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PREPARATIVE ISOLATION AND PHYTOCHEMICAL ANALYSIS OF *RHAPONTICUM CARTHAMOIDES*, *RHODIOLA ROSEA* AND *WITHANIA FRUTESCENS* CONSTITUENTS

Ph.D. Thesis

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LIST OF PUBLICATIONS RELATED TO THE THESIS

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Three novel constituents from the roots of *Rhaponticum carthamoides*
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TABLE OF CONTENTS

Abbreviations

20OHe	20-Hydroxyecdysone
a.s.l.	Above sea level
CH_2Cl_2	Dichloromethane/methylene chloride
EtOAc	Ethyl acetate
EtOH	Ethanol
GIRK	G protein-coupled inward rectifier K^+ (channel)
hERG	human ether-a-go-go-related gene (channel)
H_2O	Water
MeCN	Acetonitrile
MeOH	Methanol
MPLC	Medium Pressure Liquid Chromatography
PC12	Rat pheochromocytoma cell
RPC	Rotational Planar Chromatography
TLC	Thin Layer Chromatography

1 INTRODUCTION

The definition of stress is not unambiguous, due to the complexity of the phenomenon and has changed many times in the last decades. The first records are connected to the name of *Hans Selye* (*János Selye*, 1907-1982), a Hungarian physiologist. According to his theory, based on his animal experiments, stress is a "non-specific response of the body to a demand", in other words the answer of our body to an internal or external change to recover its homeostasis. This stress response is usually connected to a negative meaning, but Selye has distinguished two different "stress types": eustress (positive) e.g., stress caused by wedding, birth, promotion etc., and distress (negative) e.g., caused by mechanical, chemical or psychological harm. The definition of stress has been reworked and expanded throughout the next decades to be able to explain the depths of human behaviour and to understand different stress-related diseases [1]–[3].

To be able to cope with the everyday stress, to try to adapt, the human organism has a huge variety of defensive mechanisms, including enzymatic pathways, antioxidants, hormones etc. According to Selye there are three stages of stress response. In stage 1 (state of alarm) the body gives an immediate, usually nonspecific response to a stressor. When the body is exposed repeatedly or for a long time to the stressor, the body develops a specific adaptation, usually combined with anabolic processes, to withstand the stressor. This is the second phase (state of resistance). If the stress-signal exceeds the limitation of the body, either being too strong or persisting too long, the organism enters the third phase (state of exhaustion). In this phase the body runs out of resources and is no more able to cope with the noxious agent, which potentially can lead to fatal organ damages [4].

The stress-response can be supported with the so-called adaptogens either by lowering the alarm-response through non-specific mechanisms, extending the duration of resistance phase or delaying the exhaustion phase. The term of adaptogen was first introduced by the Russian scientist *Nikolay Vasilievich Lazarev* in 1947 with the discovery of dibazol (2-benzyl-benzimidazol) and its adaptogenic/tonic effects through raising the non-specific response of the body against stressors [4]. Later in a study *Israel I. Berkhman* defined further three criteria for adaptogens. An adaptogenic substance must increase the non-specific resistance of the body against noxious agents; must have normalizing effect independent of pathological or physical state; furthermore should have no or just minimal physiological effect on a healthy organism, should not influence the

normal body functions. Due to the complexity of the mechanism of action there is some overlap between adaptogens and nootropics, tonics, immunostimulants and anabolic drugs [4]. It is a generally accepted view that the effect of the adaptogens is related to the increase of serum ACTH and corticosteroid levels, however the mechanisms of action are more complex [4] [5]. Considering the complexity of the adaptogenic effect it is not surprising that this pharmacologic group almost exclusively consists of medicinal plants, which can be characterized by complex chemical composition and pharmacological profile.

In the past few decades many studies have been carried out in order to find new adaptogenic plants and discover the chemical constituents responsible for the adaptogenic effect. The most common and therapeutically exploited species are *Panax ginseng* C.A. Meyer, *Bryonia alba* L., *Eleutherococcus senticosus* Maxim, *Rhodiola rosea* L., *Schiandra chinensis* (Turcz.) Bail., *Withania somnifera* L. [5]. Due to the complexity of mechanisms of action, no individual chemical compounds responsible for the adaptogenic effect can be identified, but rather chemical groups such as certain phenolic compounds (e.g. phenylpropanoids), phenylethane derivatives, lignans, which are structurally similar to catecholamines and possibly play a role in the early stress-response phase. Further compounds belong to tetracyclic triterpenes, which are similar to the corticosteroids and oxylipins (unsaturated trihydroxy or epoxy fatty acids), showing a huge resemblance to the leukotrienes and lipoxins. [5]. However, the mechanism of actions of these compounds and the full spectrum of metabolites responsible for the adaptogenic effect has not been totally elucidated.

