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Effect-Directed Analysis of *Rhodiola Rosea* L. based on Planar Chromatography: the Search for Bioactive Components

Doctoral thesis

carried out in the Department of Chromatography

under the supervision of Dr. hab. Irena M. Choma, prof. UMCS

and Dr. Marek Studziński (auxiliary promoter)

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LIST OF ABBREVIATIONS

^{1}H NMR	Proton Nuclear Magnetic Resonance
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AS	*p*-Anisaldehyde sulphuric (reagent)
BCG	Bromocresol green reagent
BChE	Butyrylcholinesterase
DAD	Diode Array Detection
DART	Direct Analysis in Real Time
DESI	Desorption Electrospray Ionization
DPPH•	2,2-Diphenyl-1-picrylhydrazyl reagent
EDA	Effect-Directed Analysis
EDD	Effect-Directed Detection
ESI-MS	Electrospray Ionization-Mass Spectrometry
FBS	Fast Blue B salt
FDG	Fluorescein Di-β-D-Galactopyranoside
FLD	Fluorescence Detector
HESI-MS	Heated Electrospray Ionization-Mass Spectrometry
HPLC	High-Performance Liquid Chromatography
HPTLC	High-Performance Thin-Layer Chromatography
HRMS	High-Resolution Mass Spectrometry
MALDI	Matrix-Assisted Laser Desorption/Ionization
MS	Mass Spectrometry

NP–PEG	Natural Products – Polyethylene glycol (reagent)
PABA	*p*-Aminobenzoic acid (reagent)
PD	Parkinson's disease
PMA	Phosphomolybdic acid (reagent)
pYAVAS	Planar Yeast Anti- and Androgen Verified Screen
pYAVES	Planar Yeast Anti- and Estrogen Verified Screen
$\boldsymbol{R_F}$	Retardation Factor
TCM	Traditional Chinese Medicine
TLC	Thin-Layer Chromatography
USP	United States Pharmacopeia
UV	Ultraviolet light
VIS	White light illumination, visible light
§	Section

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1. LIST OF PUBLICATIONS INCLUDED IN THE DOCTORAL THESIS

The presented doctoral thesis is a series of six thematically related scientifical articles **D1-D6** published in journals from the *Journal Citation Reports* database:

D1 I.M. Choma**, H. Nikolaichuk**, TLC-bioprofiling – A tool for quality evaluation of medical plants, in: Pulok Mukherjee (Ed.) Evidence-based validation of herbal medicine. Translational research on botanicals. Second edition, Elsevier 2022, pp. 407-422.

IF **MEiN: 20**

D2 **H. Nikolaichuk**, I.M. Choma, TLC screening in searching for active components in *Rhodiola rosea* L. roots. Annales Universitatis Mariae Curie-Skłodowska, Lublin – Polonia. Section AA – Chemia LXXIV (2019), pp. 55-64.

IF **MEiN: 20**

D3 **H. Nikolaichuk**, M. Studziński, I.M. Choma, Effect directed detection of *Rhodiola rosea* L. root and rhizome extract. Journal of Liquid Chromatography and Related Technologies, 43 (2020), pp. 361-366.

IF 1.467 **MEiN: 40**

D4 **H. Nikolaichuk**, R. Typek, S. Gnat, M. Studziński, I.M. Choma, Effect-directed analysis as a method for quality and authenticity estimation of *Rhodiola rosea* L. preparations. Journal of Chromatography A, 1649 (2021) 462217.

IF 4.601 **MEiN: 100**

D5 **H. Nikolaichuk**, M. Studziński, M. Stankevič, I.M. Choma, Qualitative and quantitative evaluation of rosavin, salidroside, and *p*-tyrosol in artic root products via TLC-screening, HPLC-DAD, and NMR spectroscopy. Molecules 27 (2022) 8299.

IF 4.927 **MEiN: 140**

D6 **H. Nikolaichuk**, I.M. Choma, G.E. Morlock, Bioactivity profiles on 15 different effect mechanisms for 15 golden root products via high-performance thin-layer chromatography, planar assays, and high-resolution mass spectrometry. Molecules 28 (2023) 1535.

IF 4.927 **MEiN: 140**

Total IF = 15.882

Total MEiN = 460

2. INTRODUCTION

Medicinal plants are a natural source of bioactive compounds with healing abilities which have been used as folk medicines and cosmetics for thousands of years all over the world. Nowadays, they are experiencing their renaissance as modern pharmaceutical preparations, diet supplements, and cosmetics (or their components). Despite enormous progress in civilization, mankind is increasingly struggling with civilization diseases, such as diabetes, cancer, obesity, heart diseases, and neurological diseases such as Alzheimer's (AD) and Parkinson's (PD) [1,2]. A growing number of people currently appreciate herbal products as medicinal preparations. The development of phytotherapy, pharmacology and analytical methods provides much better results of treatment based on herbal medicine and enables the production of new herbal preparations and drugs. However, at the same time, such widespread use of herbal products has raised serious concerns about their quality, safety, and efficacy. Unlike conventional medicine, the use of plant products is based mostly on empirical knowledge coming from traditional methods. In addition, plants contain complex mixtures of chemical constituents, which are often poorly characterized and scientifically evaluated. Moreover, due to variations in chemical composition of plant products and the instability of some plant constituents, the identity of the active components is often unknown. In most cases, the emergence of synergistic, agonistic, or antagonistic effects that contribute to biological activity should be taken into account. These difficulties make it challenging to ensure that medicinal plant products are reliable, safe, and provide the expected bioactivity. Detection and identification of herbal components are crucial for understanding processes and pathways of disease treatment. The isolation and

evaluation of bioactive compounds from plant materials seems to be highly relevant and plays an essential role in discovery and development of new and efficient pharmaceuticals [3]. Since the plant composition depends on various factors *e.g.*, plant origin, climate, soil conditions, growing and harvest time, storage conditions, method of extraction, and stabilization, the quality control of herbal drugs is more difficult than that of synthesized ones. However, this does not change the fact that safe and high-quality herbal products are of great importance to consumers. This involves establishing strict protocols and regulations for the mandatory verification of quality and safety as well as herbal product registration. One of the most relevant acts in this matter in Europe is the Traditional Herbal Medicinal Products Directive (formally Directive 2004/24/EC). Still, many economically profitable plant products are available on the market without any control, at potential risks of adulteration [4–6]. One of such plants is *Rhodiola rosea* L., very often counterfeited mainly because of its wide range of bioactivities and beneficial effects on human organisms [7,8].

Rhodiola rosea L. (*R. rosea*, also known as the arctic root, roseroot, or golden root) has a long history of use in Europe and Asia, among others, in Traditional Chinese Medicine (TCM). *R. rosea* is a perennial flowering plant from the *Crassulaceae* family and grows in cold regions at high altitudes. The herb is well known as a classical ‘natural adaptogen’. This term can be defined as a nontoxic substance with the ability to eliminate damage caused by stressors and stimulate nonspecific resistance in a human organism [9–12]. Recent studies have revealed a broad range of medicinal properties and biological activities in *R. rosea*. Worth highlighting are the abilities to increase and enhance immunity, DNA repair, modulation of adaptation to hypoxia and angiogenesis, as well as anticancer, antistress, antifatigue, antiaging, anti-

inflammatory, antioxidant, and antiviral properties [13–22]. *R. rosea* is recommended for sportsmen and elders to increase their energy and reduce fatigue.

The consumption of *R. rosea* as a crude drug or processed medicines/supplements for treating mental disorders as well as improving memory and learning abilities has increased in recent years due to its potential benefits to human health [23–27]. Commercial products of *R. rosea* products are based on the root and/or rhizome of the plant.

The role of planar chromatography in plant analysis has been increasing in recent years [28–34]. The method has been developing and gaining several advantages over other separation techniques (*e.g.,* high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE)) [35–38]. The notable and unrivaled advantages are:

- low cost of single analysis;
- eco-friendliness;
- picture giving results;
- simple sample preparation, even for complex matrices;
- simultaneous separation of up to 40 samples at one (HP)TLC plate in one run;
- parallel chromatography of many plates under the same conditions;
- flexibility and possibility of rapid change of chromatographic conditions such as stationary and mobile phases;
- direct coupling to (bio)assays (*i.e*., effect-directed detection (EDD) and bioprofiling) and derivatization assays (chemical profiling);

- flexible and online coupling with various spectroscopic detection methods (*e.g.*, matrix-assisted laser desorption-ionization mass spectrometry (MALDI), desorption electrospray ionization (DESI), direct analysis in real time (DART), and high-resolution mass spectrometry (HRMS) via TLC Interface system;
- no carry-over and memory effects.

Undoubtedly, one of the most important advantages of planar chromatography is its hyphenation to direct (bio)assays followed by identification using spectroscopic methods [39–48]. This approach belongs to EDA methods *i.e.*, the methods measuring the effect emerging in a given biological system under the influence of a given substance [49]. The EDA based on planar chromatography is an extremely useful method for profiling the bioactivity and major constituents of herbs. Currently, bioprofiling of plants and their markers based on EDA is a convenient and effective way for identifying the main active components, also known as biomarkers. The detection of biomarkers helps to improve quality control, especially if the information on the authenticity of a plant is lacking. The standard strategy of EDA is the hyphenation of fast and straightforward ambient separation technique as (high-performance) thin-layer chromatography (HP)TLC with (bio)assays followed by mass spectrometry, HPLC-diode array detection (DAD) or nuclear magnetic resonance spectroscopy (NMR) [39,44,50–54]. This strategy is a merit of the last decade and is still being developed.

3. AIM AND OBJECTIVES OF THE THESIS

The aim of the thesis was to search for bioactive components of *Rhodiola rosea* L. using effect-directed analysis based on planar chromatography. In particular, this research aimed at creating bioprofiles and developing a targeted and non-targeted effect-directed screening/analysis that can separate, visualize and identify active compounds in *R. rosea*, one of the most intriguing medicinal plants.

It was expected that elaboration of such profiles would help to learn more about the plant variances and the differences in its chemical composition and bioactivities. Mainly, it allowed the confirmation of plants authenticity and quality, distinguishing among commercially available products and detecting possible adulterations.

The main objectives of the thesis were:

- optimization of chromatographic systems (both TLC and HPTLC);
- choosing and applying appropriate assays;
- identification of crucial ingredients using reference standards;
- (bio)profiling of the samples together with an authentic *R. rosea* reference sample;
- analytical characterization/tentative identification of active compounds.

The main research steps included:

- optimization of conditions for chromatographic separation; performed in the Department of Chromatography, UMCS, Lublin, Poland, and Chair of Food Science, Justus Liebig University Giessen, Giessen, Germany;

- chemical and physicochemical profiling of *R. rosea* extracts by TLC-UV-VIS-FLD using derivatization procedures with different reagents; performed in the Department of Chromatography, UMCS, Lublin, Poland;
- microchemical, biological, and biochemical profiling of *R. rosea* extracts using hyphenation of planar chromatography and effect-directed detection using various assays with indication to different effect mechanisms and active compounds *e.g.,* antioxidants, enzyme inhibitors, antibacterials, endocrine active compounds, and genotoxins; performed in the Department of Chromatography, UMCS University, Lublin, Poland, the Department of Veterinary Microbiology, University of Life Sciences, Lublin, Poland and Chair of Food Science, Justus Liebig University Giessen, Giessen, Germany;
- characterization of compounds and active zones via offline TLC micro-fractionation subjected to HPLC-ESI-MS analysis and online HPTLC-HPLC-HESI-HRMS analysis; performed in the Department of Chromatography, UMCS, Lublin, Poland and Chair of Food Science, Justus Liebig University Giessen, Giessen, Germany, respectively;
- qualitative and quantitative evaluation of marker compounds in *R. rosea* using HPLC-DAD and ^{1}H NMR analysis performed at the Department of Physical Chemistry and the Department of Organic Chemistry, Faculty of Chemistry, UMCS, Lublin, Poland, respectively.

The compact whole of the submitted doctoral thesis consists of one review chapter [**D1**] and five research publications [**D2-D6**].

4. RESEARCH METHODS

4.1. Effect-directed profiling

The effect-directed analysis based on planar chromatography was used for the screening of bioactive compounds in *R. rosea* extracts and allowing determination of their type and structure. The separation, (bio)assays, and visualization were performed directly on TLC or HPTLC plates for multiple detections under identical conditions to obtain as much information as possible. A schematic representation of EDA used for *R. rosea* profiling is shown in **Figure 1**.

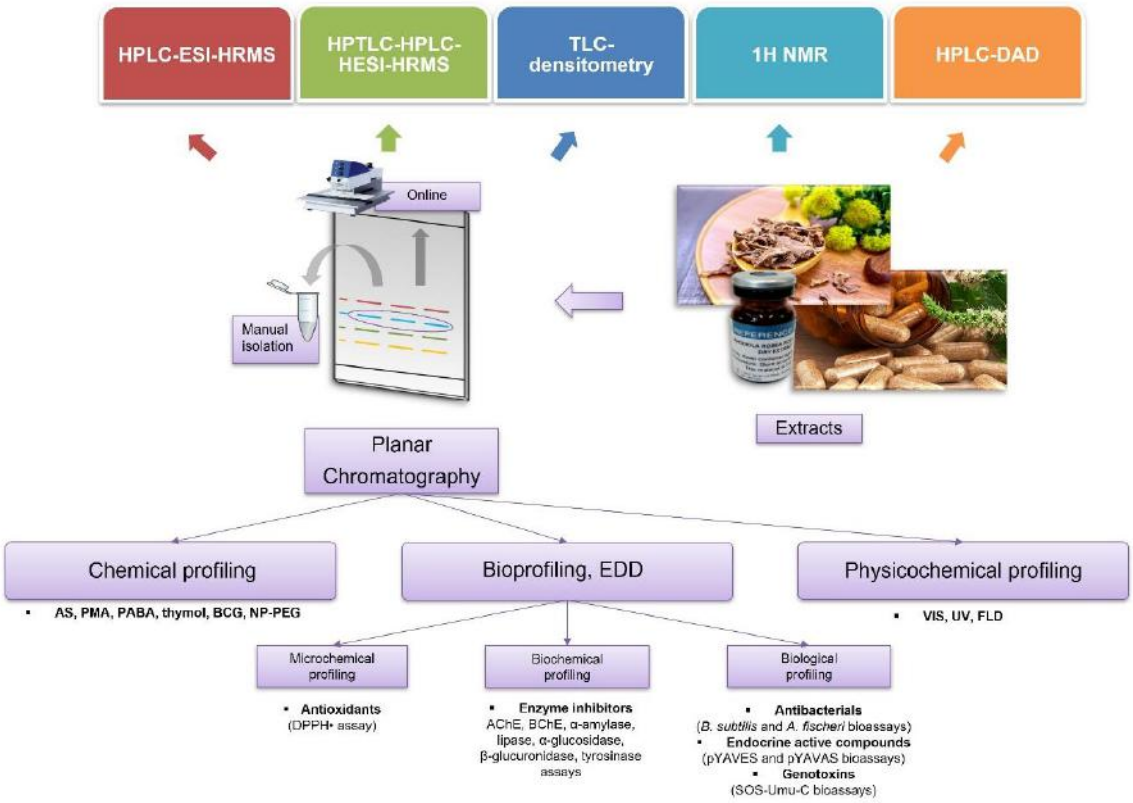

Figure 1. Schematic representation of EDA used for *R. rosea* profiling.

The planar chromatography results (chromatograms and (bio)autograms) were obtained in the form of high-resolution color images. The images provided three

parameters for analysis – the retardation factors (R_F), color intensity, and absorption/fluorescence wavelength, which allowed for the comparison and verification of sample profiles. In general, thirteen different assays were performed for *R. rosea* extracts directly on plates in the search for the seventeen bioactive effects (**Table 1**). Planar assays were selected based on the assumption that the investigated activity could be potentially useful in the treatment of civilization diseases or various ailments [**D1** and 46], *e.g.*:

- AChE and BChE inhibitors – of AD and PD;
- AChE inhibitors – of bipolar disorder;
- BChE, α-amylase and α-glucosidase inhibitors – of diabetes II type;
- α-Glucosidase inhibitors – of cancer and viral infection;
- β-Glucuronidase inhibitors – of drug-induced gastrointestinal disorders;
- Lipase inhibitors – of obesity and atherosclerosis;
- Tyrosinase inhibitors – of skin and dermatological diseases.

Planar assays used for the detection of endocrine (estrogen and androgen), antioxidant, antibacterial and genotoxic activities were also included in the research.

In general, all (bio)assays were performed in the same manner under identical conditions, at least two times to confirm their reproducibility. Additionally, respective positive controls were applied to check the assay validity. The protocols of every (bio)assay are based on a simple sequence of steps:

enzymes/cells(bacteria or yeast) → *substrate* (in case of enzymes and yeast) →

incubation (special conditions for bacteria growth or for the reaction between enzymes/yeast and substrate) →

visualization (formation of results in a visible form under UV, VIS, or FLD wavelengths).

The exception was the DPPH• assay based on the reduction of the reagent.

Table 1. Effect-directed assays and their effects.

Assay	Effect	Observation	Section/ Publication
DPPH•	Free radical scavenging and antioxidant	Yellow or colorless zones against a purple background	*§5.1.* and *§5.2.* **D2**, **D3**, **D4**, **D5**, **D6**
AChE	AChE inhibition	Yellow zones against the purple background. 1/2-Naphthyl acetate and FBS procedure protocol	*§5.1.* **D3**, **D4**
		White zones on an indigo-blue background. Indoxyl acetate procedure protocol	*§5.2.* **D6**
BChE	BChE inhibition	White zones on an indigo-blue background.	*§5.2.* **D6**
α-Amylase	α-Amylase inhibition	Brown zones against a yellow background.	*§5.2.* **D6**
α-Glucosidase	α-Glucosidase inhibition	Yellow zones against the purple background.	*§5.1.* **D4**, **D5**
β-Glucuronidase	β-Glucuronidase inhibition	Colorless zones against an indigo-blue background.	*§5.2.* **D6**
Lipase	Lipase inhibition	Yellow zones against the purple background.	*§5.1.* **D4**, **D5**
Tyrosinase	Tyrosinase inhibition	White zones against a grey background.	*§5.1.* and *§5.2.* **D4**, **D5**, **D6**
A. fischeri	Antibacterial against Gram-negative *A. fischeri* bacteria strain	Dark zones on a bioluminescent background.	*§5.2.* **D6**
B. subtilis	Antibacterial against Grain-positive *B. subtilis* bacteria strain	White zones against a purple background.	*§5.1.* and *§5.2.* **D3**, **D4**, **D6**
SOS-Umu-C	Genotoxic	Bright green fluorescent zone against a green fluorescent background.	*§5.2.* **D6**
pYAVES	Estrogen	Bright green fluorescent zone against a green fluorescent background.	*§5.2.* **D6**
	Antiestrogen	Fluorescence reduction against 17b-estradiol stripe.	
	Verified antiestrogen (false positives)	Fluorescence reduction against fluorescein stripe.	
pYAVAS	Androgen	Bright green fluorescent zone against a green fluorescent background.	*§5.2.* **D6**
	Antiandrogen	Fluorescence reduction against 17b-estradiol stripe.	
	Verified antiandrogen (false positives)	Fluorescence reduction against fluorescein stripe.	

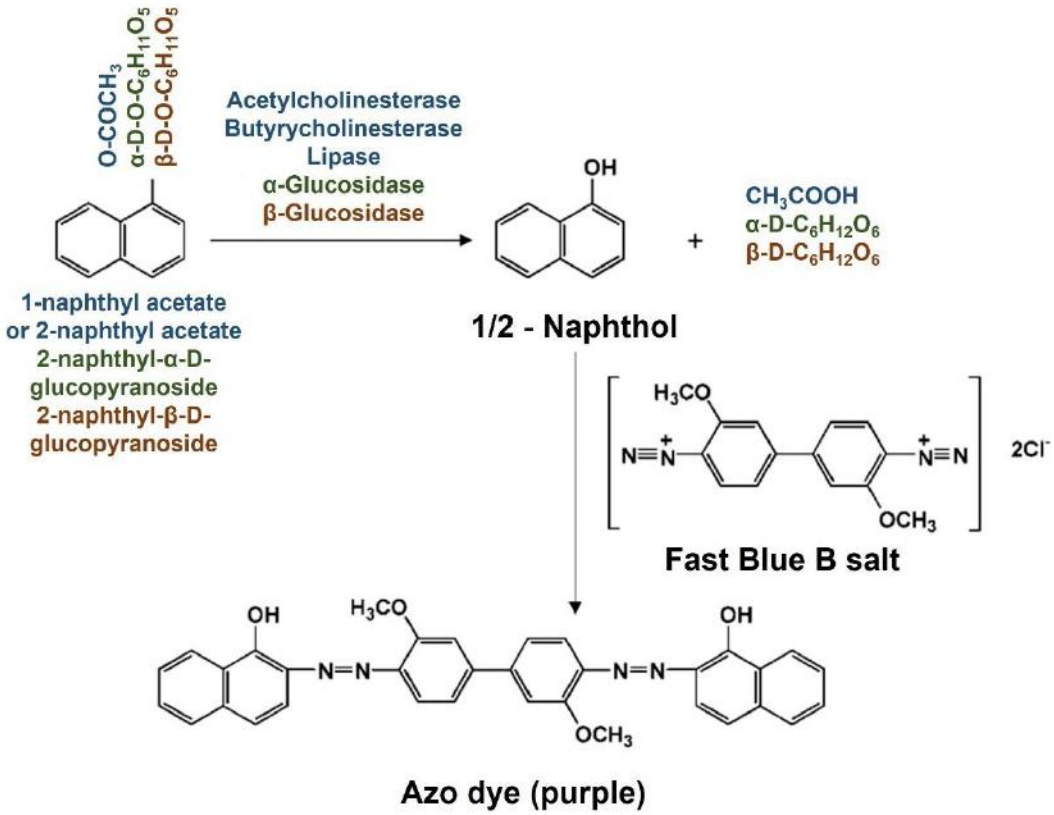

Figure 2. Scheme of enzyme inhibition assay based on diazonium reaction. *Note*. **D1**.

For example, the screening of enzymes (*i.e.*, lipase, AChE, BChE, α-glucosidase, β-glucosidase) inhibitors was based on a diazonium reaction (**Figure 2**). The enzyme cleaves the acetyl group from the substrate (*i.e.*, 1-naphthyl acetate or 2-naphthyl acetate, 2-naphthyl-α-D-glucopyranoside, 2-naphthyl-β-D-glucopyranoside) in a humid atmosphere at 37 °C, giving as a result 1-naphthol. Then, 1-naphthol reacts with Fast Blue B salt (FBS) dye forming a purple color. Enzyme inhibitors are displayed as white zones against a purple background.

Targeted and non-targeted analyses of *R. rosea* products were performed after optimizing separation conditions and parameters. The schematic representation of planar chromatography profiling is presented in **Figure 3**.

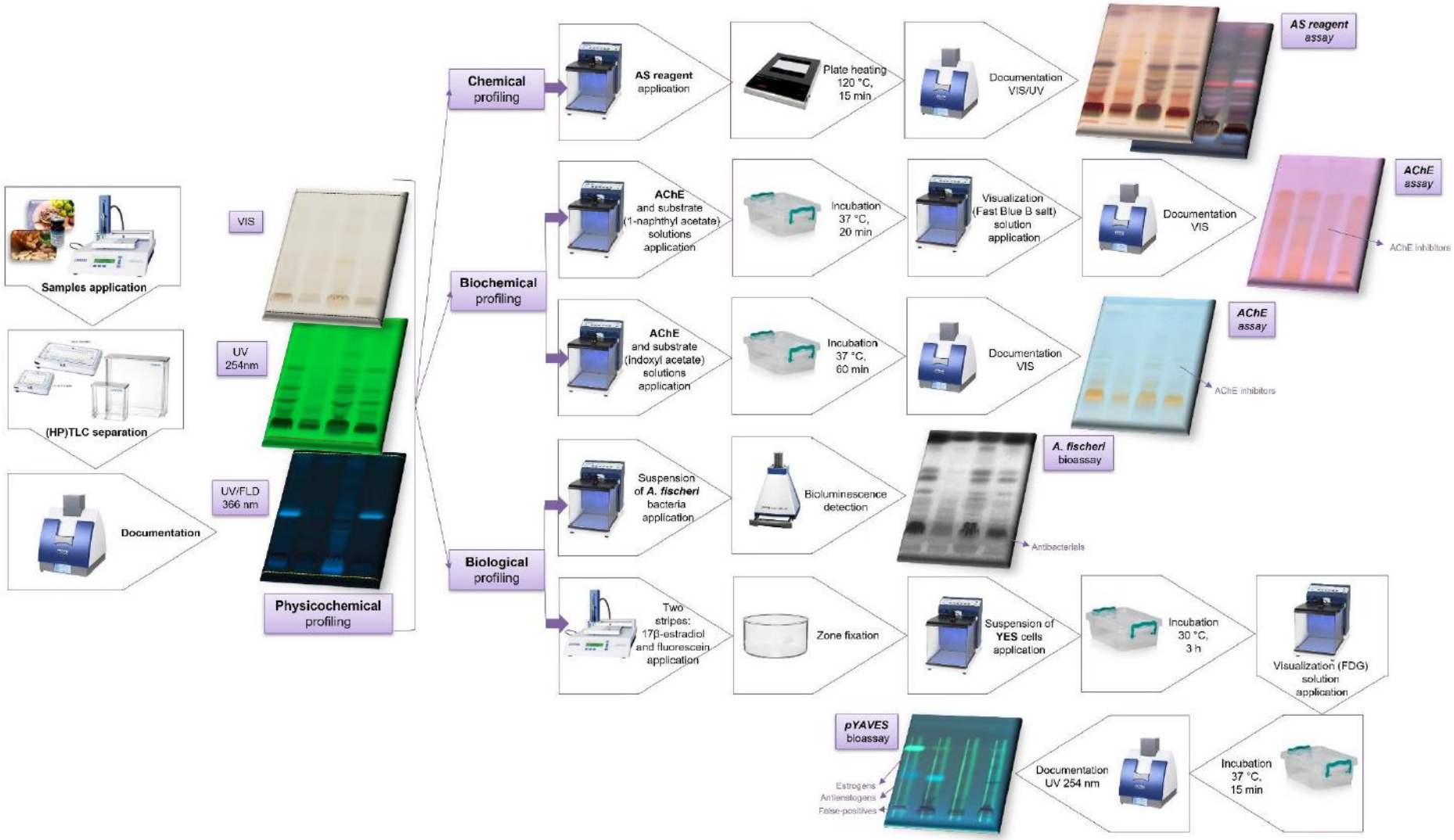

Figure 3. Scheme of physicochemical, chemical, biochemical, and biological profiling based on planar chromatography.

4.2. Targeted effect-directed detection

Targeted analyses were performed simultaneously for six *R. rosea* products (**D2-D5**, IDs S1–S7, **Table 2**, *§5.1.*), in comparison to the United States Pharmacopoeia (USP) reference standard of *R. rosea* root and rhizome and three markers; rosavin, salidroside, and *p*-tyrosol (active components characteristic of *R. rosea*, frequently used for evaluating the quality and authenticity of the plant material) [**D2-D5**].

The TLC-physicochemical profiles included the detection of natural dyes or pigments (VIS), UV absorbing compounds (UV 254 nm), and fluorophores (UV/FLD 366 nm).

The TLC-chemical profiles were based on the detection of functional groups using derivatization assays: general (AS and PMA reagents), specific (thymol and PABA reagents for sugars and glycosides detection, BCG reagent for organic acids detection, and NP–PEG reagent for polyphenols detection).

The TLC-bioprofiling was based on six non-targeted effect-directed (bio)assays which comprised TLC-microchemical profiles – antioxidant activity (1) using DPPH• assay, TLC-biochemical profiles also called enzyme inhibition profiles – enzyme inhibition activity against AChE (2), α-glucosidase (3), lipase (4), and tyrosinase (5) as well as TLC-bioprofiles – antibacterial activity (6) against Gram-positive *B. subtilis* bacteria strain.

The obtained TLC-profiles allowed the comparison of samples and the drawing of preliminary conclusions on their quality and activity. The bioactivity of rosavin, salidroside, *p*-tyrosol, and hydroquinone standards was tested simultaneously by co-application with *R. rosea* extracts followed by EDD. The presence of the abovementioned markers in *R. rosea* extracts was estimated using online TLC and TLC-densitometry as well as offline TLC-micro-preparative fractionation–HPLC–ESI-MS, HPLC-DAD, and ^{1}H NMR analysis. The quantitative estimation of extracts composition regarding the markers was performed using HPLC-DAD analysis.

Table 2. List of the *R. rosea* commercially available products studied in the thesis.

Sample form	Dosage form	Product name	Manufacturer	Country of manufacture	Country of plant origin	D6	D5	D4	D2-D3
						§5.2.	*§5.1.*	*§5.1.*	*§5.1.*
						IDs			
Root powder	Powder	Rhodiola-Rosenwurz	Vitaideal Vegan	Netherlands	Netherlands	**1**			
Root dry extract 25:1	Capsule	Rhodiola rosea	Green Naturals	Germany	Germany	**2**			
Root dry extract 20:1	Powder	Różeniec górski	Proherbis	Poland	China	**3**			
Dried root	Dried root	Korzeń różeńca górskiego	Farmvit	UK	-	**4**	**S6**		
Root dry extract, 520 mg	Capsule	Różeniec górski	Medica herbs	Poland	-	**5**			
Root dry extract, 500 mg	Capsule	Rhodiola	Now Foods	USA	-	**6**			
Dried rhizome	Dried rhizome	Kłącze różeńca górskiego	Dary natury	Poland	-	**7**	**S4**		
Root and rhizome dry extract	Powder	Rhodiola rosea	Sigma Aldrich	USA	Canada	**8**	**S5**	USP	
Dried root and rhizome	Dried root and rhizome	Różeniec górski	Natvita	Poland	Russia	**9**	**S1**	Natvita	Rhodiola
Powdered root, 400 mg	Capsule	Rhodiola	Fushi	UK	China	**10**	**S2**	Fushi	
Root dry extract, 250 mg, with additives	Capsule	Rhodiola	Solgar	USA	-	**11**			
Root dry extract, 500 mg, with inulin	Capsule	Rhodiola	ForMeds	Poland	-	**12**			
Root dry extract, 140 mg, with additives	Capsule	Rhodiola	Pharmovit	Poland	-	**13**	**S3**		
Root dry extract, 100 mg	Tablet	Różeniec górski	Herbapol	Poland	-	**14**			
Root powder, 225 mg	Tablet	Arktyczny korzeń	Altermedica Laboratories	Poland	-	**15**	**S7**		

4.3. Non-targeted effect-directed detection

Non-targeted analyses were performed simultaneously for fifteen different *R. rosea* products (**D6**, IDs S1–S15, **Table 2**, *§5.2.*), including the USP reference standard of *R. rosea* root and rhizome on HPTLC plates. The extracts were subjected to non-targeted EDD with eleven different assays to determine fifteen different bioactivities due to two triplex bioassays [**D6**].

The HPTLC-bioprofiling comprised HPTLC-microchemical profiles – antioxidant activity (1) using DPPH• assay, HPTLC-biochemical profiles – inhibition of AChE (2), butyrylcholinesterase (BChE) (3), α-amylase (4), β-glucuronidase (5), and tyrosinase (6), as well as HPTLC-bioprofiles including antimicrobial activity against Gram-negative *A. fischeri* (7) and Gram-positive *B. subtilis* (8) bacteria strains, genotoxicity using SOS-Umu-C bioassay (9) as well as agonistic and antagonistic verified effects on the endocrine activity of estrogens using pYAVES bioassay (10-12) or androgens using pYAVAS bioassay (13-15).

The detected active zones were further characterized using online HPTLC–HPLC–HESI-HRMS analysis with direct access to zones of interest at ambient and free of contamination conditions for the assignment of a potential candidate or substance that shows this activity.

Full information on the protocols, methods, techniques, and materials is presented in detail in the research methodologies in the publications [**D2-D6**].

5. OVERVIEW OF RESULTS

5.1. Targeted effect-directed analysis of R. rosea

The experiment began with the optimization of the TLC-chromatographic system and sample preparation [**D2**]. Using a visible comparison of chromatograms under UV 254 nm, UV/FLD 366 nm, as well as AS and thymol reagents along with DPPH• assay, the best sample preparation procedure was chosen – that is the maceration in 70% methanol for 72 h in a dark place at room temperature; the ratio of plant material – solvent was 1:10 g/mL. This extract had the richest composition and displayed a strong antioxidant activity. Six different mobile phases and two stationary phases were compared for two extracts (freshly prepared (S1) and one-year-old (S1*)) of *R. rosea* (**Table 2**, *§5.1.*).

Comparing the obtained chromatograms (**Figure 4**) the optimal separation system for *R. rosea* extracts was found: silica gel 60 F_{254} as a stationary phase and ethyl acetate−methanol−water (7.7:1.3:1, *V/V/V*) as a mobile phase. Additionally, the degradation of the extract was observed. In the subsequent experiments, only freshly prepared extracts were used.

Eventually, the experiment focused on the comparison of physicochemical (UV/FLD), chemical (derivatization), and biological/(bio)chemical (EDD) profiles of *R. rosea* extracts with that of the USP reference standard of *R. rosea* root and rhizome. In addition the following standards were co-applied three marker compounds (rosavin – authenticity marker inherent only in *R. rosea* species; salidroside and *p*-tyrosol – bioactivity markers) as well as hydroquinone, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, saccharose, glucose, and galactose (according to the literature constituents of *R. rosea*).

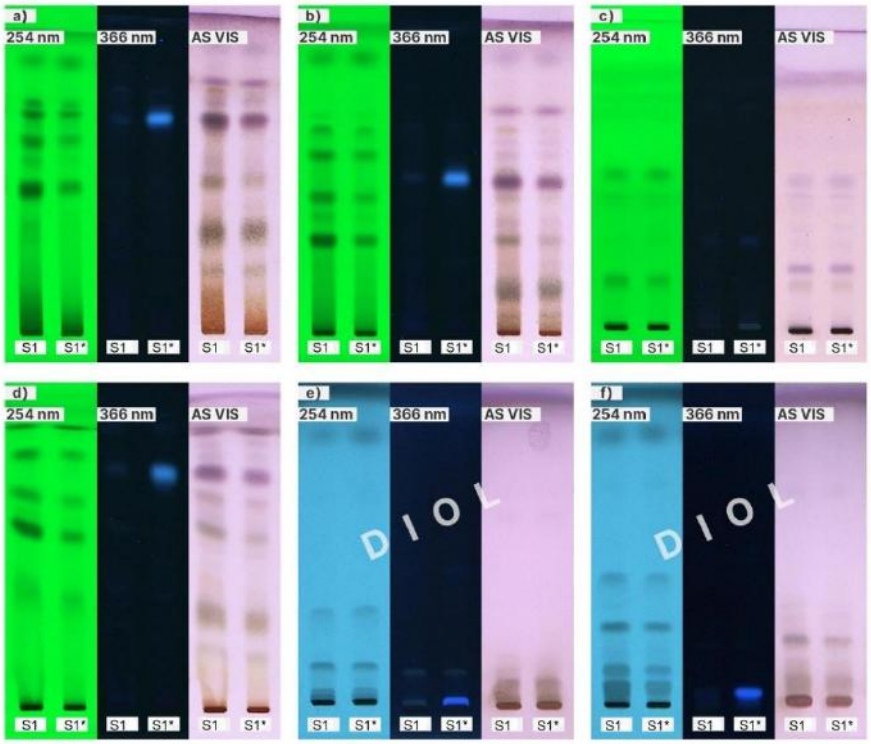

Figure 4. TLC chromatograms of the *R. rosea* extracts (S1 and S1*) separated on TLC plates silica gel 60 F_{254} (a-d) and diol (e-f) using ethyl acetate−methanol−water (7:3:1, *V/V/V*) (a); ethyl acetate−methanol−water (7.7:1.3:1, *V/V/V*) (b); toluene−ethyl acetate−formic acid (7:3:0.5, *V/V/V*) (c); chloroform−methanol−water (2.6:1.4:0.3, *V/V/V*) (d); ethyl acetate−chloroform (7.5:2.5, *V/V*) (e); ethyl acetate−chloroform−methanol (7:2.4:0.6, *V/V/V*) (f) mobile phases and detected at 254 nm, 366 nm and after AS assay white light illumination. *Note.* **D2**.

Verification and confirmation of the presence of the marker components in *R. rosea* extracts were performed using TLC assays, TLC-densitometry, and offline HPLC–ESI-MS analysis after semi-preparative fractionation [**D3-D4**].

At first, the presence of marker compounds of *R. rosea* was checked using chemical derivatization *i.e.*, AS, thymol, and NP–PEG assays (**Figure 5**). Detection of rosavin, salidroside, and *p*-tyrosol was found to be preferable using AS assay at 366 nm. In this case the compounds could be easily distinguished by their R_F values and colors; rosavin appears as a violet band at hR_F 24, salidroside as a green-brown at hR_F 47 and *p*-tyrosol as a brown band at hR_F 78. In the thymol assay, rosavin, salidroside, and *p*-tyrosol were visible as brown bands. However, the compounds could not be detected using the NP–PEG assay.

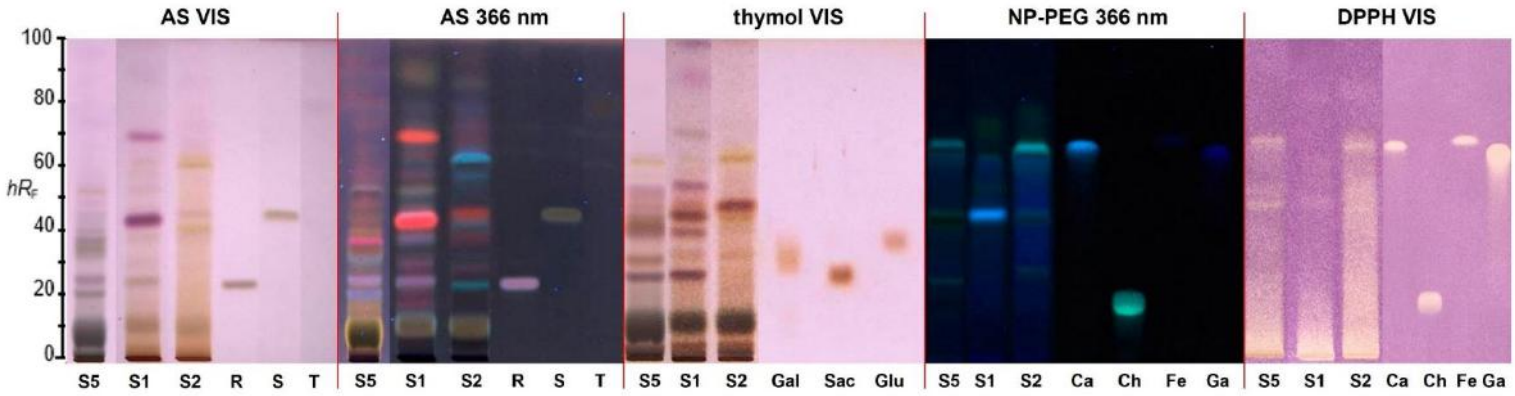

Figure 5. TLC chromatograms of the *R. rosea* extracts (IDs S5, S1−S2, **Table 2**, *§5.1.*; 500 µg/band each) and standards (5 µg/band each): rosavin (R), salidroside (S), *p*-tyrosol (T), hydroquinone (H), galactose (Gal), saccharose (Sac), glucose (Glu), caffeic acid (Ca), chlorogenic acid (Ch), ferulic acid (Fe), gallic acid (Ga) separated on TLC plates silica gel 60 F_{254} using ethyl acetate−methanol−water (7.7:1.3:1, *V/V/V*) and detected after respective assays at 366 nm and white light illumination. *Note*. Adapted from **D4**.

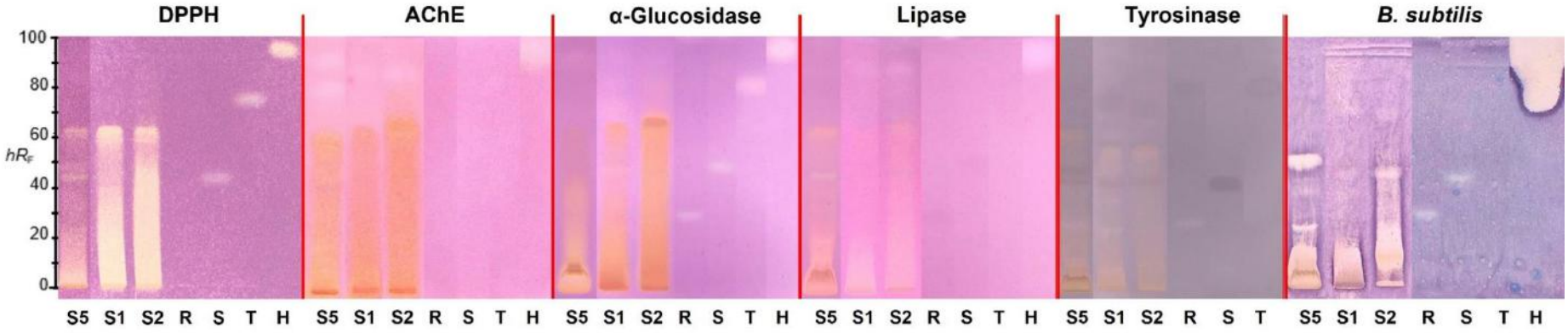

Figure 6. TLC (bio)autograms of the *R. rosea* extracts (IDs S5, S1−S2, **Table 2**, *§5.1.*; 500 µg/band each) and standards (5 µg/band each): rosavin (R), salidroside (S), *p*-tyrosol (T), hydroquinone (H) separated on TLC plates silica gel 60 F_{254} using ethyl acetate−methanol−water (7.7:1.3:1, *V/V/V*) and detected after respective (bio)assays at white light illumination. *Note*. Adapted from **D4**.

In the thymol assay, saccharose, glucose, and galactose were displayed as brown bands at hR_F 27, 36, and 31, respectively. NP–PEG and DPPH• assays were used for phenolic acids detection: caffeic acid appeared as a light blue-fluorescent band at hR_F 67, chlorogenic acid – as a green-fluorescent band at hR_F 18, ferulic acid a dark blue fluorescent band at hR_F 68, and gallic acid as a dark blue fluorescent band at hR_F 64; all acids displayed free radical scavenging activity. Interestingly, the detection of hydroquinone cannot be done using these derivatization assays, furthermore, hydroquinone was not visible at 254 nm and 366 nm wavelengths. Using AS, thymol, NP–PEG, and DPPH• assays, only rosavin could be positively confirmed in *R. rosea* extracts due to the characteristic pink color in AS 366 nm chromatogram; the presence of the other abovementioned compounds could not be stated unambiguously and it needed to be confirmed with other techniques such as TLC-densitometry or HPLC–ESI-HRMS analysis. This could be explained by low content of the compounds in extracts or the detection limits of these assays.

The bioactivity determination of standards was done for rosavin, salidroside, *p*-tyrosol, and hydroquinone using TLC-EDD assays (**Figure 6**, **Table 1**). Rosavin and *p*-tyrosol at 5 μg/band displayed α-glucosidase, lipase, and tyrosinase inhibitory activities, indicating that these components of *R. rosea* could be useful in the treatment of obesity, atherosclerosis, diabetes II type, viral infection, cancer, and skin diseases. Whereas, salidroside in the amount of 5 μg/band exhibited α-glucosidase and lipase inhibitory activity, which could be useful in the treatment of diabetes II type, viral infection, atherosclerosis, cancer, and obesity. Salidroside and *p*-tyrosol showed also antioxidant activity, while rosavin and salidroside displayed antibacterial activity against *B. subtilis*. Only hydroquinone at an amount of 5 μg/band responded positively against five effects (except tyrosinase inhibition). However, it seems that hydroquinone is not present in *R. rosea* extracts (at least not in detectable concentration) because these effects are not visible in the *R. rosea* chromatogram at the same hR_F.

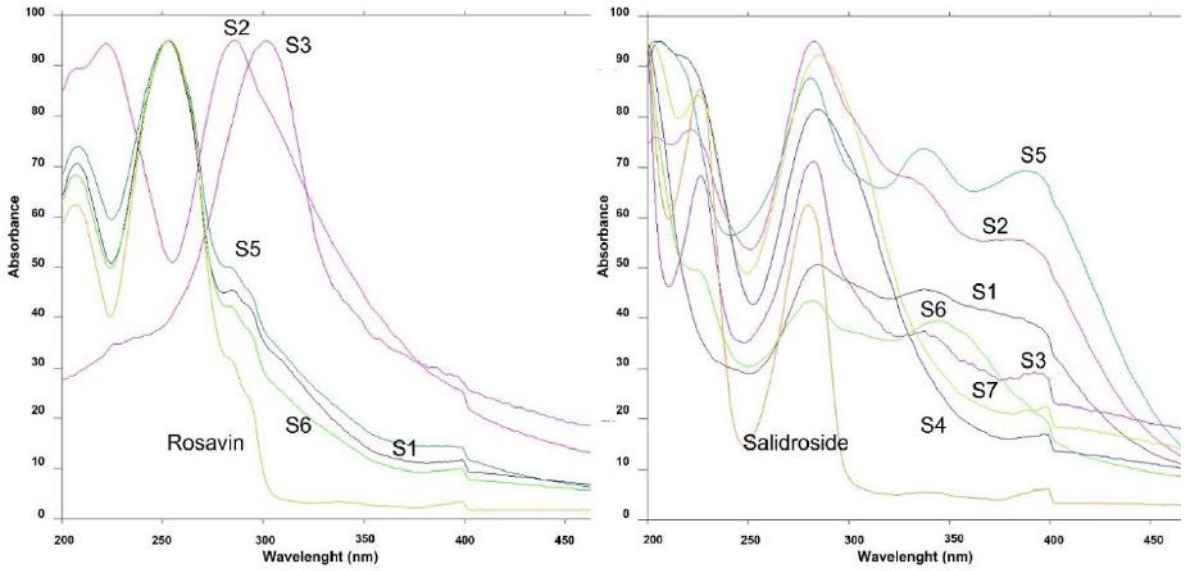

Figure 7. Spectra of rosavin (a) and salidroside (b) and corresponding to them components in the *R. rosea* extracts at the same hR_F values as for the standards (*i.e.*, 24 and 47, respectively); a) rosavin−yellow, S1−black, S2−pink, S3−violet, S5−blue, and S6−light green (**Table 2**, *§5.1.*) and b) salidroside −orange, S1−brown, S2−pink, S3−violet, S4−blue, S5−green, S6−light green, and S7−yellow (**Table 2**, *§5.1.*). *Note.* Unpublished data, adapted from **D3** (spectra of rosavin, salidroside, and S1 published) and from **D4** (spectra of rosavin, S1, S2, and S5, published).

