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Effect-directed analysis *via* hyphenated high-performance thin-layer chromatography for bioanalytical profiling of sunflower leaves

Ágnes M. Móricz[a,*], Péter G. Ott[a], Imanuel Yüce[b], András Darcsi[c], Szabolcs Béni[c], Gertrud E. Morlock[b]

[a] *Plant Protection Institute, Centre for Agricultural Research, Hungarian Academy of Sciences, Herman O. Str. 15, 1022 Budapest, Hungary*
[b] *Justus Liebig University Giessen, Institute of Nutritional Science, Chair of Food Science, and Interdisciplinary Research Center (IFZ), Heinrich-Buff-Ring 26-32, 35392 Giessen, Germany*
[c] *Department of Pharmacognosy, Faculty of Pharmacy, Semmelweis University, Üllői Str. 26, 1085 Budapest, Hungary*

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ABSTRACT

High-performance thin-layer chromatography (HPTLC) coupled with effect-directed analysis was used for non-targeted screening of sunflower leaf extract for components exhibiting antioxidant, antibacterial and/or cholinesterase enzyme inhibitory effects. The active compounds were characterized by HPTLC-electrospray ionization-high resolution mass spectrometry (ESI-HRMS) and HPTLC-Direct Analysis in Real Time (DART)-MS/MS. The latter ambient ionization technique (less soft than ESI) resulted in oxidation and fragmentation products and characteristic fragment ions. NMR spectroscopy after targeted isolation *via* preparative normal phase flash chromatography and semi-preparative reversed phase high-performance liquid chromatography supported the identification of two diterpenes to be (-)-kaur-16-en-19-oic acid and 15-α-angeloyloxy-ent-kaur-16-en-19-oic acid. Both compounds found to be multi-potent as they inhibited acetylcholinesterase and butyrylcholinesterase and showed antibacterial effects against Gram-positive *Bacillus subtilis* and Gram-negative *Aliivibrio fischeri* bacteria. Kaurenoic acid was also active against the Gram-negative pepper pathogenic *Xanthomonas euvesicatoria* bacteria.

1. Introduction

Effect-directed analysis (EDA) *via* the combination of chromatography with chemical/biological/biochemical analyses is widely used in drug discovery, especially in bioassay-guided analyses of complex samples [1,2]. Of the highest interest are antimicrobials against multi-resistant pathogens that severely impair human, animal or plant health [3,4]. Treatment of diabetes, dementia, Alzheimer's disease, hormonal and cardiovascular disorders can be improved using drugs with specific effects, *e.g.*, antioxidant, estrogenic and enzyme inhibition/induction effects. For example, the observation of chronic acetylcholine deficiency in the brain of the patients suffering from Alzheimer's disease [5] led to the introduction of cholinesterase and ß-secretase inhibitors in the treatment protocol [6]. However, in many cases, clinical administration of drugs is associated with several side effects. Cholinesterase inhibitors (*e.g.*, galantamine, rivastigmine and donepezil) can cause vomiting, nausea and dizziness [7], whereas, antibiotics can exhibit adverse effects such as diarrhoea, allergy and vomiting [8]. Hence, pharmaceutical research aims at potent candidates with a new base structure and preferably without side effects. Knowledge on the effectiveness of known compounds with regard to new activities is also needed.

EDA is extensively utilized in drug discovery processes based on the screening of natural products such as plant extracts [9]. Chemical analysis of non-volatile plant ingredients is strongly linked to the use of liquid chromatographic systems with column or planar arrangements, which enable efficient separation and isolation of constituents [10,11]. In the last two decades the technical improvements achieved in planar chromatographic hyphenations provided

* Corresponding author.
E-mail address: moricz.agnes@agrar.mta.hu (Á.M. Móricz).

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a chance for parallel bioprofiling, biodetection and characterization of individual active compounds in complex samples [2]. HPTLC offers as an open chromatographic system the opportunity to perform direct *in situ* bioassays without much effort. Contrarily, high-performance liquid chromatography (HPLC), while allowing higher separation efficiency, is operated with solvents that are often toxic to the biological cells. Nevertheless, HPLC has been coupled with bioactivity tests, which required time consuming steps including fraction collection (*e.g.*, into tubes or microtiter plates), drying or lyophilization and re-suspension in an appropriate (bioassay-proven) solution [12–14]. Online it was mainly combined with chemical reagents (*e.g.,* 2-diphenyl-1-picrylhydrazyl radical assay) [15,16] and even in some cases with enzymes (*e.g.*, cholinesterase inhibitory assay) [17,18]. So far, only cells of *Aliivibrio fischeri* bacteria have been introduced directly in the HPLC effluent [19]. The disadvantage of the HPLC-enzyme inhibition approach is the extra peak broadening due to the use of long reaction coils and dilution with reagents [17,18]. The proper activity of an enzyme in a sub-optimal environment is also questionable. In the HPLC-*A. fischeri* bioassay, cell death increased with the methanol portion in the eluent [19]. The cells in such a system are *ab ovo* in stress caused by the eluent. There are no data available whether the impact by the stressing eluent is additive, synergistic or antagonistic on the separated active components. Such drawbacks were summarized in the previous work [2]. It can be concluded that enzyme inhibitory and cell viability assays demand a special ambient milieu that is obviously easier attained in planar chromatographic systems that enable elimination of the mobile phase before bioassay application.