2 AIMS OF THE STUDY

As part of my Ph.D. research we aimed to further widen the scope of view of adaptogenic plants and to serve with data to establish possible further applications of these plants. Besides the already widely studied species *Panax ginseng*, we especially focused on the species *Rhaponticum carthamoides*, *Rhodiola rosea* and *Withania frutescens*.

Our main tasks were to

- perform a comprehensive literature overview on the chemistry and pharmacology of the species *Rhaponticum carthamoides, Rhodiola rosea*. and *Withania frutescens*
- analyse and compare the chemical composition of different plant samples according to harvest time, place and plant part, to determine optimal conditions of cultivation
- optimize the extraction process of *Withania frutescens* in order to provide an economic source of withanolides.
- isolate and identify bioactive and marker compounds of the three species.
- analyse the bioactivities of different extracts of adaptogenic plants.

Our overall goal was to provide now scientific data to facilitate the utilisation of the three plants.

3 LITERATURE OVERVIEW

3.1 *RHAPONTICUM CARTHAMOIDES*

Rhaponthicum carthamoides (Willd.) Iljin, commonly referenced as Maral root, *Leuzea carthamoides* DC. or *Stemmcantha carthamoides* is a perennial herb, indigenous to the subalpine regions (1200-2300 m a.s.l.) of Altai and Saian mountains in Russia [6] [7]. Due to its widespread ethnomedicinal use as a tonic and remedy against fatigue, the plant and its chemical composition has been in the focus of numerous studies [7].

3.1.1 CHEMICAL COMPOSITION

Thanks to the studies performed in the last decades, the chemical mapping of the plant is rather widespread and numerous compounds belonging to diverse chemical groups have been discovered. The most abundant constituents of the plant are ecdysteroids, such as 20-hydroxyecdysone (20OHe, **1**) and inokosterone (**24**), which are known from the earliest studies [7]. Besides the ecdysteroids, several sterols, flavonoids and phenolics, stilbenes, lignans, tannins, serotonin phenylpropanoids, thiophenes, polyacetylenes, sesqiuterpene lactones, triterpenes, and essential oil monoterpene compounds have also been reported from the plant [6]–[36]. The list of so far isolated compounds can be found in 1. Appendix - Table 1: 1a-k.

3.1.2 PHARMACOLOGY

Because of the adaptogenic properties of the *Rhaponticum carthamoides* numerous studies were carried out with the plant, aiming to understand its pharmacology.

IN VITRO EXPERIMENTS

Antioxidant properties

The antioxidant (free radical scavenging) activity has been examined in numerous studies with various assay methods such as DPPH, FRAP, ABTS. It was found that the flavonoid-rich extracts had strong antioxidant activity. This activity was in some cases

even stronger than the activity of some of the positive standards e.g. BHT (butylated hydroxytoluene) [37]–[39].

Immunmodulatory effect

The polysaccharide-rich fraction of the plant increased the phytohemagglutin-induced proliferation of lymphocytes and decreased the free radical release of granulocytes *in vitro* suggesting an immunomodulatory activity. Although the ecdysteroids have little to none immunomodulatory effect, other substances e.g. lignans, sesquiterpene-lactones or flavonoids could be responsible for this activity [40], [41].

ANIMAL EXPERIMENTS

Effects on work capacity, anabolic and adaptogenic properties

Various animal studies have been carried out in the past few decades with the plant, its preparations and pure compounds (mostly 20OHe). In most cases the analysed products were introduced either *per os* or intraperitoneally. 20OHe rich plant extracts caused significant increase in body and organ – such as: liver, kidney, hearth, female juveniles – mass. Other studies carried out with Japanese quails and pigs have also reported increased protein synthesis and body mass. A preparation called Leveton (*R. carthamoides* root combined with bee pollen and vitamin C) administered to mice and rats, increased work capacity in swimming and treadmill trials [42]–[46].