TLC-densitometry was used to confirm the presence of rosavin, salidroside, *p*-tyrosol, and hydroquinone in *R. rosea* extracts. The spectra were measured directly on the TLC plate at the hR_F values corresponding to the components and compared with the spectra of the standards. The obtained spectra (**Figure 7**) confirmed the presence of rosavin only in the three *R. rosea* extracts including the USP reference standard of *R . rosea* (IDs S5−S6, and S1, **Table 2**, *§5.1.*), which was consistent with TLC-AS-366 nm assay results. The absence of rosavin in the other extracts could indicate adulteration of the product, poor quality, or improper manufacturing procedures, which caused the enzymatic degradation of rosavin. The spectra of the bands referring to salidroside migration distance were more complicated to interpret and had additional signals, which could have been caused by the co-elution of compounds at the same hR_F. Unfortunately, *p*-tyrosol and hydroquinone spectra such as those of the salidroside are too difficult to correctly interpret and draw any conclusions.

To confirm the presence of rosavin, salidroside, *p*-tyrosol, and other target compounds in *R. rosea* extracts, micro-preparative fractionation of three extracts (IDs S5, S1–S2, **Table 2**, *§5.1.*) was done. The seven fractions were scrapped-off, eluted, and subjected to HPLC–ESI-MS analysis along with the standards (rosavin, salidroside, *p*-tyrosol, hydroquinone, saccharose, glucose, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, and luteolin). The selection of the target components was based on the available literature. HPLC–ESI-MS analysis (**Table 3**) provided information (presence, molecular formulas, measured and theoretical monoisotopic masses well as masses differences) on twenty-four compounds in seven fractions of S1, S2, and S5 extracts (**Table 2**, *§5.1.*). In particular, in *R. rosea* extracts rosavin, rosarin, rosin, salidroside, viridoside, *p*-tyrosol, saccharose, glucose, galactose, caffeic acid, chlorogenic, ferulic acid, gallic acid, cinnamyl alcohol, kaempherol, luteolin-7-O-Glc, luteolin, apigenin-7-Glc, herbacetin, rhodiosin, rhodionin, rhodioloflavonoside, rhodiolgin, and hydroquinone were found. Rosavin and rosin were not detected in the S2 extract (**Table 2**, *§5.1.*). The absence of rosavin in the S2 extract was also observed in TLC and TLC-densitometry results.

The obtained TLC-profiles of seven *R. rosea* extracts, including UV and FLD ones, six chemical derivatization assays, and six effect-directed assays (**Figure 8**) allowed easy comparison, differentiation, and distinguishing of products along with bioactivity detection [**D4-D5**]. Chromatograms under UV/FLD lights point to the differences between samples based on the absorption of the 254 nm wavelength, as well as fluorescence properties. For example, only in two samples, S1 and S6 (**Table 2**, *§5.1.*) blue fluorescent bands at hR_F 45 and 50, respectively, were visible. Thymol and PABA-derived chromatograms showed a high amount of sugars or glycosides in *R. rosea* extracts. On the other hand, the BCG-derived chromatogram shows a low amount of acids at the start, as white bands against the blue background. NP–PEG assay revealed the presence of polyphenols in *R. rosea* extracts (different color fluorescent bands).

Table 3. HPLC–ESI-HRMS results for the TLC fractions of the *R. rosea* extracts (**Table 2**, *§5.1.*).

Compounds	Molecular formula	Molecular formula $[M-H]^-$ ion	Calculated monoisotopic ion mass (Da)	Observed monoisotopic ion mass (Da)	Mass error (Δ ppm)	S5	S1	S2
Rosavin	$C_{20}H_{28}O_{10}$	$C_{20}H_{27}O_{10}$	427.16043	427.16044	0.03	+	+	–
Rosarin	$C_{20}H_{28}O_{10}$	$C_{20}H_{27}O_{10}$	427.16043	427.16049	0.15	+	+	–
Rosin	$C_{15}H_{20}O_6$	$C_{15}H_{19}O_6$	295.11817	295.11818	0.05	+	+	+
Salidroside	$C_{14}H_{20}O_7$	$C_{14}H_{19}O_7$	299.11308	299.11310	0.07	+	+	+
***p*-Tyrosol**	$C_8H_{10}O_2$	$C_8H_9O_2$	137.06025	137.06022	0.23	+	+	+
Viridoside	$C_{15}H_{22}O_7$	$C_{15}H_{21}O_7$	313.12873	313.12882	0.29	+	+	+
Hydroquinone	$C_6H_6O_2$	$C_6H_5O_2$	109.02896	109.02894	0.10	+	+	+
Galactose	$C_6H_{12}O_6$	$C_6H_{11}O_6$	179.05557	179.05552	0.25	+	+	+
Sucrose	$C_{12}H_{22}O_{11}$	$C_{12}H_{21}O_{11}$	341.10839	341.10843	0.12	+	+	+
Glucose	$C_6H_{12}O_6$	$C_6H_{11}O_6$	179.05557	179.05559	0.13	+	+	+
Caffeic acid	$C_9H_8O_4$	$C_9H_7O_4$	179.03444	179.03437	0.36	+	+	+
Chlorogenic acid	$C_{16}H_{18}O_9$	$C_{16}H_{17}O_9$	353.08726	353.08741	0.42	+	+	+
Ferulic acid	$C_{10}H_{10}O_4$	$C_{10}H_9O_4$	193.05009	193.05009	0.02	+	+	+
Gallic acid	$C_7H_6O_5$	$C_7H_5O_5$	169.01370	169.01366	0.24	+	+	+
Cinnamyl alcohol	$C_9H_{10}O$	C_9H_9O	133.06534	133.06529	0.38	+	+	+
Kaempferol	$C_{15}H_{10}O_6$	$C_{15}H_9O_6$	285.03992	285.03999	0.26	+	+	+
Luteolin-7-O-Glc	$C_{21}H_{20}O_{11}$	$C_{21}H_{19}O_{11}$	447.09274	447.09271	0.07	+	+	+
Luteolin	$C_{15}H_{10}O_6$	$C_{15}H_9O_6$	285.03992	285.03998	0.23	+	+	+
Apigenin-7-Glc	$C_{21}H_{20}O_{10}$	$C_{21}H_{19}O_{10}$	431.09783	431.09780	0.06	+	+	+
Herbacetin	$C_{15}H_{10}O_7$	$C_{15}H_9O_7$	301.03483	301.03489	0.20	+	+	+
Rhodiosin	$C_{27}H_{30}O_{16}$	$C_{27}H_{29}O_{16}$	609.14557	609.14556	0.02	+	+	+
Rhodionin	$C_{21}H_{20}O_{11}$	$C_{21}H_{19}O_{11}$	447.09274	447.09284	0.22	+	+	+
Rhodioflavonoside	$C_{27}H_{30}O_{17}$	$C_{27}H_{29}O_{17}$	625.14048	625.14049	0.02	+	+	+
Rhodiolgin	$C_{21}H_{20}O_{12}$	$C_{21}H_{19}O_{12}$	463.08766	463.08772	0.14	+	+	+

Note. Adapted from **D4**.

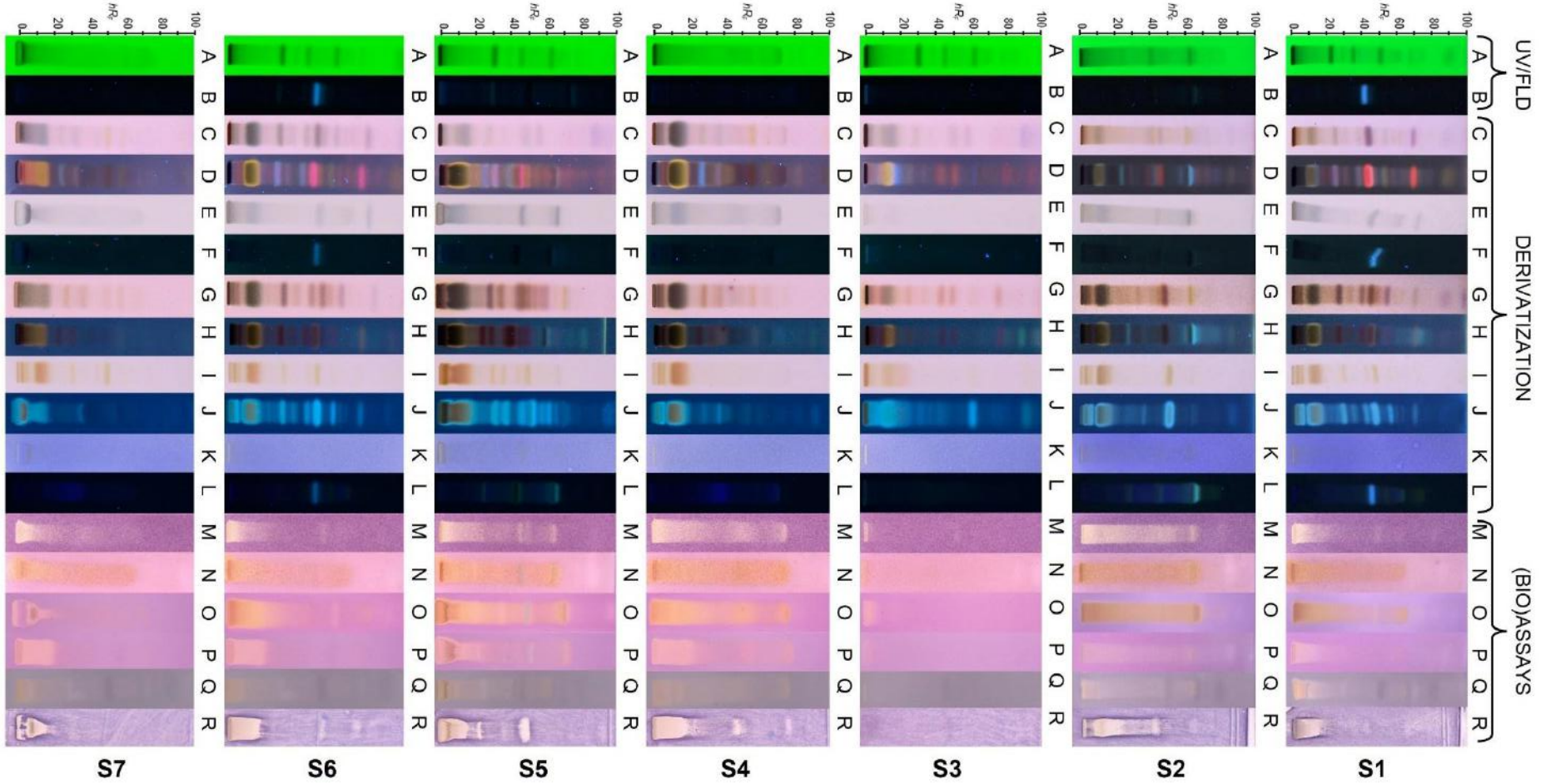

Figure 8. TLC-profiles of the *R. rosea* extracts (IDs S1−S7, **Table 2**, *§5.1.*; 500 µg/band each) separated on TLC plates silica gel 60 F_{254} using ethyl acetate−methanol−water (7.7:1.3:1, *V/V/V*) and detected at 254 nm (A) and 366 nm (B) before (for comparison) and after respective derivatization assays AS VIS (C), AS 366 nm (D), PMA VIS (E), PMA 366 nm (F), thymol VIS (G), thymol 366 nm (H), PABA VIS (I), PABA 366 nm (J), BCG VIS (K), NP–PEG 366 nm (L), and after respective (bio)assays DPPH• VIS (M), AChE VIS (N), α-glucosidase VIS (O), lipase VIS (P), tyrosinase VIS (Q), and *B. subtilis* VIS (R). *Note*. Adapted from data published in **D4** and **D5**.

Interpretation of (bio)assays was done accordingly to **Table 1**. The samples S3 and S7 (**Table 2**, *§5.1.*) showed no or little responses to (bio)assays and different chemical and UV profiles from the other samples, especially from the USP reference standard of *R. rosea* (ID S5, **Table 2**, *§5.1.*). This could be explained by a low amount of *R. rosea* in the products (**Table 2**) or their poor quality. The *R. rosea* profiles of S1, S2, S4, S5, and S6 (**Table 2**, *§5.1.*) were similar but not identical. It should be noted that the contents of components as well as their bioactivity depend on many external factors such as harvest, region, climate, *etc.*, which were not specified by the producers. Interestingly enough, the S2 extract gave strong responses to the (bio)assays although rosavin was missing in the extract. Nevertheless, the bioactivity of *R. rosea* extracts at the amount of 500 µg/band each includes the inhibition of AChE, α-glucosidase, lipase, and tyrosinase as well as free radical scavenging, and antibacterial against *B. subtilis*. Unfortunately, the interpretation of the results could interfere with the wide brown color zones resulting from the reaction between FBS and polyphenols present in *R. rosea* extracts. Especially these concerned assay protocols where FBS solution is used as a visualization reagent (**Figure 8**, tracks N, O, P). This was most visible in the case of the detection of AChE inhibitors in *R. rosea.* Nevertheless, the results indicate that *R. rosea* extracts could be very useful in the treatment of AD, PD, diabetes II type, obesity, cancer, viral infection, or skin disorders. Additionally, a strong antioxidant activity could help to prevent the aging of different tissues, osteoporosis, or stress.

Finally, quantitative estimation of rosavin, salidroside, and *p*-tyrosol in seven *R. rosea* extracts (**Table 2**, *§5.1.*) was done using HPLC-DAD (**Table 4**) [**D5**]. The absence of rosavin was observed in four extracts (IDs S2–S4, and S7, **Table 2**, *§5.1.*), which was confirmed by TLC-profiles and TLC-densitometry, as well as HPLC–ESI-MS analysis (for S2 extract only). Surprisingly, only three extracts (IDs S1, S5, and S6, **Table 2**, *§5.1.*) contained all three markers.

Table 4. Contents of rosavin, salidroside, and *p*-tyrosol in the *R. rosea* extracts by HPLC-DAD analysis.

ID	Content ± SD (µg/mL)		
	Rosavin	Salidroside	*p*-Tyrosol
S1	59.990±3.738	26.373±0.717	2.734±0.504
S2	ND	13.117±0.893	4.249±0.331
S3	ND	ND	ND
S4	ND	ND	ND
S5	100.457±5.438	37.861±3.937	4.940±0.116
S6	84.709±5.539	21.331±4.508	2.612±0.520
S7	ND	ND	ND

ND – not detected. *Note.* **D5**.

Important to note is that the highest contents of rosavin, salidroside, and *p*-tyrosol were found in the USP reference standard of *R. rosea*. The optimal standardized ratio of rosavin to salidroside *i.e.*, 3:1 was approximately preserved in three samples (IDs S1, S5, and S6, **Table 2**, *§5.1.*).

For the reliable identification of rosavin, salidroside, and *p*-tyrosol in the tested *R. rosea* extracts ^{1}H NMR spectra of extracts and standards were obtained. Based on the characteristic signals of marker compounds in the specific regions of ^{1}H NMR spectra, rosavin, salidroside, and *p*-tyrosol were identified (**Table 4**). Three products (IDs S1, S5, and S6, **Table 2**, *§5.1.*) undoubtedly contained rosavin, salidroside, and *p*-tyrosol. In the S3, S4, and S7 extracts (**Table 2**, *§5.1.*) the amounts of the markers were very low or non-detectable. ^{1}H NMR results correlate with TLC-profiles, TLC-densitometry, HPLC–ESI-MS, and HPLC-DAD results.

Table 5. Presence of marker compounds in the *R. rosea* extracts by ^{1}H NMR analysis.

ID	Rosavin	Salidroside	*p*-Tyrosol
S1	+	+	+
S2	ND	+	+
S3	ND	Traces	ND
S4	ND	Traces	ND
S5	+	+	+
S6	+	+	+
S7	ND	Traces	ND

ND – not detected. *Note.* **D5**.

5.2. Non-targeted effect-directed analysis of R. rosea

Non-targeted effect-directed profiling was performed for an extended number of *R. rosea* products. HPTLC plates were developed with slight modifications to the previously used mobile phase, such as a small variation in the solvents ratio and the addition of acetic acid, *i.e.*, ethyl acetate–methanol–water–acetic acid (7:1.5:1.5:0.1, *V/V/V/V*). The new chromatographic system yielded sharper bands; however, the presence of acid in the mobile phase required neutralization before performing the (bio)assays. The sample preparation procedure was changed from maceration to a faster sonification extraction. Using a new chromatographic system eleven different effect-directed assays were performed to evaluate bioactivity and pointed out the antioxidants, antibacterials, enzyme inhibitors as well as genotoxic, and hormone-effective compounds in the *R. rosea* extracts. The results are presented in **Figures 9–11** and their interpretation was done accordingly to **Table 1**. The six most active zones were characterized using straightforward hyphenation HPTLC–HPLC–HESI-HRMS analysis (**Table 5**) [**D6**].

The DPPH• assay (**Figure 9**) showed that *R. rosea* extracts possess very strong free radical scavenging activity even at an amount of 20 μg/band. In addition to the antioxidant potential, 400 μg/band of *R. rosea* extracts revealed antibacterial activity against Gram-positive *B. subtilis* and Gram-negative *A. fischeri* bacteria strains (**Figure 9**). However, differences in the responses to both bacteria strains were observed. The antibacterial activity of IDs 2, 5, 13 and (**Table 2**, *§5.2.*) was very weak in comparison to the other samples. The most intense response against *A. fischeri* yielded extracts with IDs 6, 11, and 12 (**Table 2**, *§5.2.*). The USP reference standard of *R. rosea* (ID 8, **Table 2**, *§5.2.*) showed medium antioxidant and antibacterial effects compared to the other extracts. The extracts of IDs 2 and 13 displayed weak free radical scavenging activity, while extract ID 14 (**Table 2**, *§5.2.*) showed neither antioxidant nor antibacterial activity. Another observation was that *A. fischeri* strains were more sensitive to *R. rosea* extracts than *B. subtilis*.

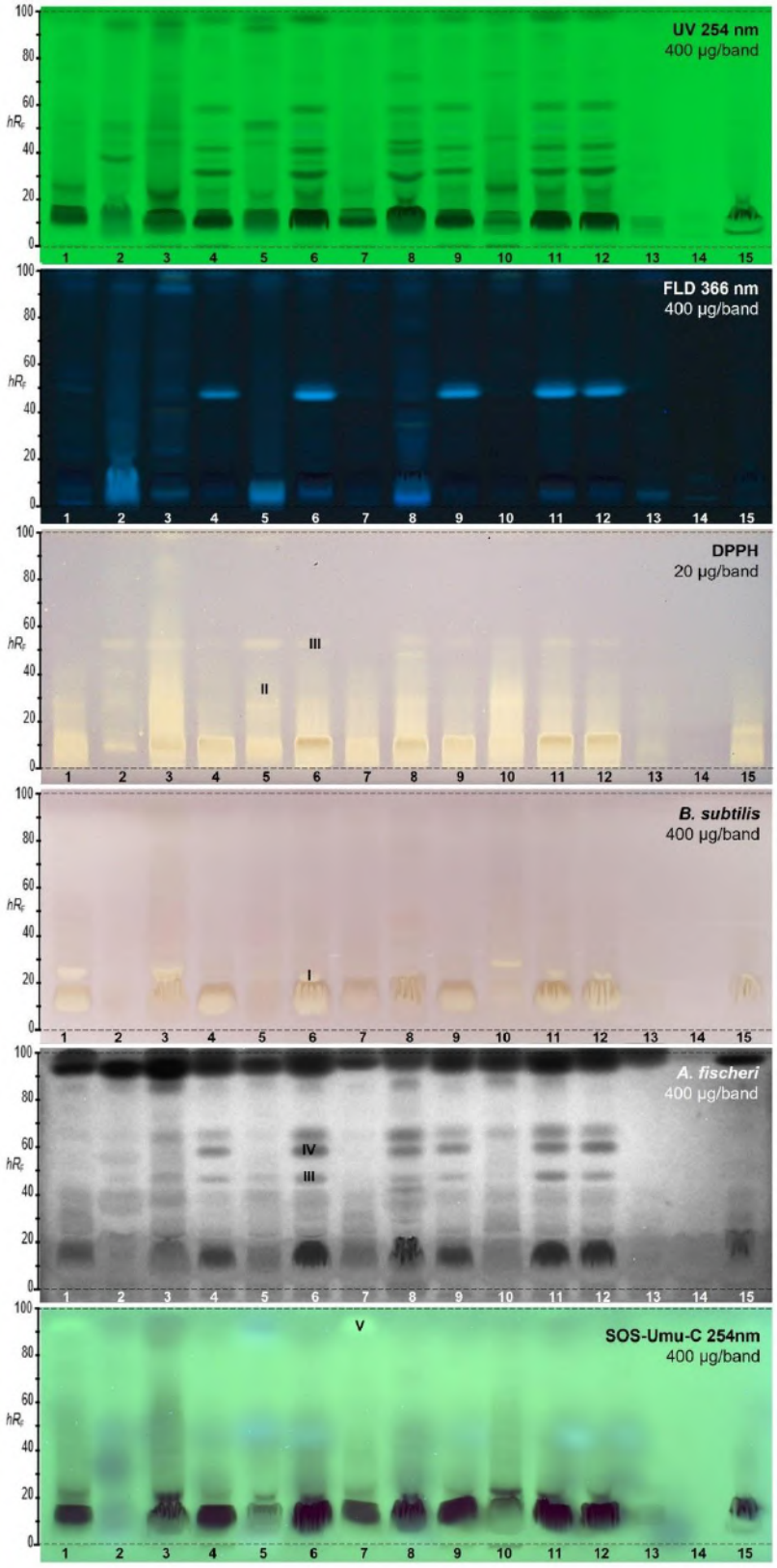

Figure 9. HPTLC-profiles of the *R. rosea* extracts (IDs 1−15, **Table 2**, *§5.2.*; 20−400 µg/band depending on the assay as indicated) separated on HPTLC plates silica gel 60 F_{254} (without F_{254} for the genotoxicity bioassay) using ethyl acetate−methanol−water−acetic acid (7:1.5:1.5:0.1, *V/V/V/V*) and detected at UV 254 nm and FLD 366 nm before (for comparison) and after the respective assays via the instant bioluminescence (*A. fischeri*), at FLD 254 nm (genotoxicity bioassay) or white light illumination. *Note.* Adapted from **D6**.

The SOS-Umu-C bioassay results (**Figure 9**) showed that two samples (IDs 1 and 7, **Table 2**, *§5.2.*) displayed genotoxicity at 400 µg/band (zone at hR_F 93, denoted as **V**). Zone **V** was later characterized by HPTLC–HPLC–HESI-HRMS analysis. The chromatogram appeared slightly diffused due to the 3.15 h incubation with the buffer (SOS-Umu-C bioassay procedure described in detail in **D6** and [55]), but genotoxic activity was still clearly visible. Fluorescein di-β-D-galactopyranoside (FDG) was used as a visualization solution because of the natural blue fluorescent compounds present in *R. rosea* extracts, which could be misleading in results interpretation. The presence of genotoxins in two *R. rosea* products emphasizes the necessity of quality evaluation of herbal products before they can be available for the consumer. Fortunately, the USP reference standard of *R. rosea* (ID 8, **Table 2**, *§5.2.*) did not contain genotoxins.

AChE and BChE inhibition assays (**Figure 10**) were performed with the indoxyl acetate substrate due to the interfering brown color caused by the FBS reagent. Two inhibition zones were observed at 100 µg/band of *R. rosea* extracts, the AChE inhibition zone at hR_F 51 (denoted as **III**) and the BChE inhibition zone at hR_F 61 (denoted as **IV**). The USP reference standard or *R. rosea* (ID 8, **Table 2**, *§5.2.*) inhibits both AChE and BChE. The extracts IDs 13–15 (**Table 2**, *§5.2.*) showed weak or no activity against AChE and BChE among all extracts. The results indicated that *R. rosea* extracts could be helpful in AD, PD, bipolar disorder, and diabetes II type treatment.

α-Amylase inhibition assay (**Figure 10**) was performed using another mobile phase. The elution power of the primary mobile phase (ethyl acetate–methanol–water–acetic acid 7:1.5:1.5:0.1 (*V/V/V/V*)) was found to be too strong for α-amylase inhibition compounds – one large active brown zone appeared on the solvent front. After mobile phase selection, the best one proved to be – ethyl acetate–hexane at a ratio of 3:7 (*V/V*). All *R. rosea* extracts showed an inhibitory activity at an amount of 400 µg/band.

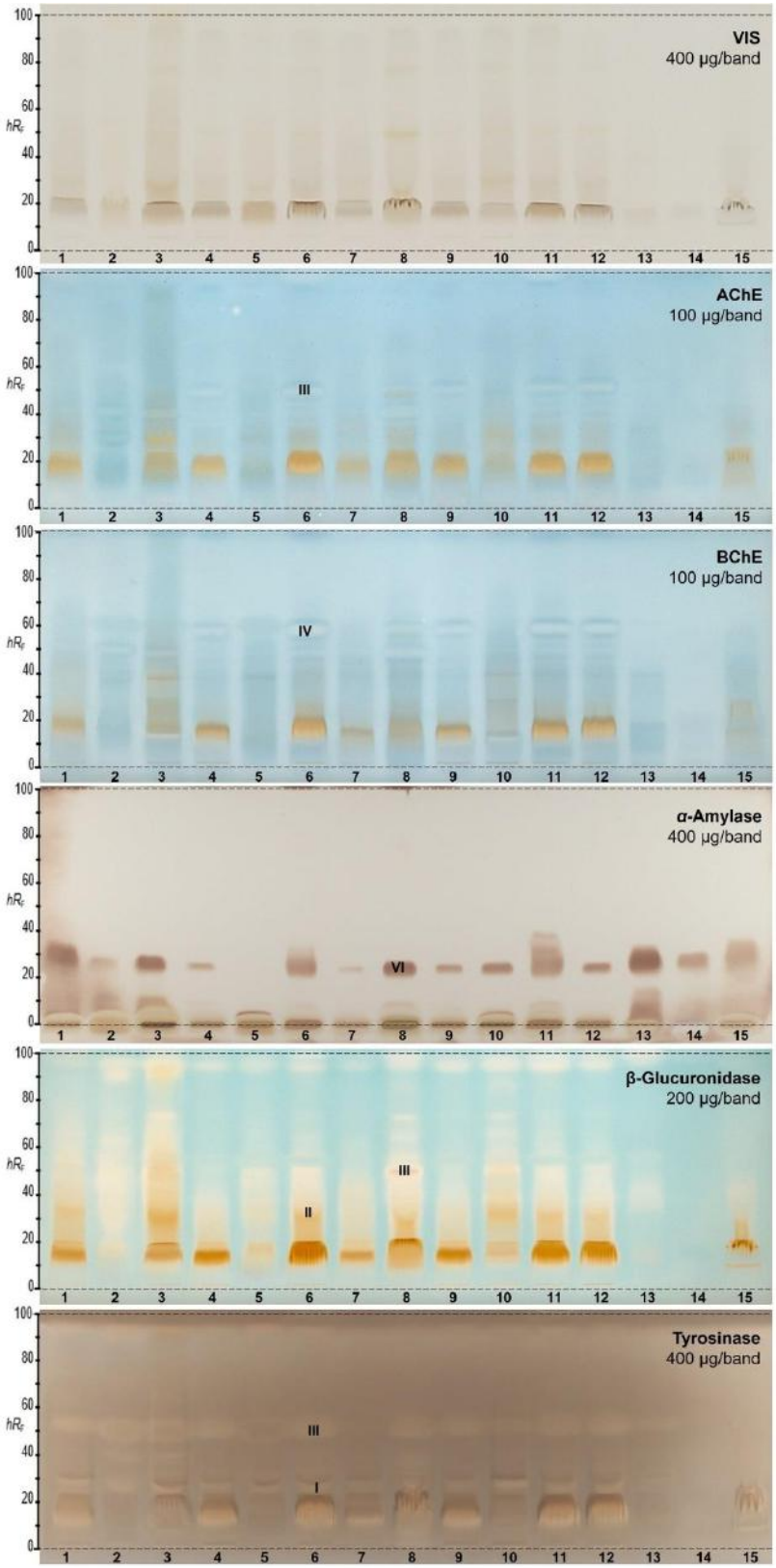

Figure 10. HPTLC-biochemical profiling of *R. rosea* (IDs 1−15, **Table 2**, *§5.2.*; 100−400 µg/band depending on the assays as indicated) separated on HPTLC plates silica gel 60 F_{254} using ethyl acetate−methanol−water−acetic acid (7:1.5:1.5:0.1, *V/V/V/V*) (for α-amylase assay was used ethyl acetate−n-hexane (3:7, *V/V*)) and detected before (for comparison) and after the respective assays at white light illumination. *Note*. Adapted from **D6**.

Two active zones were discovered at the start of a chromatogram and at hR_F 23 (denoted as **VI**). The **VI** zone was subjected to HPTLC–HPLC–HESI-HRMS recording. α-Amylase inhibition assay proved that *R. rosea* extracts could be useful in the treatment of diabetes II type.

The β-glucuronidase assay (**Figure 10**) displayed a strong inhibitory potential of β-glucuronidase at 200 µg/band of *R. rosea* extracts. Extracts IDs 1–3 and 10 (**Table 2**, *§5.2.*) showed stronger inhibition than the other extracts, while extract IDs 13–15 (**Table 2**, *§5.2.*) showed no or weak inhibitory potential against β-glucuronidase. The assay display that *R. rosea* extracts could be useful in the treatment of drug-induced gastrointestinal disorders.

The *R. rosea* extracts at an amount of 400 µg/band inhibited tyrosinase (**Figure 10**). Inhibition zones appeared at the bottom part of the track at hR_F 10 and 21 (denoted as **I**) and on the upper part at hR_F 51 (denoted as **III**) and at hR_F 90. The majority of the extracts contained every mentioned zone, but some showed weak (IDs 13 and 15) or no (ID 14) activity. The USP reference standard of *R. rosea* (ID 8, **Table 2**, *§5.2.*) displayed all the abovementioned zones. The results indicated that *R. rosea* extracts could be useful in the treatment of skin and dermatological diseases.

R. rosea extracts were also investigated for agonistic and antagonistic endocrine compounds via the planar triplex yeast antagonist-verified estrogen screen (pYAVES) and planar triplex yeast antagonist-verified androgen screen (pYAVAS) bioassays (**Figure 11**). Acetic acid addition was skipped in this mobile phase system to simplify the protocol (detailed in **D6** and [56]) and FDG was used as a substrate. The estrogen agonistic effect (pYAVES) is visible as a green fluorescent zone at hR_F 99 near the solvent front in extracts IDs 1–6, 8, 10, and 11 (**Table 2**, *§5.2.*). Extract ID 5 displayed the strongest activity compared to the other extracts. Estrogen antagonistic activity is visible as fluorescence reduction on the first stripe (17β-estradiol – known estrogen). It was observed, *e.g.*, at hR_F 90 in extracts IDs 1, 4, 6, and 7 and at hR_F 38 and 48 in extracts IDs 9, 11, and 12 (**Table 2**, *§5.2.*).

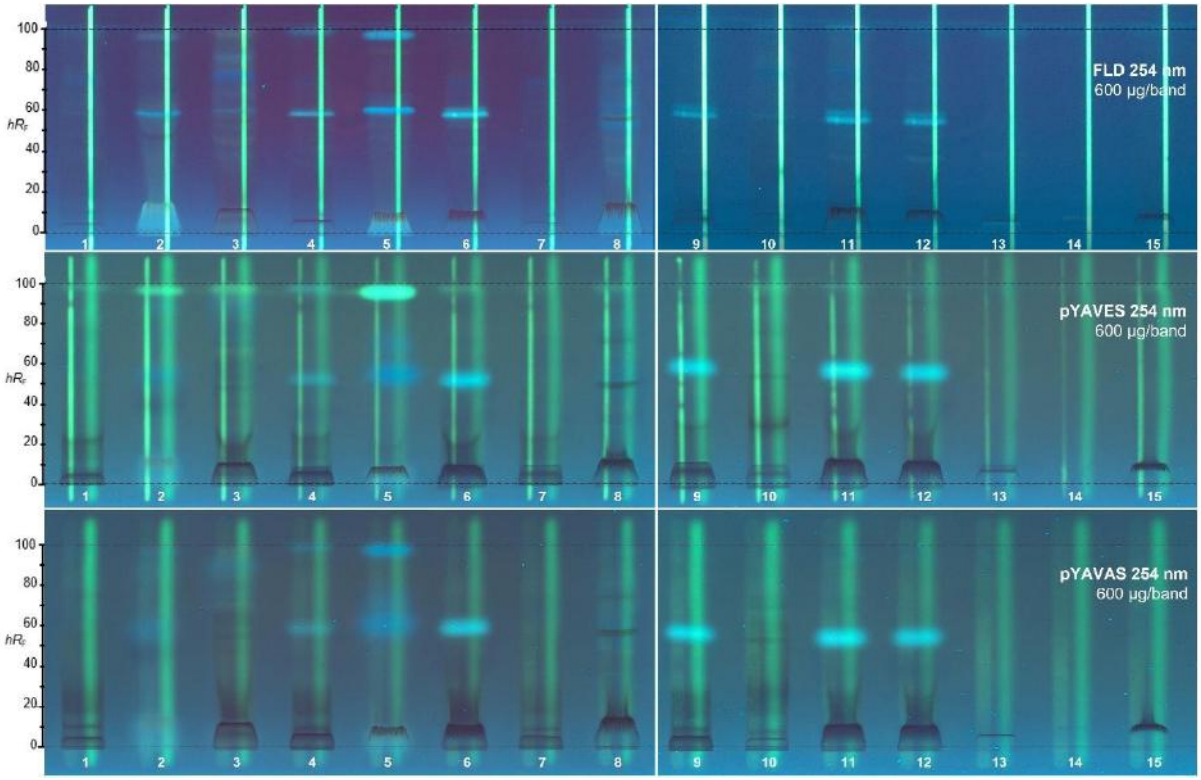

Figure 11. HPTLC pYAVES/pYAVAS bioautograms of the *R. rosea* extracts (IDs 1−15, **Table 2**, *§5.2.*; 600 µg/band each) separated on HPTLC plates silica gel 60 using ethyl acetate−methanol−water (7:1.5:1.5, *V/V/V*) and detected at FLD 254 nm before (for comparison) and after the respective bioassay. *Note*. **D6**.

Application of the second stripe (fluorescein – end-product) allows verification of the anti-estrogen effect, *e.g.*, fluorescence reduction zones at hR_F 0–10 in extracts IDs 1, 3–13 and 15 (**Table 2**, *§5.2.*) are not related to antagonist activity but are probably caused by natural dyes or pigments in *R. rosea* extracts. However, the pYAVAS bioassay displayed no androgen (no visible green fluorescent zones) or verified antiandrogen (no reduction on first (testosterone – known androgen) stripe) activity of *R. rosea* extracts at 600 µg/band.

The six most active compound zones (**I–V** in **Figure 9** and **VI** in **Figure 10**) were selected and subjected to HPTLC–HPLC–HESI-HRMS analysis. Ten different compounds were tentatively assigned to selected zones based on the HRMS spectra obtained in positive and negative ionization modes (**Table 6**).

Table 6. HPTLC−HPLC−HESI-HRMS results for the six active compound zones in the *R. rosea* extracts.

Zone ID	hR_F	Tentative assignment	Molecular formula	Calculated mass (Da)	Observed mass (Da)	Mass error (ppm)	Adduct ions
I 6	20	**Rhodioloside D**	$C_{16}H_{30}O_8$	350.1941	409.2084 373.1828	-1.09 1.13	$[M+CH_3COO]^-$ $[M+Na]^+$
II 6	35	**Rosavin/ Rosarin**	$C_{20}H_{28}O_{10}$	428.1683	427.1610 451.1576	-0.01 -0.26	$[M-H]^-$ $[M+Na]^+$
III 6	51	**Rosiridin**	$C_{16}H_{28}O_7$	332.1835	391.1978 355.1722	-1.33 1.54	$[M+CH_3COO]^-$ $[M+Na]^+$
		Viridoside	$C_{15}H_{22}O_7$	314.1365	359.1351 337.1258	-1.06 -0.10	$[M+CHOO]^-$ $[M+Na]^+$
		Salidroside	$C_{14}H_{20}O_7$	300.1209	299.1139 323.1096	-0.74 1.57	$[M-H]^-$ $[M+Na]^+$
IV 6	61	**Rosin**	$C_{15}H_{20}O_6$	296.1260	355.1399 319.1146	-0.02 1.98	$[M+CH_3COO]^-$ $[M+Na]^+$
V 1	93	**Hydroquinone**	$C_6H_6O_2$	110.0368	109.0294 111.0444	0.68 -2.74	$[M-H]^-$ $[M+H]^+$
VI 6	23	**Stearic acid**	$C_{18}H_{36}O_2$	284.2715	283.2643	-0.13	$[M-H]^-$
		Palmitic acid	$C_{16}H_{32}O_2$	256.2402	255.2330	-0.26	$[M-H]^-$

Note. Adapted from **D6**.

The active zone **I** at hR_F 20, with antibacterial activity against *B. subtilis* and tyrosinase inhibition was tentatively assigned to rhodioloside D.

The active zone **II** with strong antioxidant and β-glucuronidase inhibition activity at hR_F 35 was tentatively assigned to two compounds, rosavin, and rosarin.

The active zone **III** at hR_F 51, visible in five different assays (antioxidants, antibacterials against *A. fischeri*, and inhibitors of AChE, tyrosinase, and β-glucuronidase), was tentatively assigned to rosiridin, viridoside, and salidroside. However, our previous results of the AChE assay (**Figure 6**), proved that the standard of salidroside did not display inhibition of AChE. It means that AChE inhibitors from zone **III** are rosiridin and viridoside. Possible synergetic effect of these three compounds should be also taken into account.

Zone **IV** at hR_F 61 (BChE inhibition and antibacterial against *A. fischeri* activities) was tentatively assigned to rosin.

Hydroquinone, zone **V** at hR_F 93, was tentatively characterized as a compound responsible for the genotoxic activity in the *R. rosea* extracts.

The mass spectra for zone **VI** at hR_F 23 exhibited signals corresponding to stearic and palmitic acids. These compounds were tentatively assigned as α-amylase inhibitors in the *R. rosea* extracts.

6. CONCLUSIONS

The unique chromatographic profiles of *R. rosea* were acquired (using targeted and non-targeted effect-directed analysis based on a combination of planar chromatography, (bio)assays, and spectroscopic methods) to reveal the *R. rosea* chemical composition, and bioactivities.

The main achievements of the doctoral thesis can be summarized as follows:

- ✓ Bioprofiling *of R. rosea* extracts revealed 17 different activities of *R. rosea* including (1) antioxidant, (2-8) AChE, BChE, α-amylase, α-glucosidase, β-glucuronidase, lipase, and tyrosinase inhibition, (9-10) antibacterial against Gram-negative *A. fischeri*, and Gram-positive *B. subtilis* bacteria strains, (11) genotoxic, as well as (12-14) estrogen, and verified antiestrogen and (15-17) androgen and verified antiandrogen effects;
- ✓ The following activities of major compounds of *R. rosea* were detected:
  - rosavin – α-glucosidase, β-glucuronidase, lipase, and tyrosinase inhibition as well as an antibacterial against *B. subtilis*;
  - salidroside – antioxidant and inhibition of α-glucosidase, β-glucuronidase and lipase as well as antibacterial against *A. fischeri* and *B. subtilis*;
  - *p*-tyrosol – antioxidant as well as α-glucosidase, lipase, and tyrosinase inhibition;
  - rosarin – antioxidant and β-glucuronidase inhibition;
  - rosin – BChE inhibition and antibacterial against *A. fischeri*;
  - viridoside and rosiridin – antioxidant, and inhibition of AChE and β-glucuronidase as well as an antibacterial against *A. fischeri*;
  - rhodioloside D – tyrosinase inhibition and antibacterial against *B. subtilis*;
  - palmitic and stearic acids – α-amylase inhibition;

- hydroquinone – antioxidant, and inhibition of AChE, α-glucosidase, and lipase, and genotoxic as well as an antibacterial against *B. subtilis*.

✓ HPLC–ESI-MS offline analysis, as well as HPTLC−HPLC−HESI-HRMS online analysis, provided qualitative information on the presence of 29 compounds in *R. rosea* extracts, namely rosavin, rosarin, rosin, salidroside, *p*-tyrosol, viridoside, rhodioloside D, rosiridin, galactose, saccharose, glucose, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, palmitic acid, stearic acid, cinnamyl alcohol, kaempferol, luteolin, luteolin-7-O-Glc, apigenin, apigenin-7-Glc, herbacetin, rhodiosin, rhodionin, rhodioflavonoside, rhodiolgin, and hydroquinone.

✓ Qualitative and quantitative evaluation of rosavin, salidroside, and p-tyrosol (so-called marker compounds of *R. rosea*) by HPLC-DAD and ^{1}H NMR analysis revealed the presence of rosavin only in three, while salidroside and p-tyrosol only in four out of seven investigated extracts from commercially available formulations of *R. rosea*. The concentrations of the markers were calculated.

The obtained results, such as the presence of genotoxins in two and the absence of rosavin in four *R. rosea* products emphasize the importance of effect-directed profiling of plant materials for authenticity and quality verification as well as looking for any signs of adulterations.

Effect-directed analysis based on planar chromatography fills the gap in the relationship between chromatographic fingerprints, the efficacy of the medicinal plants, and pressing need for the quality control of herbal medicines. The method established here for *R. rosea* can be implemented for other medicinal plants, especially commercially available ones. The approaches presented in the thesis prove that planar chromatography is still updating, developing, and a challenging method of analysis as well as an excellent tool for a fast, precise, reliable, and robust detection of the activities of plants and their constituents.

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8. SCIENTIFIC BIOGRAPHY

Hanna Nikolaichuk is a PhD candidate at Maria Curie-Skłodowska University in the course of International Doctoral Study in Chemistry. Her thesis, entitled "Effect-directed analysis of *Rhodiola rosea* L. based on planar chromatography: the search for bioactive components" under the supervision of Prof. Irena Choma, explores *Rhodiola rosea* L., a medicinal plant popular in Europe and Asia and uncovers the components responsible for this activity.

In 2016, she completed a Bachelor of Science in Chemistry with a major in Chemistry from Lesya Ukrainka Volyn National University. Her thesis was entitled "Study of the fatty acid composition of avocado oil using gas chromatography". In 2018, she earned a Master of Science in Chemistry with a focus on the Chemistry of Bioactive substances and cosmetics from Maria Curie-Skłodowska University, based on the thesis entitled "Chromatography in searching for active components in *Rhodiola rosea*".

Her doctoral project was supported by a six-month research internship under supervision of Prof. Gertrud Morlock at Justus Liebig University Giessen, Germany, from September 2021 to February 2022.

In addition, she worked as a research-teaching assistant in the Department of Chromatography at Maria Curie-Skłodowska University from October 2019 to September 2022.

Hanna currently works as a research assistant at the Department of Bioanalytics at Medical University in Lublin on a project involving the identification of lipidomics markers for distinguishing the authenticity of edible oils under the supervision of Prof. Emilia Fornal.

She is the author and co-author of 8 peer-reviewed original research publications, 5 book chapters, 16 posters, and 25 oral presentations. She has received two awards, Dr. Heinz E. Hauck Young Scientist Prize, for an outstanding oral presentation at the 25th International Symposium for High-Performance Thin-Layer Chromatography and Diploma for 3rd place in The Best Oral Presentation at the UMCS Summer School for PhD students.

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9. SCIENTIFIC ACHIEVEMENTS

9.1. Publications:

1. **H. Nikolaichuk**, I.M. Choma, TLC screening in searching for active components in *Rhodiola rosea* L. roots. Annales Universitatis Mariae Curie-Skłodowska Lublin – Polonia, Section AA-Chemia LXXIV (2019) 1, pp. 55-64.
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9.2. Chapters:

1. **H. Nikolaichuk**, I.M. Choma, EDA i analiza skriningowa w poszukiwaniu biologicznie aktywnych związków w różeńcu górskim (*Rhodiola rosea* L.). Nauka i przemysł, lubelskie spotkania studenckie. Wydawnictwo UMCS, ISBN 978-83-227-9220-9, Lublin 2019, str.115-118.
2. **H. Nikolaichuk**, I.M. Choma, Dobór warunków analizy chromatograficznej związków bioaktywnych w Akebii pięciolistkowej. Nauka i przemysł, lubelskie spotkania studenckie. Wydawnictwo UMCS, ISBN 978-83-227-9370-1, Lublin 2020, str.191-194.
3. **H. Nikolaichuk**, I.M. Choma, Detekcja właściwości bioaktywnych *Clitoria ternatea* L. za pomocą bioautografii bezpośredniej. Nauka i przemysł, lubelskie spotkania studenckie. Wydawnictwo UMCS, ISBN 978-83-227-9503-3, Lublin 2021, str.165-168.
4. I.M. Choma, **H. Nikolaichuk**, TLC bioprofiling: a tool for quality evaluation of medical plants. Evidence-Based Validation of Herbal Medicine. Translational Research on Botanicals. (Pulok Mukherjee Ed. Second edition). Chapter 18, Elsevier 2022, pp. 407-422.
5. **H. Nikolaichuk**, M. Studziński, I.M. Choma, Bio-profilowanie preparatów różeńca górskiego za pomocą TLC-DB i HPLC-DAD. Nauka i przemysł, lubelskie spotkania studenckie. Wydawnictwo UMCS, ISBN 978-83-227-9603-0, Lublin 2022, str. 181-184.

