others diarrhoea and/or colitis. To reduce these side effects the use of probiotics is suggested [12]. The knowledge about the effect of the antibiotics on the various species of the flora could help to decide what kind of bacteria should be supplemented to reach the balance again. The probiotic Gram-positive *Lactobacillus plantarum* is one of the transient species in the gut flora that can grow both in anaerobic and aerobic environments [13]. *L. plantarum* is present also in many plants and fermented food and able to ferment many different carbohydrates [14]. Its high level in human gut is negatively correlated with obesity [15] and it causes immunomodulatory effect by e.g. a broad spectrum of antimicrobial activity [16].

Onopordum acanthium L. (Scotch thistle) is a biennial medicinal plant of the Asteraceae family with spiny leaves and shoots and pink flowers. The plant is native to Central and Southern Europe and Western Asia and invasive competitive species in Australia, North America and Argentina causing severe agronomic problems [17,18]. Scotch thistle has been traditionally used for the treatment of - among others - rash, cancer, ulcers, nervous complaints, antipyretic, cardiovascular and urogenital diseases and used as a bactericidal agent [19]. It is interesting that *O. acanthium* has been determined so far as a host of pathogenic fungi and a virus, but not as that of any bacteria [19]. The aerial parts of *O. acanthium* are considered as a rich source of biologically active ingredients, such as flavonoids, phenolic acids, lignans, sterols, sesquiterpenoids and triterpenoids.

The aim of this paper was to demonstrate a systematic and efficient effect-directed workflow for non-targeted tracking, and highly targeted characterization, isolation and identification of

bioactive components from complex matrices, exemplarily shown for *O. acanthium* leaf extract that contains compounds inhibiting various bacterial strains (among others plant and human pathogens). During the process, high-throughput thin-layer chromatography (TLC) and overpressured layer chromatography (OPLC) coupled with antibacterial (direct bioautography) assays, OPLC fractionation, preparative forced-flow column chromatography as well as HPLC-MS/MS and NMR were utilized for characterization and identification of active compounds. In this paper the simple and direct transfer of a mobile phase developed by analytical OPLC to preparative flash chromatography was also demonstrated.

2. Materials and Methods

2.1. Materials

Aluminum foil-backed TLC (#5554) and 0.5 mm thick glass-backed preparative TLC (PLC, #5744) plates of silica gel 60 F_{254} were purchased from Merck Millipore (Darmstadt, Germany). Solvents for extraction, TLC and flash chromatography were analytical grade from Reanal (Budapest, Hungary). Gradient grade acetonitrile (Fisher Scientific, Pittsburg, PA, USA), formic acid (Reanal) and pure water (purification equipment: Merck Millipore Direct-Q 3 UV system) were applied as HPLC eluent. For the LC-MS analyses acetonitrile and methanol of HPLC super-gradient grade were purchased from Sigma-Aldrich (Steinheim, Germany), all aqueous eluents were filtered through MF-Millipore membrane filters (0.45 μm, mixed cellulose esters) (Billerica, MA, USA). CD_3OD (99.8 atom% D) for NMR was from VWR (Budapest, Hungary). Gentamicin sulphate (purity≥99%), chloramphenicol (purity≥98%), linoleic acid (purity≥99%) and linolenic acid (purity≥99%) were from Sigma-Aldrich (Budapest, Hungary), and dye reagent 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) from Carl Roth. The bacterial strains used in the bioassays were *Aliivibrio fischeri* (DSM-7151, German Collection of Microorganisms and Cell Cultures, Berlin, Germany), *Bacillus subtilis* strain F1276 [20], *Pseudomonas syringae* pv. *maculicola* (*P. maculicola*, Jun Fan, John Innes Center, Department of Disease and Stress Biology, Norwich, UK [21]), *Xanthomonas euvesicatoria* (Hungarian isolate, János Szarka, Primordium Kft., Budapest, Hungary), *Lactobacillus plantarum* (ATCC 8014), *Staphylococcus aureus* (ATCC 29213), methicillin resistant *S. aureus* (MRSA 4262) and *Escherichia coli* (ATCC 25922). Peptone (Sigma-Aldrich Budapest, Hungary), yeast extract (Scharlau, Barcelona, Spain), glucose (Reanal) and KH_2PO_4 (Reanal) were used for preparation of *L. plantarum* cell media.