Planar layer chromatography ensures further advantages such as a minimal sample preparation (crude extracts can be applied without losing components), a parallel profiling of samples or a parallel performance of different effect-directed assays. A wide range of bacterial and fungal strains have been introduced for planar chromatography combined with bioassays, termed bioautography [20]. Several HPTLC-enzyme inhibition assays have been developed, *e.g.,* for cholinesterase and glucosidase inhibition. Estrogen-effective, antioxidant and kidney stone-inhibitory activity can also be detected *in situ* in the adsorbent layer. Important innovations in TLC/HPTLC-MS substantially contributed to this field and made possible a rapid characterization of the separated compounds. The manually operated, pneumatically driven TLC-MS Interface [21,22] equipped with an elution head, enabled the online coupling between TLC/HPTLC and MS. A pump-driven elution solvent directed the eluted zone into the MS or HPLC-MS [23] or vial for further investigations with, *e.g.*, HPLC-MS/MS, gas chromatography-MS and NMR [24]. Scanning MS of a track was demonstrated for ion sources based on desorption electrospray ionization [25], matrix-assisted laser desorption and ionization [26,27], laser ablation-inductively coupled plasma [28] and Direct Analysis in Real Time (DART)-MS [29,30]. Such an arsenal of HPTLC hyphenations allows activity screening of the separated compound zones and a subsequent targeted chemical characterization of the active zones *in situ* in the adsorbent bed [31].

Sunflower (*Helianthus annuus* L.) is an annual wildflower belonging to the *Asteraceae* family. It is native to North-America, but cultivated world-wide for its seed and seed oil. The plant is well known to possess a strong defence mechanism called allelopathy that is attributed to secondary metabolites [32]. The aerial part is rich in terpenoids [32–34], but contains also phenolics, flavonoids [35] and coumarins [36], which exhibit various biological activities [37] such as anti-inflammatory [38] and antimicrobial effects [39,40].

HPTLC-EDA-MS is the most streamlined technique for discovery of active components in complex samples and makes innovations cost-efficient. To trigger its use, this paper aimed at demonstrating the benefit of HPTLC-hyphenations in the discovery and characterization of active natural compounds. *H. annuus* leaf extract was used as an illustrative example. The bioprofiling of the extract was performed by the combination of HPTLC with an antioxidative assay, four antibacterial assays of different strains (among them two plant pathogens) and an acetylcholinesterase inhibitory assay. Multi-potent compounds underwent a subsequent targeted characterization using scanning HPTLC-DART-MS/MS and HPTLC-ESI-HRMS. Two compounds exhibiting antibacterial and acetylcholinesterase inhibitory activity were isolated by column chromatographic techniques and identified by NMR.

2. Materials and methods

2.1. Materials

HPTLC silica gel 60 F_{254} plates with glass or aluminium backing and methanol (MS grade) were purchased from Merck, Darmstadt, Germany. All solvents for HPTLC (analytical grade) were from Sigma-Aldrich, Steinheim, Germany, or Reanal, Budapest, Hungary. Pure water was produced by a Millipore Direct-Q 3 UV system (Merck). Acetonitrile (gradient grade) was supplied by Fisher Scientific, Pittsburg, PA, USA. Deuterated methanol (CD_3OD, 99.8 atom% D) was from VWR, Budapest, Hungary. Marine *Aliivibrio fischeri* bacteria (DSM-7151) were from Leibniz Institute DSMZ, German Collection of Microorganisms and Cell Cultures, Berlin, Germany. *Bacillus subtilis* subsp. *spizizenii* soil bacteria (DSM 618) were from Merck. *B. subtilis* strain F1276 was from József Farkas (Central Food Research Institute, Budapest, Hungary), a luminescent *Arabidopsis* pathogen *Pseudomonas syringae* pv. *maculicola* chromosomally tagged with lux-CDABE genes from *Photorhabdus luminescens,* was kindly provided by Jun Fan, John Innes Center, Department of Disease and Stress Biology, Norwich, UK. The pepper pathogen *Xanthomonas euvesicatoria* is a Hungarian paprika isolate from János Szarka, Primordium Kft., Budapest, Hungary. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and bovine serum albumin were from Carl Roth, Karlsruhe, Germany. Acetylcholinesterase lyophilisate (from *Electrophorus electricus*), butyrylcholinesterase (from horse serum), Fast Blue Salt B and 2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) were purchased from Sigma. α-Naphthyl acetate was from Panreac, Barcelona, Spain.

2.2. Sample preparation

Leaves of full flowering sunflowers seeded in April in the Great Plain, Hungary, were collected in August 2013. Dried (15 g; at 25 °C in the dark) and ground (Bosch MKM6000, Stuttgart, Germany) leaves were macerated in 100 mL ethyl acetate for 24 h. The filtered crude extract with 13.4 mg/mL dry weight was directly used for flash chromatographic fractionation. For HPTLC application, it was diluted 1:10 with ethyl acetate.

2.3. HPTLC method

Samples (5–20 μL/band) were applied as 8-mm bands with 11 mm track distance onto the HPTLC layer by a TLC sampler (ATS4 or Linomat IV, CAMAG, Muttenz, Switzerland) at 8 mm distance from the bottom. HPTLC separation was performed with *n*-hexane – isopropyl acetate – acetic acid (80:19:1, *V/V*) in an unsaturated 20 cm × 10 cm Twin Trough Chamber (CAMAG) up to a migration distance of 70 mm, which took 22 min. After development the plate was dried in a cold air stream and documented under UV and white light illumination by TLC Visualizer Documentation System (CAMAG) or a digital camera (Cybershot DSC-HX60, Sony, Neu-Isenburg, Germany) using a UV lamp (CAMAG). HPTLC data were processed and evaluated by winCATS software, version 1.4.7.2008

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