9.3. Research projects:

Participation in the implementation of the project „Identyfikacja lipidomicznych biomarkerów rozpoznawania autentyczności olejów jadalnych wsparta profilowaniem DSC i chemometrią"
OPUS 16 grant 2018/31/B/NZ9/02762.
August 2020 – present.

9.4. Internships:

Justus Liebig University Giessen, Giessen, Germany.
September 2021 – February 2022.

9.5. Summer schools:

- Summer School in Pharmaceutical Analysis, September 22-24, 2021, Bologna, Italy.
- UMCS Summer School for PhD Students, May 18-20, 2022, Lublin, Poland.

9.6. Conferences:

Author of:

1. **H. Nikolaichuk**, I.M. Choma, E. Sobstyl, Analiza inhibitorów acetylocholinesterazy wybranych roślin za pomocą chromatografii cienkowarstwowej sprzężonej z bioautografią. II Ogólnopolskie Sympozjum Młodych Naukowców „ProDoc", April 12, 2019, Lublin, Poland. *Oral presentation*.
2. **H. Nikolaichuk**, E. Sobstyl, I. M. Choma, Effect directed analysis and TLC screening of chosen plants used in Traditional Chinese Medicine. 48th International Symposium on High-Performance Liquid Phase Separations and Related Techniques, June 16-20, 2019, Milan, Italy. *Oral presentation.*
3. **H. Nikolaichuk**, I.M. Choma, EDA i analiza skriningowa w poszukiwaniu biologicznie aktywnych związków w różeńcu górskim (*Rhodiola rosea* L.). VII Ogólnopolskie sympozjum „Nauka i przemysł, lubelskie spotkania studenckie", June 24, 2019, Lublin, Poland. *Oral presentation.*
4. **H. Nikolaichuk**, I. M. Choma, EDA and TLC screening in searching of biologically active components in plants. 6th International conference "Plant – the source of research material", September 10-12, 2019, Nałęczów, Poland. *Oral presentation.*
5. **H. Nikolaichuk**, E. Sobstyl, R. Typek, I. M. Choma, Detection and identification of biological active compounds present in *Rhodiola rosea* L. roots by TLC/bioassay/MS. 6th International conference, "Plant – the source of research material", September 10-12, 2019, Nałęczów, Poland. *Poster presentation.*
6. **H. Nikolaichuk**, I.M. Choma, Bioprofiling of *Rhodiola rosea* L. and *Akebia quinata* D. using effect directed analysis. QUO VADIS Life Sciences, June 23-27, 2021, Opole, Poland. *Oral presentation.*
7. **H. Nikolaichuk**, R. Typek, I.M. Choma, Biological activity of *Rhodiola rosea* L. preparations. QUO VADIS Life Sciences, June 23-27, 2021, Opole, Poland. *Poster presentation.*
8. **H. Nikolaichuk**, I.M. Choma, Detekcja właściwości bioaktywnych *Clitoria ternatea* L. za pomocą bioautografii bezpośredniej. IX Ogólnopolskie sympozjum „Nauka i przemysł, lubelskie spotkania studenckie", June 28, 2021, Lublin, Poland. *Poster presentation.*
9. **H. Nikolaichuk**, I.M. Choma, Effect directed detection of enzyme inhibitors in chosen plants. 8th International Congress of the Society for Ethnopharmacology-India, August 27-29, 2021, Pune, India. *Oral presentation.*

10. **H. Nikolaichuk**, I.M. Choma, Detection and identification of biological active compounds using effect directed analysis. Summer School in Pharmaceutical Analysis, September 22-24, 2021, Bologna, Italy. *Oral presentation.*
11. **H. Nikolaichuk**, I.M. Choma, Bio-profiling of plants via effect directed analysis coupling high-resolution mass spectrometry. Modern Research Techniques for Physicochemical Characterization of the Potential Application Systems, May 18-20, 2022, Lublin, Poland. *Oral presentation.*

    Diploma for 3rd place The Best Oral Presentation
12. **H. Nikolaichuk**, I.M. Choma, Effect directed analysis of biological active compounds present in *Rhodiola rosea* L. root and rhizome by HPTLC/bioassay/MS. Modern Research Techniques for Physicochemical Characterization of the Potential Application Systems, May 18-20, 2022, Lublin, Poland. *poster presentation.*
13. **H. Nikolaichuk**, I.M. Choma, E. Fornal, Detekcja bioaktywnych metabolitów roślin za pomocą wysokosprawnej chromatografii cienkowarstwowej w połączeniu z HRMS. XI Polska Konferencja Chemii Analitycznej, June 19-23, 2022, Łódź, Poland. *Oral presentation.*
14. **H. Nikolaichuk**, A. Kozub, K. Przykaza, J. Tomaszewska-Gras, E. Fornal, Wykrywanie markerów różnicujących w olekach z konopi, lnu i lnianki za pomocą LC/QTOF. XI Polska Konferencja Chemii Analitycznej, June 19-23, 2022, Łódź, Poland. *Poster presentation.*
15. **H. Nikolaichuk**, M. Studziński, I.M. Choma, Bio-profilowanie preparatów różeńca górskiego za pomocą TLC-DB i HPLC-DAD. X Ogólnopolskie Sympozjum „Nauka i przemysł – lubelskie spotkania studenckie", June 27, 2022, Lublin, Poland. *Poster presentation.*
16. **H. Nikolaichuk**, G. Morlock, I.M. Choma, HPTLC analysis coupling high-resolution mass spectrometry of plants. 25th International Symposium for High-Performance Thin-Layer Chromatography, June 28 – July 1, 2022, Ljubljana, Slovenia. *Oral presentation.*

    Dr. Heinz E. Hauck Young Scientist Prize for an outstanding oral presentation.
17. **H. Nikolaichuk**, G. Morlock, I.M. Choma, Evaluation of the biological activity of *Rhodiola rosea* L. using HPTLC/bioassay/MS. 25th International Symposium for High-Performance Thin-Layer Chromatography, June 28 – July 1, 2022, Ljubljana, Slovenia. *Poster presentation.*
18. **H. Nikolaichuk**, A. Kozub, K. Przykaza, J. Tomaszewska-Gras, E. Fornal, Profilowanie lipidomiczne wybranych olejów niszowych za pomocą LC-QTOF. 64. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, September 11-16, 2022, Lublin, Poland. *Oral presentation.*

19. **H. Nikolaichuk**, I.M. Choma, E. Fornal, Chromatografia cienkowarstwowa w połączeniu ze spektrometria mas w poszukiwaniu substancji aktywnych w klitorii ternateańskiej (*Clitoria ternatea* L.). 64. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, September 11-16, 2022, Lublin. *Poster presentation.*
20. **H. Nikolaichuk**, G. Morlock, I.M. Choma, Screening of *Akebia quinata* D. bioactivity via effect directed analysis based on HPTLC hyphenated HRMS. 33rd International Symposium on Chromatography, September 18-22, 2022, Budapest, Hungary. *Poster presentation.*

    Nominated for the poster prize (among the 14 best posters out of 222 posters).
21. **H. Nikolaichuk**, G. Morlock, I.M. Choma, Effect directed analysis of *Clitoria ternatea* L. flower based on HPTLC-MS. 33rd International Symposium on Chromatography, September 18-22, 2022, Budapest, Hungary. *Poster presentation.*

and co-author of:

22. I.M. Choma, **H. Nikolaichuk**, E. Sobstyl, J. Skolimowska, A. Tokarzewska, Screening analysis of chosen plants used in Traditional Chinese Medicine**.** Sixteenth Polish – Ukrainian Symposium on Theoretical and Experimental Studies of Interface Phenomena and their Technological Applications, August 28-31, 2018, Lublin, Poland. *Poster presentation.*
23. I.M. Choma, **H. Nikolaichuk**, E. Sobstyl, Thin-liquid chromatography screening and effect-directed analysis of chosen plants used in dementia treatment. International Symposium for High-performance Thin-Layer Chromatography, November 28-30, 2018, Bangkok, Thailand. *Oral presentation.*
24. E. Sobstyl, **H**. **Nikolaichuk**, I.M. Choma, TLC jako metoda analizy ekstraktów roślinnych stosowanych w Tradycyjnej Medycynie Chińskiej. II Ogólnopolskie Sympozjum Młodych Naukowców ,,ProDoc", April 12, 2019, Lublin, Poland. *Oral presentation.*
25. E. Sobstyl, **H. Nikolaichuk**, I. M. Choma, Analysis and comparison of *Schisandra* species using TLC-DB and screening analysis. 6th International conference "Plant – the source of research material", September 10-12, 2019, Nałęczów, Poland. *Poster presentation.*
26. I.M. Choma, **H. Nikolaichuk**, E. Sobstyl, Effect directed detection of chosen plants used as components of pharmaceutical preparations. 25th International Symposium on Separation Sciences, September 15-18, 2019, Łódź, Poland. *Oral presentation.*

27. I.M. Choma, **H. Nikolaichuk**, E. Sobstyl, Effect directed analysis of plants used in Traditional Chinese Medicine. 25th International Symposium on Separation Sciences, September 15-18, 2019, Łódź, Poland. *Invited oral presentation.*
28. I.M. Choma, **H. Nikolaichuk**, E. Sobstyl, Effect directed detection and TLC bio-profiling as a tool to distinguish among plants from various species and origin. 7th International Congress of the Society for Ethnopharmacology, February 15-17, 2020, New Delhi, India. *Invited oral presentation.*
29. I.M. Choma, **H. Nikolaichuk**, E. Sobstyl, Bio-profiling and effect directed analysis in searching of active constituents of plants and their preparations. International Conference on Second Annual Conference of the Environment, Water, Agriculture, Sustainability and Health (EWASH-2020): expanding our vision post Covid-19, December 19-20, 2020, Kolkata, West Bengal, India. *Invited oral presentation.*
30. E. Fornal, A. Stachniuk, A. Sumara, A. Łuciuk, A. Kozub, **H. Nikolaichuk**, K. Przykaza, J. Tomaszewsk-Gras, M. Montowska, Fighting food fraud. Analytical chemistry in demand to safeguard consumers. QUO VADIS Life Sciences, June 23-27, 2021, Opole, Poland. *Oral presentation.*
31. A. Kozub, A. Stachniuk, A. Sumara, **H. Nikolaichuk**, K. Przykaza, J. Tomaszewska-Gras, E. Fornal, Authentication of hemp oil using LC-MS – lipidomics approach. QUO VADIS Life Sciences, June 23-27, 2021, Opole, Poland. *Poster presentation.*
32. A. Kozub, A. Stachniuk, A. Sumara, **H. Nikolaichuk**, K. Przykaza, J. Tomaszewska-Gras, E. Fornal, LC/MS-based detection of lipidomic markers for the monitoring of seed oils quality, QUO VADIS Life Sciences, June 23-27, 2021, Opole, Poland. *Poster presentation.*
33. I.M. Choma, **H. Nikolaichuk**, TLC-Bio profiling, TLC-Direct Bioautography, Effect Directed Detection and Effect Directed Analysis – how they differ? 8th International Congress of the Society for Ethnopharmacology-India (SFEC 2021), August 27-29, 2021, Pune, India. *Invited oral presentation.*
34. A. Kozub, A. Stachniuk, **H. Nikolaichuk**, K. Przykaza, J.Tomaszewska-Gras, E. Fornal, Application of LC-QTOF in lipidomic profiling of cold pressed seed oils. Modern Research Techniques for Physicochemical Characterization of the Potential Application Systems. Summer School for PhD Students, May 18-20, 2022, Lublin, Poland. *Oral presentation.*
35. E. Fornal, A. Stachniuk, A. Sumara, A. Trzpil, A. Kozub, **H. Nikolaichuk**, K. Przykaza, Zastosowanie LC/MS w badaniach żywności. XI Polska Konferencja Chemii Analitycznej, PoKoChA 2022, June 19-23, 2022, Łódź, Poland. *Oral presentation.*

36. A. Kozub, A. Stachniuk, A. Trzpil, **H. Nikolaichuk**, K. Przykaza, J. Tomaszewska-Gras, E. Fornal, Wpływ procesu oksydacji na profile lipidomiczne tłoczonych na zimno olejów z lnianki siewnej. XI Polska Konferencja Chemii Analitycznej, PoKoChA 2022, June 19-23, 2022, Łódź, Poland. *Oral presentation.*
37. K. Przykaza, **H. Nikolaichuk,** A. Kozub, J. Tomaszewska-Gras, E. Fornal, Spektrometria mas typu shotgun jako narzędzie do jakościowej i ilościowej oceny profili lipidomicznych wybranych olejów jadalnych. XI Polska Konferencja Chemii Analitycznej, PoKoChA 2022, June 19-23, 2022, Łódź, Poland. *Poster presentation.*
38. I.M. Choma, **H. Nikolaichuk**, E. Sobstyl, Effect directed detection and TLC screening as a method for comparing various species and preparations of selected medicinal plants. 25th International Symposium for High-Performance Thin-Layer Chromatography, June 28 – July 1, 2022, Ljubljana, Slovenia. *Invited oral presentation.*
39. A. Kozub, A. Stachniuk, **H. Nikolaichuk**, K. Przykaza, A. Trzpil, J. Tomaszewska-Gras, E. Fornal, Profilowanie lipidomiczne LC/QTOF w uwierzytelnieniu tłoczonych na zimno olei roślinnych. 64. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, September 11-16, 2022, Lublin, Poland. *Oral presentation.*
40. A. Kozub, A. Stachniuk, **H. Nikolaichuk**, K. Przykaza, A. Trzpil, J. Tomaszewska-Gras, E. Fornal, Analiza lipidomiczna LC-QTOF nierafinowanych olei konopnych wspomagana chemometrią. 64. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, September 11-16, 2022, Lublin, Poland. *Poster presentation.*
41. E. Fornal, A. Stachniuk, A. Sumara, A. Trzpil, A. Kozub, **H. Nikolaichuk**, K. Przykaza, Zastosowanie LC/HRMS i technik omicznych w naukach o żywności i żywieniu. 64. Zjazd Naukowy Polskiego Towarzystwa Chemicznego, September 11-16, 2022, Lublin, Poland. *Oral presentation.*

9.7. Conducted laboratory classes:

- Chromatographic methods of analysis (*pol. Chromatograficzne metody analizy)*;
- Instrumental analysis;
- Instrumental analysis - chromatographic methods (*pol. Analiza instrumentalna -metody chromatograficzne*);

- Methods of instrumental analysis in forensics – chromatographic methods (*pol. Metody analizy instrumentalnej w kryminalistyce -metody chromatograficzne)*;
- Methods of identification and separation of biologically active compounds (*pol. Metody identyfikacji i rozdzielenia związków biologiczne czynnych*);
- Methods of sample preparation for chromatographic analysis (*pol. Metody przygotowania próbek do analizy chromatograficznej*);
- Classical analytical chemistry – quantitative part (*pol. Klasyczna chemia analityczna - ilościowa*);
- Chromatographic methods (*pol. Metody chromatograficzne*);
- Training (Effect-directed detection) for students of the Faculty of Chemistry – Integrated UMCS.

9.8. Conducted research carried out in cooperation with:

- Chair of Food Science, Institute of Nutritional Science, Justus Liebig University Giessen, Giessen, Germany;
- Department of Veterinary Microbiology, University of Life Sciences, Lublin, Poland;
- Department of Physical Chemistry, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Skłodowska University, Lublin, Poland;
- Department of Organic Chemistry, Institute of Chemical Sciences, Faculty of Chemistry, Maria Curie-Skłodowska University, Lublin, Poland.

10. SUMMARY

Today, new medicinal preparations, dietary supplements, and cosmetics based on various plants or their extracts are constantly launched on the market. Plants are a rich source of biologically active compounds, and despite many objections towards their efficacy, in mainstream medical research, they can be utilized to successfully treat different diseases and ailments. Undoubtedly, this is thanks to the achievements of modern phytotherapy and pharmacology, as well as the application of modern analytical methods and tools. However, along with the proliferation of new products, emerge problems with their quality and authenticity.

In this thesis, effect-directed analysis was applied to the screening and (bio)profiling of *Rhodiola rosea* L. extracts and to their quality and authenticity evaluation. Effect-directed analysis was based on planar chromatography in combination with (bio)assays and chemical derivatization coupled with spectroscopic identification methods. *R. rosea* extracts were tested using 13 different assays including antioxidant, antibacterial, enzyme inhibition, endocrine, and genotoxic ones. The *R. rosea* extracts were further characterized by TLC-densitometry, HPLC–ESI-MS, HPTLC–HPLC–HESI-MS, HPLC-DAD, and ^{1}H NMR techniques. The presence of genotoxins in two *R. rosea* products, and the absence of rosavin (a major constituent and quality marker, inherent only in *Rhodiola rosea* L. species) in four *R. rosea* commercially available products were observed.

The obtained results point to the great potential of EDA based on planar chromatography, as well as to the significance of effect-directed profiling of *R. rosea* products before they are made available to the consumer.

Doctoral thesis consists of five main chapters:

1. First chapter "*Introduction"* – gives a brief outline of the current research into medicinal plants, *Rhodiola rosea* L., planar chromatography, and effect-directed analysis;
2. Second chapter "*Aim and objectives"* – sets out the main aim and detailed objectives of the thesis;
3. Third chapter *"Research methods"* – presents research methods and techniques;
4. Forth chapter *"Overview of results"* – is a concise and consistent discussion of the results;
5. Fifth chapter *"Conclusions"* – provides a discussion and summary of the main accomplishments of the thesis.

The thesis is closed by a bibliography list, copies of six publications constituting the basis of the research followed by the scientific bibliography and scientific achievements of the candidate.

11. CO-AUTHORSHIP STATEMENTS AND PUBLICATIONS

Co-authorship statements:

Hanna Nikolaichuk 59

Dr hab. Irena Choma, prof. UMCS 61

Dr Marek Studziński 63

Dr Rafał Typek 64

Dr hab. Sebastian Gnat, prof. UP 65

Dr hab. Marek Stankevič, prof. UMCS 66

Prof. Gertrud Morlock 67

Publications:

D1 68

D2 84

D3 93

D4 99

D5 110

D6 123

Lublin, 6.02.2023.

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Co-authorship statement

in connection with the submission of a PhD thesis

I hereby solemnly declare that I am a co-author of the following article/manuscripts. I have major or equal contributions to the elements of this article/manuscripts as follows: formulation of the scientific problem, planning of the experiments and methodology, involvement of the experimental work, conducting the analysis of data, interpretation of the results, writing of the first draft of the manuscript, as well as the finalization of the manuscript and submission.

[D1] I.M. Choma, H. Nikolaichuk, TLC-bioprofiling – A tool for quality evaluation of medical plants, in: Pulok Mukherjee (Ed.) Evidence-based validation of herbal medicine. Translational research on botanicals. Second edition, Elsevier 2022, pp. 407-422.

[D2] H. Nikolaichuk, I.M. Choma, TLC screening in searching for active components in *Rhodiola rosea* L. roots. Annales Universitatis Mariae Curie-Skłodowska, Lublin – Polonia. Section AA – Chemia LXXIV (2019), pp. 55-64.

[D3] H. Nikolaichuk, M. Studziński, I.M. Choma, Effect directed detection of *Rhodiola rosea* L. root and rhizome extract. Journal of Liquid Chromatography and Related Technologies, 43 (2020), pp. 361-366.

[D4] H. Nikolaichuk, R. Typek, S. Gnat, M. Studziński, I.M. Choma, Effect-directed analysis as a method for quality and authenticity estimation of *Rhodiola rosea* L. preparations. Journal of Chromatography A, 1649 (2021) 462217.

[D5] H. Nikolaichuk, M. Studziński, M. Stankevič, I.M. Choma, Qualitative and quantitative evaluation of rosavin, salidroside, and p-tyrosol in artic root products via TLC-screening, HPLC-DAD, and NMR spectroscopy. Molecules 27 (2022) 8299.

[D6] H. Nikolaichuk, I.M. Choma, G.E. Morlock, Bioactivity profiles on 15 different effect mechanisms for 15 golden root products via high-performance thin-layer chromatography, planar assays, and high-resolution mass spectrometry. Molecules 28 (2023) 1535.

Hanna Nikolaichuk

Lublin, 6.02.2023.

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I hereby declare that I am aware that the work in the following articles/manuscripts of which I am a co-author, will form part of the PhD dissertation by PhD student – Hanna Nikolaichuk. My contribution to the elements of this articles/manuscripts is as follows: supervision, formulation of the scientific problem, planning of the experiments and methodology, interpretation of the results, revision of the paper as well as the finalization of the manuscript and submission.

[D1] I.M. Choma, H. Nikolaichuk, TLC-bioprofiling – A tool for quality evaluation of medical plants, in: Pulok Mukherjee (Ed.) Evidence-based validation of herbal medicine. Translational research on botanicals. Second edition, Elsevier 2022, pp. 407-422.

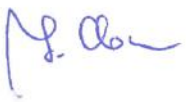
[D2] H. Nikolaichuk, I.M. Choma, TLC screening in searching for active components in *Rhodiola rosea* L. roots. Annales Universitatis Mariae Curie-Skłodowska, Lublin – Polonia. Section AA – Chemia LXXIV (2019), pp. 55-64.

[D3] H. Nikolaichuk, M. Studziński, I.M. Choma, Effect directed detection of *Rhodiola rosea* L. root and rhizome extract. Journal of Liquid Chromatography and Related Technologies, 43 (2020), pp. 361-366.

[D4] H. Nikolaichuk, R. Typek, S. Gnat, M. Studziński, I.M. Choma, Effect-directed analysis as a method for quality and authenticity estimation of *Rhodiola rosea* L. preparations. Journal of Chromatography A, 1649 (2021) 462217.

[D5] H. Nikolaichuk, M. Studziński, M. Stankevič, I.M. Choma, Qualitative and quantitative evaluation of rosavin, salidroside, and p-tyrosol in artic root products via TLC-screening, HPLC-DAD, and NMR spectroscopy. Molecules 27 (2022) 8299.

[D6] H. Nikolaichuk, I.M. Choma, G.E. Morlock, Bioactivity profiles on 15 different effect mechanisms for 15 golden root products via high-performance thin-layer chromatography, planar assays, and high-resolution mass spectrometry. Molecules 28 (2023) 1535.



Lublin, 19.01.2023.

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Co-authorship statement

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I hereby declare that I am aware that the work in the following manuscripts of which I am a co-author, will form part of the PhD dissertation by PhD student – Hanna Nikolaichuk. My contribution to the elements of this manuscripts is as follows: conducting the experimental work, and analysis of data, *i.e.* TLC-densitometry and HPLC-DAD analysis.

[D3] H. Nikolaichuk, M. Studziński, I.M. Choma, Effect directed detection of *Rhodiola rosea* L. root and rhizome extract. Journal of Liquid Chromatography and Related Technologies, 43 (2020), p. 361-366.

[D4] H. Nikolaichuk, R. Typek, S. Gnat, M. Studziński, I.M. Choma, Effect-directed analysis as a method for quality and authenticity estimation of *Rhodiola rosea* L. preparations. Journal of Chromatography A, 1649 (2021) 462217.

[D5] H. Nikolaichuk, M. Studziński, M. Stankevič, I.M. Choma, Qualitative and quantitative evaluation of rosavin, salidroside, and p-tyrosol in artic root products via TLC-screening, HPLC-DAD, and NMR spectroscopy. Molecules 27 (2022) 8299.

Marek Studziński

Lublin, 19.01.2023.

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Department of Chromatography,
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Co-authorship statement

in connection with the submission of a PhD thesis

I hereby declare that I am aware that the work in the following manuscript of which I am a co-author, will form part of the PhD dissertation by PhD student – Hanna Nikolaichuk. My contribution to the elements of this manuscript is as follows: conducting the experimental work and analysis of data, *i.e.* HPLC-ESI-MS analysis.

[D4] H. Nikolaichuk, R. Typek, S. Gnat, M. Studziński, I.M. Choma, Effect-directed analysis as a method for quality and authenticity estimation of *Rhodiola rosea* L. preparations. Journal of Chromatography A, 1649 (2021) 462217.

Rafał Typek

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Co-authorship statement

in connection with the submission of a PhD thesis

I hereby declare that I am aware that the work in the following manuscript of which I am a co-author, will form part of the PhD dissertation by PhD student – Hanna Nikolaichuk. My contribution to the elements of this manuscript is asfollows:conducting the experimental work,*i.e.Bacillus subtilis*TLC-bioassay.

[D4] H. Nikolaichuk, R. Typek, S. Gnat, M. Studziński, I.M. Choma, Effect-directed analysis as a method for quality and authenticity estimation of *Rhodiola rosea* L. preparations. Journal of Chromatography A, 1649 (2021) 462217.

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Co-authorship statement

in connection with the submission of a PhD thesis

I hereby declare that I am aware that the work in the following manuscript of which I am a co-author, will form part of the PhD dissertation by PhD student – Hanna Nikolaichuk. My contribution to the elements of this manuscript is as follows: conducting the experimental work and analysis of data, *i.e.* ^{1}H NMR analysis.

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I hereby declare that I am aware that the work in the following manuscript of which I am a co-author, will form part of the PhD dissertation by PhD student – Hanna Nikolaichuk. My contribution to the elements of this manuscript is as follows: supervision, formulation of the scientific problem, planning of the experiments and methodology, interpretation of the results, revision of the paper as well as the finalization of the manuscript and submission.

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Sincerely yours,

G. Morlock

Prof. Dr. Gertrud Morlock

CHAPTER

16

TLC bioprofiling—A tool for quality evaluation of medicinal plants

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1 Introduction

Medicinal plants are abundant in bioactive constituentswhich can be used in cosmetics, pharmaceutical preparations, and drugs. Especially, the healing abilities of herbs cannot be overestimated. Medicinal plants and their preparations are used not only in the treatment of human diseases but also of animal and plant ones. It should be stressed that almost half of the contemporary drugs are derived from medicinal plants. The growing interest in natural healing entails also growing interest in medicinal plants. The attention is focused on the pharmacological properties of herbs used in folk medicine all over the world and especially of those used in great healing systems like Ayurveda or Traditional Chinese Medicine (TCM). Although there are over 750,000 plants on earth, a small number have been studied for healing purposes. Even in the case of such popular herbs as thyme or mint, many potentially interesting components are still not discovered.

Quality assessment of plant material is of great importance for the pharmaceutical industry, especially when the bulk plant material comes not from big industrialized farms but from small farmers or wild-crops. In many cases, people buy herbs, herbal drugs, and supplements not from reputable manufactures but from unknown producers via internet, completely out of control. This imposes a great challenge for government authorities to supervise this market.

In the traditional healing systems, organoleptic, morphological, and taxonomical assessments were used for identification and authentication of medicinal plants. Nowadays, microscopy, genetics as well as immunological and analytical methods are applied additionally for their evaluation. The most important are the last ones, especially chromatographic and spectroscopic analysis. Chromatography (HPLC, GC, CCC, CE, OPLC, TLC) enablesseparation of sample constituents while spectroscopy (UV-VIS, IR, NMR, MS) their identification. Usually, hyphenated techniques are used as HPLC-UV, HPLC-MS, GC-MS, TLC-UV, TLC-MS, etc. [1,2]. To obtain relevant information from many chromatographic and spectroscopic data, chemometrics is advised to use [3–6].

Undoubtedly, HPLC is the most powerful technique in natural product analysis. It is used both for quality assessment and for quantitation of marker compounds. HPLC fingerprints (also multiple) are popular in screening analysis [7–9]. However, the most important are pharmacological properties of medicinal plants, in particular correlation of bioactivity of their constituents with structural information. In case of HPLC, this is a great challenge for analysts and instrumentation. The bioassays can be performed directly on a column [8] or in post-column assays: on-line, at-line, and off-line [2,10,11]. The most promising is the post column on line assay [10–13]. However, in relation to TLC, it is a complicated, tedious, and expensive method that cannot be performed in the ordinary laboratory.

Although HPLC ensures huge separation power and provides sophisticated profiles, thin-layer chromatography seems to be the method of choice in herbal profiling. Thin-layer chromatography (TLC), including its highperformance version (HPTLC), is a well-established method for qualitative and quantitative analysis. Its great predominance over other chromatographic methods is related to its simplicity, flexibility, moderate cost, low organic solvent consumption, and the possibility of analyzing many samples in parallel. TLC is an open, decentralized system that allows to perform derivatization/

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bioassays directly on the plate [14,15] and for convenient hyphenation with spectroscopic methods [16]. Therefore, TLC can be used for comparing samples of various species, different origins, harvesting, and storage conditions. TLC profiles, so-called "fingerprints", allow to find in an easy way, visual differences among the samples and to reject those of other types, poor quality or adulterated.

2 TLC profiling

As it was already stated in the Introduction, medicinal plants can replace or support many conventional drugs. However, the problem with their use in modern medicine is mostly connected with a lack of adequate research methodology for their evaluation, standardization, and authentication. Various pharmacopoeia monographs recommend comparing macroscopic, microscopic, and chemical characteristics with an authenticated reference sample [1,5]. Usually, a few markers (sometimes only one), i.e., pharmacologically active components, characteristic for given species, are used for evaluating the quality and authenticity of herbal medicines [17,18]. Such methodology does not give a full assessment of the herbal material; not to mention that chosen markers can be characteristic also for other species. Moreover, the amounts of markers as well as other constituents may vary, depending on habitat, harvest season, drying procedure, storage and transportation.

It is impossible to identify all chemical constituents of a given herb, to assign individual biological properties, and all the more to predict possible synergistic effects. So the best way for quality control is the determination of full profiles of plant metabolites using chromatographic methods in so-called untargeted analysis [17]. This is also advised by global regulatory authorities such as World Health Organization, European Medicine Evaluation Agency, European Commission, and American Food and Drug Administration [19,20]. If it is possible, the untargeted analysis should be completed with targeted quantification of the officially recognized markers [17,21,22]. According to Cieśla, herbal sample fingerprint can be define as a set of characteristic chromatographic or spectroscopic signals, whose comparison leads to an unambiguous sample recognition [8]. In other words, a chromatographic fingerprint is a chromatographic pattern of pharmacologically active and/or chemically characteristic constituents present in the extract [23,24]. Because of the complexity of herbs, some small differences between closely related species may not be visually observed in fingerprints but can hardly influence pharmacological properties of herbs and herbal medicines. The problem can be solved by chemometric methods such as Principal Component Analysis (PCA) and Soft-Independent Modeling of Class Analogy (SIMCA), which bring out information included in fingerprints [3–6,19,25,26]. Interesting concept which allows for more comprehensive data analysis is two-dimensional TLC (2D-TLC) [27], multiple fingerprints (more than one chromatographic profile), or multidimensional fingerprinting (more than one detection mode) [8,19].

Generally, TLC profiling can be done using chemical, biological and physicochemical/spectroscopic screening approaches [28].

2.1 TLC chemical profiling

TLC chemical profiling (micro-chemical tests, derivatization) of medicinal plants is based on the chemical reactivity of the constituents. Derivatization reagents can be divided into two groups — general and specific reagents. General reagents can react with many compounds from different groups and classes. Among them are: p-anisaldehyde (AS), Fast Blue B salt (FBB), iodine, sulfuric acid, phosphoric acid, phosphomolybdic acid (PMA), rhodamine B, vanillin, etc. Basically, this type of derivatization is used for the estimation of sample complexity. Specific (group) reagents are those that can interact only with one or several related groups of chemical compounds. For example, bromocresol green (BGG) is used for detection of organic acids, bromothymol blue (BTB) for lipids and phospholipids, Dragendorff reagent for detection of alkaloids, ninhydrin for detection of amino acids and amines, thymol and o-phosphomolybdic acid reagent (PABA) for detection of sugars, natural products-polyethylene glycol reagent (NP-PEG) for detection of polyphenols, dinitrophenylhydrazine (DNPH) for ketones and aldehydes (Fig. 1).

Some specific reagents can point to possible biological properties of plant components, e.g., the presence of pharmacologically active polyphenols can be detected by NP-PEG reagent. Besides, different colors of fluorescent spots, obtained as a result of derivatization with NP-PEG, allow characterizing the type of compounds in a sample. In particular red spots in NP-PEG correspond to chlorophylls, purple spots-to anthocyanins, intense blue ones-to phenol carboxylic acids, yellow/orange-to flavanols, and green-to flavanones [14,29].

TLC profiling using many derivatization reagents can give preliminary information on the composition and biological activity of investigated plant extracts [17,30–34]. Compounds being detected using NP-PEG usually have antioxidant properties. This can be confirmed by derivatization with DPPH reagent, which is used to detect antioxidants. Although DPPH (2,2-diphenyl-1-picrylhydrazyl radical) is a typical chemical reagent, it is used for bioassays which are classified as microchemical ones.

TLC profiling can be also used for optimization of chromatographic systems, especially for choosing mobile phase composition with a proper elution strength and

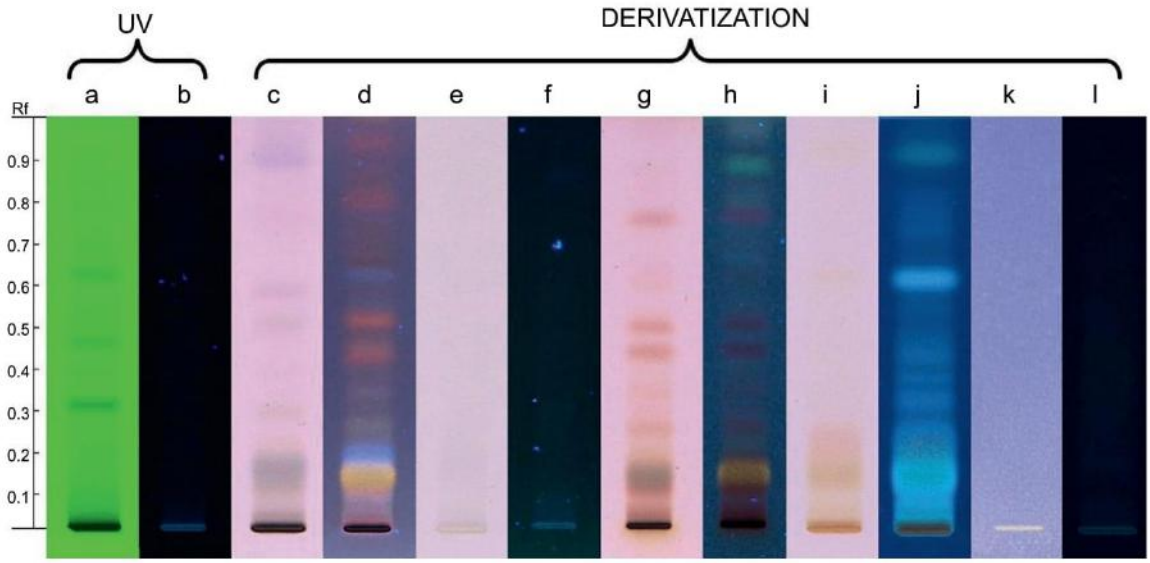

FIG. 1 TLC chromatograms of the root and rhizome extract of *Rhodiola rosea* L. at UV 254 nm (a), UV 366 nm (b), AS VIS (c), AS UV 366 nm (d), PMA VIS (e), PMA UV 366 nm (f), thymol VIS (g), thymol UV 366 nm (h), PABA VIS (i), PABA UV 366 nm (j), BGG (k), NP-PEG (l). Silica gel, mobile phase—ethyl acetate-methanol-water 77:13:10 (v/v/v).

with good selectivity. Fig. 2 presents NP-PEG chromatograms and related to them TLC-DPPH bioautograms for chosen herbs using two different mobile phases.

What should be stressed, TLC profiling can be used for comparison of many samples in parallel to assess their quality and to reject those of pure quality, containing impurities or adulterated [17].

2.2 TLC bioprofiling

Bioassays are screening or semi-quantitative methods measuring/detecting an effect emerging in a given biological system under action of the biologically active substance(s) [35–38]. As pointed out in the Introduction, thin-layer chromatography is the most convenient chromatographic method to be connected with bioassays because of the possibility of running many samples in parallel and open system which enables evaporation of the mobile phase.

TLC can be hyphenated with bioassays performed directly on a TLC plate (TLC-direct bioautography) to detect, e.g., antimicrobials, antioxidants, estrogenic compounds, enzyme inhibitors. TLC-DB followed by spectroscopic identification (MS, NMR, surface-enhanced Raman spectroscopy, SERS) belongs to the effect directed analysis (EDA) and provides full information of sample components showing a given effect on a TLC plate [39–41]. When identification is not necessary or is too difficult, it can be skipped, and such a method should be named effect directed detection (EDD) [35,36,39,42]. So, TLC-DB, when it is not followed by spectroscopic identification of bioactive components of the sample, should be classified as the EDD method. Biological profiles of plant extracts performed on a TLC plate (i.e., TLC-DB, EDD) can be used to compare many samples in parallel. It may concern various plants, various species from the same family or the same species but differing in e.g. geographical location, harvesting or storage conditions (Fig. 3).

TLC-DB was historically used for the detection of antimicrobial activity using bacteria and fungi as test organisms [43,44]. Antimicrobial activities have also been estimated using other TLC bioautographic (TLC-B) methods: contact bioautography and agar overlay/immersion [45–47]. Out of these three TLC-bioautographic methods: contact, overlay, and direct, only the last one can be easily adapted for other types of bioassays, e.g., antioxidants. Now, TLC-DB is mainly used for detection of:

1. Antimicrobials,
2. Antioxidants,
3. Enzyme inhibitors,
4. Estrogens.

2.2.1 Antimicrobials

In TLC-DB, based on microbiological detection, a developed and dried TLC plate is dipped in or sprayed with a suspension of microorganisms growing in a proper broth. Bacteria or fungi form a very thin layer at the surface of a TLC plate. Then, a plate is incubated under optimized for a given test organism conditions. Microorganisms grow

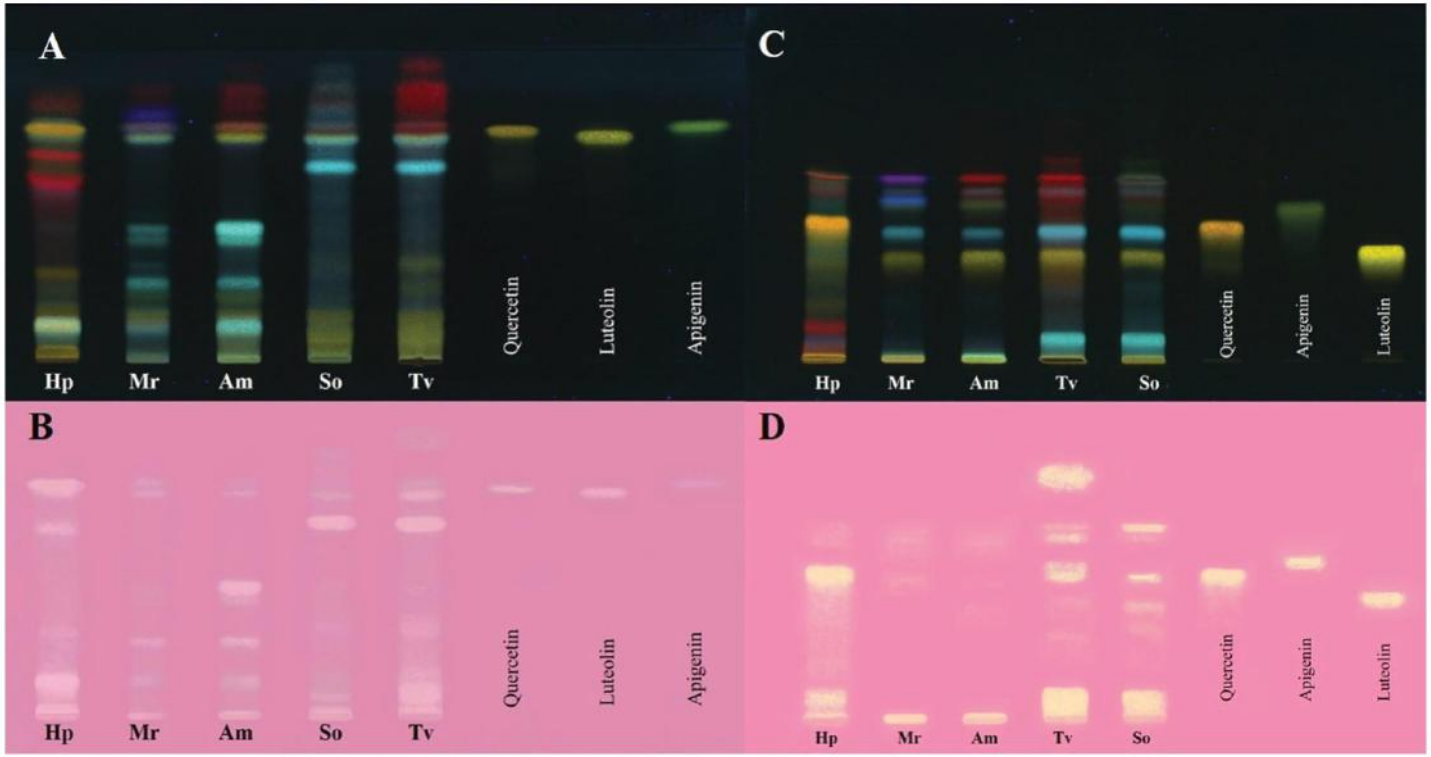

FIG. 2 Chromatograms of *Hypericum perforatum* L (Hp), *Matricaria recutita* (Mr), *Achillea millefolium* (Am), *Salvia officinalis* (So), *Thymus vulgaris* (Tv); detection with NP-PEG reagent at 366 nm (A, C) and DPPH assay (B, D). Silica gel, mobile phases: A, B–ethyl acetate-toluene-formic acid 70:30:10 (v/v/v); C, D–toluene-diethyl ether-acetic acid 60:40:10 (v/v/v).

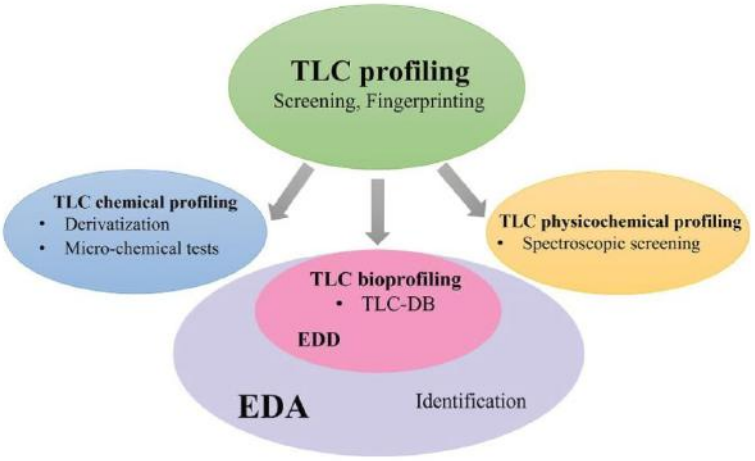

FIG. 3 Mutual relation among TLC profiling, EDD, and EDA.

directly on the surface of a TLC plate, excluding the places where antibacterial or antifungal agents are located. Zones of microbial growth inhibition are visualized using tetrazolium salts, usually, MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), which is converted by dehydrogenases of living microorganisms into purple formazan. Creamy spots appearing against a purple background point to the presence of antimicrobial agents. The most popular bacteria strains applied as test microorganisms for TLC-DB are *Escherichia coli*, *Bacillus* sp. *Pseudomonas* sp., *Mycobacterium* sp., *Staphylococcus* sp., and luminescent *Aliivibrio fischeri*, while the most popular fungi strains are *Candida albicans*, *Cladosporium* sp., *Aspergillus* sp., *Fusarium* sp., *Penicillium* sp., and *Colletotrichum* species [47].

The commercial TLC-DB test based on *B. subtilis*, Chrom Biodip Antibiotics, was launched by Merck in 2000 year [48]. The kit consisted of a culture medium, *B. subtilis* spore suspension, nutrient medium, and MTT reagent. However, the incubation time for conversion of *B. subtilis* spores to their vegetative form, given in the recipe, was insufficient, resulting in poor staining of TLC plates with MTT. The test is not available at present.

The TLC-DB tests against *E. coli* and *B. subtilis* were developed and fully validated by Choma group [49,50]. Optimization of growth conditions for test bacteria/fungus cultures used in TLC-DB (*B. subtilis*, *E. coli*, and *C. albicans*) were described in three papers of Nagy et al. [51–53]. There are many papers on antibacterial and antifungal properties of selected plants usually tested against one or two bacterial strains [34,54]. The antibacterial properties of *Hypericum perforatum*, *Matricaria chamomilla* L., *Achillea millefolium* L., *Thymus vulgaris* L., and *Salvia officinalis* were bioprofiled against eight bacterial strains, including four pathogenic bacteria (e.g., methicillin-resistant *Staphylococcus aureus*) and luminescent bacteria (*Pseudomonas syringae* pv. *maculicola* and *Aliivibrio fischeri*) [33,55,56]. Numerous review papers on TLC-DB are available [35,36,45–48,57–61].

TLC-bioluminescence

TLC-bioluminescence test works on a different basis than described before. The test bacteria are *Aliivibrio fischeri* or genetically modified strains as *Pseudomonas syringae* pv. *maculicola* [33,62–72]. Quenching of their luminescence is observed instead of growth inhibition. The compounds disturbing metabolism of luminescent bacteria are visible as dark spots against a bright background. Bioluminex, TLC bioluminescence test based on *A. fischeri*, is commercially available from Chroma-Dex [67]. Besides antimicrobials, the test detects natural, biological, and chemical toxins. Bioluminizer from Camag (Muttenz, Switzerland) with CCD camera is dedicated for documentation of results in TLC-bioluminescence test.

The luminescent test is based on *Pseudomonas syringae* pv. *maculicola*, a plant pathogenic bacterium, was used to search compounds active against bacterial plant infections in plant material [63,64,68,72].