2.2. Sample preparation

Leaves of fully flowered *Onopordum acanthium* L. were collected in June 2015, in the Great Plain, Hungary and identified by Péter Csontos. Voucher plant specimen was deposited at the Herbarium of the Hungarian Natural History Museum, Budapest, Hungary, under accession number BP754798. For pre-investigation 150 mg of fresh leaves were extracted with 1 mL of *n*-hexane, toluene, chloroform, ethyl acetate, ethanol or methanol by maceration for 24 h. For further experiments 100 g of fresh leaves were macerated for 24 h with 500 mL of ethyl acetate in a glass bottle. The supernatant was filtered, dried by the use of a rotary evaporator (Büchi Rotavapor R-134, Flawil, Switzerland) at 40 ^{o}C and the residue was dissolved in ethanol (16 mg/mL) providing the crude extract. TLC-based antibacterial assays were performed with 10-times diluted crude extract (with ethanol).

2.3. Layer chromatography

TLC separation of the leaf extract components was made with *n*-hexane – ethyl acetate (45:55, v/v) as a mobile phase in an unsaturated 20 cm × 10 cm twin trough chamber (CAMAG). A total of 1–10 µL of the sample were deposited as 8 mm bands onto the plate by a Linomat IV (CAMAG) at 8 mm distance from the bottom. The plate was developed to a distance of 8 cm, dried in a cold air stream (5 min) and cut with a blade. The parallel tracks were used for direct bioautography, antioxidant assay and chemical characterization. Zones were universally visualized by immersion into the vanillin-sulphuric acid reagent (mixture of 40 mg vanillin, 10 mL ethanol and 200 µL concentrated sulphuric acid), followed by heating to 110 ^{o}C for 5 min and documented under white light illumination. The native and the visualized chromatograms were documented using a digital camera (Cybershot DSC-HX60, Sony, Neu-Isenburg, Germany).

Fully off-line analytical OPLC separation was performed on a 20 cm x 20 cm TLC layer (Merck) sealed at all four edges by the use of Personal automatic OPLC BS50 system (OPLC-NIT, Budapest, Hungary). Samples (5 µL) were applied in 5 mm wide bands with 15 mm spaces at 3 cm from the lower edge with a Linomat IV sample applicator (CAMAG). The separation conditions were: 50 bar external pressure, 400 µL rapid mobile-phase flush, 400 µL min^{-1} mobile phase flow rate, 4500 µL mobile phase, 685 s development time. The mobile-phase was chloroform - ethyl acetate - 2-propanol 90:5:5 (v/v). The chromatograms were visualized under UV illumination at 254 and 365 nm as well as by direct bioautography. The analytical OPLC method was expanded to a semi-preparative scale by applying 500 µL sample in a 16 cm wide band at 3 cm from the lower edge onto a 20 cm x 20 cm PLC sealed at all four edges and the

rapid mobile-phase flush and the mobile phase flow rate were changed to 1000 µL and 2000 µL min^{-1}, respectively. Infusion-transfusion mode [10,11,22] was used and after opening the outlet the detection was achieved using an on-line coupled flow-through UV detector at a wavelength of 220 nm. The collected peaks were concentrated by cold air stream and then tested by OPLC-*B. subtilis* assay. Fractions containing active compounds were further analysed by LC-MS/MS.

2.4. Direct bioautography

B. subtilis F1276, *X. euvesicatoria* and *P. maculicola* cells were grown according to [23,24]. *A. fischeri* was maintained with a method described by Krüger et al. [25]. Experiments with human pathogenic bacteria (*S. aureus*, MRSA and *E. coli)* were performed as described by Horváth et al. [26]. *L. plantarum* cells were grown in a culture medium containing 5 g/L peptone from meat, 20 g/L yeast extract, 10 g/L glucose and 2 g/L KH_2PO_4 at 28 °C on an orbital shaker with the speed of 130 rpm to reach the late exponential phase ($5x10^8$ cells/mL, OD_{600} = 1.2).

In TLC/OPLC-direct bioautography experiments, the plates with the separated substances were immersed into one of the cell suspensions using a home-made cassette [7] to prepare the bioautograms. When working with luminescent bacteria (*A. fischeri* and *P. maculicola*), the bioautograms were documented under a glass plate allowing sufficient humid air above the layer using a cooled low-light camera IS-4000 (Alpha Innotech, San Leandro, CA, USA) at an exposure time of 15 min (*P. maculicola*) or 3 min (*A. fischeri*). Dark spots against a luminous background indicated the inhibitory activity. In the case of *B. subtilis* F1276, *X. euvesicatoria* and *L. plantarum* bacterial strains, after 2 h incubation time in a vapor chamber at 28 °C the chromatographic zones with antibacterial effect were visualized by dipping the bioautograms into an aqueous solution of MTT vital dye (1 mg/mL) for 1 s and dried at 60 °C for 5 min. Similar method was utilized for human pathogenic bacteria, just the incubation was performed at 37 °C for 5 h and followed also after dipping into MTT for 2 h. Metabolically active cells reduce the yellow MTT to bluish MTT-formazan, revealing the inhibition zones as bright spots against bluish background, which was documented by a Sony Cybershot DSC-HX60 digital camera.