Effects on the central nervous system

Experiments with *R. carthamoides* extract have shown beneficial effects on the nervous system such as increased locomotor activity, decreased neuronal destruction in cerebral ischemia, improved learning and memory capacity in maze tests even in scopolamine-induced memory impairment studies. The *per os* administration of the plant extract increased EEG activation and reduced the negative effects of Na-thiopental and chloralhydrate induced hypnosis [7], [47]–[49].

Effects on the cardiovascular system and blood levels

In the trials with the plant and its extracts decreased blood pressure and coagulation capability, improved rheological properties (such as increased erythrocyte deformability and decreased blood viscosity), decreased blood sugar, cholesterol IgA and IgG levels

were reported. A further study has reported significant antiplatelet activity of the plant extract [7], [26], [48], [50].

HUMAN TRIALS

Effects on work capacity, anabolic and adaptogenic properties

In human clinical studies with athletes treated with Leveton (*R. carthamoides* root combined with bee pollen and vitamin C), Ecdysten (20OHe) and Prime plus (20OHe, casein and unrefined sugar) significant muscle mass increase was observable in the treated groups compared with the control subjects. In another study with Leveton and *R. carthamoides* tinctures decreased blood coagulation and increased immunoglobulin (IgA and IgG) levels, furthermore 10-15% increase in work capacity were reported [7], [42], [51].

Effects on the central nervous system

In a study with alcoholics the decoction of *R. carthamoides* root alleviated organ-related aches and depressive episodes [7].

3.2 *RHODIOLA ROSEA*

Rhodiola rosea L. (Crassulaceae) (common names: Roseroot, Rosenroot, Golden root, Arctic root) is an amphi-Atlantic, arctic-alpine species indigenous in North Asia, Europe (Scandinavia, UK, Iceland, Greenland) and North-America [52]. Due to its beneficial medicinal properties, the plant is widely cultivated and its preparations from the roots and rhizomes are sold as adaptogen, tonic and anti-stress remedies [53], [54].

3.2.1 CHEMICAL COMPOSITION

The roots and rhizomes of *Rhodiola rosea* (L) are extensively examined, thus in the last decades a huge variety of compounds have been isolated from the plant. The most abundant chemical groups are the phenylpropanoids and phenylpropenoids and their

glycosides (which are also possibly responsible for the adaptogenic effect of the plant) such as viridoside (**4**), sachalinol A (**23**), rosin (**9**), rosavin (**8**), rosarin (**10**), p-tyrosol (**2**), and salidroside (**3**). The latter two are often used as marker molecules to standardize dietary supplement preparations of the plant and distinguish *R. rosea* from other *Rhodiola* species [53], [55]. In addition, the plant contains terpenoids, their glucosides, phenolic acids, flavonoids, sterols cyanogenic glucosides, and essential oil [54], [56]–[66]. The full list of components is in 2. Appendix - Table 2: 2a-k.

3.2.2 PHARMACOLOGY

Rhodiola rosea (L.) has a long history in European and Asian medicine. Many of the traditional indications though are not closely related with its adaptogenic properties, such as adstringent, remedy against hysteria, against head- and joint aches, wound treating agent, treatment against kidney stones, freckles and different skin problems. In traditional medicine the tonic properties of the plant such as effects to enhance mental and physical performance, resistance at high altitude, to strengthen the nerves, to alleviate pain, fatigue and depression symptoms, and to help in recovery after illnesses were also described. The roots were used among the Lapps as a stimulant during long journeys and as a strengthening remedy by Vikings [54], [55], [67]. Nowadays the preparations of the plant are part of numerous pharmacopoeia for its adaptogenic properties and are widely studied in order to find possible new constituents and application fields [54].

IN VITRO EXPERIMENTS

Effects on the central nervous system, neuroprotective and adaptogenic properties

In *in vitro* studies *R. rosea* preparations expressed beneficial effects on the central nervous system such as slowing down/inhibition of H_2O_2 induced apoptosis in rat pheochromocytoma (PC12) cell lines, and protective effect on cultured PC12 cells against hypoglycaemia and beta-peptide induced cytotoxicity. Protection of neuronal cells from sodium-azide and glutamate induced injuries was also observed. Salidroside expressed antioxidant activity in numerous experiments preventing the neuronal damage

induced with iNOS, IL-1β, TNF-α and cytokines and through stabilizing of Ca^{2+} homeostasis [54][55].