2.2.2 *Antioxidants*

Antioxidant/radical scavenging activity can be measured using stable 2,2-diphenyl-1-picrylhydrazyl radical ($DPPH^{\bullet}$) or 2,2′-azino-bis (3 ethylbenzothiazoline-6-sulphonic acid) diammonium radical cation ($ABTS^{\bullet+}$). The developed TLC plate is sprayed with/dipped in the $DPPH^{\bullet}$/$ABTS^{\bullet+}$ solution, and a color change related to reagents reduction is observed. The plates should be

scanned at 517 nm (DPPH$^{\bullet}$ absorption maximum) or at 734 nm (ABTS$^{\bullet+}$ absorption maximum). In TLC-DPPH$^{\bullet}$, antioxidants appear as pale yellow spots against a purple background, while in TLC-ABTS$^{\bullet+}$ as pale pink spots against blue/green background [15,73,74]. Less popular is the TLC-β-carotene test [73]. Inhibition of β-carotene bleaching is observed under VIS or UV 366 nm light in places where antioxidants are located. TLC-DPPH$^{\bullet}$ and TLC-ABTS$^{\bullet+}$ were compared by Danciu et al. [75], who found that TLC-ABTS$^{\bullet+}$ was a more sensitive method than TLC-DPPH$^{\bullet}$. Nevertheless, TLC-DPPH$^{\bullet}$ is by far more popular (Fig. 4) [73].

The DPPH and ABTS$^{\bullet+}$ tests are standard colorimetric methods used to evaluate the radical-trapping efficiency of antioxidants [76]. They were easily adopted for TLC bioprofiling. Recently, our group adopted for TLC another colorimetric method—FRAP—measuring the ability of electron transfer from an antioxidant to a radical [76], which, according to our knowledge, was previously used only for a dot-blot [77]. Fig. 5 presents the detection of antioxidants using TLC-FRAP, TLC-DPPH, and TLC-ABTS bioassays of *Schisandra rubriflora* extracts.

Ciesla et al. [78] introduced so-called "binary chromatographic fingerprint" combining chemical (vanillin reagent) and biological (DPPH) profiling. Using freely available image processing program—ImageJ—videoscans were converted into real chromatograms without the need to use densitometers. 2D-TLC-DB (DPPH assay) of *Spirulina platensis* metabolites was done by Cid-Hernández et al. [27].

2.2.3 *Enzyme inhibitors*

Recently, enzyme inhibition bioassays performed on TLC plates have become very popular mostly due to the

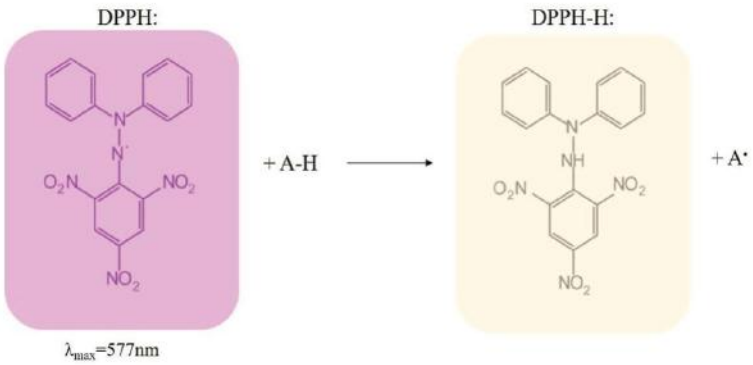

FIG. 4 DPPH assay.

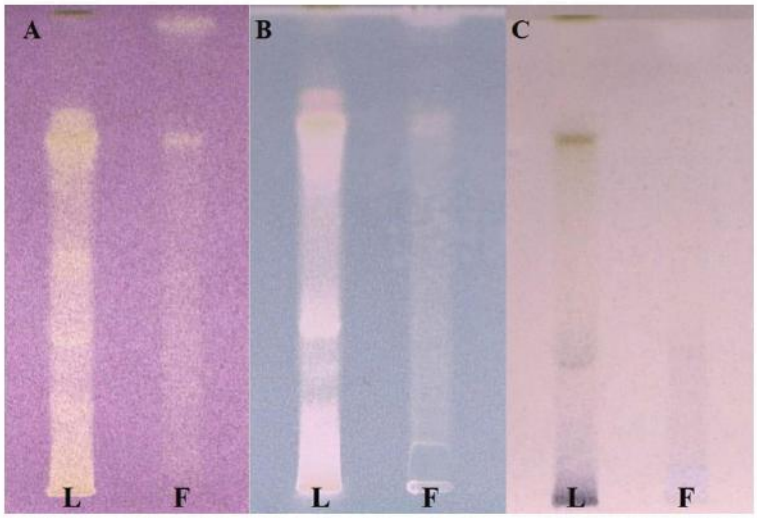

FIG. 5 Bioautograms of *Schisandra rubliflora* leaf (L) and *Schisandra rubliflora* fruit (F); detection of antioxidants with DPPH assay (A), ABTS assay (B) and FRAP assay (C). Silica gel, mobile phase: ethyl acetate-methanol-water 70:30:10 (V/V/V).

hypothesis that enzyme inhibitors may be successfully used in the treatment of civilization diseases such as Alzheimer's and Parkinson's, obesity, diabetes, cancer, depression, etc. The enzymes most frequently used in TLC-DB are: acetylcholinesterase and butyrylcholinesterase (AChE/BChE), α- and β-glucosidase, lipase, tyrosinase, α-amylase, xanthine oxidase, peroxidase, β-glucuronidase, arginase, cutinase, and rabbit liver enzyme. The bioassays based on them have already been introduced as TLC-DB tests, improved, modified, and reviewed in various articles [15,31,79–85].

Acetylcholinesterase and butyrylcholinesterase

Alzheimer's Disease (AD) is the most common form of dementia in our aging society. The pathogenesis of AD has not yet been explained. It is widely accepted that a combination of genetic predisposition and environmental factors -is responsible for the inception of AD. According to cholinergic hypothesis, AChE inhibitors may be helpful therapeutic agents for symptomatic improvement in AD. Most of currently available drugs for AD treatment, as rivastigmine and donepezil are AChE inhibitors. Searching for natural AChE inhibitors, possibly less toxic and more efficient than conventional drugs in the treatment of AD, seems to be very important. There are two types of cholinesterases, AChE and BChE. AChE is found primarily in the blood and neural synapses, while BChE — in the liver. Inhibitors of BChE may be useful in the treatment of 2 type diabetes [86].

TLC-DB for detection of AChE/BChE inhibitors is based on two methods introduced by Marston [79] and Rhee [87]. Rhee adopted for TLC-DB spectrophotometric Ellman's method [88], which is based on catalytic decomposition of acetylcholine to tricholine and acetate. Tricholine reacts with DNTB (5,5-dithio-bis-2-nitrobenzoic acid) to form a yellow dye (5-thio-2-nitrobenzoate anion). As a consequence, AChE inhibitors are visible as white zones on a yellow background. The main disadvantage of the assay is the lack of contrast between inhibition zones and the background.

The method by Marston is used for the detection of AChE and BChE inhibitors. The assay procedure is based on a diazonium reaction. AChE or BChE enzyme reacts with 1-naphthyl acetate (or 2-naphthyl acetate) during the incubation (20 min, 37°C in humid conditions). As a result, 1-naphthol (2-naphthol) is produced. Further, 1-naphtol reacts with the diazonium dye Fast Blue B salt to form a purple azo dye. AChE/BChE inhibitors appear as creamy spots on the purple background. One of the disadvantages of this method is the high consumption of enzymes, which causes the procedure to be rather expensive.

In the assay, modified by Zhong-Duo Yang [89], 4-methoxyphenyl acetate was used as a substrate. A mixed solution of potassium ferricyanide and iron chloride hexahydrate is used as a visualization agent. The inhibitors of AChE appear as light-yellow spots on an aquamarine blue background. A disadvantage of the method is the high possibility of a false-positive results.

Nowadays, AChE/BChE bioassays based on TLC-DB are the most frequently employed screening methods of AChE/BChE inhibitors in complex matrices. Usually, Marston method in original or modified version is used [79,89–91] (Fig. 6).

α-Glucosidase and β-glucosidase

Glucosidase inhibitors have promising therapeutic potential in the treatment of many disorders such as diabetes 2 type, human immunodeficiency virus (HIV) infection, metastatic cancer, and lysosomal storage diseases. Glucosidases (α- and β-) catalyze the hydrolysis of polysaccharides to simple sugars, which leads to the increased glucose levels in blood [92].

Initially, α- and β-glucosidase inhibition assay was based on esculin as a substrate. Inhibitors of α- and β-glucosidase appear as a white spot against dark brown background [81]. The disadvantage of this method is, difficult to eliminate false-positive results. Later, Simoes-Pires [83] introduced a method similar to the Marston one for AChE/BChE inhibitors. They used 2-naphthyl-α-D-glucopyranoside or 2-naphthyl-β-D-glucopyranoside as substrates for α-glucosidase and β-glucosidase enzymes, respectively. After incubation (37°C in a humid atmosphere), 2-naphthol yields in the reaction between substrate and enzyme and reacts with Fast Blue B salt to give a purple-colored diazonium dye. Inhibitors of α-/β-glucosidase are seen as yellow spots on a purple background (Fig. 6).

Lipase

Lipase inhibitors are important for the digestion of triglycerides. They reduce the absorption of monoglycerides and free fatty acids, leading to the loss of body weight. Orlistat — an anti-obesity drug approved for long-term administration — is a pancreatic lipase inhibitor. Lipase inhibitors are also helpful in the treatment of atherosclerosis. According to the World Health Organization, approximately 2 billion people worldwide suffer from overweight or obesity. For this reason, it is very important to find effective lipase inhibitors [93].

There are three TLC-DB methods for screening lipase inhibitors. Two of them are based on diazonium reactions using different specific substrates for lipase. In the method described by Hassan [80], 1-naphthyl acetate was used as a substrate and Fast Blue B salt as a visualization solution. Lipase inhibitors appear as yellow spots against a purple background. The Tang [94] assay, which is a modified Hassan method, uses 2-naphthyl myristate as a substrate for lipase. The Bayineni method [95] is

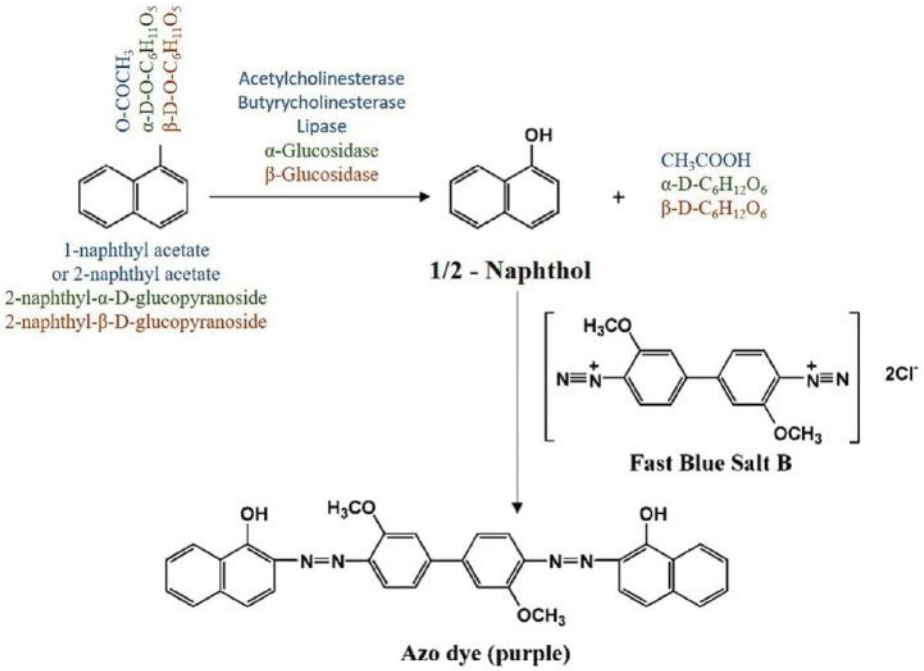

FIG. 6 Scheme of diazonium reaction of different enzymes and substrates.

another approach for the detection of lipase inhibitors. The products of the reaction between enzyme and substrate (*p*-nitrophenyl butyrate) are p-nitrophenol and butyric acid. Bromothymol blue, used as a visualization solution, reacts with p-nitrophenol. Lipase inhibitors appear as a blue spot on the greenish-yellow background. The Bayineni method has higher sensitivity than the one of Hassan (Fig. 6).

Tyrosinase

Tyrosinase is a multifunctional enzyme, which controls the production of melanin from tyrosine in animals. It catalyzes the hydroxylation of monophenols to o-diphenols and their subsequent oxidation to o-quinones. It is also responsible for the browning of damaged fruits or plant tissues. Melanin is responsible for skin color as well as for several skin diseases and esthetic characteristics such as freckles, melasma, and age spots. Uncontrolled tyrosinase activity can increase melanin synthesis associated with dermatological diseases. Tyrosinase inhibitors have become increasingly important as whitening agents in cosmetics [96].

The TLC-DB method for the detection of tyrosinase inhibitors is based on two types of substrate—L-tyrosine [97] or L-DOPA [82]. Tyrosinase reacts with a substrate immediately after spraying. After short incubation (room temperature, humid conditions), tyrosinase inhibitors appear as white spots on the dark gray background. A disadvantage of using L-tyrosine as a substrate is its low solubility in water.

α-Amylase

α-Amylase inhibitors could be potentially used in the treatment of 2 type diabetes, related to the fact that α-amylase catalyzes the hydrolysis of starch [98]. TLC-DB for detection of α-amylase inhibitors is based on the Sonkamble method [99,100], which uses the starch solution as a substrate and Gram Iodine solution for visualization. The α-amylase inhibitors are detected as blue spots against a white background. Another method [101] is based on enzymatic hydrolysis of 2-chloro-*p*-nitrophenyl-a-D-maltotrioside (CNP-G3) to 2-chloro-4-nitrophenol. Amylase inhibitors appear as white spots against the yellowish-green background. It seems to be more convenient to use the starch solution as a substrate due to the high contrast between spots of inhibitors and a background.

Xanthine oxidase inhibition

The xanthine oxidase (XO) inhibitors run down oxidative stress and inflammation, slow down aging, act against cancer. Additionally, they reduce gout and kidney stones caused by hyperuricemia. The XO catalyzes

the oxidation of xanthine to uric acid. Superoxide radicals that are produced in this reaction reduce the pale-yellow tetrazolium salt to purple formazan. The XO inhibitors are visible as white spots against the purple background [102,103]. Because the enzyme should be suspended in agar, agar overlay method is advised for this assay.

2.2.4 Estrogens

Besides natural estrogens, there are many compounds with estrogenic activities not generated within the endocrine system, e.g., pesticides, herbicides, insecticides, polychlorinated biphenyls, cosmetic components, UV filters, preservatives, mucoestrogens and phytoestrogens. The last ones are plant-derived xenoestrogens such as lignans, coumestans, prenylflavonoids, and isoflavones. The best-known phytoestrogens are isoflavones, which are commonly found in soy and red clover. Besides the health benefits of phytoestrogens on CNS, cardiovascular system, metabolism, and menopausal symptoms, there is also a concern that phytoestrogens may act as endocrine disruptors.

The planar yeast estrogen screen (pYES) has been adopted from the cuvette YES assay developed for the measurement of estrogenic activity [104]. Both versions of bioassays are based on genetically modified yeast cells containing the human estrogen receptor gene incorporated into the main genome. Activation of the receptor by estrogen/xenoestrogen molecule leads to the expression of the reporter gene Lac-Z, which encodes β-galactosidase. In pYES, the dried developed TLC plate is dipped into the test yeast culture and incubated in a moisture box. Then, the substrate, usually 4-methylumbelliferyl-β-D-galactopyranoside (MUG), is sprayed onto the plate, and the plate is incubated once more. Estrogenic compounds are observed as fluorescent zones of 4-methylumbelliferone (MU), the product of enzymatic cleavage of MUG (Fig. 7). The enzyme can also hydrolyze X-β-Gal (5-bromo-4-chloro-3-indoxyl-β-D-galactopyranoside) into β-galactose and 5-bromo-4-chloro-3-indoxyl. The indoxyl compound is oxidized by oxygen to the deep-blue dye 5,5β-dibromo-4,4β-dichloro-indigo. This variant is preferable when the sample contains native fluorescing compounds.

The pYES test is mostly applied for water and food samples [105–108]. However, it can also be used for plant components [109].

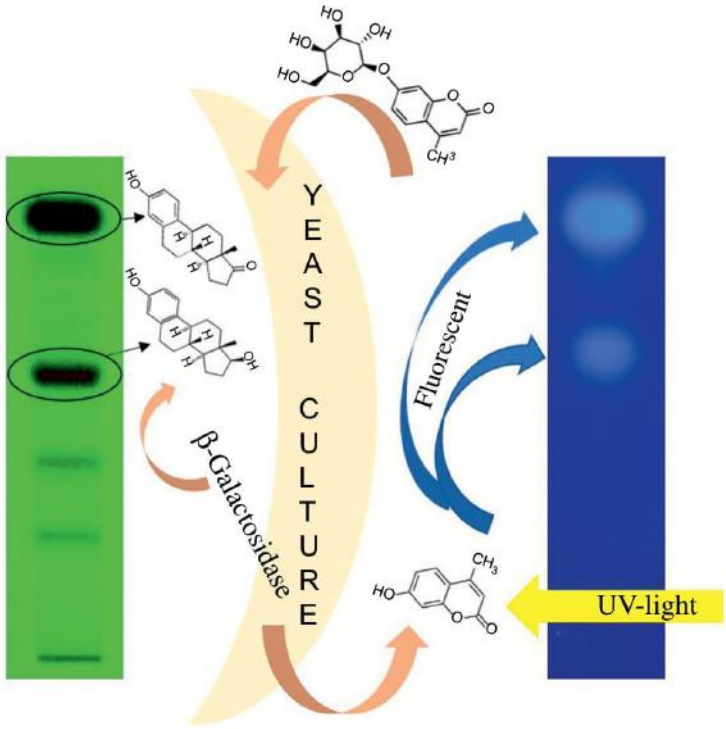

FIG. 7 pYes scheme.

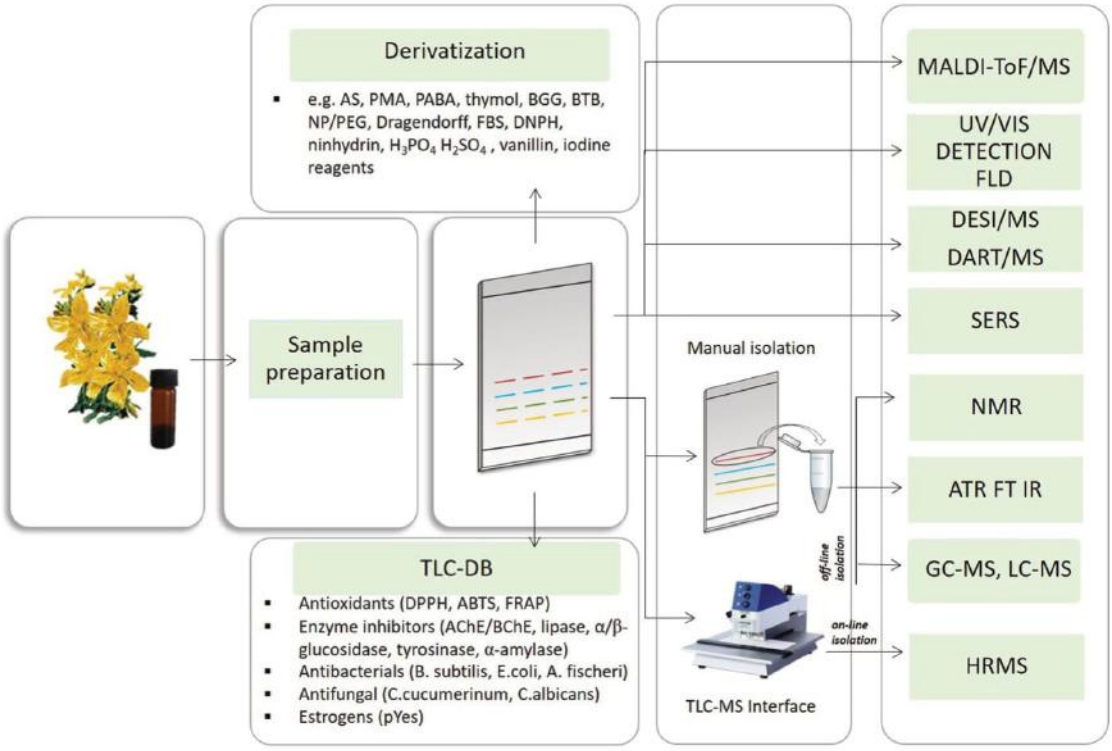

FIG. 8 Scheme of hyphenations of TLC, bioassays, derivatization, and identification techniques. *Modified from Choma IM, Jesionek W. Effect-directed detection in chromatography. Reference module in chemistry, molecular sciences and chemical engineering; 2017, p. 1–10. https://doi.org/10.1016/b978-0-12-409547-2.12679-4.*

3 Hyphenations

As it was stated above, TLC bioprofiling or TLC-DB belongs to the so-called EDD. If TLC bioprofiling is followed by spectroscopic methods, it can be classified as EDA (Fig. 3). Spectroscopic measurements can be done directly at the TLC plate (UV-VIS, FLD, DESI, DART MALDI, SERS) or for extracts from chromatographic zones [16,35,40,61,110]. TLC-MS interface (Camag, Muttenz) enables on-line HRMS analysis from a given bioactive zone. It is also possible to elute the target compounds from the zones to vials. If more analyte is needed, semi-preparative or micro-preparative (using analytical plate) separation is advised. The zones of interest should be scrapped/eluted to vials, and the subsequent extracts can be subjected to any spectroscopic method (UV, IR, MS, NMR) [111]. Fig. 8 shows possible TLC hyphenations with spectroscopic methods as well as with bioassays and derivatization methods. Nowadays, many papers are constructed according to the scheme shown in Fig. 8. It means that TLC separation is followed by TLC bioprofiling and/or chemical profiling (derivatization) and then hyphenated to spectroscopic identification of bioactive zones. The latest chosen papers on plant analysis, presenting various modes of hyphenations, are collected in Table 1.

TABLE 1 Effect directed analysis of chosen plants—TLC-bioprofiling followed by spectroscopic identification.

Plant	Bioassays	Identification	References
Salvia miltiorrhiza	*A. fischeri*; *B. subtilis*; AChE; DPPH	HPTLC-UV/Vis/FLD-(bio)assay-HRMS, (PLC)-^{1}H NMR	[111]
Peganum harmala L. seed extract	AChE; BChE	HPTLC-FLD	[112]

TABLE 1 Effect directed analysis of chosen plants—TLC-bioprofiling followed by spectroscopic identification.—cont'd

Plant	Bioassays	Identification	References
30 samples of French propolis	UV 366 nm	RP-HPTLC-FLD; RP-HPTLC-DART-MS; PCA	[113]
Salicaceae bud extracts (*Populus* L. and *Salix* L.)	AChE; BChE; RLE; NP-PEG; *A. fischeri*; *B. subtilis*; pYES	HPTLC-MS	[31]
Stevia	AChE; BChE; *A. fischeri*; *B. subtilis*; DPPH; α-glucosidase; β-glucosidase; lipase; tyrosinase; β-glucuronidase	HI-HPTLC-UV/Vis/FLD-assay HILIC separation	[114]
Salvia officinalis leaf	AChE; *A. fischeri*; β-glucuronidase; Dragendorff's reagent; 2,4-dinitrophenylhydrazine reagent	HPTLC-ESI-MS	[115]
Sambucus nigra L.	*A. fischeri*; *B. subtilis*; pYES; DPPH; AChE; tyrosinase	HPTLC-ESI-MS	[116]
Sunflower leaves	AChE; *A. fischeri*; *B. subtilis*	HPTLC-DART-MS/MS; HPTLC-ESI-HRMS	[117]
Seaweeds	AS; DPPH; *A. fischeri*; *E. coli*	UHPLC-LTQ-MS/MS; GC/ESI-MS	[118]
Egyptian propolis	NP-PEG	NIR-HPTLC	[119]
Mexican *Plectranthus amboinicus* Lour. essential oil	α-glucosidase; β-glucosidase; AChE; DPPH; tyrosinase; α-amylase; *A. fischeri*; *B. subtilis*	HPTLC-UV/Vis/FLD-EDA; HPTLC-DART-HRMS	[120]
Seaweed	α-glucosidase	HPLC-HRMS-SPE-NMR	[121]
Tansy root	*A. fischeri*; *B. subtilis*; DPPH; PABA	OPLC-DART-HRMS	[122]
Chamomile tea	NP-PEG	HPTLC with multivariate analysis	[123]
Morus alba	*A. fischeri*; *B. subtilis*; DPPH	HPLC-LTQ-MS/MS; HPTLC-bioautography/UPLC-MS/MS	[124]
Ginger (*Zingiber officinale*)	*A. fischeri*; *B. subtilis*; DPPH; pYES; AChE; tyrosinase; AS	HPTLC-UV/Vis/FLD-bioassay; HPTLC-ESI-HRMS	[125]
Ficus religiosa leaf	Tillman's reagent; NP-PEG; Dragendorff's reagent; DPPH; ABTS; tyrosinase; α-glucosidase; β-glucosidase, *A. fischeri*; *B. subtilis*; AChE; BChE; α-amylase; pYes; SOS-UMU-C	HPTLC-UV/Vis/FLD-EDA-HESI-HRMS/MS	[91]
Musa spp. (Bananas)	DPPH; AChE; BChE; FRAP; α-glucosidase; β-glucosidase; α-amylase; *A. fischeri*; *B. subtilis*, SOS-UMU-C; NP, ninhydrin reagent; diphenylamine reagent; AS	HPTLC-EDA-HRMS	[126]
Linden tea	DPPH	HPTLC-UHPLC-HRMS	[127]
77 Botanical extracts	DPPH; *A. fischeri*; *B. subtilis*; AChE; tyrosinase; $AlCl_3$	HPTLC-UV/VIS/FLD-EDA-ESI-HRMS	[128]
	AChE; AS		[129]

Continued

TABLE 1 Effect directed analysis of chosen plants—TLC-bioprofiling followed by spectroscopic identification.—cont'd

Plant	Bioassays	Identification	References
Anarrhinum pubescens		HPTLC-HRMS/MS; NMR	
Primula boveana	*A. fischeri*; *B. subtilis*; AChE; BChE, AS	HPTLC-UV/VIS/FLD-EDA-ESI-HRMS; NMR	[130]
Solidago spp.	*A. fischeri*; *B. subtilis*; AChE; BChE, α-glucosidase; β-glucosidase; α-amylase; vanillin sulfuric reagent	HPLC-DAD-ESI-MS; HPTLC-EDA; HPTLC-HRMS/MS	[101]
Cinnamons	DPPH; AChE; BChE; α-glucosidase; β-glucosidase; tyrosinase	HPTLC-UV/Vis/FLD-EDA-HRMS	[131]
Rhodiola rosea L.	DPPH; AChE; *B. subtilis*; α-glucosidase; lipase; tyrosinase; AS; NP-PEG; PMA; PABA; Thymol; BGG	TLC-UV-VIS; HPLC-ESI-MS	[17]
Schisandra chinensis L.	DPPH; AChE; *B. subtilis*; PMA; PABA; thymol; AS; BGG	HPLC-ESI-MS	[30]
17 fortified plant extracts	DPPH; AChE; BChE; α-glucosidase; β-glucosidase; tyrosinase; β-glucuronidase; *A. fischeri*	HPTLC-HESI-HRMS; HPTLC-UV/Vis/FLD	[132]

4 Conclusions and future perspectives

TLC bioprofiling enables comparing chromatographic data and biological activities of many samples in parallel. The method belongs to EDD which covers all methods (not only chromatographic ones) to detect effects emerging in a given biological system. TLC bioprofiling followed by spectroscopic identification provides knowledge on chemical and biological properties of analytes correlated with structural information and belongs to EDA which covers all methods to identify compounds responsible for these effects. The most popular TLC bioprofiling method is TLC-DB, when biological tests are performed directly on a TLC plate.

The chapter emphasizes the nomenclature and interdependences of the described methods such as TLC profiling, TLC chemical profiling, TLC bioprofiling, TLC-DB, EDD, and EDA. We focused on chosen subjects of TLC bioprofiling from the enormous scope of issues hidden under this term. The main idea of our chapter is to present the current status of TLC bioprofiling and its future perspectives, which are connected to chemometrics, spectroscopic hyphenations, and enzyme inhibition bioassays which are described in more detail in the chapter. Nowadays, multidimensional and multidetection methods allow to obtain new sophisticated fingerprints and improved separations. We believe that plant analysis based on TLC bioprofiling is a convenient, quick, and useful tool for evaluation of their quality, pharmacological properties, and corresponding to the chemical structures of bioactive constituents.

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TLC screening in searching for active components in *Rhodiola rosea* L. roots

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Thin layer chromatography (TLC) has been used for screening analysis of extracts from *Rhodiola rosea* roots. TLC is a fast, simple and inexpensive screening technique, often used in qualitative analysis of plant substances. The results of TLC analysis confirmed to the possible presence of sugars, terpenes, terpenoids, saponines and propylpropanoids in *Rhodiola* roots. The extracts of *Rhodiola rosea* revealed strong antioxidant activity.

1. INTRODUCTION

Rhodiola rosea L. has been used for many years in traditional medicine to treat diarrhea, migraine, hysteria and cognitive dysfunction. The infusion of the root and rhizome of the *Rhodiola* reduces back pain and pain of kidney caused by stones, cures stomatitis and swelling. It also stimulates hair growth and alleviates the symptoms of various skin diseases. *Rhodiol*a has also been known as a plant that helps adapt to external conditions. *Rhodiola*, like other adaptogens, increases the ability of organism to adapt to environmental stressors and generates non-specific resistance [1, 2].

Summarizing, *Rhodiola* (mainly its roots and rhizome), is an adaptogen with anti-stress, antidepressant and antioxidant effects. It also has anticancer and immunostimulatory properties. Thanks to the content of numerous biologically active compounds, it has also a positive effect on memory, thinking and cognitive processes. These properties can be very important in the treatment of diseases associated with central nervous system disorders such as Alzheimer's disease.

Initially, it was believed, that for the adaptogenic properties of *Rhodiola* only two constituents were responsible, that is p-tyrosol and salidroside. Later, the important group of compounds was discovered, the so-called rosavins, which includes: rosin, rosavin and rosarin. In fact, both groups are responsible for the adaptogenic properties of *Rhodiola rosea*. The ratio of rosavins to salidroside in the raw material is 3:1 [2].

Rhodiola rosea biological properties have been investigated using spectrophotometric methods for bulk samples, e.g. DPPH assay [3], acetylcholinesterase (AChE) assay [4] revealing its antioxidant and AChE inhibiting properties, respectively. However, the composition of the plant has been analyzed mostly using chromatographic methods especially gradient HPLC [5-7].

Recently, chromatographic methods have been usually hyphenated with spectroscopic technics as HPLC-LC-MS [8] and GC-MS (essential oils) [9]. Thin layer chromatography (TLC) has been used as an alternative to HPLC, also in testing quality and authenticity of *Rhodiola* samples of different origin [10, 11]. However, there are no papers on separation and detection of bioactive compounds in parallel.

The method of choice is TLC, which is a simple and rapid method for analysis and screening for plant material. The great predominance of TLC over column chromatography is possibility to compare many samples in the one chromatographic run and to perform biological assays directly on TLC plate [12-14]. The aim of the study was TLC screening analysis for biologically active substances in the *Rhodiola rosea*, using chemical and biological-chemical detection.

2. EXPERIMENTAL

2.1. Chemicals

Methanol (99,8%), ethanol (96%), ethyl acetate (99,8%), toluene, chloroform, formic acid (85%), acetic acid (99,5-99,9%) and sulfuric acid (96-98%), all of the analytical grade, were from POCH (Gliwice, Poland), while p-anisaldehyde, thymol, DPPH (2,2-diphenyl-1-picrylhydrazyl) were from Sigma Aldrich (Poznań, Poland).

2.2. Sample preparation

The samples were prepared by maceration of grounded to a powder *R. rosea* dry root (manufactured by NatVita, Poland) with water/alcohol mixtures (Table 1). The plant (g) to solvent (mL) ratio was 1:10 (w/v). All extracts were stored in a refrigerator in dark glass vials.

Table 1. The extracts of *Rhodiola* used for further investigation.

Sample	Solvent	Maceration time	Extraction conditions
S1	Methanol	24h	Dark place, room temperature
S2	Methanol	72h	Dark place, room temperature
S3	Methanol	24h	Shaking
S4	Methanol	72h	Shaking
S5	Ethanol	72h	Dark place, room temperature
S6	70% methanol	72h	Dark place, room temperature
S7	70% ethanol	72h	Dark place, room temperature
S6*	70% methanol	72h	Dark place, room temperature *extract stored in a refrigerator for one year

2.3. Chromatography

Chromatography was performed on 20 cm × 10 m silica gel 60 F_{254} TLC plates and on 10 cm × 10 cm Diol F_{254} HPTLC plates (Merck, Germany). The plates were not activated. The samples (3 mm^3 and

5 mm³) were applied using the Linomat 5 automatic applicator (Camag, Switzerland) as 5 mm and 10 mm bands, respectively, with application velocity at 0.15 mm³/s. TLC plates were developed with mobile phases listed in the Table 2 [15, 16] to a distance of 8 cm using an unsaturated DS horizontal chamber (Chromdes, Poland). The air-dried developed plates were documented using TLC Visualiser (Camag, Switzerland) at UV and VIS light.

Table 2. The mobile phases used in experiments.

Number	Components	Proportions (v/v)
I	ethyl acetate: methanol: water [16]	70/30/10
II	ethyl acetate: methanol: water [15]	77/13/10
III	toluene: ethyl acetate: formic acid [16]	7/3/0,5
IV	chloroform: methanol: water [15]	26/14/3
V	ethyl acetate: chloroform [15]	75/25
VI	ethyl acetate: chloroform: methanol [16]	70/24/6

2.4. Chemical derivatization

2.4.1. AS

AS (p-anisaldehyde) is a good general reagent, used mostly for detection of terpenes, terpenoids, saponines and propylpropanoids. Plates were sprayed with solution of 0.5 cm³ p-anisaldehyde in 85 cm³ methanol, 10 cm³ glacial acetic acid and 5 cm³ concentrated sulfuric acid. Then plates were heated to 105°C for maximum visualization of spots. Chromatograms were documented in VIS light and UV light (366 nm) [17].

2.4.2. Thymol

Thymol reagent is used for detection of sugars. Plates were sprayed with a solution of 2-isopropyl-5-methylphenol (0.5 g) in 95 cm³ ethanol and 5 cm³ concentrated sulfuric acid. For visualization of sugars plates were heated 15-20 min at 120°C. Chromatograms were documented in VIS light [17].

2.4.3. DPPH

DPPH reagent is used for detection of antioxidants. Plates were sprayed with 0,2% 2,2-diphenyl-1-picrylhydrazyl solution in methanol. Results were observed after 30 min in VIS light. Radical scavengers appeared as yellow spots on the purple background [17].

3. RESULTS AND DISCUSSION

3.1. Chromatographic conditions

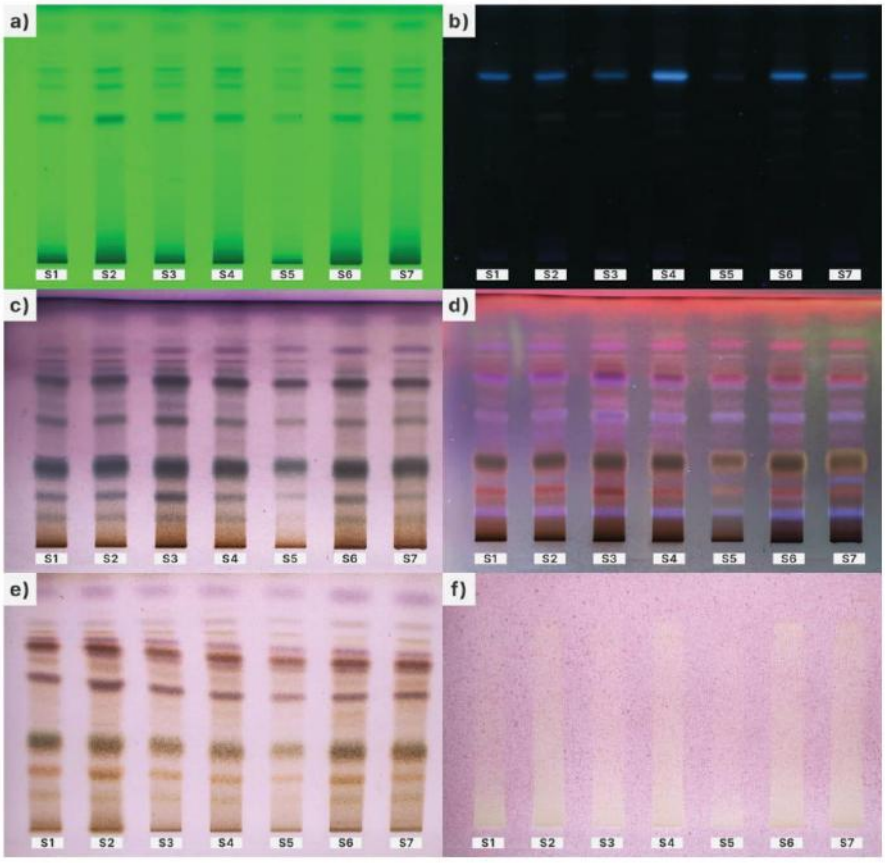

Fig. 1. Set of chromatograms documented using TLC Visualiser at UV and VIS light for *Rhodiola* extracts. Silica gel, mobile phase I. a) 254nm, b) 366nm, c) AS VIS, d) AS 366nm, e) Thymol VIS, f) DPPH VIS.

Preliminary TLC experiments were performed for various extracts (Table 1) to compare their composition and activity. The plant constituents were detected without any derivatization in UV light as well as after spraying with AS, Thymol and DPPH reagents. The mobile phases I and II (Table 2) were used according to the literature [15, 16].

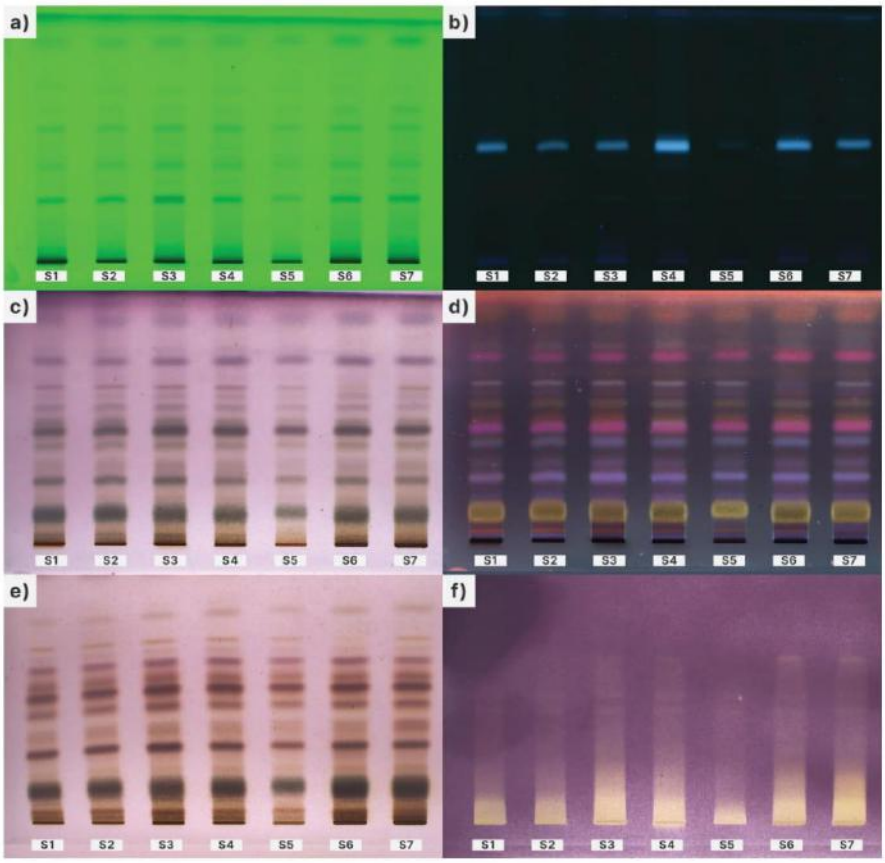

Figure 2. Set of chromatograms documented using TLC Visualiser at UV and VIS light for *Rhodiola* extracts. Silica gel, mobile phase II. a) 254nm, b) 366nm, c) AS VIS, d) AS 366nm, e) Thymol VIS, f) DPPH VIS

Figure 1 and Figure 2 present sets of chromatograms performed for *Rhodiola* roots extracts (Figure 1 – mobile phase I and Figure 2 – mobile phase II). Under these two conditions, the good separation of compounds was achieved. The investigated extracts revealed positive

reactions with AS and Thymol reagents pointing to the possible presence of terpenes, saponines, propylpropanoids and sugars. The DPPH test (Figure 1f and Figure 2f) confirmed antioxidant activity in all *Rhodiola* extracts. The S6 extract is the most rich in components and revealed the strongest antioxidant properties. Basing on these results (Figure 1 and Figure 2), the extract S6 were used for further analysis.

The samples (S6 and S6*) were analyzed using different mobile and stationary phases to find the optimal conditions for analytes. Besides the sample S6, i.e. fresh 70% methanol extract of *Rhodiola* roots, also the S6* (one year old 70% methanol extract of *Rhodiola* roots) was analyzed to compare the stability of the *Rhodiola* extract.

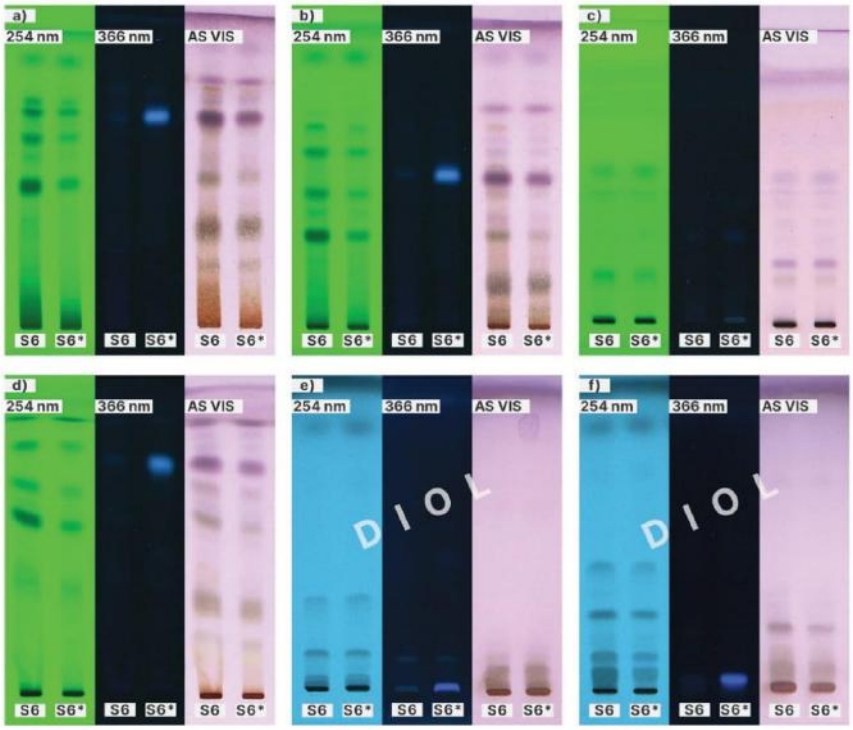

Figure 3. Set of chromatograms documented using TLC Visualiser at UV and VIS light for *Rhodiola* extracts. Silica gel: a-d; diol: e,f; the mobile phases: a) I, b) II, c) III, d) IV, e) V, f) VI.

Figure 3 showed that optimal chromatographic conditions for *Rhodiola* roots samples were obtained with the mobile phases I and II and silica gel 60 as the stationary phase. It is also evident, that the 70% methanol extract of *Rhodiola* roots slightly decomposed throughout a year. The S6 is richer in components compared to the S6*. Additionally, the fluorescence of the compound at R_f 0,6 is much stronger for the S6* (Figure 3a).

4. CONCLUSIONS

Rhodiola rosea has become very popular plant in the recent years. The investigated samples revealed strong antioxidant activity. The optimal chromatographic conditions were achieved with ethyl acetate: methanol: water 70/30/10 (v/v) I and ethyl acetate: methanol: water 77/13/10 (v/v) II mobile phases on silica gel 60 stationary phase.

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Effect directed detection of *Rhodiola rosea* L. root and rhizome extract

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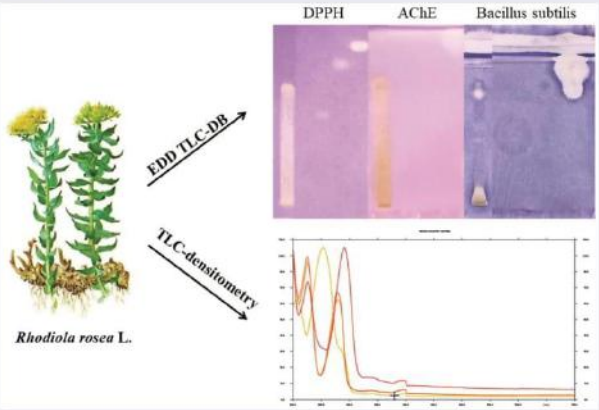
ABSTRACT

Rhodiola rosea is a well-known adaptogen plant with antioxidant, antidepressant, anti-stress, and anti-dementive properties that seems to be important in the potential treatment of disorders associated with central nervous system such as Alzheimer's disease. The aim of this study was to effect directed detection (EDD) of biologically active substances in the root and rhizome of *R. rosea* L. based on chemical and biological assays performed directly on a thin-layer chromatography (TLC) plate. TLC-direct bioautography revealed the presence of both antioxidants (DPPH assay) and antibacterials (*Bacillus subtilis* assay) in the plant methanol extract. Unfortunately, the presence of acetylcholinesterase (AChE) inhibitors was not evident. The marker compounds, typical for *R. rosea* L. that is salidroside, rosavin, p-tyrosol, and hydroquinone were searched in the extract using TLC densitometry. In fact, only rosavin was found in the investigated extract.