2.5. HPLC-DAD-ESI-MS/MS

The active fractions obtained by semi-preparative OPLC were transferred to HPLC-DAD-ESI-MS/MS analysis. Chromatographic separation and on-line mass spectral analyses were

performed with an Agilent 6410B triple quadrupole equipped with an electrospray ionization source (ESI) (Agilent Technologies, Palo Alto, CA, USA) coupled to an Agilent 1100 HPLC system equipped with a G1379A degasser, G1312A binary gradient pump, G1329A autosampler, G1316A column thermostat and G1315C diode array detector (Agilent Technologies, Waldbronn, Germany). Samples were separated on a Zorbax SB-C18 (150 mm length, 3.0 mm inner diameter (ID), 3.5 μm particle size) column (Agilent Technologies, Santa Clara, CA, USA), maintained at 25 ºC. Detection wavelengths were 220 and 290 nm; UV spectra of characteristic compounds were recorded between 200 and 600 nm. Injection volume was 5 μL.

For the analysis of onopordopicrin eluent A was formic acid in water (0.5%, v/v), and eluent B was acetonitrile. The following gradient elution was used at a flow rate of 0.4 mL/min: 0 min 15% (v/v) B, 30 min 100% (v/v) B, 33 min 100% (v/v) B, and 34 min 15% (v/v) B. For the analysis of fatty acids eluent A was formic acid in water (0.1%, v/v), and eluent B was methanol. The following gradient elution was used at a flow rate of 0.3 mL/min: 0-20 min, 30-100% (v/v) B; 20-30 min 100% (v/v) B.

ESI conditions were as follows: temperature: 350 °C, nebulizer pressure: 45 psi (N_2), drying gas flow rate: 9 L/min (N_2), fragmentor voltage: 120 V, capillary voltage: 4000 V, collision energy was changed between 15-50 eV, according to structural differences. High purity nitrogen was used as collision gas. Full mass scan spectra were recorded in negative and in positive ionization mode over the range of *m/z* 50-1000 (1 scan/sec). The Masshunter B.01.03 software was used for data acquisition and qualitative analysis.

2.6. Isolation by flash chromatography

Low pressure flash chromatograph (Macherey-Nagel, Düren, Germany) was used to fractionate 10 mL of crude extract through a column (Macherey-Nagel, 3 cm ID, 12 cm length) filled in-house with preparative silica gel (Sigma-Aldrich, St. Louis, MO; No. 60752, high-purity grade, 60 Å pore size, 230–400 mesh particle size). To the bottom and top of the stationary phase 1 cm of sand was applied. The extract was dried onto 5 g of silica, layered to the top of the column and first washed with 80 mL of diethyl ether. The fractionation was carried out with chloroform – ethyl acetate – 2-propanol 90:5:5 (v/v) as a mobile phase, which was accelerated by nitrogen gas pressure. The eluate was collected in 9 fractions of 15–16 mL each. The fractions were tested by TLC-direct bioautography using *B. subtilis*.