Hepatoprotective effect

The extract of *R. rosea* showed protective effects on mouse hepatic cells against D-galactosamine induced injuries [55]. In other studies hepatoprotective effect has been reported, where the extract inhibited peroxidation and oxidation in hepatic cells and against tacrine-induced hepatotoxicity in human Hep G2 cells [54].

Anti-inflammatory effect

In *in vitro* studies the extract of *R. rosea* inhibited the activity of inflammation-related enzymes such as cyclooxygenase 1 and 2 (COX-1 and 2) and phospholipase A_2 (PLA_2). [55]

Animal experiments

Effects on the central nervous system, neuroprotective and adaptogenic properties

In rat experiments the water extract of *R. rosea* increased serotonin, and decreased norepinephrine and dopamine levels in the cerebral cortex [67]. In further studies with rats reduction of cerebral oedema in global cerebral ischemia-reperfusion injury and increased cognitive functions were reported [54]. In animal experiments, small doses of *Rhodiola rosea* (100-600 mg/d) enhanced the swimming capacity in mice, although higher doses (>600 mg/d) had rather sedative effects [67]. The alcoholic extract of the plant has improved neurogenesis and inhibited oxidative damage through scavenging activity [68]. In rat experiments, salidroside raised exercise tolerance by increasing liver glycogen and reducing malonaldehyde levels. In addition the level of antioxidant enzymes such as catalase and superoxide dismutase were also elevated [55].

Hepatoprotective effect

In mice experiments, salidroside treatment dampened the increase of serum aspartate aminotransferase and alanine aminotransferase activities, increased glutathione, SOD, catalase, and glutathione peroxidase levels and decreased MDA (malondialdehyde) levels in liver tissue. In addition, reduction of the size of necrotic regions of liver was also observable [55].

Anti-inflammatory effect

Orally administered *R. rosea* tinctures decreased carrageenan-, formaldehyde-, and nystatin-induced paw oedema in rats in dose-dependent manner. Salidroside lowered D-galactosamine- and LPS-induced increases of serum nitric oxide, tumour necrosis factor-α, interleukine-1β and 6 and reduced cell infiltration in animal experiments. [55]

HUMAN TRIALS

Effects on the central nervous system, neuroprotective and adaptogenic properties

In a study carried out with patients with asthenia (fatigue, irritability, decreased work capacity) and healthy subjects, a long term treatment (10 days to 4 months) with *Rhodiola* 3 times 50 mg a day, the vegetative symptoms decreased by 64% [67]. In another study *Rhodiola rosea* tincture (10 drops equivalent to 100-150 mg *R. rosea*) was given 1-2 times a day for 2-3 weeks before intense intellectual work to healthy subjects. Improvement in work capacity was observed, and the fatigue-related decompensation after the work was decreased [67]. In a trial, burnout patients treated with SHR-5 [Swedish Herbal Rhodiola: *Rhodiola rosea* extract, drug-extract ratio 4:1, 70% EtOH, which contains approx. 2.5% rhodioloside (salidroside), 3.9% rosavin and 0.8% tyrosol] showed increased mental performance and concentration and reduced cortisol response to awakening stress and alleviated depressive symptoms [55]. In a study with biathletes, the group treated with *R. rosea* had a significant increase in shooting accuracy, better coordination and lowered hearth-rate [67].

3.3 *WITHANIA FRUTESCENS*

Withania frutescens (L.) Pauquy [syn: *Atropa frutescens*, *Hypnoticum frutescens* (L.) Rodati et Boiss. and *Physalis frutescens* (L.) DC.] is a perennial plant native to Northwest Africa (Algeria, Morocco) and Southwest Europe (Portugal, Spain) [69], [70]. Due to its similar chemical composition to *Withania somnifera* (L.) Dunal, indigenous in India, the plant might be an alternative European source of medicinally valuable withanolides [71].