KEYWORDS

Densitometry; EDD; *Rhodiola rosea*; TLC-DB

GRAPHICAL ABSTRACT



Introduction

Rhodiola rosea, known also as the golden root, is one of the most studied species of the Crassulaceae family. The herb has been used as an important crude drug for promoting mental health and enhancing the ability to avoid damage from stressors. The healing properties of *R. rosea* are well documented by many scientific reports proving its effectivity in treating numerous diseases, such as neurodegenerative disorders, depression, immunodeficiency, cancer, heart diseases, hyperlipidemia, and obesity.[1,2] The plant is also known as antiviral, antioxidant, and antimicrobial agent.[3,4] In addition, *Rhodiola* has also been used as skincare and wound healing agents.[5] What is the most important, *Rhodiola* has been classified as the most active plant adaptogen. It means that the plant stimulates nonspecific resistance of organism against chemical, physical, and biological stressors.[1,2,6] According to the literature, root and rhizome of *Rhodiola* contain polyphenols, proanthocyanidins, organic acids, glycosides, waxes, essential oils, tannins, and proteins. The main identified active marker components are: salidroside, p-tyrosol, and rosavins (such as rosavin, rosarin, and rosin).[7,8] These compounds are responsible for the pharmacological effects of *R. rosea*. The plant demonstrates very low side effects and low clinical toxicity.[9] For that reason, in recent years, the root and rhizome extracts of *Rhodiola* have been used as additives for drinks, supplements, and pharmaceutical preparations. Most of the products have standardized levels of the marker compounds.

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The optimal ratio of rosavins to salidroside should be equal approximately 3:1.[10]

The research papers on *Rhodiola* focus either on chromatographic analysis and identification of its constituents or on biological properties of full extracts (e.g., antibacterial and antioxidant).[11–15] In order to find and identify active substances in *Rhodiola*, it is necessary to choose the right method capable both for screening biological properties and for structural identification. Among present strategies, the method of choice is the combination of planar chromatography with effect directed detection (EDD), eventually followed by spectroscopic methods.[16,17] The visual interpretation of the plate image enables a fast comparative bioprofiling of various samples.[18–21]

The aim of this study was to determine activity of the root and rhizome of *R. rosea* L. using thin-layer chromatography (TLC) hyphenated with EDD that is antioxidant, antibacterial, and enzyme inhibition tests performed directly on chromatographic plates. The attempt of the marker compounds identification (salidroside, rosavin, p-tyrosol, and hydroquinone) was done using TLC densitometry.

Materials and methods

Chemicals and reagents

Methanol, ethanol, ethyl acetate, acetic acid, sulfuric acid, and hydrochloric acid were from POCH (Gliwice, Poland), while p-anisaldehyde, thymol, 2,2-diphenyl-1-picrylhydrazyl (DPPH), rosavin, salidroside, p-tyrosol, hydroquinone, MTT, Hepes buffer, diphenylboryloxyethylamine (NP), polyethylene glycol-4000 (PEG-4000), acetylcholinesterase (AChE) from *Electrophorus electricus*, Fast Blue B salt, β-naphthyl acetate and bovine serum albumin (BSA) were purchased from Sigma Aldrich (St. Louis, MO). The Gram-positive bacteria, *Bacillus subtilis* (ATCC 6633), were from American Type Culture Collections.

Sample preparation

The sample (extract) of *R. rosea* (dried root and rhizome, NatVita, Mirków, Poland) was obtained by 72 hr maceration of 1 g plant material with 10 mL 70% methanol. Maceration was done in the darkness at room temperature. Standard solutions of rosavin, salidroside, p-tyrosol, and hydroquinone were prepared by dilution of the appropriate amounts of the substance in methanol at a concentration of 1 mg mL^{-1}.

Chromatography

The samples (plant extract and standards) (5 µL) were applied as 5 mm bands 10 mm from the lower edge of TLC silica gel 60 F254 plate (20 × 10 cm, 1.05715.0001, Merck, Germany) using the automatic TLC applicator Linomat 5 (Camag Chemie-Erzeugnisse und Adsorptionstechnik AG, Muttenz, Switzerland). The chromatogram development was performed in DS sandwich chamber (Chromdes, Lublin, Poland) with ethyl acetate:methanol:water 77:13:10 (v/v). The development distance was 8 cm. After that, chromatograms were dried on air. TLC sprayer (Merck, Darmstadt, Germany) was used for the derivatization, while Visualizer (Camag Chemie-Erzeugnisse und Adsorptionstechnik AG, Muttenz, Switzerland) with Digistore 2 Documentation System, VideoScan version 1.1 and WinCATS version 1.4.7 software (Camag Chemie-Erzeugnisse und Adsorptionstechnik AG, Muttenz, Switzerland) was used for documentation. Densitometric scans were performed using TLC Scanner version 4 (Camag Chemie-Erzeugnisse und Adsorptionstechnik AG, Muttenz, Switzerland) (spot dimensions were 4 × 0.3 mm, scanning speed was 20 mm s^{-1}, and data resolution was 100 µm per step). Spectral detection was performed in range the 200–700 nm with scanning speed 100 nm s^{-1} and data resolution 1 nm per step. Both operations were under control of Camag WinCATS version 1.4.9 Software.

Derivatization

AS

Plates were sprayed with solution of p-anisaldehyde (0.5 mL) in 85 mL methanol, 10 mL acetic acid and 5 mL sulfuric acid. Then plates were heated on the TLC Plate Heater (Camag Chemie-Erzeugnisse und Adsorptionstechnik AG, Muttenz, Switzerland) to 105 °C for 5–7 min. Chromatograms were documented in VIS (reflected light) and UV light by the Visualizer.[22]

Thymol

Plates were sprayed with a solution of 2-isopropyl-5-methylphenol (0.5 g) in 95 mL ethanol and 5 mL sulfuric acid. For visualization of sugars, plates were heated for 15–20 min on the TLC Plate Heater (Camag Chemie-Erzeugnisse und Adsorptionstechnik AG, Muttenz, Switzerland) at 120 °C. Chromatograms were documented in VIS (reflected light).[22]

NP-PEG. The plate was sprayed with NP solution (1 g diphenylboryloxyethylamine in 100 mL methanol) and PEG solution (5 g PEG-4000 in 100 mL ethanol), subsequently. Chromatograms were documented in UV 366 nm light.[22]

Effect directed detection

DPPH assay

Plates were sprayed with 0.2% 2,2-diphenyl-1-picrylhydrazyl solution in methanol. Results were observed after 30 min in VIS (reflected light). Radical scavengers appeared as yellow spots on the purple background.[21]

AChE assay

The chromatograms were sprayed in the enzyme solution (20 units of AChE and 150 mg BSA in 150 mL 0.05 M TRIS buffer, pH 7.8) and substrate solution (150 mg 2-naphthyl acetate solution was dissolved in 50 mL ethanol),

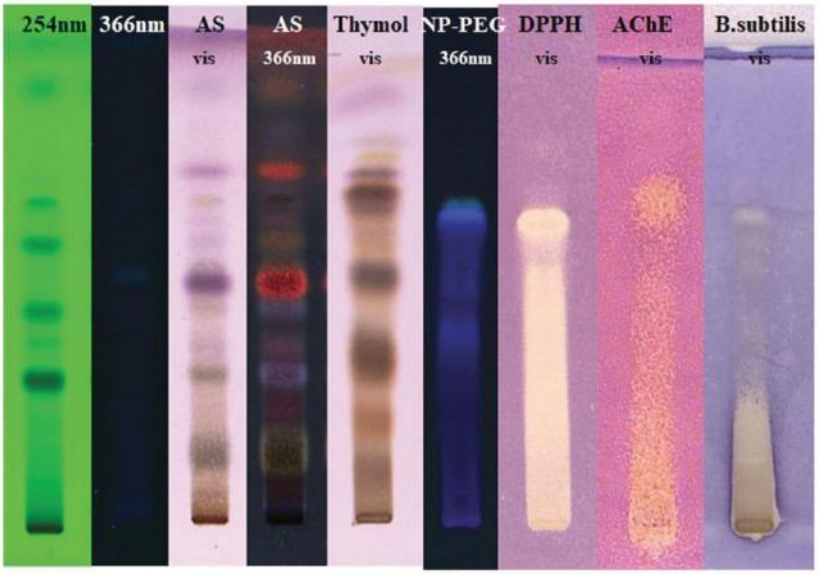

Figure 1. The set of chromatograms and bioautograms of 70% methanol extract of *Rhodiola rosea*. Mobile phase: ethyl acetate:methanol:water 77:13:10 (v/v).

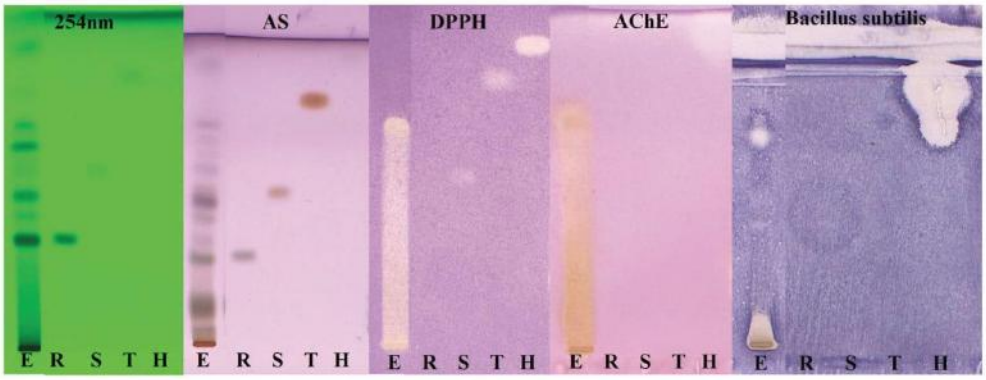

Figure 2. The set of chromatograms and bioautograms of 70% methanol extract of *Rhodiola rosea* and the standard solutions. Mobile phase: ethyl acetate:methanol:water 77:13:10 (v/v). E-extract, R-rosavin, S-salidroside, T–p-tyrosol and H-hydroquinone.

subsequently. After spraying, TLC plate was dried quickly, because ethanol might inhibit acetylcholine activity. The plate was incubated in the humid atmosphere in a closed vessel at 37 °C for 20 min. After incubation, the plate was sprayed with 50 mg Fast Blue B salt diluted in 100 mL of water. AChE inhibitors appeared like white spots on the purple background. Results were documented in VIS (reflected light).[23]

Bacillus subtilis bioassay

The chromatograms were immersed for 8 s in the bacterial suspension (8.0×10^7 CFU mL^{-1}) using the TLC Immersion Device. Then, the plate was placed in a plastic box lined with the wetted paper and incubated at 37 °C for 17 h. For visualization, the bioautogram was sprayed with 0.2% MTT aqueous solution. To improve the intensity of the color, one drop of Triton X-100 was added per 10 mL of aqueous MTT solution. After re-incubation at 37 °C for 0.5 h, white zones of bacterial growth inhibition were visible against the purple background. The bioautogram was documented in VIS (reflected light) using the Visualizer.[24]

Results and discussion

Screening analysis

The samples of *Rhodiola* were chromatographed and further derivatized with various chemical reagents to observe the groups of substances present in the 70% methanol extracts of root and rhizome (Figure 1). The chromatograms derivatized with AS reagent pointed to the possible presence of saponins, essential oils, and terpenes as yellow-brown, violet, green, blue, and red spots. In the thymol test, glycosides and

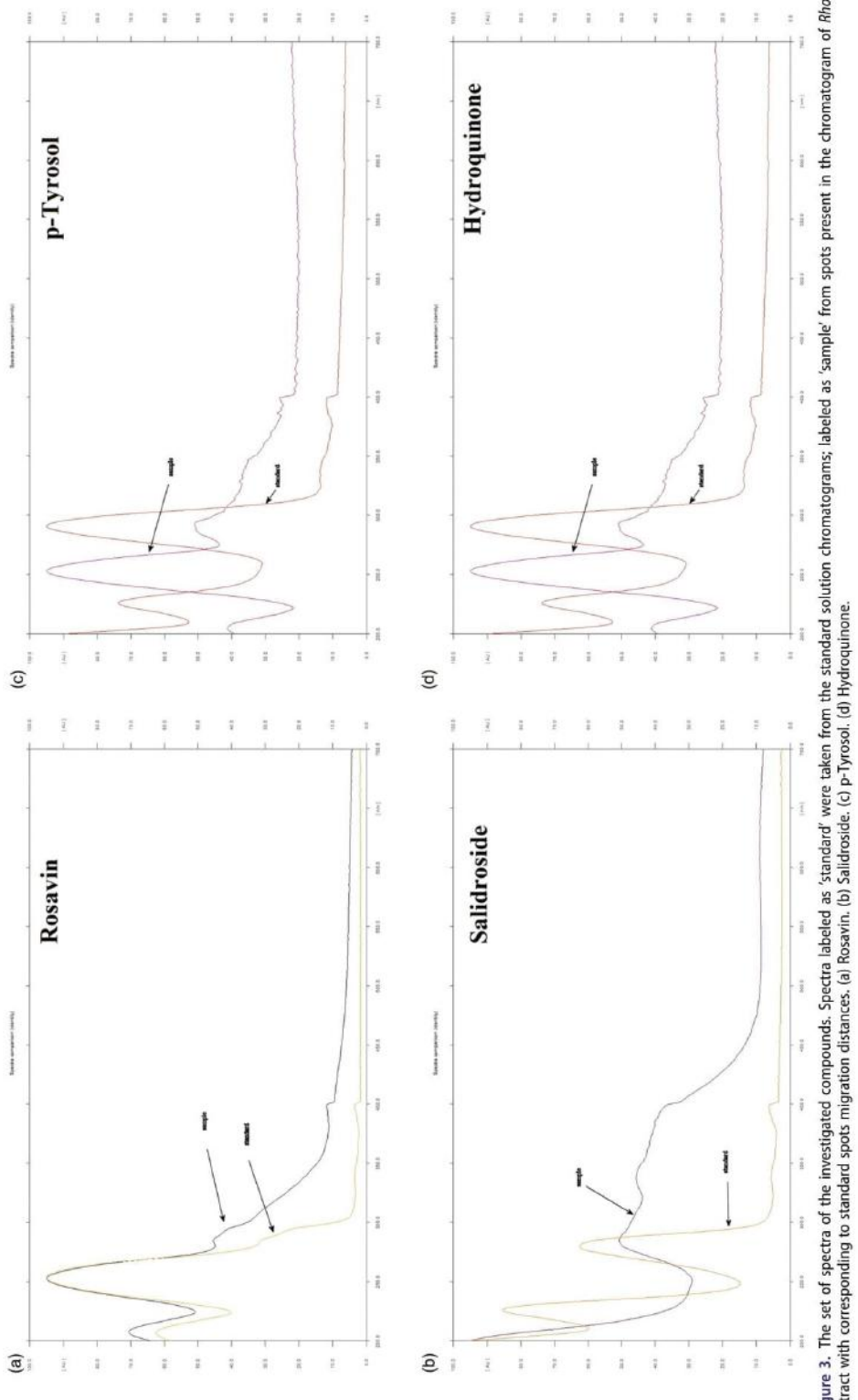

Figure 3. The set of spectra of the investigated compounds. Spectra labeled as 'standard' were taken from the standard solution chromatograms; labeled as 'sample' from spots present in the chromatogram of *Rhodiola* extract with corresponding to standard spots migration distances. (a) Rosavin. (b) Salidroside. (c) p-Tyrosol. (d) Hydroquinone.

sugars were detected as brown zones. The intense blue spots appeared due to the presence of phenol carboxylic acids, while the dark green spots – of flavanones in NP-PEG test.[22]

Effect directed detection

TLC-screening analysis gave general information on the composition of *R. rosea*. The presence of polyphenols suggested its possible antioxidant properties. To confirm it, DPPH assay was performed directly on TLC plate with separated extract of *Rhodiola* root and rhizome (Figure 1). Oxidative stress leads to serious diseases such as osteoporosis, cancer, dementia, and Alzheimer's illness.[4,5] The important natural source of antioxidants are plants reach in carotenoids, tocopherols and polyphenols. The extract of *R. rosea* showed strong radical-scavenging activity proved by the presence of large pale confluent zones between $hR_F = 0$ and $hR_F = 65$. This activity may be due to the high content of phenolic compounds, which presence was confirmed in the NP-PEG test.

Two other TLC-DB tests were also performed to investigate the biological activity of *Rhodiola*. These were TLC-DB against *Bacillus subtilis* for detection of antimicrobials and TLC-DB-AChE assay for enzyme inhibition activity.

In traditional Russian and Mongolian medicine, *Rhodiola* is well known as antimicrobial and antinflammatory agent with regenerative properties, therefore, it is used to treat skin diseases and diarrheas caused by bacteria.[2] Components with antibiotic effect against *Bacillus subtilis* were detected in the extract using TLC-DB. They were seen on TLC plate as bright zones between $hR_F = 0$ and $hR_F = 65$. The similar ranges were noticed both for polyphenols visualized by NP-PEG and for antioxidants in DPPH assay. Probably both effects i.e., antioxidant and antibacterial are related to the presence of polyphenols in the investigated extract.

AChE is a very important enzyme controlling the level of choline – the neurotransmitter of great impact on central and peripheral nervous system. According to the cholinergic theory, inhibitors of AChE are potential drugs for Alzheimer's disease.[4]The AChE inhibitors of plant origin are especially appreciated in this matter. They may be also used in glaucoma treatment. In the AChE assay, AChE inhibiting compounds should be detected as yellowish zones on a purple background, however, in the case of AChE assay for investigated extract from *Rhodiola*, the inhibiting zones were masked by brown color which could result from reaction between polyphenols and Fast Blue B salt reagent.[25]

The obtained results confirm a strong antioxidant and antibacterial activity of the root and rhizome of *R. rosea* ascribed to it in the literature. However, the AChE activity of the plant is not evident. Thus, it is possible that antidementive properties of *Rhodiola*, known from the literature, are based on other, than AChE inhibition, effect.

For qualitative analysis purpose, the investigated extract was applied on one TLC plate together with standards (rosavin, salidroside, p-tyrosol, and hydroquinone) and after development subjected to chemical derivatization and TLC-DB (Figure 2). According to the literature, the earlier mentioned standards, are marker compounds responsible for the biological activity of *Rhodiola*. The derivatization with AS reagent shows where the spots of standards are located. Rosavin is seen as a dark green spot at $hR_F = 32$, salidroside as a brown spot at $hR_F = 53$ and p-tyrosol as orange-brown spot at $hR_F = 82$. Hydroquinone was not detected in the AS derivatization. In DPPH assay, standards of salidroside and p-tyrosol reveal medium antioxidant activity, in contrast to the standard of hydroquinone which gives the strong pale zone. The standard of rosavin does not show any antioxidant activity. In AChE and *Bacillus subtilis* assays, only hydroquinone shows both antimicrobial and enzyme inhibition activity.

In order to confirm the presence of the markers in the investigated *Rhodiola* extract, TLC densitometry was used. The obtained spectra (Figure 3a–d) confirmed only the presence of rosavin in the investigated *Rhodiola* extract. The absence of salidroside, p-tyrosol, and hydroquinone can be caused by improper harvesting and/or storage of plant material. The important factor, influencing the presence of the marker substances is also the place of harvest and the age of the plant subjected to the analysis.

Conclusions

EDD based on TLC is a rapid and simple method used to acquire bioprofiling information about a given plant (in this case – *R. rosea*). The obtained results showed that the *Rhodiola* root and rhizome extract has strong antioxidant and antibacterial activity probably due to the presence of polyphenols. Unexpectedly, AChE inhibition assay did not unanbigously confirm its antidementive properties described in the literature. TLC densitometry was used for checking the presence of the marker compounds (rosavin, salidroside, p-tyrosol, and hydroquinone) in the plant sample. The spectra confirmed only the presence of rosavin in the investigated *Rhodiola* extract.

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Effect-directed analysis as a method for quality and authenticity estimation of *Rhodiola rosea* L. preparations

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ABSTRACT

Adulterations of food and pharmaceutical preparations are the important global problem. On the one hand, fraud practices are becoming more and more sophisticated while on the other, monitoring and uncovering falsifications are insufficient. One of the most common consumer concern is the quality and authenticity of the purchased products, related to the confidence that they have composition and properties in accordance with the manufacturer's declaration on the label. This refers also to pharmaceuticals potentially delivering great health benefits such as *Rhodiola rosea* L. supplements. The aim of this study was defining authenticity and possible adulterations of two *R. rosea* preparations basing on their TLC-bioprofiles and the presence of biomarker compounds characteristic for this plant. The effect-directed analysis (EDA), i.e. TLC hyphenated with micro-chemical and biological assays performed directly on TLC plates followed by HPLC-ESI-MS was used for the bioprofiling of antioxidants, antibacterials, and inhibitors of lipase, acetylcholine, α-glucosidase and tyrosinase as well as for the identification of the biomarkers. The results pointed to the possible adulteration of one of the tested products related to the absence of two rosavins, the most important quality markers of *R. rosea*.

1. Introduction

Rhodiola rosea L. *(R. rosea)* is a commonly known herbal drug with a long history of using in several traditional healing systems, such as Traditional Chinese Medicine. *R. rosea* has received the attention as an adaptogenic and ergogenic agent and has been used for various medical purposes linked to acute and chronic stress, including physical performance. Herbal products containing the root and/or rhizome of *R. rosea* are widely available on the European market, mainly as so-called food supplements. These products have to be proved for their quality, efficacy and safety prior to gaining access to the market. Unfortunately, most of the supplements are sold without any registration via Internet. The European Pharmacopoeia focuses on authenticity standardization of *R. rosea* products based on a ratio of marker compounds i.e. salidroside and rosavins, which should equals 1:3. In many cases the level of markers is too low what can be related to adulterations. It especially concerns rosavins. However, the absence of rosavins may not always be an indicative for falsification. Enzymatic degradation of rosavins can be caused by improper harvesting or processing of the plant material. Another concern regarding the authenticity of *R. rosea* is the admixture of root and/or rhizome from other *Rhodiola* species. In the European herbal supplement industry, *R. rosea* raw material from Asia is often mixed with other *Rhodiola* species, for example *R. crenulata*. Taking into account the above mentioned facts, the *R. rosea* supplements may carry a high risk potential due to insufficient definition, problems with identity, purity and falsifications [1–3].

According to the literature, *R. rosea* is rich in polyphenols, flavonoids, proanthocyanidies, phenolic glycosides, organic acids, sugars, tannins, terpenes and essential oils [4,5]. The plant contains also so-called marker compounds characteristic for this species:

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phenylpropanoids (rosavin, rosin, rosarin – the group of rosavins) and phenylethanoids (salidroside, viridoside and p-tyrosol). The adaptogenic properties of *R. rosea* are mostly related to the presence of these marker compounds [6,7], while antioxidant activity mainly to organic acids and flavonoids.

Effect-directed analysis (EDA) based on planar chromatography allows for screening of bioactive compounds in complex matrices and identification of the type and structure of distinguishing substances. The separation, bioassays and visualization are performed directly on a TLC plate for many samples in parallel, which allows comparing them as well as the target and/or non-target detecting compounds responsible for the bioactive response [8]. This effect-directed detection (EDD) step of EDA, followed by spectroscopic identification gives full information on both structure and bioactivity of the sample constituents.

The aim of the paper is the investigation of authenticity, possible adulteration and bioactivity of two *R. rosea* preparations, available on the market in Europe, using TLC-fingerprints and EDA. The bioprofiling of antioxidants and antibacterials as well as inhibitors of lipase, acetylcholinesterase, α-glucosidase and tyrosinase, combined with TLC-UV-VIS, followed by TLC micro-fractionation and HPLC-ESI-MS was performed to provide information about the presence of marker and authenticity constituents such as salidroside, p-tyrosol and rosavins known for their therapeutic efficacy. Additionally, for comparison, TLC fingerprinting, bioprofiling and HPLC-ESI-MS of the *R. rosea* reference standard was performed.

2. Material and methods

2.1. Reagents

All reagents were of the analytical grade. Acetone, acetic acid, ethanol, ethyl acetate, methanol, o-phosphoric acid, sodium hydroxide, sulfuric acid, phosphomolybdic acid (PMA), 2-isopropyl-5-methyl-phenol (thymol), p-aminobenzoic acid (PABA), diphenylboryloxyethylamine (NP), polyethylene glycol – 4000 (PEG-4000) sodium acetate buffer and phosphate buffer were from POCH (Poland). p-Anisaldehyde (AS), bromocresol green (BCG), 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2-naphthyl acetate, bovine serum albumin (BSA), acetylocholinesterase (AChE) from *Electrophorus electricus*, Fast Blue B salt, TRIS buffer, 2-naphthyl α-D glucopyranoside, α-glucosidase from *Saccaromyces*, 1-naphthyl acetate, lipase from *Porcine pancreas*, L-DOPA, Triton X, tyrosinase from mushroom, 3-(4,5-dimethyldiazol-2-yl)-2,5 diphenyltetrazolium bromide (MTT) dye, Hepes buffer, rosavin, salidroside, p-tyrosol, hydroquinone, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, luteolin, saccharose, glucose and galactose were purchased from Sigma Aldrich (Poland). Mueller-Hinton (M-H) agar and M-H broth were purchased from Biocorp (Warsaw, Poland). The Gram-positive bacteria, *Bacillus subtilis* (ATCC 6633) were from American Type Culture Collections. Pure water was from Milipore Q system (Millipore, Bedford, MA, USA).

2.2. Sample preparation

Two samples of *R. rosea* diet supplements were purchased from NatVita, Poland (dry root and rhizome) and Fushi, Great Britain (dry root) while the United States Pharmacopeia (USP) reference standard of *R. rosea* (dry root and rhizome) from Sigma Aldrich. The samples were macerated in 70% methanol (1 g per 10 ml) for 72 h in the darkness at room temperature, then filtered through a paper filter. After filtration, extracts were used for TLC application. The standards of rosavin, salidroside, p-tyrosol, hydroquinone, caffeic acid, chlorogenic acid, ferulic acid, gallic acid, luteolin, saccharose, glucose and galactose were dissolved in methanol at the concentration of 1 mg mL^{-1}. All samples were stored at -8°C.

2.3. Planar chromatography

The samples (plant extracts and standards) were applied as 8 mm bands (10 mm from the lower and left edge, at the distance of 13 mm between tracks) on TLC plates (silica gel 60 F_{254}, 20 × 10 cm, 1.05715.0001, Merck, Germany) using the automatic TLC applicator Linomat 5 (Camag, Switzerland). The application volume was 5 μL. TLC separation was carried out with ethyl acetate-methanol-water 77:13:10 (v/v/v) as a mobile phase in the DS sandwich chamber (Chromdes, Poland) up to the migration distance of 8 cm. After separation, chromatograms were dried on air. Then, dry chromatograms were documented at UV 254 nm, UV 366 nm and white light illumination (at a reflectance mode) using Visualiser with DigiStore 2 Documentation System, VideoScan 1.1 and winCATS 1.4.7 software (Camag, Switzerland). The chromatograms were derivatized by automatic piezoelectric spraying (TLC Derivatizer for 20 × 10 cm plates, Camag, Switzerland). The blanks control was done to screen false positive results.

2.4. Dot-blot

The samples (plant extracts and standards) were applied manually on the TLC plate silica gel 60 F_{254} (20 × 10 cm, 1.05715.0001, Merck, Germany) in 5 μL volume using microsyringe (Hamilton, Switzerland). After application the plates were documented at UV 254 nm and UV 366 nm using Visualiser and subjected to bioassays.

2.5. Derivatization

2.5.1. AS reagent

The chromatogram was derivatized with 4 mL of p-anisaldehyde sulfuric acid reagent (0.5 mL of p-anisaldehyde was dissolved in 85 mL of methanol, then 10 mL of acetic acid and 5 mL of sulfuric acid were added) by automatic piezoelectric spraying (red nozzle, speed 6). After that, the plate was heated on the TLC Heater (Camag, Switzerland) at 105°C for 7 min and documented at VIS and UV 366 nm light using the Visualiser [9].

2.5.2. PMA reagent

The chromatogram was derivatized with 4 mL of PMA reagent (20 g of phosphomolybdic acid dissolved in 100 mL of ethanol) by automatic piezoelectric spraying (blue nozzle, speed 6). After that, the plate was heated at 100°C for 5 min and documented at VIS light using the Visualiser [9].

2.5.3. Thymol reagent

The chromatogram was sprayed with 4 mL of thymol (0.5 g) solution in 95 mL of ethanol and 5 mL of sulfuric acid by automatic piezoelectric spraying (red nozzle, speed 6). After that, the plate was heated at 120°C for 15 min and documented at VIS light and UV 366 nm using the Visualiser [9].

2.5.4. PABA reagent

The chromatogram was sprayed with 4 mL of PABA reagent (0.5 g of p-aminobenzoic acid dissolved in 18 mL of glacial acetic acid diluted with 20 mL of water, then added 60 mL of acetone and 1 mL of o-phosphoric acid) by automatic piezoelectric spraying (red nozzle, speed 6). After that, the plate was heated at 140°C for 5 min and documented at VIS light and UV 366 nm using the Visualiser [10].

2.5.5. BCG reagent

The chromatogram was derivatized with 4 mL of BCG solution (40 mg BCG dissolved in 100 mL of ethanol and 5 mL of 0.1 M NaOH) by automatic piezoelectric spraying (green nozzle, speed 6).

After that, chromatogram was documented at VIS light using the Visualiser [9].

2.5.6. NP-PEG reagent

The chromatogram was sprayed with 4 mL of the NP solution (1 g of NP dissolved in 100 mL of methanol) followed by 4 mL PEG solution (5 g of PEG-4000 dissolved in 100 mL of ethanol) by automatic piezoelectric spraying (red nozzle, speed 6). After that, chromatogram was documented at UV 366 nm light using the Visualiser [9].

2.6. Effect directed detections

2.6.1. DPPH assay

The chromatogram was sprayed with 4 mL of 0.2 % DPPH solution in methanol by automatic piezoelectric spraying (blue nozzle, speed 6). Results were observed after 30 min at VIS light. Radical scavengers appeared as white bands against the purple background [11].

2.6.2. AChE assay

The chromatogram was sprayed with 4 mL of substrate solution (150 mg of 2-naphthyl acetate solution dissolved in 50 mL of ethanol) by automatic piezoelectric spraying (green nozzle, speed 6). After that, the plate was dried with a stream of cold air. Subsequently the plate was sprayed with 4 mL of the enzyme solution (20 units of AChE and 150 mg of BSA in 150 mL of 0.05 M TRIS buffer, pH 7.8) (red nozzle, speed 6). The plate was incubated horizontally in the humid atmosphere in a closed vessel at 37°C for 20 min. After incubation, the plate was sprayed with 2 mL of visualization solution (50 mg of Fast Blue B salt diluted in 100 mL of water) (blue nozzle, 6 speed). AChE inhibitors appeared as white bands against the purple background. Results were documented at VIS light [12].

2.6.3. α-Glucosidase assay

The chromatogram was sprayed with 2 mL of substrate solution (60 mg of 2-naphthyl α- D glucopyranoside in 50 mL of ethanol) by automatic piezoelectric spraying (green nozzle, speed 6) and subsequently dried for removing ethanol using a stream of cold air. Subsequently. the plate was sprayed with 4 mL of enzyme solution (500 units of α-glucosidase in 50 mL of sodium acetate buffer pH 7.5) (red nozzle, 6 speed). After spraying, the plate was incubated horizontally in the humid atmosphere in a closed vessel at 37°C for 10 min. After incubation the plate was sprayed with 0.5 mL of visualization solution (10 mg Fast Blue B salt diluted in 10 mL of water) (blue nozzle, speed 6). α-Glucosidase inhibitors appeared as white bands against the purple background. Results were documented at VIS light [13].

2.6.4. Lipase assay

The chromatogram was sprayed with 3 mL of substrate solution (150 mg of 1-naphthyl acetate in 100 mL ethanol) by automatic piezoelectric spraying (green nozzle, 6 speed) and dried using a stream of cold air to remove ethanol. After that, the plate was sprayed with 4 mL of enzyme solution (500 units of lipase and 50 mg of BSA in 50 mL of 0.05 M TRIS-HCl buffer pH 7.4) (red nozzle, speed 6). Then, the plate was incubated in the humid atmosphere in a closed vessel at 37°C for 20 min. After incubation the plate was sprayed with 2 mL of visualization solution (50 mg of Fast Blue B salt in 100 mL of water) (blue nozzle, 6 speed). Lipase inhibitors appeared as white bands against the purple background. Results were documented at VIS light [14].

2.6.5. Tyrosinase assay

The chromatogram was sprayed with 2.5 mL of substrate solution (0.1183 g of L-DOPA diluted in 49.5 mL of 0.02 M phosphate buffer pH 6.8 and 0.5 mL of Triton X) by automatic piezoelectric spraying (red nozzle, speed 6). Subsequently, the plate was sprayed with 3.0 mL of enzyme solution (400 units of tyrosinase in 1 mL of 0.02 M phosphate buffer, pH 6.8) (red nozzle, speed 6). After that, the plate was incubated for 10 min at room temperature in the closed vessel in humid atmosphere. Tyrosinase inhibitors appeared as white bands against grey background. Results were documented at VIS light [15].

2.6.6. Bacillus subtilis *bioassay*

The chromatogram was immersed for 8 s in the bacterial suspension (8.0×10^7 CFU mL^{-1}) using the TLC Immersion Device (Camag, Switzerland). Then, the plate was placed in a plastic box lined with the wetted paper and incubated at 37°C for 17 h. The bioautogram was sprayed with visualization solution (0.2 % of MTT aqueous solution with one drop of Triton X-100). After re-incubation at 37°C for 0.5 h, white zones of bacterial growth inhibition were visible against the purple background. The bioautogram was documented at VIS light [16].

2.7. Micro-preparative analysis

The extracts (80 μL each) of the USP standard, NatVita and Fushi were applied as 8 cm band on the analytical TLC plate and separated into fractions with ethyl acetate-methanol-water 77:13:10 (v/v/v). The fractions were scraped, placed in the Eppendorf tubes and eluted with 2200 μL of methanol. After filtration through the paper filter, the eluates were concentrated by evaporation of the solvent under a stream of nitrogen to dryness and reconstituted with 200 μL of methanol. Then, the fractions (Table 1S; Fig. 1S) and the standards were subjected to HPLC-ESI-MS analysis in negative ionization mode.

2.8. Mass spectrometry

The HPLC-MS analysis was performed using the HPLC-MS system composed of the UHPLC chromatograph (UltiMate 3000, Dionex, Sunnyvale, CA, USA), the linear trap quadrupole-Orbitrap mass spectrometer (LTQ-Orbitrap Velos, Thermo Fisher Scientific, San Jose, CA), the ESI ionization source operating in the negative polarization mode at the needle potential equal 4.5 kV. Nitrogen (> 99.98%) was used as a sheath gas - 40 arbitrary units, an auxiliary gas - 10 arbitrary units, a sweep gas - 10 arbitrary units; capillary temperature equals 320°C. The scan cycle used a full-scan event at the resolution of 60.000. For chromatographic separation Gemini C18 column (4.6 × 100 mm, 3 μm; Phenomenex, USA) was used. Mobile phase components were: A - 25 mM formic acid in water and B - 25 mM formic acid in acetonitrile. The gradient program started at 5% B increasing to 95% for 60 min and was followed by isocratic elution (95% B) for 10 min. The total run time was 70 min at the mobile phase flow rate 0.5 mL min^{-1}. The MS spectra were continuously collected in the range of 100-1000 m/z at 50 ms scan rate in the course of each run.

2.9. TLC densitometry

Densitometric scans were performed using TLC Scanner version 4 (Camag, Switzerland). The spot dimensions were 4 × 0.3 mm, scanning speed was 20 mm s^{-1}, and data resolution was 100 μm per step. Spectral detection was performed in the range of 200–700 nm with scanning speed 100 nm s^{-1} and data resolution 1 nm per step. Both operations were under control of Camag WinCATS version 1.4.9 Software.

Table 1
HPLC-ESI-MS results for USP standard, Fushi and NatVita samples. Negative ionization mode.
F1-F7 micro-preparative fractions (see the Supplementary material Fig. 1S and Table 1S).

Compounds	Molecular formula	Theoretical monoisotopic ion mass [Da]	Measured monoisotopic ion mass [Da]	Mass accuracy [Δ ppm]	USP standard	NatVita	Fushi
*Rosavin	$C_{20}H_{27}O_{10}$	427.16043	427.16044	0.03	F2 F3	F2 F3	NI
Rosarin	$C_{20}H_{27}O_{10}$	427.16043	427.16049	0.15	F2 F3	F2 F3	NI
Rosin	$C_{15}H_{19}O_6$	295.11817	295.11818	0.05	F5	F4 F5	F4 F5
*Salidroside	$C_{14}H_{19}O_7$	299.11308	299.11310	0.07	F4	F4	F4
*p-Tyrosol	$C_8H_9O_2$	137.06025	137.06022	0.23	F6 F7	F6 F7	F6 F7
Viridoside	$C_{15}H_{21}O_7$	313.12873	313.12882	0.29	F4	F3 F4	F3 F4
Galactose	$C_6H_{11}O_6$	179.05557	179.05552	0.25	F1 F2	F1 F2	F1 F2
*Saccharose	$C_{12}H_{21}O_{11}$	341.10839	341.10843	0.12	F2 F3	F1 F2 F3	F2 F3
*Glucose	$C_6H_{12}O_6$	179.05557	179.05559	0.13	F1 F2	F1 F2	F1 F2
*Caffeic acid	$C_9H_7O_4$	179.03444	179.03437	0.36	F6	F6	F6
*Chlorogenic acid	$C_{16}H_{17}O_9$	353.08726	353.08741	0.42	F2 F3	F2 F3	F2 F3
*Ferulic acid	$C_{10}H_9O_4$	193.05009	193.05009	0.02	F6 F7	F6 F7	F6 F7
*Gallic acid	$C_7H_5O_5$	169.01370	169.01366	0.24	F6	F6	F6
Cinnamyl alcohol	C_9H_9O	133.06534	133.06529	0.38	F6 F7	F7	F7
Kaempferol	$C_{15}H_9O_6$	285.03992	285.03999	0.26	F5 F6	F5 F6	F5 F6
Luteolin-7-O-Glc	$C_{21}H_{19}O_{11}$	447.09274	447.09271	0.07	F4 F5	F4 F5	F4 F5
*Luteolin	$C_{15}H_9O_6$	285.03992	285.03998	0.23	F5 F6	F5 F6	F5 F6
Apigenin-7-Glc	$C_{21}H_{19}O_{10}$	431.09783	431.09780	0.06	F4	F4	F4
Herbacetin	$C_{15}H_9O_7$	301.03483	301.03489	0.20	F7	F6 F7	F6 F7
Rhodiosin	$C_{27}H_{29}O_{16}$	609.14557	609.14556	0.02	F2 F3	F2 F3	F2 F3
Rhodionin	$C_{21}H_{19}O_{11}$	447.09274	447.09284	0.22	F3 F4	F4	F4
Rhodioflavonoside	$C_{27}H_{29}O_{17}$	625.14048	625.14049	0.02	F2 F3	F2 F3	F2 F3
Gossypetin -7-O-rhamnopyranoside	$C_{21}H_{19}O_{12}$	463.08766	463.08772	0.14	F3	F3	F2 F3
*Hydroquinone	$C_6H_5O_2$	109.02896	109.02894	0.10	F6 F7	F6 F7	F6 F7

NI - not identified
* The standards subjected to HPLC-ESI-MS analysis

3. Results and discussion

3.1. TLC fingerprints combined with TLC-UV-VIS

The preliminary experiments were focused on comparing fingerprints of two supplements of *R. rosea*: NatVita and Fushi in reference to the USP standard. The UV, micro-chemical (derivatization) and biological (EDD) profiles were compared, revealing their differentiations (Fig. 1). The differences are already seen on the chromatograms at UV 254 and 366 nm. The distinctive fingerprints are revealed by most of the derivatization procedures – generally, NatVita gives more sophisicated fingerprints similar to those obtained for the USP standard (Fig. 1).

The major bioactive compounds of *R. rosea, i.e.* rosavin, salidroside and p-tyrosol, are often used for the quality evaluation of *R. rosea* preparations [15,17,24]. After derivatization with AS reagent, standards of rosavin, salidroside and p-tyrosol were detected as color bands at hRf 24 - rosavin (violet), at hRf 47 - salidroside (green brown) and at hRf 78 - p-tyrosol (brown) (Fig. 2). The presence of rosavin was confirmed by the AS test both in the USP standard and the NatVita extracts (Fig. 2). However, this spot was missing in the Fushi extract. To check the possible presence of rosavin in the extracts, UV-VIS spectra were taken using TLC densitometry (Fig. 3). The obtained spectra confirmed the presence of rosavin in the NatVita extract and the USP standard (overlapped spectra of rosavin standard and related to it compound both in the NatVita and the USP extracts, taken at the same hRf value i.e. 24). The spectrum obtained for the band at hRf 24 of the Fushi extract was different from that of rosavin. The results pointed to the absence of rosavin in the Fushi sample, which may suggest the adulteration of this supplement or improper manufacturing procedures [1,2].

The presence of the salidroside and p-tyrosol in the extracts was hard to confirm using derivatization tests due to low intensity of the spots, what can be related to their low content in the extracts. Additionally, the derivatization tests pointed to the presence of sugars (detected using thymol and PABA tests), acids (detected by BCG test at the start position) and polyphenols (revealed by NP-PEG reagent) in all *R. rosea* extracts (Fig. 1). In particular, saccharose, glucose and galactose were detected using thymol test as brown bands at hR_f 27, 36 and 31, respectively (Fig. 2). According to the literature [5], *R. rosea* contains phenolic acids including caffeic, chlorogenic, ferulic and gallic acids with strong antioxidant properties. In the NP-PEG test (Fig. 2) the standards of caffeic (hR_f 67), chlorogenic (hR_f 18), ferulic (hR_f 68) and gallic (hR_f 64) acids were detected, however their presence in the extracts of *R. rosea* couldn't be confirmed, probably because of too low sensitivity of TLC-NP-PEG test. Finally, DPPH test (Fig. 2) was done which confirmed antioxidant properties of the aforementioned acids. However, it was impossible to detect them unequivocally in the extracts. The bioassays, including DPPH, are discussed in more detail in the next paragraph.

3.2. EDD - TLC bioactivity assays

The main experiments focused on biological properties of two *R. rosea* preparations which were investigated using EDD that is TLC-bioactivity assays. The bioassays were performed both for the extracts and the standards (marker constituents - rosavin, salidroside, p-tyrosol and hydroquinone). The antioxidants, enzyme inhibitors and antimicrobials were revealed on bioautograms as bright zones. The biological assays were performed both on the developed (Fig. 4) and not developed plates (the dot-blots, Fig. 2S). The dot-blots gave general information on biological properties of the standards and of the whole, non-separated samples taking into account possible synergistic or antagonistic effects in the plant extracts. Table 2S contains detailed information on the bioactive zones found in *R. rosea* extracts and those of the standards.

The DPPH assay showed antioxidative activities (Fig. 4) of all three *R. rosea* extracts and standards of salidroside, p-tyrosol and hydroquinone. The extract of Fushi had stronger antioxidant activity comparing to the NatVita and the USP standard ones. The standards of salidroside (hR_f 47) and p-tyrosol (hR_f 78) revealed rather

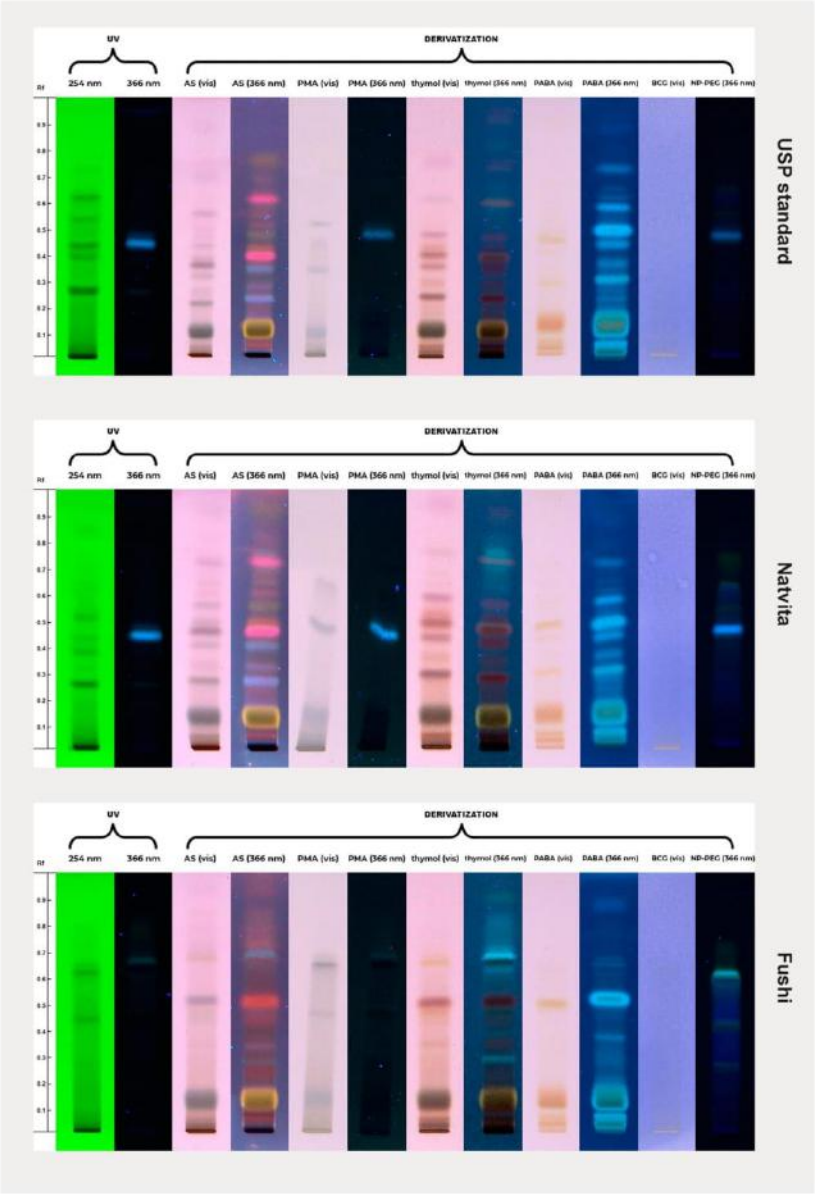

Fig. 1. TLC chromatograms of the *R. rosea* extracts (USP standard, Natvita, Fushi).

weak antioxidant activity. These bands are also slightly seen at the bioautograms of plant extracts. Rosavin had no antioxidant properties, which were confirmed also by the dot-blot test (Fig. 2S). Strong antioxidative properties of *R. rosea* may play an essential role in the adaptogenic, anti-aging and antifatigue activities of the plant [17].