2.7. Purification by semi-preparative HPLC

The main antibacterial component was isolated from the combined flash chromatography fractions by a semi-preparative HPLC. First, an analytical method was developed on a Shimadzu LC-MS-2020 equipped with an SPD-M20A UV/VIS photodiode array detector and a single quadrupole mass analyzer with an electrospray ionization (ESI) interface (Shimadzu, Kyoto, Japan). Data acquisition and processing were accomplished using Shimadzu LabSolution software (Ver. 5.72). The separation was achieved at 35 ^{o}C, on a Gemini C18 column (250 mm length, 4.6 mm ID, 5 µm particle size) purchased from Phenomenex (Torrance, CA, USA). Eluent A was 5% aqueous acetonitrile with 0.05% formic acid and eluent B was acetonitrile with 0.05% formic acid. The following gradient program was applied at a flow rate of 1 mL/min: 0-8 min, 20-35% B; 8-15 min, 35-45% B; 15-16 min, 45-100% B and 16-20 min, 100% B. The injection volume was 2 µL. ESI conditions were as follows: temperature: 350 ^{o}C, applied voltage: 4.5 kV, desorption liquid temperature: 250 ^{o}C, heat block temp: 400 ^{o}C, nebulizer gas (N_2) flow rate: 1.5 L/min, drying gas flow rate: 15 L/min. Full mass scan spectra were recorded in the positive ionization mode over the range of *m/z* 50–800. The analytical HPLC method was scaled up by increasing the diameter of the column (Gemini C18, 250 mm length, 10 mm ID, 10 µm particle size, Phenomenex) and the mobile phase flow rate to 6 mL/min. The injection volume was 100 µL, and the appropriate fraction was collected based on UV chromatogram. It was repeated five-times; the combined fractions were dried by the use of a rotary evaporator (Büchi Rotavapor R-134) at 40 ^{o}C.

2.8. NMR

All NMR experiments were carried out on a 600 MHz Varian DDR NMR spectrometer equipped with a 5 mm inverse-detection gradient (IDPFG) probehead. Standard pulse sequences and processing routines available in VnmrJ 3.2 C/Chempack 5.1 were used for structure identifications. The complete resonance assignments were established from direct ^{1}H–^{13}C, long-range ^{1}H–^{13}C, and scalar spin–spin connectivities using 1D ^{1}H, ^{13}C,^{1}H–^{1}H gCOSY, ^{1}H–^{1}H NOESY, ^{1}H–^{13}C gHSQCAD (J = 140 Hz), ^{1}H–^{13}C gHMBCAD (J = 8 Hz) experiments, respectively. The probe temperature was maintained at 298 K and standard 5 mm NMR tubes were used. The ^{1}H chemical shifts were referenced to the applied NMR solvent CD_3OD (δ (CD_2HOD) = 3.310 ppm) and ^{13}C chemical shifts were referenced to 49.00 ppm.

2.9. Method of microdilution test

Minimal inhibitory concentration (MIC) of *O. acanthium* leaf extract (16 mg/mL in ethanol) and its isolated components (10 mg/mL in ethanol) was determined against *B. subtilis*, *X. euvesicatoria*, *L. plantarum*, *A. fischeri* and *P. maculicola* by a broth microdilution method. The experiments were made in triplicate. Gentamicin (0.1 mg/mL in ethanol) or chloramphenicol (0.1 mg/mL in ethanol) was used as positive and ethanol as negative control. Ethanolic two-fold dilution series of the samples were prepared and 5 µL of each was mixed with 140 µL of cell suspension (10^5 CFU/mL) in 96-well sterile microtiter plates. MIC values were obtained after incubation and shaking at 28 °C for 16 h by: optical density measurement at 630 nm (StatFax 2100 microplate reader, Awareness Technology); dark photography (luminescent *A. fischeri* and *P. maculicola*); or adding 35 µL of 0.5 mg/mL MTT dye solution (*B. subtilis*, *X. euvesicatoria* and *L. plantarum*). The MICs were determined as the lowest concentrations of tested samples that completely inhibited bacterial growth, which was indicated by the absence of the formation of bluish MTT-formazan.

3. Results and discussion

3.1. TLC-DB

A non-targeted tracking of antibacterial components of the *O. acanthium* leaf extracts was carried out with a fast and effective direct bioautographic method that comprises a planar layer chromatographic separation and a subsequent bioassay for the detection of separated compounds with the desired effect. The development of the TLC method was guided by direct bioautography using the Gram positive soil bacterium *B. subtilis*, resulting in the mobile phase *n*-hexane – ethyl acetate (45:55, v/v). The ethyl acetate extract was found to be the most prospective, containing the active compounds in the least matrix. However, its solvent was changed to ethanol, which is more compatible with agar diffusion and microdilution tests.

The components of *O. acanthium* leaf extracts separated by TLC were tested against further seven bacterium strains: pepper pathogen *X. euvesicatoria*, marine *A. fischeri*, *Arabidopsis* pathogen *P. maculicola*, probiotic *L. plantarum*, (Fig. 1) and human pathogens *S. aureus*, MRSA and *E. coli* (Fig. 2). Two characteristic antibacterial zones were found at hR_F 37 and 82. Both chromatographic zones could be visualized by primulin TLC-reagent, which is applicable for lipophilic compounds, whereas the universally used vanillin-sulphuric acid reagent gave visible zone only at hR_F 37 (Fig. 1).