3.3.1 CHEMICAL COMPOSITION

Unlike *Withania somnifera*, *W. frutescens* is not widely examined, in total only 41 articles reported chemical data on the plant. The main constituents of the species are the withanolides, strongly oxygenated C28 steroidal lactones [70]. Nortropane polyhydroxylated alkaloids (calystegines) have also been identified in solanaceous plants including *W. somnifera* and *W. frutescens*, although *W. somnifera* contained only calystegine B2 (**11**) and C1 (**13**), but in the latter plant calystegine A3 (**9**), B1 (**10**), B3 (**12**), and N1 (**14**) was also observable [72]. Numerous flavonoids and phenolic acids have also been identified. The so far isolated compounds are to be found in references [70], [72]–[75]. The full list of components with chemical structures are shown in 3. Appendix - Table 3: 3a-d.

3.3.2 PHARMACOLOGY

W. frutescens is used in traditional medicine against various diseases and conditions, e.g. dysentery and intoxication [70], [75], [76]. It is considered as a safe alternative to other adaptogens having low to none acute toxicity [76], [77]. In the last decades a few studies have been carried out to explore its medicinal effects and possible utilisation.

IN VITRO EXPERIMENTS

Antimicrobial activity

The methanolic fraction of *W. frutescens* leaf extract showed good, the ethyl acetate and butanol fractions moderate antiplasmodial activity against chloroquine-resistant *Plasmodium falciparum* [78]. In another study, the ethyl acetate and *n*-butanol fractions exhibited good antibacterial activity against both Gram-positive and negative strains [79]. A study proved moderate antimicrobial activity against *S. aureus*, *E. faecalis* and *E. cloacae* strains [80]. Antifungal activity against *Penicillium digitatum* was also reported [81].

Antioxidant activity

Several studies have reported moderate to high activity of the plant extracts in various antioxidant assays such as Folin-Ciocalteu, DPPH, FRAP, CAT and β-carotene discoloration methods [79], [81] [82].

Cytotoxic activity

In an activity-controlled isolation experiment the plant extract prepared with methylene chloride exhibited high cytotoxic activity against cancer cell lines HepG2 and HT29 in 3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide (MTT) assay [70].

ANIMAL EXPERIMENTS

In rat experiments, *W. frutescens* extract expressed anti-inflammatory and analgesic activities, furthermore promoted wound healing [75]. A toxicology study reported neither toxic histological or biochemical effects nor symptoms in extreme high (up to 2000 mg/kg) doses in mice [77]. In another study, *W. frutescens* exerted hepatoprotective activity (both preventive and curative) against CCl_4-induced hepatotoxicity in rats [83].

3.4 BIOLOGICAL ASSAYS

3.4.1 ROTIFER ASSAY

In search for biologically active substances pharmaceutical toxicological *in vivo* assays are inevitable. Such *in vivo* experiments can be based on using small model organisms, or small animals e.g. mice, rats or rabbits. Ideally, an experiment with small model organisms should be non-invasive, sensitive, inexpensive and easy to maintain. Such model is the nematode *Caenorhabditis elegans* (CE), however, its major drawback is that it has no homology with mammal genome. [84] Rotifers (phylum *Rotifera*) have the advantage of CE in terms of culturing, physiology and anatomy, and in addition have an approximately 10% similarity to vertebrae genome [85]. Rotifers are inexpensive to maintain, have short lifespan and have multicellular structure with individual nervous system and organs such as ciliated head structure with defined jaw-like mastax and

photosensitive and tactile organs, differentiated ovaries, muscles, digestive and secretory systems [86]. On the ground of the previous described attributes, rotifers have several values that can be measured, such as toxicity and survival lifespan (TSL), bright-light disturbance (BLD), mastax contraction frequency (MFC), body size index (BSI) and cellular reduction capacity (CRC) [86]. Due to these characteristics, rotifers are an excellent model for toxicologic and lifespan evaluations.