Based on the cholinergic hypothesis, AChE inhibitors may be used in the Alzhemer's disease treatment [12]. Several studies of *R. rosea* pointed out salidroside, p-tyrosol, gossypetin-7-O-L-rhamnopyranoside and rosarin as potential AChE inhibitors [18–20]. Bioautogram of the USP standard extract revealed two bands of AChE inhibition: at hR_f 76 and 90. However, only single white bands at hR_f 80 were detected on AChE bioautograms of both NatVita and Fushi extracts (Fig. 4). Among standards, only hydroquinone showed AChE inhibition. It was also confirmed by the dot-blots (Fig. 2S). All *R. rosea* extracts showed weak anti-AChE activity in the dot-blot. The detection of inhibitors in the extracts was disturbed by interfering brown zones seen on AChE bioautograms (Figs. 4, 2S).

α-Glucosidase enzyme inhibitors are potential drugs in the therapy of the type 2 diabetes. According to the literature, *R. rosea* can be considered as a drug for the diabetes treatment [17]. However, the investigated herb samples revealed only a few slightly visible white bands of α-glucosidase inhibition, probably related

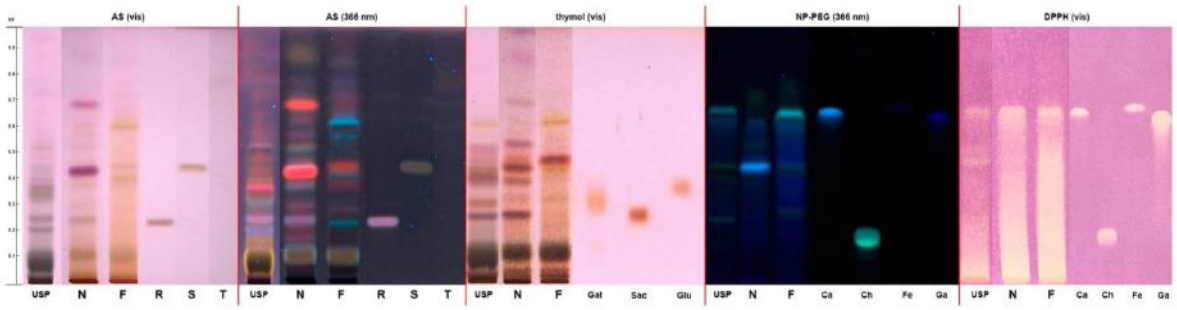

Fig. 2. TLC chromatograms of the *R. rosea* extracts: USP standard (USP), NatVita (N) and Fushi (F) and standards: rosavin (R), salidroside (S), p-tyrosol (T), hydroquinone (H), galactose (Gal), saccharose (Sac), glucose (Glu), caffeic acid (Ca), chlorogenic acid (Ch), ferulic acid (Fe), gallic acid (Ga).

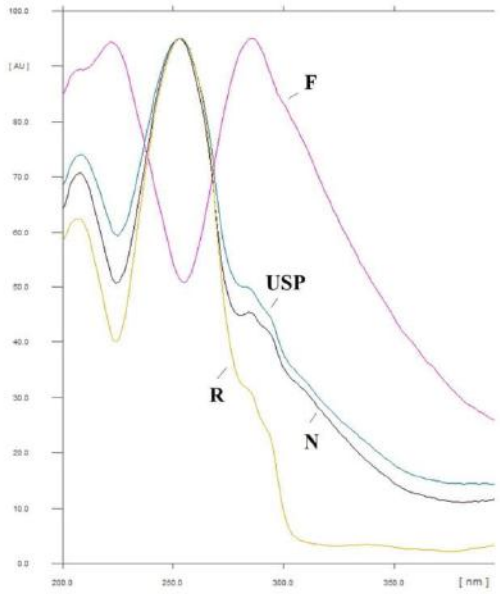

Fig. 3. The spectra of the rosavin standard (R) and corresponding to it zones in *R. rosea* extracts: USP standards (USP), NatVita (N) and Fushi (F) at the same hR_f value i.e 24.

to the marker compounds: rosavin, salidroside and tyrosol (Fig. 4). The inhibition zones of α-glucosidase are partially masked by brown zones at hR_f range from 0 to about 65 in the tracks of all extracts. The brown zones, already observed in the AChE assay, may be the effect of reaction between the Fast Blue B salt reagent applied as a visualization solution and polyphenols (detected as wide zones in NP-PEG and DPPH tests, Fig. 2) [21]. However, the reaction of polyphenols, leading to the emergence of brown zone, was not observed in the lipase assay, where the Fast Blue B salt was also used as a visualization solution. This effect should be further investigated. This problem does not concern standards: rosavin, salidroside, p-tyrosol and hydroquinone were seen as bright zones in the α-glucosidase assay. In the dot-blot test (Fig. 2S) bright inhibition zones were visible both for the *R. rosea* extracts as well as for standards.

Lipase inhibitors could be effective in the treatment of obesity. According to the literature, *R. rosea* and p-tyrosol may prevent obesity [17,22]. The lipase assay resulted in bright zones of inhibition in all three extracts of *R. rosea* (Fig. 4). The Fushi extract revealed stronger anti-lipase activity then that of the USP standard and NatVita extracts. The standards, except hydroquinone, had weak inhibition activities better visible in the lipase dot-blot assay (Fig. 2S). Hydroquinone showed strong anti-lipase activity.

Tyrosinase inhibitors may be useful as skin whitening agents in cosmetics and for treatment of some skin disorders related to melanin hyperpigmentation [17]. The all three *R. rosea* extracts and rosavin revealed tyrosinase inhibition (Fig. 4). Besides bright zones of the tyrosinase inhibition also dark bands were observed on bioautograms, what could be related to the enhancing tyrosinase activity. Bright bands related to rosavin and hydroquinone proved their inhibition property (Figs. 4, 2S). The salidroside and p-tyrosol were observed on bioautograms as dark bands. These results are in contrast with those reported by Wen who observed tyrosinase inhibition effect of salidroside and p-tyrosol [23].

The antibacterial activity against *Bacillus subtilis* was revealed both in three analyzed extracts and standards, with exception of p-tyrosol (Figs. 4, 2S). The antibacterial zones are visible at hRf ranges of 0 – 20, 0 – 20 and 0 – 25 for the USP standard, NatVita and Fushi, respectively. Besides these wide-range antibacterial zones, three bands at hRf 25, 40 and 50 were detected for the USP standard while two bands at hRf 45 and 70 were detected for the NatVita and Fushi extracts. The rosavin and salidroside stan-

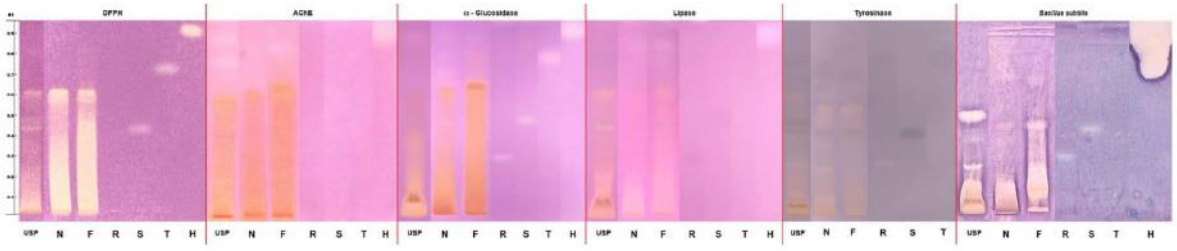

Fig. 4. TLC bioautograms of the *R. rosea* extracts: USP standard (USP), NatVita (N) and Fushi (F) and standards: rosavin (R), salidroside (S), p-tyrosol (T), hydroquinone (H).

dards showed weak antimicrobial activity, while hydroquinone – very strong.

3.3. Detection of the target compounds in Rhodiola rosea *L. by HPLC-ESI-MS*

The HPLC-ESI-MS technique was used for identification of the target compounds in the *R. rosea* extracts in the fractions with biological activity. Table 1 lists compounds, their molecular formulas, measured and teoretical monoisotopic masses as well as mass differences in parts per million (ppm) and the fractions in which a given compound was found. The HPLC-ESI-MS analysis of the fractions provided information on twenty four constituents of the analyzed extracts detected in seven fractions. In NatVita and the USP standard extracts three phenylpropanoids were identified – rosavin (F2, F3), rosarin (F2, F3) and rosin (F4, F5), although in the Fushi extract - only rosin (F4, F5). This is another evidence (besides TLC-bioprofiling and TLC-UV-VIS) of possible adulteration of the Fushi supplement. The presence of salidroside (F4), viridoside (F3, F4) and p-tyrosol (F6, F7) was confirmed in all three *R. rosea* extracts. The saccharose, glucose and galactose were detected in the NatVita (F1, F2, F3), Fushi (F1, F2, F3) and USP standard (F1, F2, F3) extracts. The presence of phenolic acids was confirmed by the standards of caffeic (F6), chlorogenic (F2, F3), ferulic (F6, F7) and gallic (F6) acids also in all three *R. rosea* extracts. The HPLC-ESI-MS analysis identified also herbacetin (F6, F7), rhodioflavonoside (F2, F3), rhodiosin (F2, F3), rhodionin (F3, F4), gossypetin-7-O-rhamnopyranoside (F2, F3), cinnamyl alcohol (F6, F7), kaempferol (F5, F6), luteolin-7-O-Glc (F4, F5), luteolin (F5, F6), apigenin-7-Glc (F4) and hydroquinone (F6, F7) in all three *R. rosea* samples. *In vitro* and *in vivo* studies showed that herbacetin exerts an unspecific effect on membranes and enzyme activities linked to cancer progression [24]. Gossypetin-7-O-rhamnopyranoside and rhodioflavonoside are compouds with antibacterial and anticancer activity [6]. Hydroquinone shows strong anti-AChE activity [7]. Herbacetin, rhodiosin, rhodionin, cinnamyl alcohol, kaempferol, luteolin-7-O-Glc, luteolin and apigenin-7-Glc are polyphenol compounds with strong antioxidant properties [5,24].

4. Conclusions

The TLC fingerprinting and EDD followed by TLC-UV-VIS and HPLC-ESI-MS are proved to be useful methods for defining authenticity, quality control, differentiation and possible adulterations of herbal samples. The EDA of the supplements and the USP standard of *Rhodiola rosea* L. pointed to the adulteration of the Fushi sample, what was related to the absence of rosavin and rosarin. The absence of these rosavins could serve as an evidence of falsification or poor quality of the supplement, which questions its authenticity. We believe that control and standardization of the herb supplements is very important for understanding its activity and for prevention of falsifications. The results revealed also biological activity of *R. rosea* preparation and the chosen standards. The *R. rosea* extracts inhibit acetylcholinesterase, tyrosinase, lipase and α-glucosidase and show antibacterial and antioxidant activity. The rosavin standard show inhibition effect of α-glucosidase, tyrosinase and activity against *Bacillus subtilis*. The salidroside and p-tyrosol standards are proved to be antioxidants as well as α-glucosidase inhibitors. Additionally, salidroside shows antibacterial activity against *Bacillus subtilis*.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.chroma.2021.462217.

CRediT authorship contribution statement

Hanna Nikolaichuk: Conceptualization, Methodology, Investigation, Writing – original draft, Writing – review & editing. **Rafał Typek:** Investigation. **Sebastian Gnat:** Investigation. **Marek Studziński:** Investigation. **Irena Maria Choma:** Supervision, Conceptualization, Writing – original draft, Writing – review & editing.

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The supplementary material

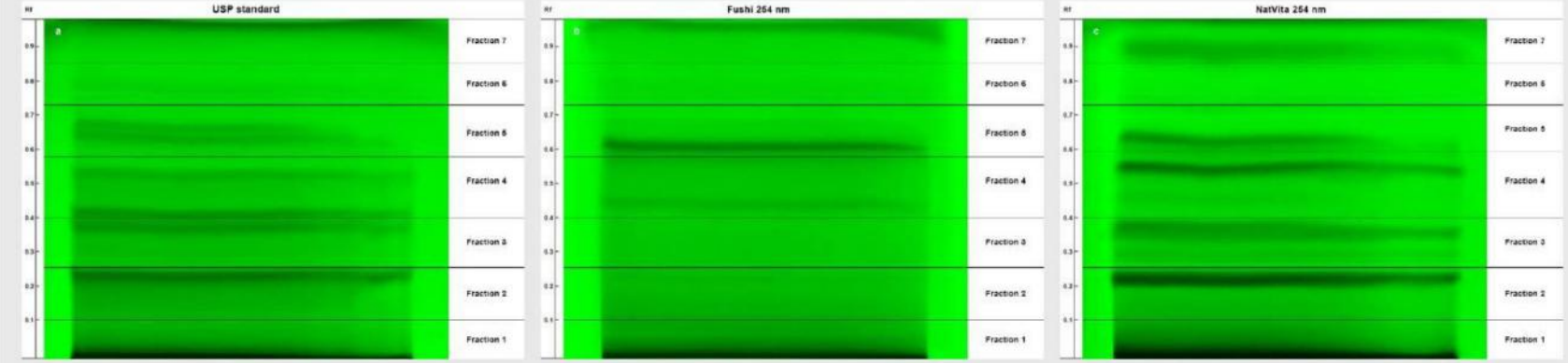

Figure 1S. Micro-preparative chromatograms at UV 254 nm a) USP standard, b) Fushi, c) Natvita

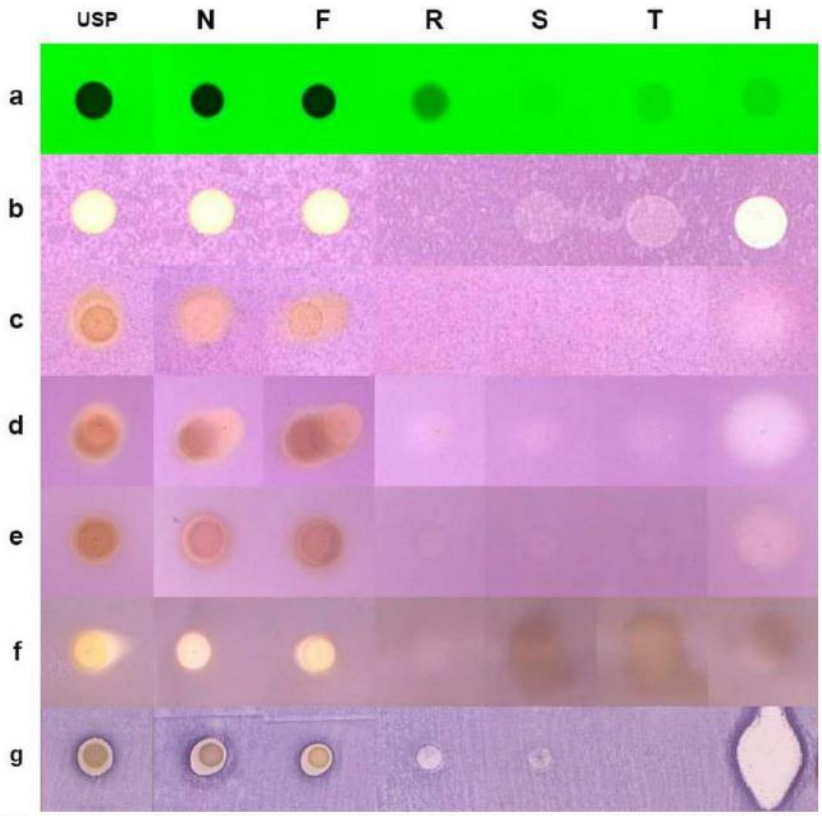

Figure 2S. TLC dot-blot of *R. rosea* extracts USP standard (USP), NatVita (N) and Fushi (F) and standards: rosavin (R), salidroside (S), p-tyrosol (T), hydroquinone (H) at 254 nm (a), DPPH (b) AChE (c), α-glucosidase (d), lipase (e), tyrosinase (f), *B. subtils* (g) assays

Table 1S

R_f ranges of the fractions obtained by micro-preparative TLC.

Fractions	**USP standard** $\mathbf{hR_f}$	**NatVita** $\mathbf{hR_f}$	**Fushi** $\mathbf{hR_f}$
Fraction 1	0-10	0-10	0-10
Fraction 2	12-25	12-25	12-25
Fraction 3	26-40	26-40	26-40
Fraction 4	42-58	42-58	42-58
Fraction 5	59-73	59-73	59-73
Fraction 6	75-85	75-85	75-85
Fraction 7	86-100	86-100	86-100

Table 2S
The detailed information on the bioactive bands.

	DPPH	**AChE**	**Glucosidase**	**Lipase**	**Tyrosinase**	***Bacillus subtilis***
USP standard	white zone at hR_f range 0-25; white bands at hR_f 44, 48 and 60	brown zone at hR_f range 0-62; white bands at hR_f 78 and 90	brown zone at hR_f range 0-67; white bands at hR_f 78 and 90	white zone at hR_f range 0-62; white bands at hR_f 20, 40 and 78	white zone at hR_f range 0-23; white bands at hR_f 24, 62, 68; dark bands at hR_f 49, 52 65 and 81	white zone at hR_f range 0-20; white band at hR_f 24, 50
NatVita	white zone at hR_f range 0-67; white bands at hR_f 49 and 78	brown zone at hR_f range 0-64; white band at hR_f 86	brown zone at hR_f range 0-64; white bands at hR_f 45, 78 and 90	white zone at hR_f range 0-23; white band at hR_f 78	white zone at hR_f range 0-23; white bands at hR_f 24, 62, 68; dark bands at hR_f 49, 65 and 81	white zone at hR_f range 0-20; white band at hR_f 46
Fushi	white zone at hR_f range 0-67; white band at hR_f 78	brown zone at hR_f range 0-67; white band at hR_f 86	brown zone at hR_f range 0-67; white bands at hR_f 78 and 90	white zone at hR_f range 0-67; white band at hR_f 78	white zone at hR_f range 0-10; white band at hR_f 68; dark bands at hR_f 49, 65 and 81	white zone at hR_f range 8-26; white bands at hR_f 46,70
Rosavin	-	-	white band hR_f 24	-	white band hR_f 24	white band hR_f 24
Salidroside	white band hR_f 47	-	white band hR_f 47	-	dark band hR_f 47	white band hR_f 47
p-Tyrosol	white band hR_f 78	-	white band hR_f 78	-	dark band hR_f 78	-
Hydroquinone	white zone at hR_f range 85-100	white zone at hR_f range 85-100;	white zone at hR_f range 85-100;	white zone at hR_f range 85-100;	dot-blot	white zone at hR_f range 70-100;

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Qualitative and Quantitative Evaluation of Rosavin, Salidroside, and p-Tyrosol in Artic Root Products via TLC-Screening, HPLC-DAD, and NMR Spectroscopy

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Abstract: Artic root is a well-known plant adaptogen with multipotential pharmacological properties. Thin-layer chromatography (TLC)—screening followed by diode-array high-performance liquid chromatography and nuclear magnetic resonance spectroscopy proved to be a reliable and convenient method for the simultaneous determination of the quality of various herbal raw materials and supplements. This combination allowed for comparing and differentiating arctic root samples as well as defining their authenticity. The study provided information on the chemical and biological properties of the seven chosen samples as well as qualitative and quantitative evaluation of the quality markers: rosavin, salidroside, and p-tyrosol. The absence of rosavin, salidroside, and p-tyrosol in three samples was detected using TLC screening and confirmed by HPLC-DAD and NMR. The paper highlighted the importance of quality control and strict regulation for herbal medicine supplements and preparations.

Keywords: TLC-screening; HPLC-DAD; NMR; arctic root

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1. Introduction

Artic root, also known as *Rhodiola rosea* (*R. rosea*), is a worldwide known medicinal and functional plant with multipotential healing abilities. It has been used in European and Asiatic traditional medicines primarily as an adaptogen, which means that arctic root helps a human organism to produce a non-toxic and non-specific response to physical, biological, and chemical stressors [1,2]. This activity does not disturb homeostasis and has normalizing and health-promoting effects regardless of changes caused by the stressors. With the development of pharmaceutical technology, the application of *R. rosea* rhizome and root has been expanded. Arctic root material is added to various products, including food supplements and health and beauty products, which are increasingly accepted in European countries. In recent years, the research on arctic root has mainly concerned chemical and biological profiling as well as pharmacological activity. Various biological activities of arctic root, including antioxidant, antibacterial, antiviral, anticancer, immunostimulant, antidepressant, and ergogenic, were identified [3–6]. In clinical practice, arctic root has been investigated to improve physical and mental performance and treat fatigue and depression.

The safety and quality of herbal medicines available to European consumers have been vital for medicine regulations. The introduction of the Traditional Herbal Medicinal Products Directive (formally Directive 2004/24/EC) and herbal registration gave confidence to consumers that they have access to a wide range of popular herbal medicines with high

quality and safety. However, many unregulated drugs are still widely available on the market [7]. Primarily, this concerns economically profitable products when raw plant material shows high bioactivity, short expiry dates, and possible enzymatic degradation. Arctic root is one of these kinds of items. Moreover, due to the popularity of arctic root products among sportspersons, they may be adulterated with performance-enhancing synthetic stimulants, e.g., steroids. It should also be mentioned that other cheap species of *Rhodiola*, such as *R. crenulata*, *R. kirilowii*, *R. quadrifida*, *R. sacra*, and *R. yunnanensis*. *R. crenulata* are widespread in China and thus can be of main economic interest. *R. crenulata* is cheaper than arctic root and relatively easy to gather, which causes the falsification of *R. rosea* products. Therefore, the potential risks of this kind of adulteration are lack of effectiveness and beneficial effect on organisms [8,9].

This study used TLC—screening, HPLC-DAD, and ^{1}H-NMR spectroscopy to compare the identity and composition of the arctic root products. These techniques provide complementary data, which allowed us to discriminate among tested samples. TLC analysis gives qualitative data to determine the marker compounds and made visual comparisons among different products relatively quickly. Moreover, TLC-bioassays enable an estimation of the biological properties of the samples.

The focus was on the presence of rosavin, salidroside, and p-tyrosol in the tested arctic root samples using the methods mentioned above. Since these constituents play a critical role in arctic root bioactivity and are regarded to be responsible for their pharmacological properties, they are used to standardize the raw materials. The lack of marker compounds indicates possible adulteration and poor quality of samples. HPLC and spectroscopic methods confirmed the obtained TLC results.

2. Results and Discussion

2.1. TLC Screening

TLC screening, a fast and straightforward tool, was used to compare visual differences between the chosen arctic root samples, which may be caused by adulteration or poor quality. The content of rosavin, salidroside, and p-tyrosol in arctic root depends on various factors such as a different origin, harvest, age of the plant, method of raw material stabilization, storage conditions, and even the extraction method.

Available literature data on the last item, i.e., solvent and extraction method, are contradictable and mainly concern the aqueous and ethanol extraction [10]. However, our results indicated that 70 % methanol extract from a maceration of the dry arctic root shows the highest bioactivity among water, ethanol, methanol, and ethyl acetate [11,12]. Additionally, in our previous work [11], TLC screening pointed to the absence of rosavin, one of the major compounds and quality markers, in one of the tested samples. To acquire more information, the AS, thymol, NP-PEG, DPPH (chemical screening), and tyrosinase, lipase, and α-glucosidase (biochemical screening) assays were done for seven arctic root samples (including United States Pharmacopeia standard of *R. rosea* root and rhizome denoted by S6) together with the standards: rosavin—authenticity marker, and salidroside and p-tyrosol as bioactivity markers. The obtained results (Figures 1 and 2) pointed to the high variability of constituents (sugars, glucosides, polyphenols, etc.—see AS, thymol, and NP-PEG), different inhibition activities against enzymes (lipase, tyrosinase, and α-glucosidase assays) and different antioxidant (DPPH assay) properties. The S2, S4, and S5 samples showed potent inhibition of lipase, α-glucosidase, and tyrosinase, as well as strong antioxidant activities, whereas the S1, S6, and S7 samples revealed weaker inhibition of lipase, tyrosinase, and α-glucosidase along with the antioxidant activity. The S3 sample differs in chemical and biological profiles from the other samples, which could be explained by the low content of *R. rosea* material or the falsification of this supplement.

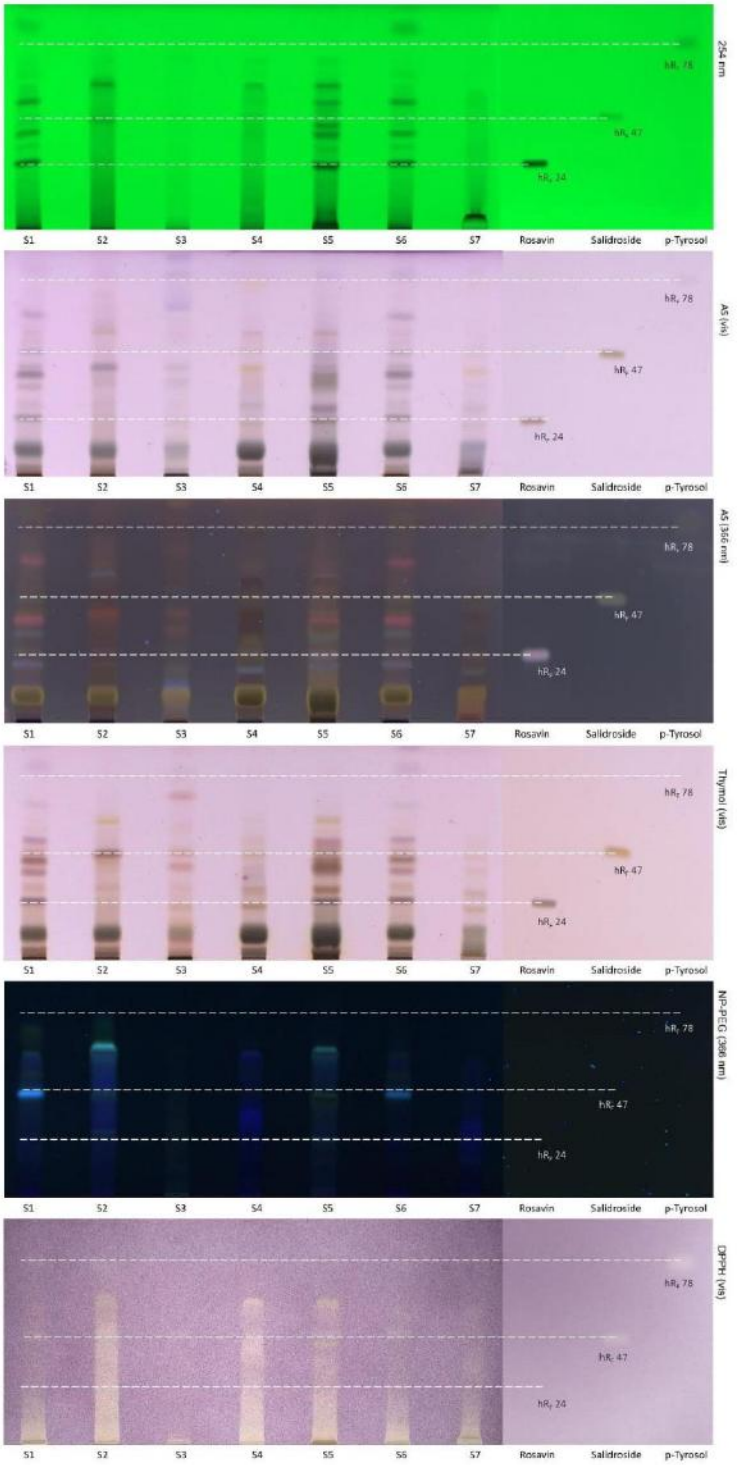

Figure 1. TLC-chemical screening of the arctic root extracts and standards. MP: ethyl acetate-methanol-water (77:13:10, *V*/*V*/*V*).

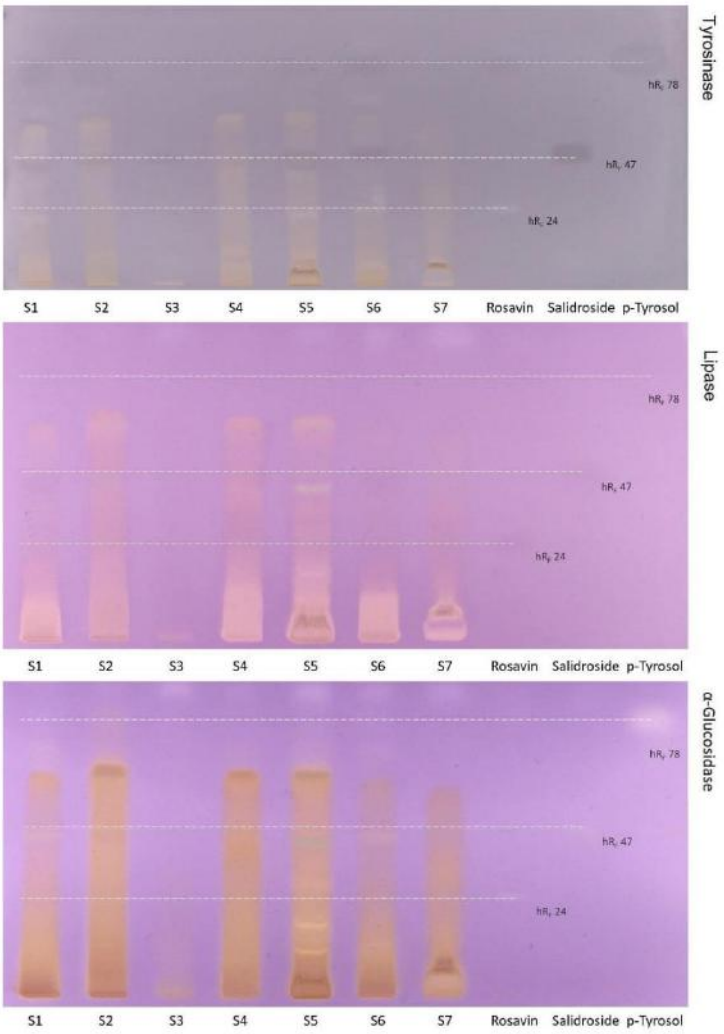

Figure 2. TLC-biochemical screening of the arctic root extracts and standards. MP: ethyl acetate-methanol-water (77:13:10, *V/V/V*).

The band of the rosavin standard was visible at hR_F 24 under UV 254 nm and after chemical visualization with AS and thymol reagents (Figure 1). Moreover, rosavin revealed inhibition of tyrosinase and α-glucosidase at 5 μg (Figure 2). Based on these chromatograms, it can be concluded that rosavin was present only in three samples (S1, S5, S6). The absence of rosavin in the S2 was already confirmed in our previous paper by HPLC-ESI-MS, which suggested the adulteration of that product or improper manufacturing procedures [11]. However, in the S3, S4, and S7 samples, the absence of rosavin had to be confirmed (it is possible that the amount of rosavin in samples could be under the detection limit in the assays).

Similarly to rosavin, the presence of salidroside and p-tyrosol compounds are tough to evaluate in arctic root samples using only TLC screening. The salidroside standard band at hR_F 47 was visible at 254 nm as well as in AS and thymol assays. Moreover, the 5 μg amount of salidroside showed antioxidant activity as well as tyrosinase and α-glucosidase inhibition. Salidroside was visible in four samples (S1, S4, S5, and S6) in AS assay under 366 nm. Concerning p-tyrosol, related bands were visible at hR_F 78 at 254 nm and in

the AS assay. The marker showed antioxidant and enzyme inhibition (tyrosinase and α-glucosidase) activities at 5 µg. The presence of p-tyrosol was stated in four samples, S1, S2, S5, and S6, in AS assay at 366 nm. Rosavin, salidroside, and p-tyrosol were not detected using the NP-PEG test. Besides, none of the markers showed lipase inhibition activity at 5 µg. In order to verify TLC screening results and observations regarding the presence of marker constituents in arctic root samples, HPLC-DAD and NMR were used.

2.2. HPLC-DAD Analysis

The HPLC-DAD method was used to detect and quantify four marker components (rosavin, salidroside, and p-tyrosol) in seven arctic root samples. The results indicated differences in the presence of markers and their amounts in the *R. rosea* samples. The contents of the markers in the extracts were calculated from the calibration curves constructed for four standards. The calibration ranges, regression equations, correlation coefficients, and LOD and LOQ values are given in Table 1.

Table 1. HPLC calibration parameters for chosen standards.

Compound	Retention Time (min)	Calibration Range [2] (µg/mL)	Regression Equation [1]	R^2 (n = 3)	LOD (µg/mL)	LOQ (µg/mL)
Rosavin	17.2	1–200	y = 33071x + 413,929	R^2 = 0.993	3.76	11.41
Salidroside	4.0	1–200	y = 13110x + 14,399	R^2 = 0.989	1.04	3.16
p-Tyrosol	5.4	1–100	y = 57136x − 186,643	R^2 = 0.998	0.01	0.02

[1] y: peak area, x: concentration (µg/mL),[2] five calibration points.

HPLC-DAD analysis confirmed the results from TLC screening regarding the presence/absence of rosavin, salidroside, and p-tyrosol in arctic root extracts (Table 2). The TLC results concerning the absence of rosavin in S2, S3, S4, and S7 samples were confirmed. Only three samples (S1, S5, and S6) contain all three markers, while the S3, S4, and S7 samples have none. The content of rosavin, the authenticity marker of arctic root extracts, varied between 59.99 ± 3.74 µg/mL (S1) and 100.46 ± 5.44 µg/mL (S5). The amount of salidroside was the highest in the reference standard S5 (37.86 ± 3.94 µg/mL), followed by S1 and S6 samples, while the lowest content was in the S2 sample. The quantities of p-tyrosol varied between 2.61 ± 0.52 µg/mL (S6) and 4.94 ± 0.12 µg/mL (S5). That differs from the results of Marchev et al. [9], where only trace amounts of p-tyrosol were detected in *R. rosea* samples. Salidroside and p-tyrosol were not detected in S3, S4, and S7. The HPLC-DAD results suggest that the contents of the marker constituents of *R. rosea* depend on the morphology of plant material (rhizome or/and root) and the type of products (supplements, preparations).

Table 2. The contents of rosavin, salidroside, and p-tyrosol in arctic root extracts.

ID	Content ± SD (µg/mL)		
	Rosavin	**Salidroside**	**p-Tyrosol**
S1	59.99 ± 3.74	26.37 ± 0.72	2.73 ± 0.50
S2	ND	13.12 ± 0.89	4.25 ± 0.33
S3	ND	ND	ND
S4	ND	ND	ND
S5	100.46 ± 5.44	37.86 ± 3.94	4.94 ± 0.12
S6	84.71 ± 5.54	21.33 ± 4.51	2.61 ± 0.52
S7	ND	ND	ND

ND—not detected.

2.3. NMR

For the more reliable identification of the compounds, NMR analysis was used. First, ^{1}H NMR spectra of standard compounds: rosavin, salidroside, and p-tyrosol (Figure 3) were obtained (Figure 4). This allowed identifying the most characteristic signals for each compound for the subsequent analysis of extracts. The seven samples of arctic root were analyzed by ^{1}H NMR, and the fingerprint profiles were obtained (Figures 5 and 6). The results revealed qualitative and quantitative differences among arctic root samples. Based on the characteristic signals of marker compounds in specific regions of NMR spectra, rosavin, salidroside, and p-tyrosol were identified (detailed information in Table 3).

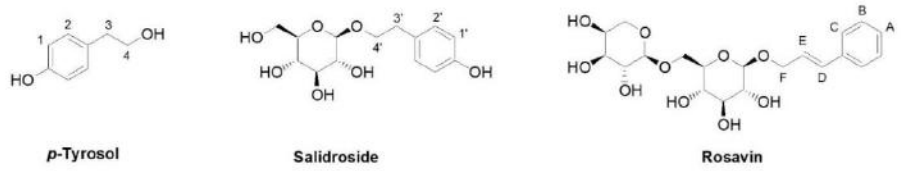

Figure 3. Structures of standard samples p-tyrosol, salidroside, and rosavin) with markings of characteristic signals.

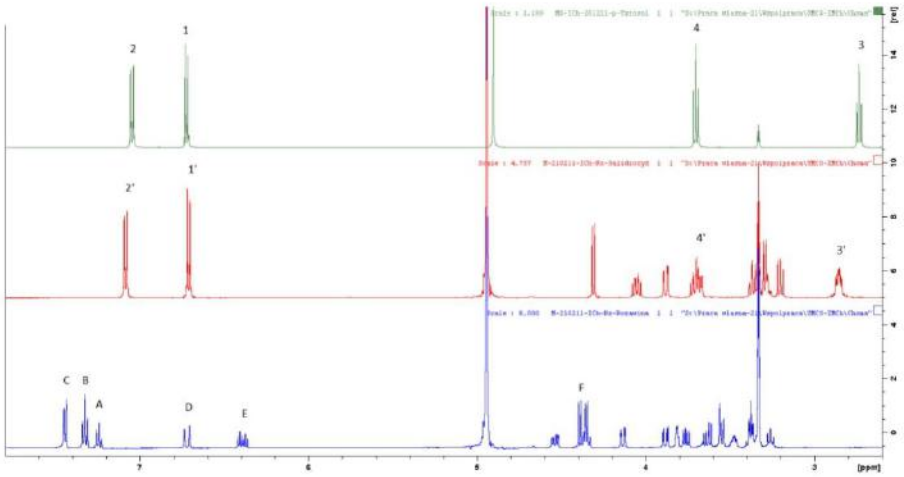

Figure 4. ^{1}H NMR spectra of standard samples of rosavin (blue), salidroside (red), and p-tyrosol (green) (from the bottom to the top) with markings of characteristic signals.

Table 3. Presence of marker compounds in the arctic root samples analyzed by NMR.

ID	Rosavin	Salidroside	p-Tyrosol
S1	+	+	+
S2	ND	+	+
S3	ND	Traces	ND
S4	ND	Traces	ND
S5	+	+	+
S6	+	+	+
S7	ND	Traces	ND

+—present; ND—not detected.

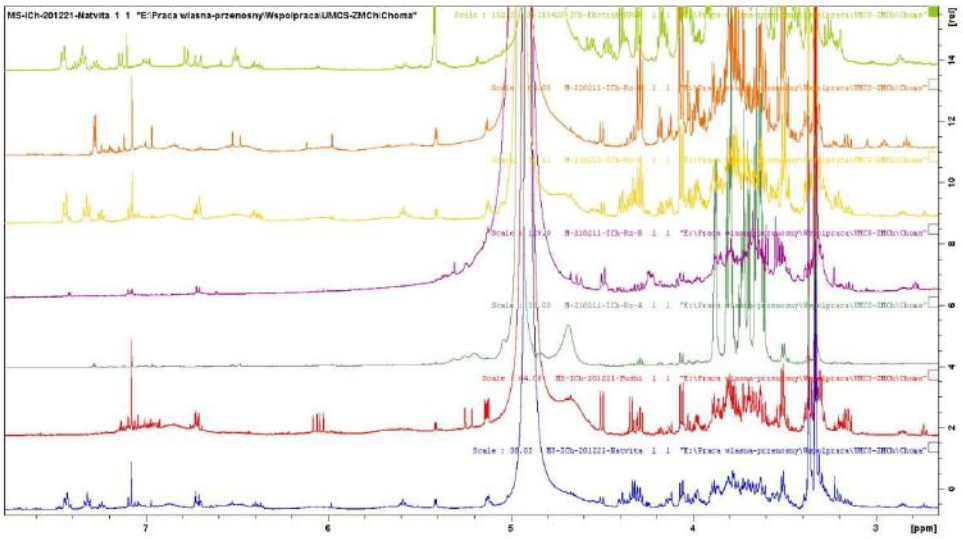

Figure 5. ^{1}H NMR spectra of S1 (blue), S2 (red), S7 (green), S3 (purple), S6 (yellow), S4 (orange), and S5 (light green) (from the bottom to the top).

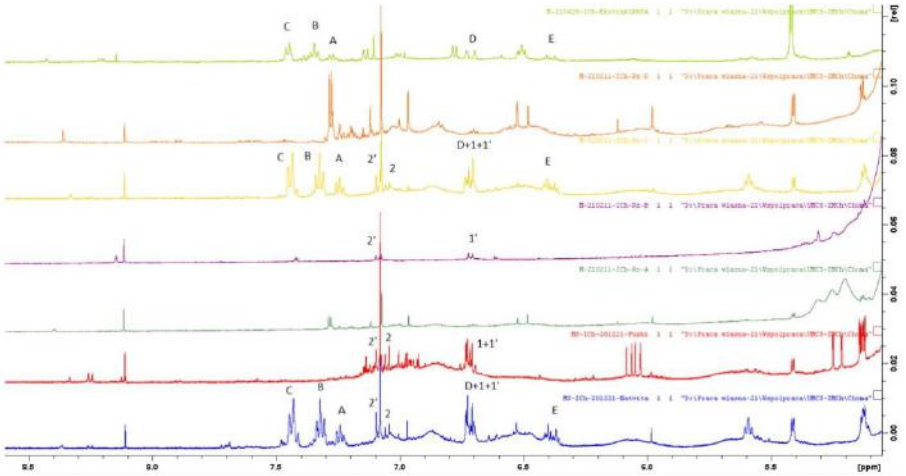

Figure 6. Fingerprint region of ^{1}H NMR spectra of S1 (blue), S2 (red), S7 (green), S3 (purple), S6 (yellow), S4 (orange), and S5 (light green) (from the bottom to the top) with markings of characteristic signals.

As seen from ^{1}H NMR spectra of analyzed extracts and standards, three samples (S1, S5, and S6) undoubtedly contain rosavin, salidroside, and p-tyrosol). In three other samples, S3, S4, and S7, the amounts of the markers are very low or non-detectable. Sample S2 lacks rosavin, as was also proved by TLC, HPLC-DAD, and previous HPLC-ESI-MS results [11]. The detection of p-tyrosol in the analyzed samples was also challenging as the signals in aromatic regions overlay with those from the salidroside. In general, small amounts of the investigated compounds are difficult to confirm due to the lower sensitivity of NMR compared to chromatographic methods. Additionally, the presence of a complex matrix may strongly influence the chemical shifts of hydrogen atoms present in the molecules; therefore, a simple comparison with a pure standard rarely gives a sure answer.

3. Materials and Methods

3.1. Reagents and Materials

Acetone (99.5%), acetic acid (99.5–99.9%), diphenylboryloxyehtylamine (NP) (≥97.0%), ethanol (96%), ethyl acetate (99.8%), 2-isopropyl-5-methyl-phenol (thymol) (≥98.5%), methanol (99.8%), o-phosphoric acid (85%), polyethylene glycol—4000 (PEG-4000), phosphate buffer, sodium hydroxide (≥98%), sulfuric acid (96–98%), and sodium acetate buffer were from POCH (Gliwice, Poland). P-Anisaldehyde (AS) (≥98%), bovine serum albumin (BSA) (≤100%), 2,2-diphenyl-1-picrylhydrazyl (DPPH) (≤100%), 3,4-dihydroxy-L-phenylalanine (L-DOPA) (>98.0%), α-glucosidase from *Saccharomyces cerevisiae* (≥10 units/mg protein), lipase from porcine pancreas (30–90 units/mg protein), 1-naphthyl acetate (≥98%), 2-naphthyl acetate (≤100%), 2-naphthyl α- D glucopyranoside ((≤100%), polyethylene glycol tert-octylphenyl ether (Triton X) (≤100%), rosavin (≥98.0%), salidroside (≥98.0%), p-tyrosol (98%), Fast Blue B salt (95%), tris(hydroxymethyl)aminomenthane hydrochloride (TRIS) buffer, and tyrosinase from mushroom (≥1000 unit/mg solid) were purchased from Sigma Aldrich (Poznań, Poland). Pure water was from the Millipore Q system (Millipore, Bedford, MA, USA). All reagents were of the analytical grade. Acetonitrile used in HPLC-UV/Vis-DAD quantification procedure was purchased from Romil (Cambridge, UK). TLC silica gel 60 F_{254} (20 × 10 cm) plates were purchased from Merck, (Darmstadt, Germany).

3.2. Sample Preparation

The seven samples (Supplementary Material, Table S1) of arctic root were purchased from different Polish vendors; one of the samples is the United States Pharmacopeia reference standard of root and rhizome from Sigma Aldrich (Poznań, Poland) (S5). The samples (0.1 g) were filled with 70 percent methanol (1 mL), and maceration took place in the darkness at room temperature for 72 h. After maceration, the extracts were filtered through a paper filter. Obtained extracts were used for TLC application. The standards of rosavin, salidroside, and p-tyrosol were prepared at a concentration of 1 mg/mL in methanol for TLC analysis. All samples were stored at −8 °C.