The antibacterial effect at hR_F 82 was apparent with *B. subtilis, X. euvesicatoria, A. fischeri, S. aureus* and MRSA (Figs. 1 and 2). All bacterial strains, even the probiotic one, were sensitive against compound(s) present at hR_F 37, however *P. maculicola* was inhibited only by a relatively higher applied amount. Comparing the applied amount used to obtain the inhibition zones it was found that the most sensitive was the *A. fischeri*, and that *S. aureus* and MRSA were more sensitive than *B. subtilis* or *X. euvesicatoria*.

3.2. OPLC-DB and OPLC-UV-fractionation

Our next aim was to obtain fractions with the active components (at hR_F 37 and 82 in TLC) to make possible their further mass spectrometric characterization. For this reason a fully off-line OPLC separation was established using chloroform - ethyl acetate - 2-propanol 90:5:5 (v/v) as a mobile phase. The presence of the antibacterial zones in the OPLC chromatoplate was confirmed by *B. subtilis* and *P. maculicola* bioassays (Fig. 3). The main active zone was found below the gamma-front at 6.2 cm height and the other one was moved with the gamma front (at 10.35 cm). This method could easily be further developed to a mixed mode infusion-transfusion process comprising on-line detection and fractionation. Ten fractions were collected based on UV detection at 220 nm, concentrated and transferred to OPLC – *B. subtilis* assay (Figs. 3 and S-1). The first fraction contained the compounds detected at hR_F 82 with TLC and the main active compound (at hR_F 37) was found in fractions 5-7, which were combined.

3.3. HPLC-DAD-ESI-MS/MS analysis of the active OPLC fractions

The OPLC fractions containing antibacterial compounds were transferred to HPLC-MS/MS analysis. In the first fraction linoleic and linolenic acids were present, as identified by the use of standards. These fatty acids are essential for humans. So far linoleic acid has been reported as a constituent of *O. acanthium* seed oil, but linolenic acid not [17]. Both detected fatty acids displayed antibacterial activity in bioautographic assay at the appropriate hR_F (Fig. S-2) and the MIC values were determined against *B. subtilis* as 43.1 µg/mL for both fatty acids and against *A. fischeri* to be 21.5 µg/mL for linoleic acid and 43.1 µg/mL for linolenic acid. The antibacterial activity of these long-chain unsaturated fatty acids is well-known and they are applied as food additives to repress infections [27-31].

The analysis of the combined OPLC fractions 5-7 showed that there is only one major component in the chromatographic zone, which gave mass signals in the negative ionization mode at *m/z* 347 and 393 that were assigned to be the deprotonated molecular ion $[M-H]^-$ and

the adduct ion $[M+HCOO]^-$, respectively (Fig. S-3). In positive ionization mode the protonated molecular ion at *m/z* 349 $[M+H]^+$, the adduct ion at *m/z* 371 $[M+Na]^+$ and the sodium adduct of the dimer at *m/z* 719 $[2M+Na]^+$ were detected (Fig. S-3). In negative ionization mode the MS/MS fragmentation of *m/z* 393 resulted in ions at *m/z* 243, 191 and 99. The compound absorbed a wide range of UV light (190-260 nm) with the absorbance maximum at 225 nm (Fig. 4c).

3.4. Isolation procedure

As the main antibacterial substance could not be identified by MS/MS analysis, its preparative scale isolation from 10 mL crude extract was planned to provide appropriate amount for NMR investigation. Such amount of the compound is also sufficient for the determination of minimal inhibitory concentrations with microdilution tests. The mobile phase developed for OPLC separation was successfully utilized for flash chromatographic fractionation. It has to be noted that the transfer of the mobile phase between the two forced-flow techniques is very simple, directly applicable. While TLC separation is adaptable to flash chromatography (also to OPLC) when the interesting compounds are in a proper hR_F range (10-40) as well as they are not close to the demixation and total wetting fronts, which can cause distortion of the zones and/or co-elution of the substances. Nine fractions were collected by flash chromatography. According to their TLC fingerprints (Fig. S-4) No. 5 and 6 were combined. A part of their combination was used for the final purification step. An analytical HPLC separation was scaled up to a semi-preparative one by changing the column diameter (4.6 to 10 mm), the particle size (5 to 10 µm) of the column packing, the flow rate (1 to 6 mL/min) and the injected volume (2 to 100 µL), which provided appropriate resolution for the isolation of the desired main active compound (Fig. 4). The isolation process was repeated five times to yield 21.3 mg pure compound. The NMR assignments (Table S-1) allowed its identification as the germacranolide sesquiterpene lactone onopordopicrin (Fig. S-5).