3.4.2 GIRK CANNEL INHIBITORY ASSAY

Ion channels are pore-forming proteins belonging to a large and diverse family of transmembrane structures. They conduct a rapid transport of ions (e.g. Na^+, K^+, Ca^{2+}, Cl^-) through cell membranes [87]. These channels are divided into two larger groups: either voltage-gated (activated through plasma membrane potential and usually specific to various cations or anions), or ligand-gated (activated by ligands). Ion channels have an important role in various physiological processes in the central nervous and cardiovascular system and muscles. Their dysfunction can lead to severe pathological conditions, such as cardiac arrhythmia, hypertension, multiple sclerosis, epilepsy, migraine, depression, and schizophrenia. Since these ion channels are „highly druggable" (their function can easily influenced by drugs) the previously mentioned ion channel dysfunctions theoretically are optimal targets for pharmaceutical research [88]. The G protein-coupled inward rectifier K^+ (GIRK) channels are a type of ligand-gated channels. They are mainly expressed in the brain, skeletal muscle, endocrine tissues and hearth. Their activation triggers a flow of K^+ out of the cells causing the membrane potential be more negative, which decreases spontaneous action potential formation. In the hearth tissue this means anti-pacemaker activity. Thus inhibition to these channels causes anti-arrhythmic effect. However the pharmacology behind these channels is largely unexplored, they are important targets also in the treatment of neuropathic pain and cardiac arrhythmia [89]. In the field of drug-safety research human *ether-a-go-go-related gene* (hERG) coded ion channels located in hearth tissues have an important role. The inhibition to these channels has the opposite action of GIRK channels and can lead fatal ventricular arrhythmia [88].

4 MATERIALS AND METHODS

4.1 PLANT MATERIALS AND ANALYTICAL STANDARDS

For the experiments ginsenoside Rb1 was purchased from HWI Analytik Gmbh (Tübingen, Germany), withanolide A, withanolide B, and withaferin A were purchased from Phytolab (Vestenbergsgreuth, Germany), rosin, rosavin, salidroside, tyrosol, and cinnamyl alcohol were purchased from Sigma Aldrich (Düsseldorf, Germany), rhodiosin and herbacetin were purchased from Carbosynth (Compton-Burkshire, UK), and 20-hydroxyecdysone and ajugasterone were isolated previously in our Institute. The structures and purities of the isolated materials have been verified via NMR and MS analyses.

Rhaponticum carthamoides roots ("Roots of Leuzea carthamoides [Lujza]" 2,0 kg) were purchased from Herbosus Finnland Espoo. The plant material was identified by dr. Zsuzsanna Hajdú (University of Szeged, Institute of Pharmacognosy).

For the experiments with *Rhodiola rosea*, in total 28 root and rhizome samples were collected. Sample set A (p01-p20): seeds from 17 wild provenances of *R. rosea* originated from Northwestern European Islands (NW, n=4), Northeastern Europe (NE, n=3), Alps/Pyrenees (ALP/PYR, n=6) and Southern Siberia (ALTAI, n=4). They were completed by one example of another *Rhodiola* species from habitats in the Eastern Alps that could not be identified (p18) and two rhizome/roots of wild alpine plants of unknown age. Voucher specimens have been sent to the herbarium of the Biocenter Linz (Austria). Sample set B (R1–R10 (samples R6 and R9 were excluded from trial), for harvest season experiment: plants from 8 wild provenances have been previously identified and described [53]. The samples are listed in 4. Appendix: *Rhodiola rosea* samples.

For the GIRK channel inhibitory assay extracts of various parts of *W. frutescens*, e.g. leaves, rhizomes and roots, twigs and fruits were used. The list is completed with *R. rosea* extract and Withaferine A isolated by our group. The *W. frutescens* samples were collected and identified by Wieland Peschel. In total 66 samples were utilised, which are listed in 5. Appendix: samples for GIRK channel inhibitory assay.

For the trial to evaluate two possibly new markers from the plant, additional six samples were purchased in addition to the samples mentioned in the third paragraph of this section. Four dried drugs (1: 'Rhizomata et radices Rhodiolae roseae'; 2: unlabelled drug

from a market stand; 3: ‘*Rhodiola rosea* (rhizoma)’; and 4: ‘Rhodiolae radix concissus’) and two products (1: *Rhodiola rosea* extract) and 2: ‘Arctic root – *Rhodiola rosea*’, powder in capsules, originating from UK/sold in Szeged, Hungary) [90].

Withania frutescens (L.) Pauquy samples (6 leaves, 4 roots, and 8 twigs) were collected from 3-year-old cultivated plants from Reading, United Kingdom and also wild specimens from Spain for the evaluation of the optimal extraction process of the main constituents. The *Rhodiola rosea* and *Withania* sp. plant materials were identified by dr. Wieland Peschel.

Due to its well characterised properties *Panax ginseng* and its macerate were used as a comprehensive standards for the analyses. The pharmacopoeia grade plant material was purchased from a local pharmacy (Szeged, Hungary).