3.3. TLC-Chromatography

The TLC chromatograms were obtained as follows. Samples application was conducted using the automatic TLC applicator Linomat 5 (Camag, Muttenz, Switzerland). On the NP-TLC plate, 5 µL of samples (plant extracts and standards) were applied as 8 mm bands (10 mm from the lower and left edge, at a distance of 13 mm between tracks). As the mobile phase, ethyl acetate-methanol-water 77:13:10 (*V*/*V*/*V*) was used. The development of the mobile phase was up to 8 cm in the DS sandwich chamber (Chromdes, Lublin, Poland). After development, chromatograms were dried on a cold stream of air for 20 min. Documentation of chromatograms was at UV 254 nm, UV 366 nm, and white light illumination (at the reflectance mode) using Visualiser with DigiStore 2 Documentation System, VideoScan 1.1, and winCATS 1.4.7 software (Camag, Muttenz, Switzerland). All chromatograms were prepared in the same way under the same conditions and further used for chemical and biochemical assays. The assay reagents/solutions were sprayed on TLC plates using automatic piezoelectric spraying (TLC Derivatizer for 20 × 20 cm plates, Camag, Muttenz, Switzerland).

3.4. TLC-Chemical Screening

3.4.1. AS Reagent Assay

AS reagent was prepared as follows 0.1 mL of p-anisaldehyde was dissolved in 17 mL of methanol, then to the mixture were added 2 mL of acetic acid and 1 mL of sulfuric acid. The plate was sprayed with 4 mL (red nozzle, speed 6) of AS reagent and then heated at 105 °C for 7 min on the TLC Heater (Camag, Muttenz, Switzerland). The results were documented at VIS and UV 366 nm light. AS, an excellent general reagent,

is used to detect organic compounds such as terpenes, terpenoids, saponins, sugars, and propylpropanoids [13].

3.4.2. Thymol Reagent Assay

Thymol reagent solution was prepared by dissolving 0.1 g of thymol in 19 mL of ethanol and mixing it with 1 mL of sulfuric acid. The plate was sprayed with 4 mL (red nozzle, speed 6) of thymol reagent, and the plate was heated at 120 °C for 15 min. The results were documented at VIS light [13].

3.4.3. NP-PEG Reagent Assay

The plate was sprayed (green nozzle, speed 6) with 4 mL of 10 mg/mL NP methanol solution. After drying, the plate was sprayed (green nozzle, speed 6) with 4 mL of 50 mg/mL PEG ethanol solution. The results were documented at UV 366 nm light. NP-PEG reagent is used for the detection of polyphenols [13].

3.4.4. DPPH Assay

The plate was sprayed (blue nozzle, speed 6) with 4 mL of 0.2% DPPH methanol solution. Results were documented after 30 min at the VIS light. Radical scavengers appeared as white bands against the purple background [14].

3.5. TLC-Biochemical Screening

3.5.1. Tyrosinase Bioassay

The plate was sprayed (red nozzle, speed 6) with 2.5 mL of substrate solution (0.1183 g of L-DOPA diluted in 49.5 mL of 0.02 M phosphate buffer (pH 6.8) and added 0.5 mL of Triton X). Subsequently, the plate was sprayed (red nozzle, speed 6) with 3.0 mL of enzyme solution (400 units of tyrosinase in 1 mL of 0.02 M phosphate buffer, pH 6.8). The incubation lasted 10 min at room temperature in the closed vessel with a humid atmosphere in a dark place. Results were documented at VIS light. Tyrosinase inhibitors are revealed as white bands on grey background [15].

3.5.2. Lipase Bioassay

The plate was sprayed (green nozzle, 6 speed) with 3 mL of 1.5 mg/mL of 1-naphthyl acetate of ethanol solution. After that, the plate was sprayed (red nozzle, speed 6) with 4 mL of enzyme solution (50 units of lipase and 5 mg of BSA dissolved in 5 mL of 0.05 M TRIS buffer, pH 7.4). The incubation was at 37 °C for 20 min in a closed vessel in a humid atmosphere. After incubation, the plate was sprayed (blue nozzle, 6 speed) with 2 mL of 0.5 mg/mL of Fast Blue B salt water solution. Results were documented at VIS light. Lipase inhibitors emerged as white bands on a purple background [16].

3.5.3. α-Glucosidase Bioassay

The plate was sprayed (green nozzle, speed 6) with 2 mL of 1.2 mg/mL 2-naphthyl α- D glucopyranoside ethanol solution. Then, the plate was sprayed (red nozzle, 6 speed) with 4 mL of enzyme solution (50 units of α-glucosidase dissolved in 5 mL of sodium acetate buffer, pH 7.5). The plate was incubated in the humid atmosphere at 37 °C for 10 min in a closed vessel. After incubation, the plate was sprayed (blue nozzle, speed 6) with 0.5 mL of 1 mg/mL of Fast Blue B salt aqueous solution. Results were documented at VIS light. α-Glucosidase inhibitors are visible as white bands on the purple background [17].

3.6. HPLC-DAD

The HPLC-UV/VIS-DAD chromatograms were collected using the following equipment purchased from Shimadzu Corporation (Kyoto, Japan): control unit CBM-20A, two pumps LC-20AD (high-pressure gradient), degasser DGU-20A5R, autosampler SIL-20AC HT, column oven CTO-20AC, and detector SPD-M20A. Column: Phenomenex Kinetex C18 100, dimensions: 4.6 mm × 150 mm. The system was working under the control of

LabSolutions software (Shimadzu Corporation) Version 5.71SP2. The mobile phase was water (A) and acetonitrile (B) with a flow rate—of 1 mL/min. The injection volume was set to 10 µL for the standards and 1 µL for the extracts. Each run started at 10% of B and increased to 12% in 7 min and then 100% in 25 min. The column temperature was set at 30 °C. In order to purge the highly lipophilic compounds present in the extracts, the flow was held from 25 min to 45 min at 100% B, at 60 °C. Then, the B concentration was linearly lowered to 10%, and the temperature was cooled down to 30 °C to restore initial conditions. Every development was triplicated, and the presented results are averages from the obtained results (Figure S1). The RSD values of retention times and peak areas were lower or equal to 5%. Diode-array detection was set to collect data at 218 nm and 254 nm.

3.7. NMR

The ^{1}H NMR spectra (zg30 pulse program) were recorded with Bruker Ascend 500 MHz spectrometer in MeOH-d4 (Deutero, 99.8 atom %D) as a solvent at room temperature. Chemical shifts are reported in ppm relative to the residual solvent peak. Samples for analysis were prepared as follows: 10 mg of dry analyzed sample was placed in a 5 mm NMR tube, followed by adding MeOH-d4 (0.7 mL). The tube was closed by a stopcock and shacked intensely until a homogeneous solution was obtained.

4. Conclusions

TLC-screening combined with HPLC-DAD and NMR proved the reliable and comprehensive method for evaluating the identity, pharmacological value, and composition of the arctic root chosen products. The results indicated the absence of marker compounds in three samples confirmed by TLC, HPLC-DAD, and NMR methods. In four samples presence of rosavin was not detected, which may indicate falsification or enzymatic degradation of the marker. Besides Pharmacopeia standard (S5), only two samples contain all important arctic root markers, which proves their good quality and authenticity. The results indicated the importance of quality control and strict regulations for herbal supplements and preparations.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules27238299/s1, Table S1: The sample list of arctic root; Figure S1: HPLC-DAD chromatogram of arctic root sample (S5).

Author Contributions: Conceptualization, H.N. and I.M.C.; methodology, I.M.C. and H.N.; software, H.N., M.S. (Marek Studziński) and M.S. (Marek Stankevič); formal analysis, H.N., M.S. (Marek Studziński) and M.S. (Marek Studziński); investigation, H.N., M.S. (Marek Studziński) and M.S. (Marek Stankevič); data curation, H.N.; writing—original draft preparation, H.N.; writing—review and editing, H.N. and I.M.C.; visualization, H.N. and M.S (Marek Stankevič); supervision, I.M.C.; All authors have read and agreed to the published version of the manuscript.

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Sample Availability: Samples are available from the authors.

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Supplementary material

Qualitative and quantitative evaluation of rosavin, salidroside, and p-tyrosol in artic root products via TLC-screening, HPLC-DAD, and NMR spectroscopy

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Table S1. The sample list of arctic root

ID	Sample form
S1	Dry root and rhizome
S2	Dry root
S3	Supplement
S4	Rhizome
S5	United States Pharmacopean reference standard of root and rhizome (Sigma Aldrich)
S6	Dry root
S7	Supplement

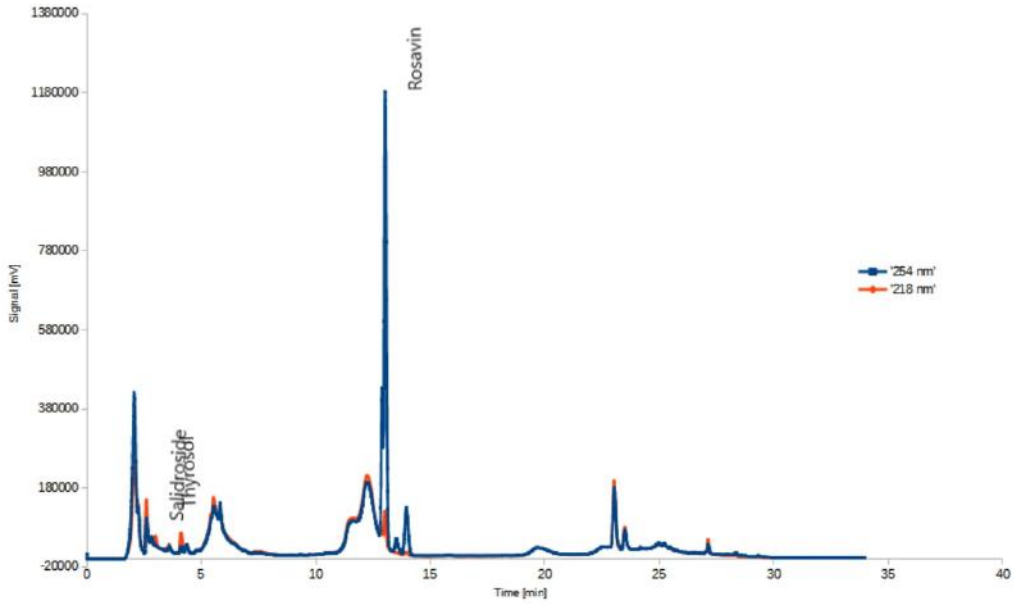

Figure S1. HPLC-DAD chromatogram of arctic root sample (S5); at λ=218 nm (red) and 254 nm (blue).

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Article

Bioactivity Profiles on 15 Different Effect Mechanisms for 15 Golden Root Products via High-Performance Thin-Layer Chromatography, Planar Assays, and High-Resolution Mass Spectrometry

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Abstract: Planar chromatography has recently been combined with six different effect-directed assays for three golden root (*Rhodiola rosea* L.) samples. However, the profiles obtained showed an intense tailing, making zone differentiation impossible. The profiling was therefore improved to allow for the detection of individual bioactive compounds, and the range of samples was extended to 15 commercial golden root products. Further effect-directed assays were studied providing information on 15 different effect mechanisms, i.e., (1) tyrosinase, (2) acetylcholinesterase, (3) butyrylcholinesterase, (4) β-glucuronidase, and (5) α-amylase inhibition, as well as endocrine activity via the triplex planar yeast antagonist-verified (6–8) estrogen or (9–11) androgen screen, (12) genotoxicity via the planar SOS-Umu-C bioassay, antimicrobial activity against (13) Gram-negative *Aliivibrio fischeri* and (14) Gram-positive *Bacillus subtilis* bacteria, and (15) antioxidative activity (DPPH• radical scavengers). Most of the golden root profiles obtained were characteristic, but some samples differed substantially. The United States Pharmacopeia reference product showed medium activity in most of the assays. The six most active compound zones were further characterized using high-resolution mass spectrometry, and the mass signals obtained were tentatively assigned to molecular formulae. In addition to confirming the known activities, this study is the first to report that golden root constituents inhibit butyrylcholinesterase (rosin was tentatively assigned), β-glucuronidase (rosavin, rosarin, rosiridin, viridoside, and salidroside were tentatively assigned), and α-amylase (stearic acid and palmitic acid were tentatively assigned) and that they are genotoxic (hydroquinone was tentatively assigned) and are both agonistic and antagonistic endocrine active.

Keywords: *Rhodiola rosea*; antibacterial; antimicrobial; enzyme inhibitor; antioxidant; genotoxin; estrogen; androgen; agonist; antagonist; endocrine activity; bioassay; biochemical assay; effect-directed analysis; HPTLC-EDA; HPTLC−HPLC−HESI-HRMS

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1. Introduction

Golden root, botanically known as *Rhodiola rosea* L., is a herbal remedy commonly used in traditional Chinese medicine and in European and Asiatic healing systems [1]. It is a perennial flowering plant from the Crassulaceae family. Its extracts are well-known as natural adaptogens, which increase adaptability, resilience, and organisms' ability to survive stress [2–4]. Recent in vitro and in vivo studies indicated that *R. rosea* extracts exhibit a wide variety of medicinal properties and biological activities. These include anti-aging [5], anti-inflammatory [6], anti-stress [7], anti-depression [8], antioxidant [9,10], anti-fatigue [11], anti-viral [12], and anti-osteoporosis [13] properties, as well as effects that promote increased

immunity [14–17]. The ergogenic properties of golden root extracts include the improvement of mental and physical conditions, memory, mood, energy metabolism, and cognitive function [18–22]. Further studies reported a possible role for *R. rosea* extracts in the treatment of cardiovascular [23] and neurodegenerative diseases [24–28], type 2 diabetes [29,30], obesity [31], and cancer [32,33]. Most of these properties are related to salidroside, one of the known major bioactive components in golden root.

Furthermore, *R. rosea* extract was highlighted as a natural selective estrogen receptor modulator beneficial in treating and preventing menopause and related symptoms such as fatigue, stress, depression, osteoporosis, and cancer. In comparison with its synthetic counterparts, *R. rosea* possesses fewer side effects [34]. Another study indicated that constituents of golden root such as gossypetin, herbacetin, and (+)-lariciresinol docked strongly to both of the two estrogen receptors ERα and ERβ [35]. However, further studies are needed, as reports of the estrogenic properties of *R. rosea* are not consistent, and the differences are probably related to different plant sources, extraction protocols, and modes of administration. Moreover, golden root polysaccharides are reported to have protective effects on boar sperm; these include improved motility, mitochondrial activity, acrosomal integrity, and plasma membrane integrity [36]. Though its protective mechanisms remain unclear, *R. rosea* extract could be a potential cryoprotectant in freezing semen. At very high minimum inhibitory concentration values ranging from 1 to 32 mg/mL determined by using the serial microdilution method, *R. rosea* extract inhibited *Staphylococcus epidermidis*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Bacillus cereus*, *Bacillus subtilis*, *Listeria monocytogenes*, *Enterobacter aerogenes*, *Escherichia coli*, *Proteus mirabilis*, and *Pseudomonas aeruginosa*. The results indicated that strains of Gram-positive bacteria were more sensitive to the preparations of golden root than Gram-negative bacteria, a finding attributed to the presence of essential oils [37]. In another study of *R. rosea* [38], gossypetin-7-*O*-L-rhamnopyranoside and rhodioflavonoside, which are antibacterial against *Staphylococcus aureus*, were detected at minimum inhibitory concentrations of 50 µg/mL and 100 µg/mL, respectively. However, there is a lack of information on the compounds responsible, as the antibacterial activity of *R. rosea* is rarely studied.

Thin-layer chromatography–effect-directed analysis (TLC–EDA) via six different assays (i.e., acetylcholinesterase, lipase, α-glucosidase, and tyrosinase inhibition assays in addition to antibacterial and antioxidant assays) had already been used for authenticity and bioactivity screening of the golden root samples and the marker compounds rosavin, salidroside, and *p*-tyrosol [39]. However, the effect profiles obtained were unsatisfactory, since no compound differentiation was possible, owing to a highly intense zone tailing. Nevertheless, seven fraction areas were scraped off, eluted, and analyzed offline using high-performance liquid chromatography–electrospray ionization mass spectrometry (HPLC–ESI-MS) to match them with known compounds. The co-applied marker rosavin showed activity against α-glucosidase, tyrosinase, and *Bacillus subtilis*. Salidroside and *p*-tyrosol were proven to be antioxidants, as well as α-glucosidase inhibitors. Salidroside also exhibited antibacterial activity. However, the dominant effect responses in the sample zone tailing could not be explained by the three reference compounds; this suggested that other compounds were responsible for the main bioactivity.

This study aimed to improve the profiling and to develop a non-target effect-directed screening that is able to separate and thus visualize the individual bioactive compounds in the complex golden root samples. The profiling was extended to 15 golden root samples available on the market, including a United States Pharmacopeia (USP) reference product, in order to learn more about product variances and differences in the bioactivity profiles. The samples were investigated using high-performance thin-layer chromatography (HPTLC) combined with 11 different assays (among these were 2 triplex bioassays), indicating 15 effect mechanisms. The six most bioactive zones were further characterized via a straightforward online hyphenation, i.e., via heart-cut zone elution, a desalting loop (freed from assay salts), and orthogonal HPLC separation to heated electrospray ionization high-resolution mass spectrometry (HPTLC–HPLC–HESI-HRMS).

2. Results and Discussion

2.1. Optimization of the Effect-Directed Profiling Method

To widen the sample range, 15 golden root samples were bought from different Polish and German vendors (Table 1). Thus, information was obtained on the current product variants on the market and the differences in their bioactivity profiles. Similarly to the previous study [39], the samples were extracted with methanol–water 4:1 (*v*/*v*), and 4 µL of each extract was applied (400 µg/band). The non-target effect-directed profiling was developed with no standard or marker compounds in mind. The Gram-negative *Aliivibrio fischeri* bioassay, which, based on our experience in other studies, detects a high number of bioactive zones by reducing bioluminescence in real time, was used to evaluate the 20 mobile phases studied with regard to bioactive compound separation (Supplementary Material Table S1). The mobile phase ethyl acetate–methanol–water 77:13:10 [39] was used as a basis for optimization. The zone tailing was substantially reduced by adding acetic acid to the mobile phase system. However, this required an additional neutralization step prior to the assay application, since most assays do not tolerate acidic traces that remain adsorbed (after plate drying). The zones were comparatively sharper, and when the proportion of water in the mobile phase system was increased, the polar compounds migrated out of the start zone, as is evident in the HPTLC chromatogram at UV 254 nm (Supplementary Material Table S1). Based on the *A. fischeri* bioautogram, the mobile phase consisting of ethyl acetate–methanol–water–acetic acid 70:15:15:1 was found to be suitable in initiating the intended effect-directed profiling. After the application and separation of all 15 products (Table 1), plate neutralization was performed in order to neutralize any remaining traces of acidic solvent.

Table 1. The list of the 15 commercially available golden root samples studied and respective information provided on the label.

ID	Sample Form	Dosage Form	Product Name	Manufacturer, City, Country	Plant Origin	Batch Number	Expiration Date
1	Root powder	Powder	Rhodiola-Rosenwurz	Vitaideal Vegan, Enshede, Netherlands	Netherlands	-	-
2	Root dry extract 25:1	Capsule	Rhodiola rosea	Green Naturals, Berlin, Germany	Germany	-	-
3	Root dry extract 20:1	Powder	Różeniec górski	Proherbis, Zarzecze, Poland	China	-	04/2023
4	Dried root	Dried root	Korzeń różeńca górskiego	Farmvit, Peterborough, UK	-	-	-
5	Root dry extract, 520 mg	Capsule	Różeniec górski	Medica herbs, Cracow, Poland	-	ROZ-0-01	02/2024
6	Root dry extract, 500 mg	Capsule	Rhodiola	Now Foods, Chicago, USA	-	3196374	02/2026
7	Dried rhizome	Dried rhizome	Kłącze różeńca górskiego	Dary natury, Grodzisk, Poland	-	-	06/2023
8	Root and rhizome dry extract	Powder	Rhodiola rosea root and rhizome dry extract	Sigma Aldrich	Canada	F00100	-
9	Dried root and rhizome	Dried root/ rhizome	Różeniec górski	Natvita, Mirków, Poland	Russia	29995	02/2023
10	Powdered root, 400 mg	Capsule	Rhodiola	Fushi, London, UK	China	FOHC6180/ 12970CN	09/2023
11	Root dry extract, 250 mg, with additives	Capsule	Rhodiola	Solgar Leonia, USA	-	530923-02	12/2023
12	Root dry extract, 500 mg, with inulin	Capsule	Rhodiola	ForMeds, Poznań, Poland	-	K220221	02/2023
13	Root dry extract, 140 mg, with additives	Capsule	Rhodiola	Pharmovit, Płock, Poland	-	RG0719/ PH	07/2023
14	Root dry extract, 100 mg	Tablet	Różeniec górski	Herbapol, Lublin, Poland	-	010621	06/2023
15	Root powder, 225 mg	Tablet	Arktyczny korzeń	Altermedica Laboratories, Żywiec, Poland	-	A0419/7	05/2023

The neutralized chromatogram was prepared 11 times (with adjustments for specific assays as described), as 11 effect-directed assays were applied, indicating 15 different response mechanisms due to 2 triplex bioassays. Considering the medicinal properties previously mentioned, the activity of the 15 golden root samples was evaluated with regard to

their effects as antibacterials against Gram-positive *B. subtilis* and Gram-negative *A. fischeri*, and inhibitors of tyrosinase, acetylcholinesterase, butyrylcholinesterase, β-glucuronidase, and α-amylase, as well as antioxidants detected via the 2,2–diphenyl–1–picrylhydrazyl radical (DPPH•) scavenging assay. In particular, information was sought on the presence of genotoxic compounds in the golden root samples via the SOS-Umu-C bioassay and agonistic and antagonistic endocrine compounds via the planar triplex yeast antagonist-verified estrogen/androgen screen (pYAVES/pYAVAS) bioassay.

2.2. *Effect-Directed Profiling of 15 Golden Root Samples*

2.2.1. *Aliivibrio fischeri* Bioassay

The 15 golden root extracts (400 µg/band each) revealed antibacterial activity against Gram-negative *A. fischeri* in the bioautogram (Figure 1). Up to five different prominent antibacterial (dark) zones were observed on the bioluminescent plate background, where the intrinsic (instant) green-blue bioluminescence was depicted as a grayscale image. The different products showed clear differences in the antibacterial profiles; e.g., ID 14 did not provide a response at all, and IDs 2, 5, 7, 13, and 15 reacted with a much weaker antibacterial effect in comparison with the other products. The USP reference standard of *Rhodiola rosea* L. root and rhizome (ID 8) showed antibacterial effects comparable to product IDs 6, 11, and 12.

2.2.2. *Bacillus subtilis* Bioassay

The incubation time for the *B. subtilis* bioassay was reduced by 15 h, from the 17.5 h used previously [39] to 2.5 h [40]. The overall antibacterial response against Gram-positive *B. subtilis*, observed in the bioautogram under white light illumination as colorless (white) zones against a purple plate background, was comparatively weaker than that against *A. fischeri*. The main response was evident in the polar compound range and was similar to the previous dark zones active against *A. fischeri*. Again, the observed antibacterial profiles differed clearly between the products. The USP reference product ID 8 showed only a weak zone at hR_F 10, which was not as intense as in IDs 1, 3, 4, 6, 9, 11, and 12. Some products (IDs 2, 5, 13, and 14) were not active at all at the given amounts.

2.2.3. SOS-Umu-C Genotoxicity Bioassay

The planar SOS-Umu-C bioautogram indicated a genotoxic compound at hR_F 93 as a bright green fluorescent zone against a less green fluorescent plate background in two samples (Figure 1, IDs 1 and 7). The genotoxic zone was clearly detectable despite the diffuse zones in the bioautogram caused by the 3.5 h long incubation. Later, this genotoxic compound zone was identified using HPTLC−HPLC−HESI-HRMS. Fluorescein-di-β-D-galactopyranoside (FDG) was chosen as a substrate for the glucosidase released upon contact with the genetically modified *Salmonella typhimiurium* strain with a genotoxin, since it provided the fluorescein, which is the green fluorescent end product. This was advantageous owing to the given natively blue fluorescent compounds in the separated golden root samples (HPTLC chromatogram at FLD 366 nm), which are able to shine through in the bioautogram (Figure 1). The formed fluorescein was detected at FLD 254 nm; this required the use of HPTLC plates silica gel 60 without the fluorescence indicator F_{254}, in order to avoid any measurement-signal interference. The presence of genotoxic compounds in two of the golden root supplement products on the market highlighted the importance of the effect-directed profiling as a quality control measure for plant-based supplements before they are made available to consumers. Further research is needed to clarify whether the genotoxic response is also detected in other batches. Luckily, the USP reference product ID 8 did not contain a genotoxin.

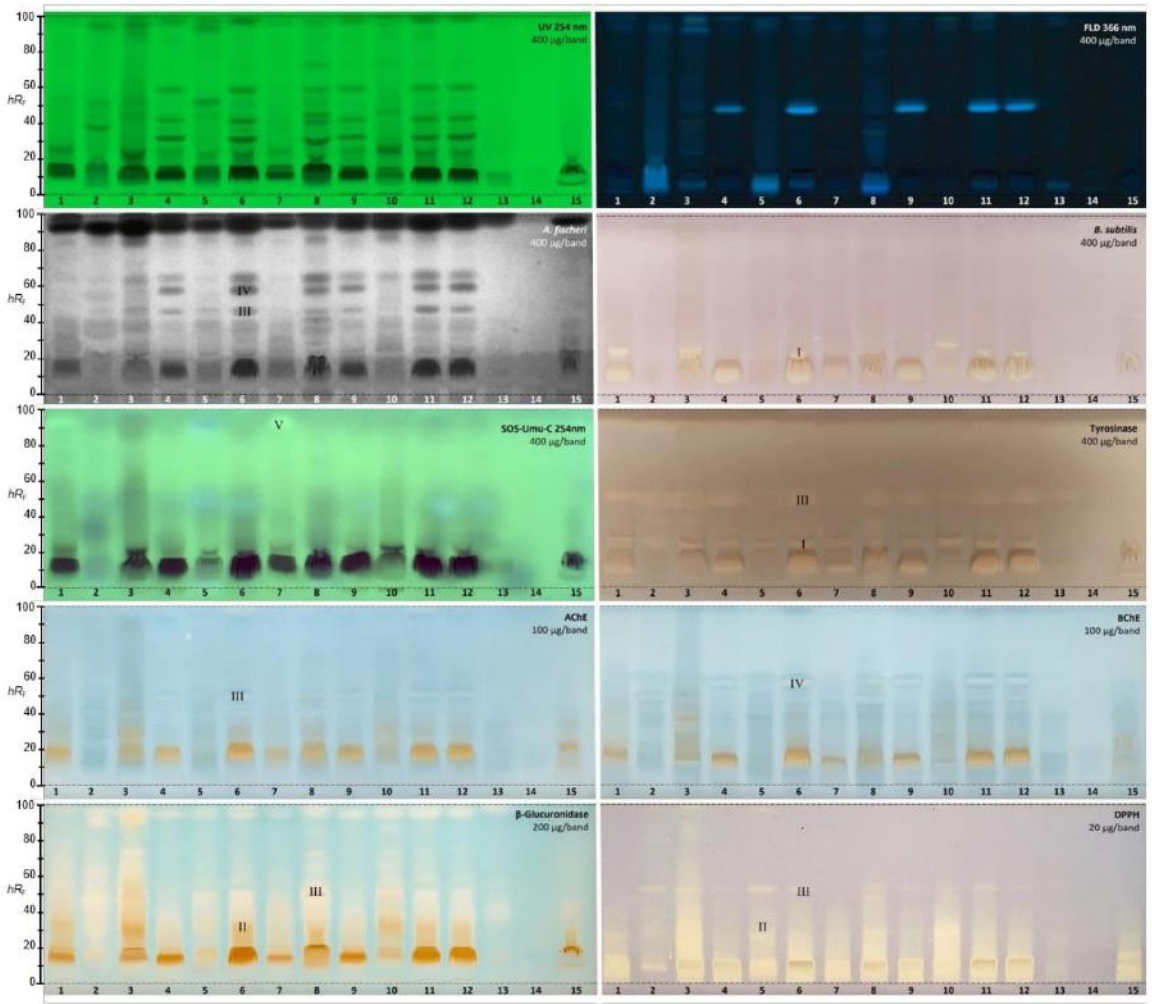


Figure 1. HPTLC chromatograms at UV 254 nm and FLD 366 nm and (bio)autograms of 15 golden root product extracts (Table 1, IDs 1−15, 20−400 µg/band depending on the assay as indicated) separated on HPTLC plates silica gel 60 F_{254} (without F_{254} for the SOS-Umu-C bioassay) using ethyl acetate–methanol–water–acetic acid 70:15:15:1 (*v*/*v*/*v*/*v*) and detected after the respective assay application via the instant bioluminescence (*A. fischeri*) at FLD 254 nm (SOS-Umu-C bioassay) or white light illumination; zones marked (I−V) were subjected to HPTLC−HPLC−HESI-HRMS recording.

2.2.4. Tyrosinase Inhibition Assay

The tyrosinase inhibition autogram of the 15 golden root products (Figure 1) revealed (as colorless inhibiting zones on a grey plate background) several more polar inhibition zones at hR_F 10 and 20, and further such zones at hR_F 51 and 90. Again, in contrast to most samples with up to three intense responses, product ID 14 showed no activity at all, and product IDs 13 and 15 only a very weak inhibition. The USP reference product ID 8 revealed all the above-mentioned inhibition zones (hR_F 10, 20, 51, and 90). The inhibiting compound zones at hR_F 20 and 51 were subjected to HPTLC−HPLC−HESI-HRMS.

2.2.5. Acetyl- and Butyrylcholinesterase Inhibition Assays

The acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) inhibition assays were often applied using Fast Blue B salt as a chromogenic reagent for the enzyme−substrate reaction. However, the sample matrix can react with polyphenols and generate an interfering brown color. A test of the reaction between golden root extract and Fast Blue B salt confirmed our hypothesis (Supplementary Material Figure S1). Thus, in contrast to [39], the AChE and BChE inhibition assays were performed using indoxyl acetate as the substrate, resulting in colorless inhibiting zones against an indigo blue plate background (Figure 1). The amount of golden root applied was reduced by a factor of 4 from 400 to 100 µg/band to avoid signal overload. In half of all the samples, up to two clear inhibitors were observed: the lower zone at hR_F 51 was an AChE inhibitor, as evident in the AChE autogram, in contrast to the upper zone at hR_F 61, which preferably inhibited the BChE, as observed in the respective BChE autogram. The USP reference product ID 8 showed inhibition of the AChE at hR_F 40 and 51 and of BChE at hR_F 51 and 61. Again, the same products (IDs 13−15) showed either no inhibition potential or the weakest inhibition potential among all the samples studied. The two inhibiting compound zones at hR_F 51 and 61 were subjected to subsequent HPTLC−HPLC−HESI-HRMS analysis.

2.2.6. β-Glucuronidase Inhibition Assay

For the β-glucuronidase inhibition assay (Figure 1), the applied amount of golden root was reduced to 200 µg/band in each case (to avoid signal overload). In the autogram, β-glucuronidase inhibitors were revealed as colorless inhibiting zones against an indigo blue plate background when 5-bromo-4-chloro-3-indonyl-β-D-glucuronide was used as the substrate for the enzyme. Diffuse tailing inhibition zones were detected. The 1% acetic acid content of the mobile phase was too low to sharpen the very polar active compound zones. In a future study, these will be able to be focused by means of using a stronger acid and a higher acid proportion in the mobile phase (e.g., using 10% formic acid). Since this would alter the separation and profile, we tolerated the zone tailing for reasons of comparison. Nevertheless, clear inhibition differences were evident between the products, and one half of the products exhibited greater potency than the other half. The USP reference product ID 8 was medium in activity compared with all other samples. Again, the same products (IDs 13−15) showed either no β-glucuronidase inhibition potential or the weakest β-glucuronidase inhibition potential.

2.2.7. DPPH• Scavenging Assay

The DPPH• autogram (Figure 1) showed that golden root extracts possessed high radical scavenging, and thus antioxidant, activity, evident as yellow zones on a purple plate background. For an initial extract amount of 400 µg/band, the autogram was totally overloaded (Supplementary Material Figure S2). Consequently, the applied extract amount was reduced by a factor of 20, and even with only 20 µg/band applied, many tracks still appeared overloaded. Similarly to the β-glucuronidase inhibition assay, tailing antioxidative zones were detected, but, based on our experience in other projects, these could be sharpened in future using 10% formic acid instead of 1% acetic acid. Again, the USP reference product ID 8 was medium in activity compared with all other samples. Product ID 14 showed no antioxidant activity even at 400 µg/band (Supplementary Material Figure S2). Product ID 13 showed almost no activity, and ID 2 was third weakest product in antioxidant activity.

2.2.8. α-Glucosidase and β-Glucosidase Inhibition Assays

The α/β-glucosidase inhibition activity was studied using 4-methylumbelliferyl-α/β-D-glucopyranoside as the substrate. However, previous results [41] had already revealed that substrate detection was not functioning correctly, owing to the direct reaction of sample compounds with the substrate. Here, this assumed false positive reaction was studied in detail and proven by performing the detection with only the substrate present, and with

no enzyme (Supplementary Material Figure S3). Consequently, this assay detection still requires further optimization for its application to golden root samples.

2.2.9. α-Amylase Inhibition Assay

A different mobile phase was required for the α-amylase inhibition assay (Figure 2), since a pretest showed that all the sample responses (observed as brown zones on a bright plate background) were present in the solvent front (Supplementary Material Figure S4). The elution power was too strong for the α-amylase inhibiting compounds, and it was thus reduced in order to obtain ethyl acetate–*n*-hexane 3:7 (*v*/*v*). All 15 golden root products revealed α-amylase inhibition activity. In the autogram, two active zones were detected: one at the start zone and another prominent one at hR_F 23. The latter compound zone (hR_F 23) was subjected to HPTLC−HPLC−HESI-HRMS recording.

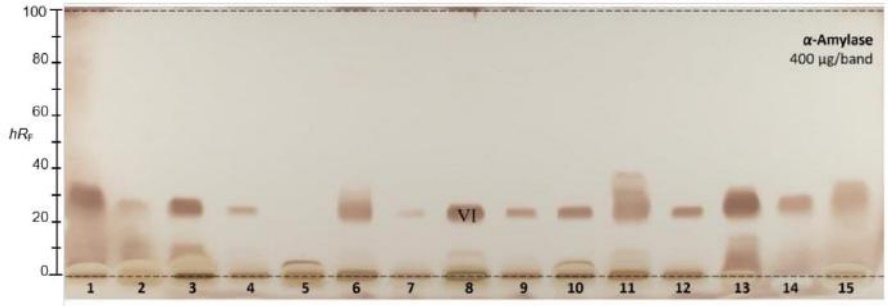


Figure 2. HPTLC–α-amylase inhibition autogram of 15 golden root product extracts (400 µg/band each) separated on HPTLC plates silica gel 60 F_{254} using ethyl acetate–*n*-hexane 3:7 (*v*/*v*) and detected at white light illumination after the assay application; the zone marked (VI) was subjected to HPTLC−HPLC−HESI-HRMS recording.

2.2.10. pYAVAS and pYAVES Bioassays

Possible endocrine effects in the 15 golden root products were studied in the triplex agonistic/antagonistic pYAVES/pYAVAS bioassays (Figure 3) [42]. Acetic acid was eliminated from the mobile phase to simplify the protocol for the following triplex bioassays. In respect of zone fixation, two additional layer treatments were newly included to avoid diffusion during the 3 or 4 h long incubation. Thus, the two stripes, which were applied along each separated sample track before the bioassay application, remained sharp, which was helpful for the evaluation of the agonistic/antagonistic response profiles in the triplex bioautogram [42]. As for the SOS-Umu-C genotoxicity bioassay, FDG was chosen as a substrate for the released glucosidase (upon contact of the respective genetically modified *Saccharomyces cerevisiae* strain with an agonist), since it advantageously generated the green fluorescent fluorescein end product as previously mentioned. Consequently, plates without F_{254} had to be used here as well. The 15 products were applied at higher amounts (600 µg/band each) owing to the longer application band (12 mm band) required to provide sufficient space for the application of the two stripes. To provide the detection of both the estrogenic and the antiestrogenic activity on the same plate via the triplex pYAVES bioassay, two stripes were applied along each separated sample track before the bioassay application. The first agonist stripe was 17β-estradiol (detectable first after the bioassay), and the second end-product stripe was fluorescein (directly detectable; see Figure 3).

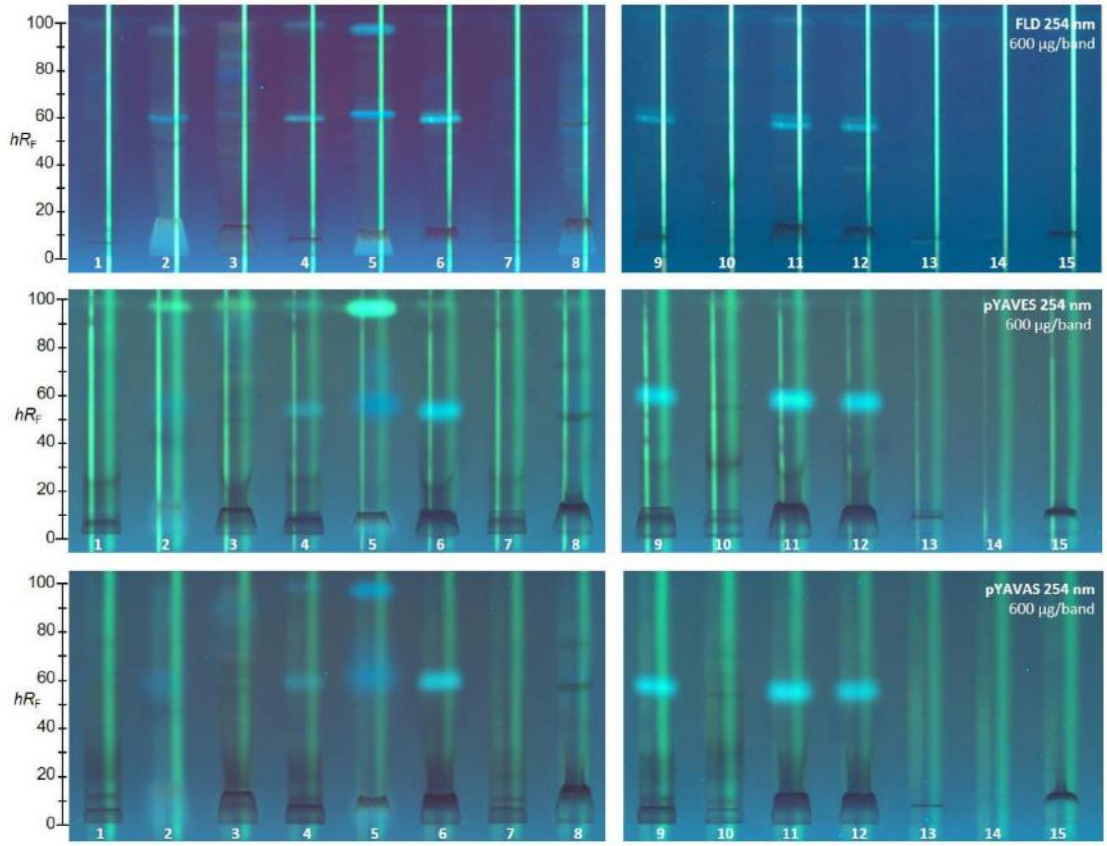


Figure 3. HPTLC chromatograms and HPTLC−pYAVES/pYAVAS−FLD triplex bioautograms showing agonistic, antagonistic, and false-positive antagonistic endocrine effects in 15 golden root extracts (12 mm band, 6 µL/band, 600 µg/band each) separated on HPTLC plates silica gel 60 using ethyl acetate–methanol–water 70:15:15 (*v*/*v*/*v*) and detected at FLD 254 nm before (for comparison) and after the respective bioassay.

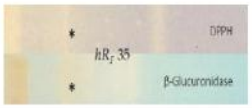
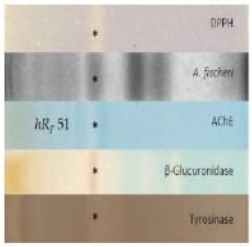
After the bioassay application, a clear estrogenic compound zone was detected as a green fluorescent zone at hR_F 99 close to the solvent front. In particular, in product ID 5 the response was strongest, followed by product ID 2, then ID 3. However, further products (IDs 1, 4, 6, 8, 10 and 11) also showed a weak response. A verified antiestrogenic activity, evident as a reduction in fluorescence on the first 17β-estradiol stripe (biologically-induced reduction), was observed in many samples, for example, in product IDs 1, 4, 6, and 7 at hR_F 90. Product ID 9 showed several (and comparatively the most) antiestrogenic compounds. These antagonistic effects were unequivocally verified by the co-applied second fluorescein stripe, which was not reduced in the fluorescence. In case of false positive responses (caused physico-chemically), the second fluorescein stripe would be reduced in fluorescence. In order to evaluate androgenic and antiandrogenic activity, the triplex pYAVAS bioassay was used analogously. However, the first agonist stripe was the testosterone. As a result, no androgens or antiandrogens were revealed, even for the applied 600 µg/band golden root extract (Figure 3).

2.3. Characterization of Six Active Compound Zones Using HPTLC−HPLC−HESI-HRMS

The six most active compound zones (**I–V** in Figure 1 according to ascending hR_F, as well as **VI** in Figure 2 with a mobile phase of reduced solvent strength) were selected and

subjected to HPTLC–HPLC–HESI-HRMS recording [40,42,43]. Based on data from the literature and the HRMS spectra obtained in the negative and positive ionization modes, molecular formulae were tentatively assigned (Table 2, Supplementary Material Figure S5).

Table 2. HPTLC–HPLC–HESI-HRMS signals obtained in the positive and negative ionization modes and the tentative assignment of the active compound zones **I**–**VI** in the golden root product IDs 1 and 6 (400 µg/band each).

Zone ID	hR_F	Bioactivity	Tentative Assignment	Formula	Calculated Mass [Da]	Observed Mass [Da]	Mass Error (Δ ppm)	Adduct Ions
I 6	20		Rhodioloside D	$C_{16}H_{30}O_8$	350.1941	409.2084 373.1828	−1.09 1.13	$[M+CH_3COO]^-$ $[M+Na]^+$
II 6	35		Rosavin/rosarin	$C_{20}H_{28}O_{10}$	428.1683	427.1610 451.1576	−0.01 −0.26	$[M-H]^-$ $[M+Na]^+$
III 6	51		Rosiridin	$C_{16}H_{28}O_7$	332.1835	391.1978 355.1722	−1.33 1.54	$[M+CH_3COO]^-$ $[M+Na]^+$
			Viridoside	$C_{15}H_{22}O_7$	314.1365	359.1351 337.1258	−1.06 −0.10	$[M+CHOO]^-$ $[M+Na]^+$
			Salidroside	$C_{14}H_{20}O_7$	300.1209	299.1139 323.1096	−0.74 1.57	$[M-H]^-$ $[M+Na]^+$
IV 6	61		Rosin	$C_{15}H_{20}O_6$	296.1260	355.1399 319.1146	−0.02 1.98	$[M+CH_3COO]^-$ $[M+Na]^+$
V 1	93		Hydroquinone	$C_6H_6O_2$	110.0368	109.0294 111.0444	0.68 −2.74	$[M-H]^-$ $[M+H]^+$
VI 6	23		Stearic acid Palmitic acid	$C_{18}H_{36}O_2$ $C_{16}H_{32}O_2$	284.2715 256.2402	283.2643 255.2330	−0.13 −0.26	$[M-H]^-$ $[M-H]^-$

The first active zone at hR_F 20 with antibacterial activity against *B. subtilis* and inhibition of tyrosinase was tentatively assigned to rhodioloside D, since the recorded mass signals at m/z 409.2084 $[M+CH_3COO]^-$ and m/z 373.1828 $[M+Na]^+$ matched this marker compound. The second active zone at hR_F 35 with strong antioxidant and β-glucuronidase inhibition activity revealed two mass signals at m/z 427.1610 $[M-H]^-$ and m/z 451.1576 $[M+Na]^+$, tentatively assigned to the two marker compounds, rosavin and rosarin. The third active zone at hR_F 51 with antioxidant, antibacterial (against *A. fischeri*), and AChE, tyrosinase, and β-glucuronidase-inhibiting activity indicated three constituents of golden root, i.e., rosiridin with mass signals at m/z 391.1978 $[M+CH_3COO]^-$ and m/z 355.1722 $[M+Na]^+$, viridoside at m/z 359.1351 $[M+CHOO]^-$ and m/z 337.1258 $[M+Na]^+$, and salidroside at m/z 299.1139 $[M-H]^-$ and 323.1099 $[M+Na]^+$. The fourth active zone at hR_F 61 inhibiting BChE and active against *A. fischeri* was tentatively assigned to rosin with mass signals at m/z 355.1399 $[M+CH_3COO]^-$ and m/z 319.1146 $[M+Na]^+$. All the compounds

mentioned were responsible for the activity and used for the standardization of golden root raw material [1,2]. The mass spectra from the fifth genotoxic zone at hR_F 93 exhibited two mass signals at m/z 109.0294 $[M\text{-}H]^-$ and 111.0444 $[M+H]^+$. Thus, hydroquinone was tentatively assigned as the compound responsible for the genotoxic activity in the golden root of which the genotoxic potential is known [44]. The mass spectra in the negative and positive ionization modes for the sixth α-amylase inhibiting compound zone at hR_F 23 (via mobile phase reduced in elution strength) exhibited signals corresponding to stearic acid and palmitic acid. These compounds were tentatively assigned as prominent α-amylase inhibitors in almost all the golden root products.

Rosavin, rosarin, rosin, salidroside, viridoside, and rosiridin are the major constituents of golden root with important activities. In particular, rosavin and salidroside have frequently been used for the standardization of golden root products, as well as for quality evaluation and the detection of possible adulteration. The standard concentration ratio of rosavin to salidroside is 3 to 1. The content of these compounds in golden root and rhizome extracts depends on several factors. However, the factors that have the most impact on the content of these compounds are harvest time, long-term vegetative propagation, and genetic diversity [39,45–47].