3.5. Antibacterial activity of onopordopicrin

The activity of pure onopordopicrin was first tested by TLC-direct bioautography, proving its hR_F value and antibacterial effect against all used bacterial strains (Figs. 1 and 2). Only *P. maculicola* was resistant to 20 µg onopordopicrin. Further confirmation of its *in vitro* antibacterial effect was performed by microdilution tests. The minimum inhibitory concentration (MIC) against different strains was found by microdilution test for the extract and the sesquiterpene lactone 27.6-275.9 and 2.2-172.4 µg/mL, respectively (Table S-2).

Onopordopicrin has been previously reported in the literature as a constituent of *O. acanthium* leaves [32]. It was established that sesquiterpene lactones exert several biological properties, including cytotoxic, antibacterial, anti-inflammatory, antimalarial, and hypotensive actions [33]. There are only few results available in the literature to support the bioactivity of onopordopicrin, and contradictory data have also been published. Onopordopicrin possessed antibacterial effect against *S. aureus* [34] and antimalarial activity against *Plasmodium falciparum* and *Trypanosoma brucei rhodesiense* parasites [35]. In contrast, Bach et al. [36] established no antibacterial effect of onopordopicrin when testing a wide range of opportunistic and pathogenic bacteria including *S. aureus*, MRSA, *E. coli* and *B. subtilis* with disc diffusion test. However, it has to be noted, that disc diffusion assay is not preferable to test compounds with poor solubility and slow diffusion into agar (like onopordopicrin), while in direct bioautography there are no such limits [7].

4. Conclusions

In this study an effect-directed workflow for the screening, isolation and identification of bioactive plant ingredients was demonstrated. In this approach TLC/OPLC-UV/bioassays were established as a high-throughput and effective biomonitoring tool for the detection of separated components from a plant matrix with antibacterial effect against various plant and human pathogens as well as a human gut bacterium. The detected bioactive compounds can be subjected to highly targeted methods including e.g. OPLC-UV/fractionation, column liquid chromatography, MS/MS and NMR to achieve their identification. The easy and direct transfer of OPLC method to flash chromatography was also shown. It was clearly demonstrated that this analytical toolbox is beneficial in the discovery of antibiotic compounds, allowing a fast, purposive, thus cost-effective tracking, isolation and identification of antibacterial compounds that can become promising drug candidates. This workflow enabled us to find and identify two fatty acids and the germacranolide sesquiterpene lactone onopordopicrin in the scotch thistle leaf.

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The authors would like to dedicate this paper to the memory of Prof. Ernő Tyihák, the main inventor of OPLC.

Declaration

The authors declare no conflict of interest.

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List of figures:

Figure 1

HPTLC chromatograms of bioactive compounds in *O. acanthium* leaf extract (1), and its isolated main component onopordopicrin (2), documented (a) at 254 nm, (b) under white light illumination after derivatization with vanillin sulphuric acid reagent and (c) at 365 nm after derivatization with primulin as well as bioautograms using (d) *B. subtilis*, (e) *A. fischeri*, (f) *L. plantarum*, (g) *X. euvesicatoria* and (h) *P. maculicola*.

Figure 2

The antibacterial activity test of *O. acanthium* leaf extract components (1) and the isolated main component onopordopicrin (2) against human pathogenic bacteria. HPTLC chromatogram was documented (a) after derivatization with vanillin sulphuric acid reagent as well as bioautograms using (b) *S. aurelius* , (c) MRSA and (d) *E. coli*.

Figure 3

Detection of bioactive *O. acanthium* leaf extract components separated by (a-d and f) fully off-line analytical OPLC and (e) infusion-transfusion preparative OPLC; a and b – chromatoplate documented at 254 and 365 nm, respectively; c and d – bioautograms using *B. subtilis* and luminescent *P. maculicola*, respectively; e – OPLC-UV chromatogram with the collected fractions; f – OPLC-*B. subtilis* assay of the collected fractions.

Figure 4

Chromatograms at 225 nm obtained by (a) analytical HPLC-DAD-MS and (b) semi-preparative HPLC-DAD of the flash chromatographic bioactive fraction of *O. acanthium* leaf extract; (c) the UV spectrum of the main active component (onopordopicrin) eluted at 10.73 and 11.15 min, respectively.