3. Materials and Methods

3.1. Chemicals

Acetic acid, bovine serum albumin, caffeine, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS), citrate buffer, dimethyl sulfoxide, 2,2–diphenyl–1–picrylhydrazyl (DPPH), Dulbecco's phosphate buffered saline (DPBS), ethanol, ethyl acetate, fluorescein di-β-D-galactopyranoside (FDG), gallic acid, glycerol, hexane, hydrochloric acid (HCl), indoxyl acetate, kojic acid, methanol, phosphate buffer, polyethylene glycol (PEG) 8000, D-saccharolactone, tetracycline, thiazol blue tetrazolium bromide (MTT), and tris(hydroxymethyl)aminomethane hydrochloride buffer (TRIS) were obtained from Carl Roth (Karlsruhe, Germany); acarbose, acetylcholinesterase (AChE) from *Electrophorus electricus*; α-amylase from hog pancreas; butyrylcholinesterase (BChE) from equine serum; β-glucuronidase from *Escherichia coli*; and Gram's iodine solution, lysogeny broth powder (containing 5 mg/mL sodium chloride), rivastigmine, testosterone, and tyrosinase from mushrooms were delivered by Sigma-Aldrich (Steinheim, Germany). 5-Bromo-4-chloro-3-indonyl-β-D-glucuronide was purchased from Carbosynth (Compton-Berkshire, UK). (2S)-2-Amino-3-(3,4-dihydroxyphenyl) propionic acid (levodopa) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA). 17β-Estradiol was obtained from Dr. Ehrenstorfer (Augsburg, Germany). 4-Nitroquinoline-1-oxide was purchased from TCI (Eschborn, Germany). *Aliivibrio fischeri* bacteria (NRRI–B11177, strain 7151) and *Bacillus subtilis* bacteria (DSM-618) were purchased from the German Collection of Microorganisms and Cell Cultures (Leibniz Institute DSMZ, Berlin, Germany). *Salmonella typhimurium* strain TA1535, genetically modified to contain the plasmid pSK1002, was purchased from Trinova Biochem (Giessen, Germany). *Saccharomyces cerevisiae* strain BJ1991 containing the human androgen receptor was obtained from Xenometrix (Allschwil, Switzerland). *Saccharomyces cerevisiae* cells equipped with hERβ were obtained from Erwin Herberle-Bors, University of Vienna, Austria. Additional chemicals and reagents used for pYAS/pYES cell culture have been reported previously [40,42,48]. All the chemicals are of analytical grade, and all the solvents are of chromatographic grade. Bidistilled water was prepared using a Heraeus Destamat Bi-18 E (Thermo Fisher Scientific, Dreieich, Germany). HPTLC plates silica gel 60 F_{254} (20 cm × 10 cm) and HPTLC plates silica gel 60 (20 cm × 10 cm) were provided by Merck (Darmstadt, Germany). The 15 commercially available golden root samples were purchased in Poland and Germany from different vendors (Table 1).

3.2. Sample Preparation

The samples (Table 1) were ground (8000 rpm, 5 min, Tube Mill, IKA, Staufen, Germany) and stored in a dark, well-ventilated place at room temperature. Each sample

(500 mg) was extracted with 5 mL methanol–water 4:1 (*v*/*v*) in a conical 15-mL Eppendorf tube, vortexed for 1 min, ultrasonicated for 15 min (20 °C, 100%, 480 W, 35 kHz, Sonorex Digi plus DL 255H, Bandelin, Germany) and centrifuged for 5 min (3000× *g*, Heraeus Labofuge 400, Thermo Scientific, Dreieich, Germany). Each supernatant was stored at −20 °C.

3.3. HPTLC–UV/Vis/FLD–EDA Profiling Method

HPTLC plates were pre-washed (developed) with methanol–water 4:1 (*v*/*v*) up to the upper plate edge (Simultan Separating Chamber, biostep, Burkhardtsdorf, Germany) and dried in an oven at 110 °C for 20 min and subsequently wrapped in aluminum foil and stored in a desiccator. The samples (0.2–6.0 µL, as mentioned) were applied as 8 mm bands or, for triplex assays, 12 mm bands (dosage speed 200 nL/s, distance from the lower edge 10 mm, from side edge 16 mm, and between tracks 12 mm, or 22 mm for triplex assays, ATS 4, CAMAG, Muttenz, Switzerland) on HPTLC plates silica gel 60 with or without F_{254}. After the samples were dried with a hairdryer for 3 min, development was carried out with ethyl acetate–methanol–water–acetic acid 70:15:15:1 (*v*/*v*/*v*/*v*) up to 70 mm migration distance (Twin Trough Chamber 20 cm × 10 cm, CAMAG). After separation, the chromatogram was dried for 10 min (ADC 2, CAMAG). Then the chromatogram was documented (TLC Visualizer, CAMAG) at 254 nm (UV), 366 nm (FLD), and under white light illumination (VIS).

Eleven chromatograms were prepared analogously with a few adjustments depending on the assay, as mentioned. Each chromatogram was neutralized with 5% sodium bicarbonate pH 8) or sodium acetate buffer (pH 7) by means of piezoelectric spraying (2.5 mL, yellow nozzle, level 6, Derivatizer, CAMAG) followed by drying for 3 min (hairdryer) and 20 min (ADC 2, CAMAG). The neutralized chromatograms were sprayed with the respective assay solutions and incubated in a humid atmosphere (KIS polypropylene box, 27 cm × 16 cm × 10 cm, ABM, Wolframs–Eschenbach, Germany). For each assay, a respective positive control was applied [40,41]. Each assay was performed at least twice to confirm the reproducibility of the response.

The *A. fischeri* bioassay was performed according to [41,49]. After spraying the *A. fischeri* suspension (4 mL, red nozzle, level 6), the still-humid plate was transferred to the BioLuminizer (CAMAG). Ten images were recorded over 30 min (exposure time 60 s, trigger interval 3.0 min). Caffeine was used as a positive control (1 mg/mL in methanol; 0.5, 1.5, and 3 µL/band).

The *B. subtilis* bioassay was performed as previously described [40]. The bacteria suspension (100 µL cryostock in 20 mL 2.3% Müller–Hinton broth incubated overnight at 37 °C and adjusted to optical density 1.1 at 600 nm) was sprayed (3 mL, red nozzle, level 6), and this was followed by incubation at 37 °C for 2 h. The MTT substrate solution (0.2% in DPBS buffer) was sprayed (0.5 mL, blue nozzle, 6 level), and this was followed by incubation at 37 °C for 30 min, plate drying (50 °C, 10 min, TLC Plate Heater, CAMAG), and documentation at white light illumination. Tetracycline was used as a positive control (0.005 mg/mL in ethanol; 0.5, 1.5 and 3 µL/band).

The planar SOS-Umu-C genotoxicity bioassay was performed on HPTLC plates without a fluorescence indicator as previously described [50–52]. The *Salmonella* suspension was sprayed (2.5 mL, yellow nozzle, level 3) on the plate, and this was followed by incubation at 37 °C for 3 h. The FDG substrate solution (25 µL of 0.5% FDG in dimethyl sulfoxide in 2.5 mL phosphate buffer) was sprayed (2.5 mL, red nozzle, level 6), and this was followed by incubation at 37 °C for 15 min, plate drying and documentation at 254 nm. 4-Nitroquinoline-1-oxide was used as a positive control (1 ng/mL in methanol; 10 µL/band).

The tyrosinase inhibition assay was performed as previously described [53]. The substrate solution (4.5 mg/mL levodopa in 20 mM phosphate buffer pH 6.8 plus 2.5 mg of CHAPS and 7.5 mg PEG 8000) was sprayed (2 mL, blue nozzle, level 5). After plate drying (2 min), the tyrosinase solution (400 U/mL in phosphate buffer) was sprayed (2 mL,

blue nozzle, level 5), and this was followed by incubation at room temperature in the dark for 20 min. Kojic acid was used as a positive control (0.1 mg/mL in ethanol; 1, 3, and 6 µL/band).

The AChE/BChE inhibition assays were performed as previously described [54]. The substrate solution (1 mg/mL indoxyl acetate in ethanol) was sprayed (2 mL, green nozzle, level 6), and this was followed by drying for 3 min and then spraying with 3 mL enzyme solution (6.66 U/mL AChE or 3.34 U/mL BChE, each in Tris–HCl buffer plus 1 mg/mL bovine serum albumin). The incubation at 37 °C took 1 h. After drying (10 min in ADC 2), the plate was documented at white light illumination. Rivastigmine was used as a positive control (0.1 mg/mL in methanol; 2, 4, and 8 µL/band).

The β-glucuronidase inhibition assay was performed as previously described [40]. The enzyme solution (50 U/mL in 0.1 M potassium phosphate buffer pH 7 plus 1 mg/mL bovine serum albumin) was sprayed (2 mL, yellow nozzle, level 6), and this was followed by incubation at 37 °C for 15 min, spraying with 1.5 mL substrate solution (2 mg/mL 5-bromo-4-chloro-3-indonyl-β-D-glucuronide in water), incubation at 37 °C for 1 h, plate drying (10 min in ADC 2), and documentation at white light illumination. D-saccharolactone solution was used as a positive control (0.1 mg/mL in water; 0.8, 1.5, and 3 µL/band).

The DPPH• assay was performed by spraying the chromatogram with DPPH• solution (4 mL, 0.04% in methanol, green nozzle, 4 level) and then drying (10 min in ADC 2) and documenting it at white light illumination. Gallic acid was used as a positive control (0.25 mg/mL in methanol; 0.2, 0.6, and 1.0 µL/band).

The α-amylase inhibition assay was performed as recently described [40]. The enzyme solution (62.5 U/mL in sodium acetate buffer, pH 7) was sprayed (2 mL, red nozzle, level 6), and this was followed by incubation at 37 °C for 30 min. Then, the substrate solution (2% starch in water) was sprayed (1 mL, red nozzle, level 6), and this was followed by another incubation at 37 °C for 20 min and by spraying with Gram's iodine solution (0.5 mL, yellow nozzle, level 6). Acarbose was used as a positive control (0.1 mg/mL in methanol; 0.3, 0.6, and 0.9 µL/band).

The triplex pYAVES/pYAVAS bioassays were performed on HPTLC plates without a fluorescence indicator, according to [42]. The samples were applied as a 12 mm band (6 µL/band, track distance 22 mm) and developed with ethyl acetate–methanol–water 70:15:15 (*v*/*v*/*v*). After plate drying, two stripes (1 mm × 70 mm) were sprayed along each separate sample track (Freemode option, winCATS software). The first agonist stripe (considered also as a positive control) was 17β-estradiol (5 µL, 10 ng/mL in ethanol) for the pYAVES, while testosterone was used (4 µL, 5 µg/mL in methanol) for the pYAVAS. The second end-product stripe was fluorescein (2 µL, 50 µg/mL in methanol), which was used to detect false-positive responses. The dried chromatogram was immersed in a fixation solution (0.25% Degalan in *n*-hexane) for 10 min, dried for 10 min, sprayed with 2.5 mL Tween 20 solution (0.02% in ethanol), and dried for 10 min. The yeast cell suspension was sprayed (2.8 mL, red nozzle, level 6) on the plate, incubated at 30 °C for 3 h (pYAVES) or 4 h (pYAVAS), sprayed with FDG solution (2.5 mL, yellow nozzle, level 6), incubated at 37 °C for 15 min, and dried for 10 min. The resulting bioautogram was documented at FLD 254 nm.

3.4. HPTLC–HPLC–HESI-HRMS

Golden root extract IDs 1 and 6 (4 µL/band) were applied in triplicate and separated as described for the respective assay. The zones marked were eluted with 10% methanol in an aqueous solution at a flow rate of 0.1 mL/min for 1 min (open-source modified autoTLC-MS Interface [43]). Subsequently, the analytes were transferred through a 50 µL sample loop and Accucore RP-MS, 10 mm × 2.1 mm, 2.6 µm (Thermo Scientific, Bellefonte, PA, USA) desalting cartridge to the HPLC separation [40,42]. An Accucore RP-MS (100 mm × 2.1 mm, 2.6 µm, Thermo Scientific, Bellefonte, PA, USA) analytical column was used. Eluent phase A (2.5 mM ammonium acetate in water, pH 4.5) and eluent phase B (methanol) were used for gradient elution over 12 min using the following program: 0–2 min 2% B, 2–7 min increase

from 2% to 100% B; 10–12 min from 100 to 2% B to restore initial gradient composition. The flow rate was 0.4 mL/min, and the column temperature was set to 40 °C. Full scan mass spectra were in the positive and negative ionization modes at mass range m/z 100–1100 recorded using the HESI-HRMS system (Q Exactive Plus mass spectrometer, Thermo Fisher Scientific, Bellefonte, PA, USA). The following parameters were used: capillary temperature 270 °C, spray voltage ± 3.5 kV, sheath gas 20 arbitrary units, and aux gas 10 arbitrary units (S–Lens RF level 50). The spectra were evaluated using Xcalibur 3.0.63 software (Thermo Fisher Scientific, Bellefonte, PA, USA).

4. Conclusions

The differences and variances in the 15 characteristic bioactivity profiles of 15 different golden root products obtained on the market highlighted the importance of effect-directed profiling as a quality control measure for plant-based supplements. Interestingly, the USP reference product showed medium activity in most assays. The activities of three samples (IDs 13–15) were comparably poor, except in respect of α-amylase inhibition. This was partially explained by the lower amount of dry root extract or root powder contained in the products, whereby root powder is comparatively less active than root extract. In particular, the genotoxicity bioassay pointed to a genotoxic zone in two products (IDs 1 and 7) tentatively assigned as hydroquinone. These results point to the need for bioactivity profiling of supplements before they are made available to consumers. Antioxidants and antibacterials against *B. subtilis* and *A. fischeri* were detected, in addition to inhibitors of acetylcholinesterase, butyrylcholinesterase, β-glucuronidase, α-amylase, and tyrosinase. Information was obtained for the first time about β-glucuronidase, α-amylase and butyrylcholinesterase inhibitors, and genotoxic compounds, in addition to individual estrogens and antiestrogens in golden root products. Even in 600 µg of the golden root extract, no androgenic or antiandrogenic activity was observed. The main bioactive compounds of the golden root detected and tentatively assigned were salidroside, viridoside, rosavin, rosarin, rosin, rosiridin, rhodioloside D, stearic acid, and palmitic acid.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/molecules28041535/s1, Figure S1: HPTLC–Vis chromatogram via Fast blue B salt reagent; Figure S2: DPPH•–Vis autogram; Figure S3: HPTLC–FLD–α–/β–glucosidase inhibition autograms; Figure S4: HPTLC–Vis–α-amylase inhibition autogram; Figure S5: HPTLC−HPLC−HESI-HRMS spectra; Table S1: Mobile phase optimization.

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Supplementary Material

Bioactivity Profiles on 15 Different Effect Mechanisms for 15 Golden Root Products via High-Performance Thin-Layer Chromatography, Planar Assays, and High-Resolution Mass Spectrometry

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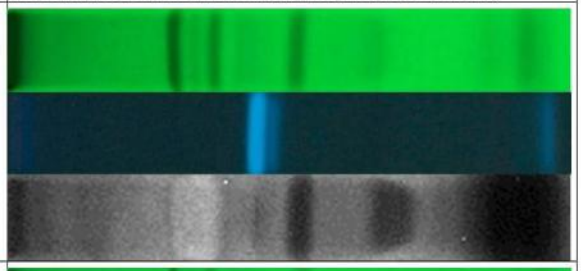
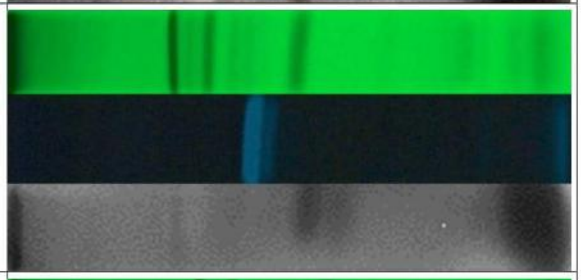
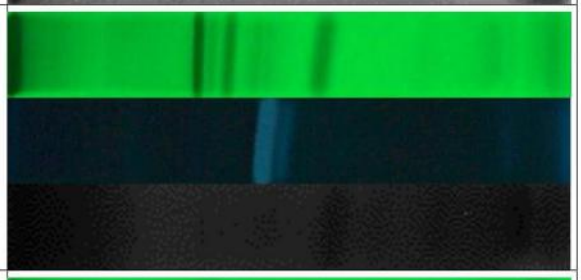
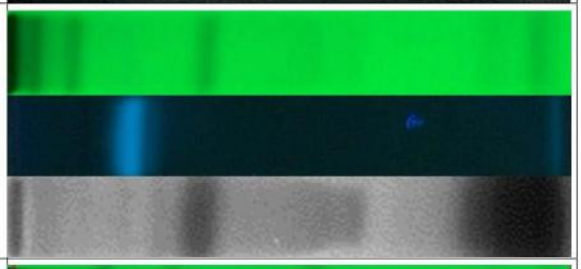
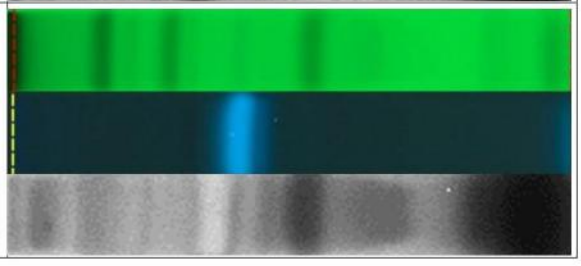
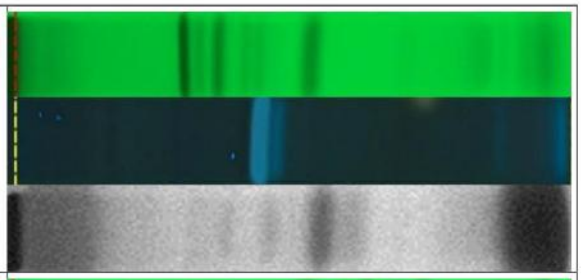
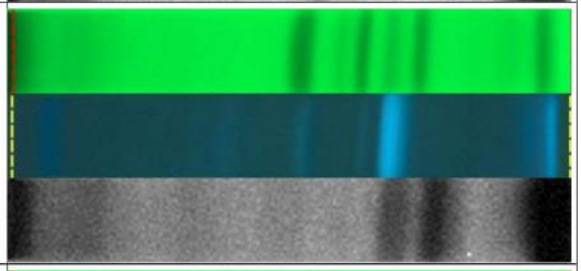
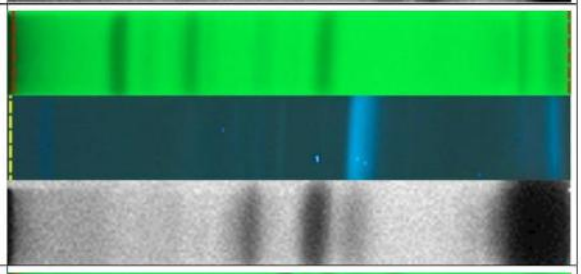
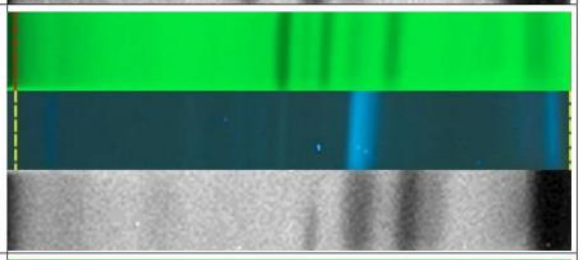
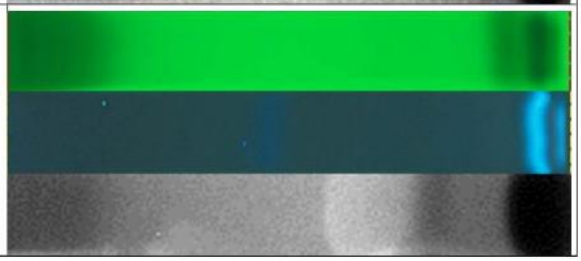
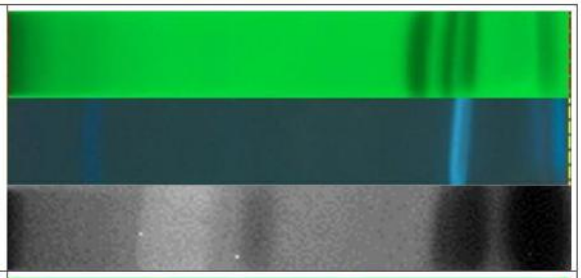
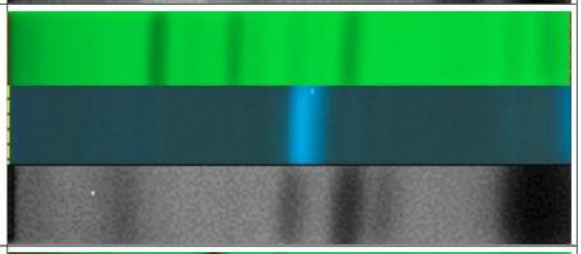
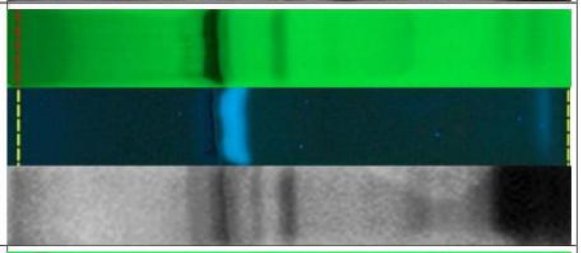
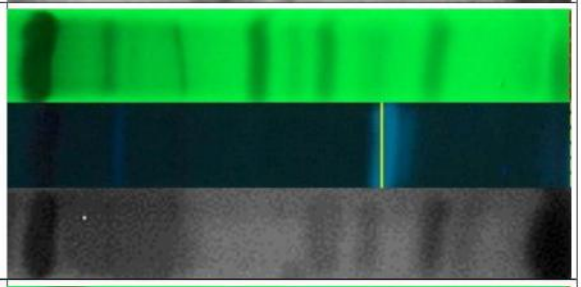
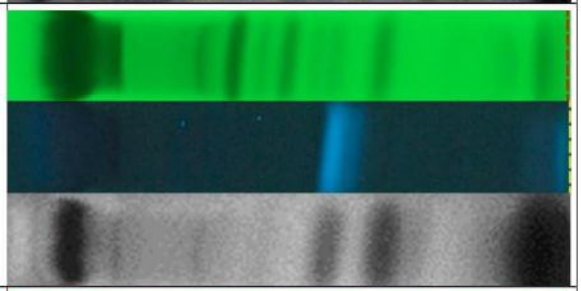
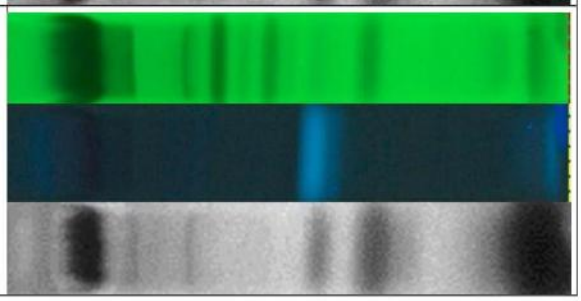
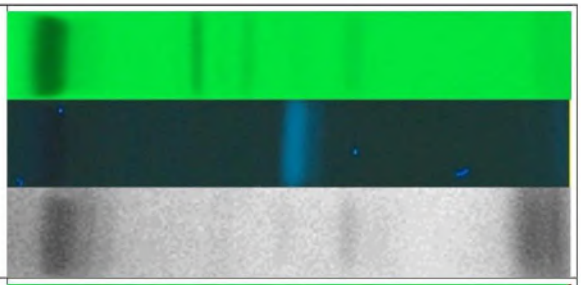
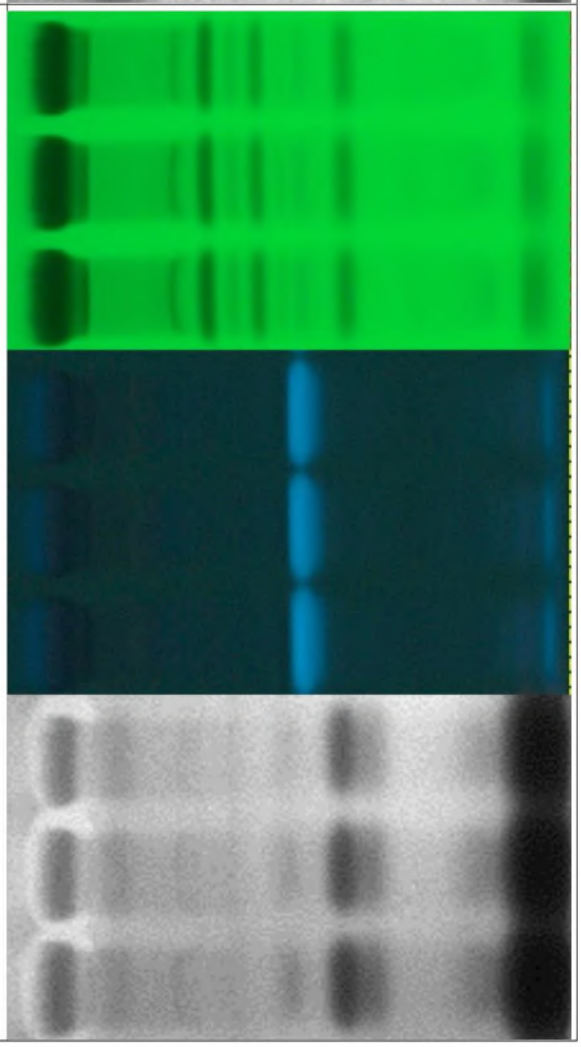
Table S1 Mobile phase optimization for a golden root extract (5 µL/band or 3–4 µL/band) separated on the HPTLC plate silica gel 60 F_{254} with the specified mobile phase system

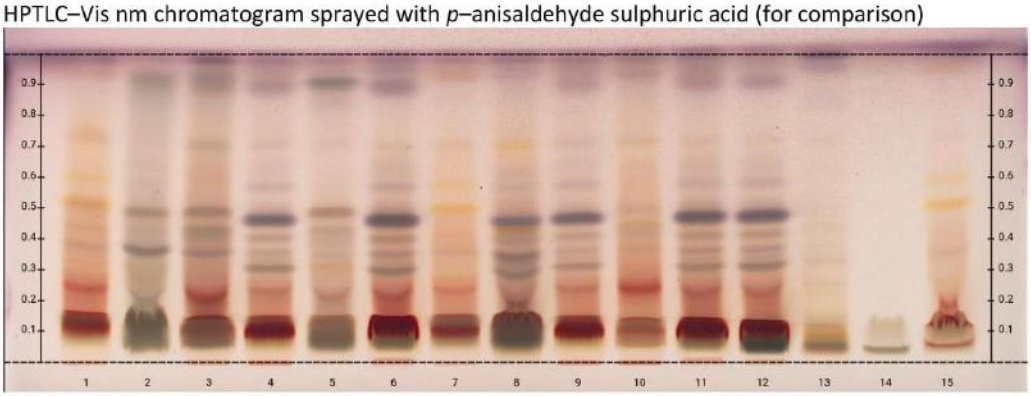
MP	Solvent composition	Ratio (*V/V*)	Chromatogram at 254 nm, FLD 366 nm and bioluminescence after *Aliivibrio fischeri* bioassay
1	Ethyl acetate-methanol-water	7.7:1.3:1	
2	Ethyl acetate-methanol-water-acetic acid	7.7:1.3:1:0.1	
3	Ethyl acetate-methanol-water-acetic acid	7.7:1.3:1:0.2	
4	Ethyl acetate-acetonitrile-water	7:2:1	
5	Ethyl acetate-acetonitrile-water	5:4:1	

6	Ethyl acetate-methanol-water-acetic acid	7.7:1.3:1:0.15	
7	Ethyl acetate-methanol-water-acetic acid	7:1.5:1:0.2	
8	Ethyl acetate-acetonitrile-water-acetic acid	5:4:1:0.1	
9	Ethyl acetate-methanol-water-acetic acid	7:1.5:1:0.1	
10	Ethyl acetate-methanol-water-acetic acid	5:4:1:0.1	
11	Ethyl acetate-methanol-water-acetic acid	6:3:1:0.1	

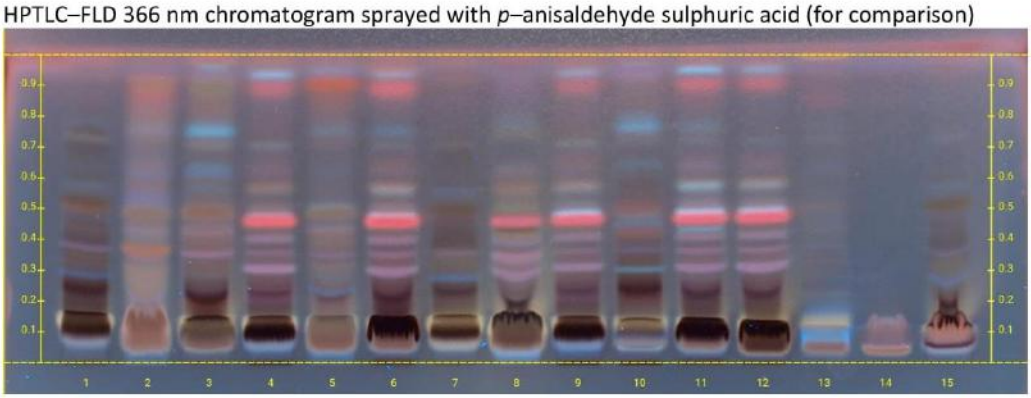
12	Ethyl acetate-methanol-water-acetic acid	7:2:1:0.1	
13	Ethyl acetate-acetonitrile-water-acetic acid	4:5:1:0.1	
14	Focusing (methanol), 3 cm Ethyl acetate-water	10:1	
15	Focusing (acetone, 2 x methanol), 2 cm Ethyl acetate-methanol-water-acetic acid	7:1.5:1.5:0.1	
16	Ethyl acetate-methanol-water-acetic acid	7:1.5:1.5:0.1	
17	Focusing (2 x acetone, methanol), 2 cm Ethyl acetate-methanol-water-acetic acid	7:1.5:1.5:0.1	

18	Ethyl acetate-methanol-water-acetic acid	7:1.5:1.5:0.1	
19	Focusing (2 x acetone, methanol), 2 cm Ethyl acetate-methanol-water-acetic acid	7:1.5:1.5:0.1	
20	Ethyl acetate-methanol-water-acetic acid	7:1.5:1.5:0.1	

HPTLC–Vis nm chromatogram sprayed with *p*–anisaldehyde sulphuric acid (for comparison)



HPTLC–FLD 366 nm chromatogram sprayed with *p*–anisaldehyde sulphuric acid (for comparison)



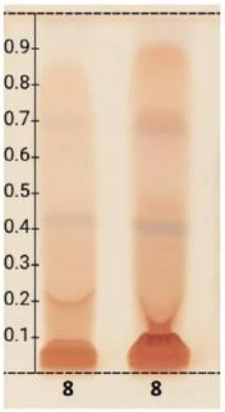


Figure S1. HPTLC–Vis chromatogram after derivatization via Fast Blue B salt reagent (0.5% aqueous solution) of a golden root extract ID 8 (200 and 400 µg/band) separated on HPTLC plate silica gel 60 F_{254} with ethyl acetate-methanol-water-acetic acid 70:15:15:1 (*V/V/V/V*) [GM1]and detected at white light illumination.

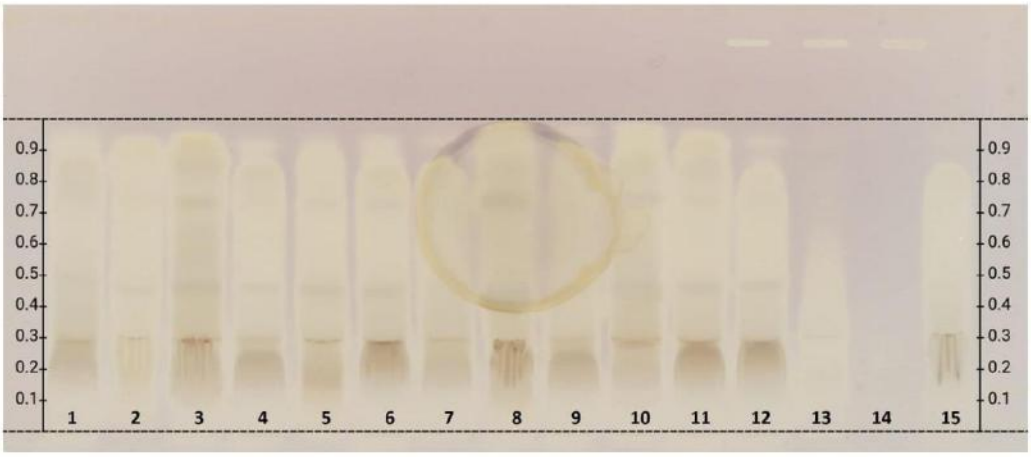
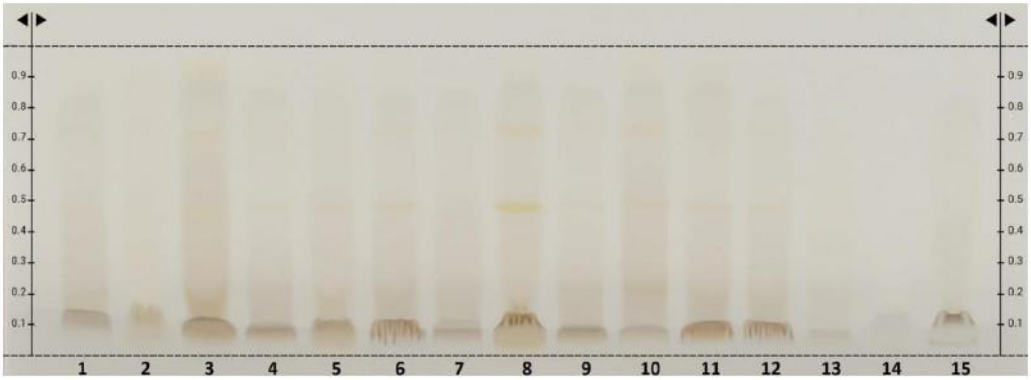
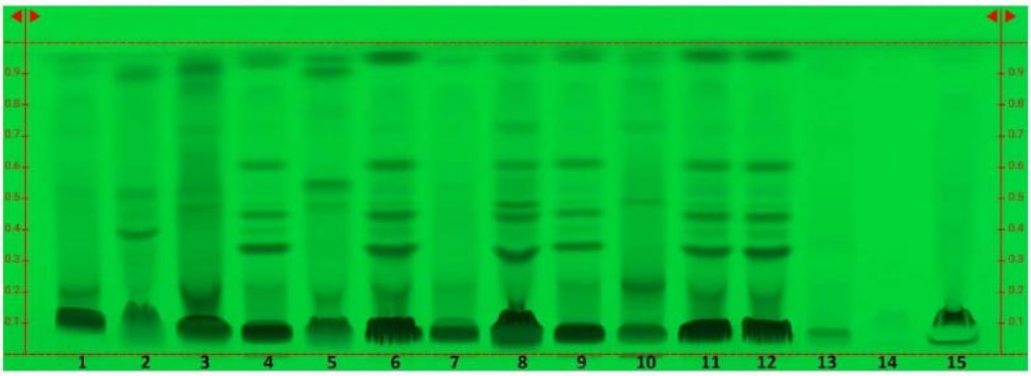


Figure S2. DPPH•–Vis autogram of golden root extract IDs 1–15 (400 µg/band each) separated on HPTLC plate silica gel 60 F_{254} with ethyl acetate-methanol-water-acetic acid 70:15:15:1 (*V/V/V/V*) and detected at white light illumination after the DPPH• assay. Positive control gallic acid in the right upper plate corner. The ring in the plate middle was caused by misfunction of the Derivatizer nozzle.

HPTLC–Vis nm chromatogram (for comparison)



HPTLC–UV 254 nm chromatogram (for comparison)



HPTLC–FLD 366 nm chromatogram (for comparison)

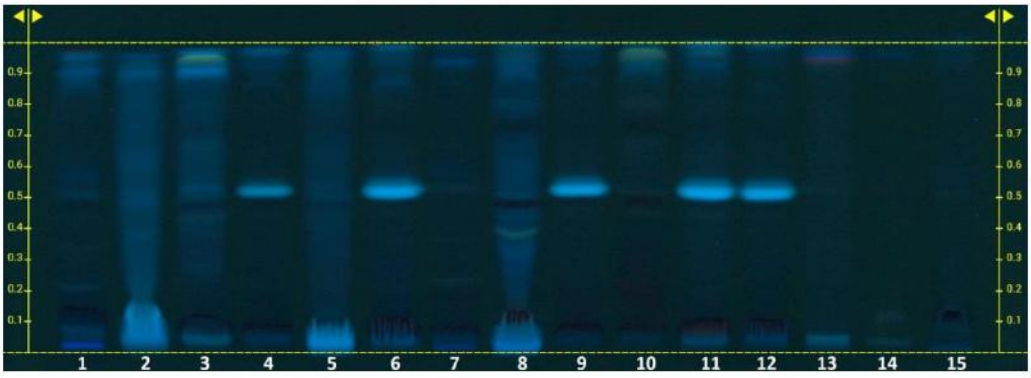
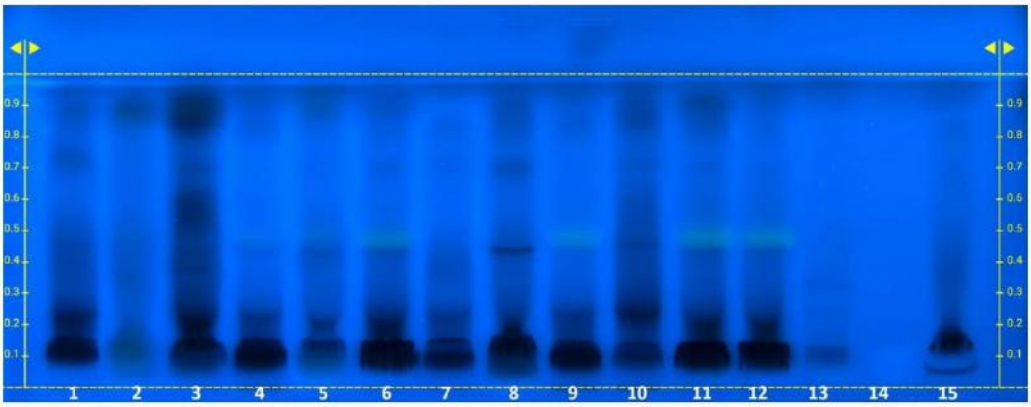
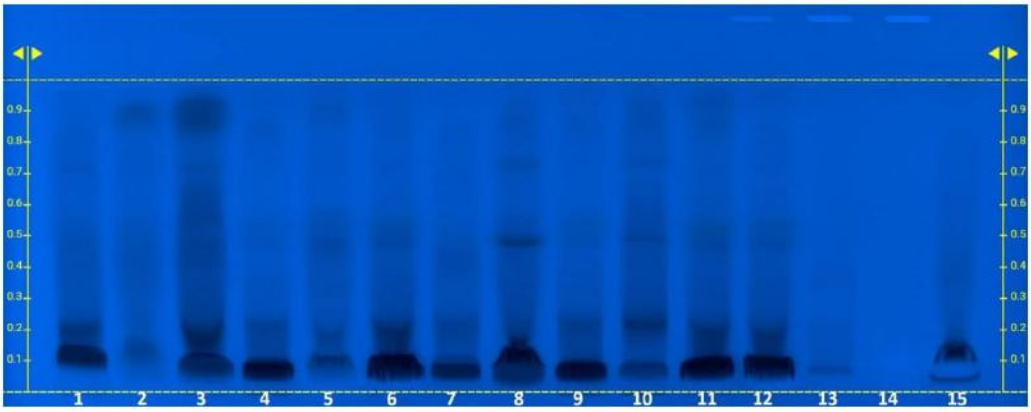


Figure S3. HPTLC–α/β-glucosidase inhibition autograms at FLD 366 nm in comparison to HPTLC–Vis/UV/FLD chromatograms of golden root extracts IDs 1–15 (400 µg/band each) separated on HPTLC plate silica gel 60 F_{254} with ethyl acetate-methanol-water-acetic acid 70:15:15:1 (*V/V/V/V*) and detected at FLD 366 nm after the respective glucosidase assay *versus* only sprayed with 4-methylumbelliferone solution. The positive controls (applied at plate top as ethanolic solution) were acarbose (3, 9, and 18 µg/band) and imidazole (2, 5, and 8 µg/band) for the α/β–glucosidase, respectively.

HPTLC–α-glucosidase inhibition autogram at FLD 366 nm



HPTLC–β-glucosidase inhibition autogram at FLD 366 nm



HPTLC–FLD 366 nm chromatogram sprayed with 4-methylumbelliferone solution

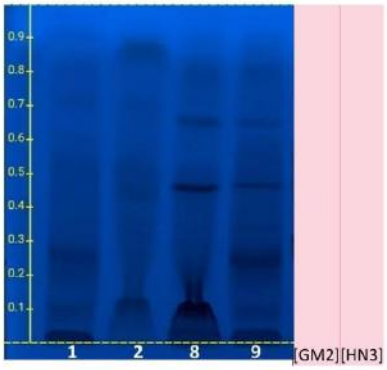


Figure S3. Continued.

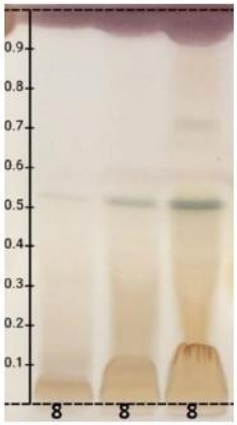


Figure S4. HPTLC–α-amylase inhibition autogram of golden root extract ID 8 (100, 200 and 400 µg/band) separated on HPTLC plate silica gel 60 F_{254} with ethyl acetate-methanol-water-acetic acid (70:15:15:1, *V/V/V/V*) and detected at white light illumination after the α-amylase inhibition assay.

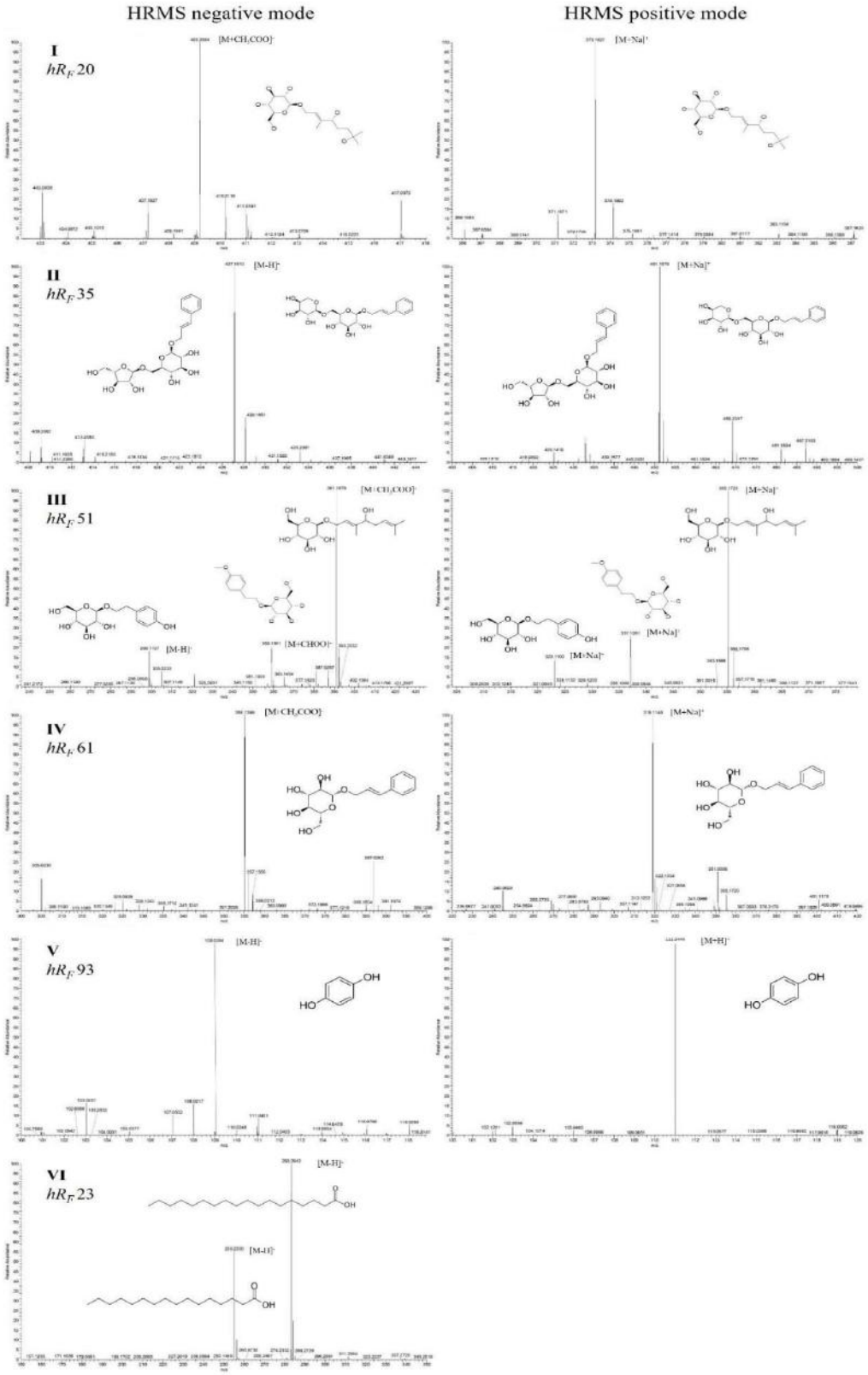


Figure S5. HPTLC–HPLC–HESI-HRMS spectra of selected bioactive zones