4.2 PHYTOCHEMICAL ANALYSIS OF *RHAPONTICUM CARTHAMOIDES*

The whole flowchart of the extraction process of the plant is to be found in 6. Appendix - Figure A1.

The dry roots (1.75 kg) were ground with a Retsch cutting mill SM 100 to ≥2mm particle size, then was extracted five times with 2000 mL MeOH each cycle, using an ultrasonic bath at room temperature. The extract then was filtered and evaporated to dryness (Büchi rotavapor) to obtain 112.52 grams of dry extract (Leuzea stock extract).

The dry residue was redissolved in 1000 mL of MeOH – H_2O (1:1), then extracted with *n*-hexane, EtOAc and CH_2Cl_2, resulting in dry fractions 9.91 g, 28.79 g and 103.37 g, respectively. The TLC analysis of the main fractions showed a remarkable amount of ecdysteroids in the ethyl acetate fraction, thus further experiments have focused on this extract. For the next step of the isolation process, atmospheric column chromatography was used. A stationary phase SiO_2 (Merck, 0.045–0.063 mm, 300 g) was utilised, with a gradient elution using CH_2Cl_2 – MeOH (95:5–0:100). The resulting fractions were combined after TLC analysis to subfractions **A-T**.

Purification of ethyl acetate-subfraction **E** by RPC on 4 mm SiO_2 plates *n*-hexane – EtOAc (100:0–0:100), finished with a MeOH wash, (8 mL/min) led to subfractions **E I-**

XXI. **EX** was further purified by HPLC on a Phenomenex Kinetex C18 column (250×21.1 mm, 100 Å, 5 μm) eluting with H_2O – MeOH (0 min: 40:60, 15 min: 0:100, 16 min: 0:100, 20 min: 40:60), resulting subfractions "**Leuzea EtOAcEX a-e**". "**c**" was further purified with an atmospheric pressure column with a stationary phase SiO_2, (25.0g), as eluent CH_2Cl_2 – EtOAc (100:0–0:100 in 10% increments 20mL each) was used. As result two pure compounds, Leuzea **EtOAcEXc γ** and **β** were collected, experimentally named **LEUr 14** and **LEUr 32** respectively**.** The subfraction **EXX** was further purified with help of RPC (1 mm SiO_2 CH_2Cl_2 – EtOH (100:0–0:100, 3.0 mL/min)). In this separation step, subfractions **Leuzea EtOAcEXX a-h** were gained. Latter was found to be a pure compound named **LEUr 10**. The further preparative TLC separations of subfractions **c**, **d**, **e** and **f** with EtOAc – EtOH – H2O = 4:0.5:0.25 on silica plate yielded the subfractions **Leuzea EtOAcEXXc α**, **Leuzea EtOAcEXXd α-γ**, **Leuzea EtOAcEXXe α-δ**, **Leuzea EtOAcEXXf α-γ** respectively. According to preliminary NMR data the subfractions **cα**, **dγ**, **eδ** and **fγ** were merged into fraction **Leuzea EtOAcEXX Σ**, which was named **LEUr 8**.

Subfraction **L** was further fractionated by MPLC on SiO_2 (0.043-0.060 mm) using a CH_2Cl_2 – EtOAc gradient (100:0–0:100) for elution, followed by EtOAc – MeOH wash (100:0–90:10). After TLC analysis the gained fractions were merged to five subfractions named **Leuzea EtOAcL I-V**.

The subfraction **LII** was further processed with help of an atmospheric column filled with SiO2 (0.043–0.060 mm). Gradient elution EtOAc – MeOH (100:0–0:100) was used. The collected fractions, after TLC check were merged into subfractions **L II a-d**. From the subfraction **b** and **c** with help of HPLC, comprising LiChrospher RP-C18e column (250×4.6mm, 5 μm) and gradient eluent composition of H_2O – MeOH (90:10–50:50) in fifteen minutes, the subfractions **Leuzea EtOAcLIIb α-ζ** and **Leuzea EtOAcLIIc α-β** were collected respectively. According to the HPLC data, two pure compounds (**bα = LEUr 27 and cα = LEUr 29**) were isolated. . The NMR analysis confirmed the two compounds (**LEUr 27 and LEUr 29**) to be identical, thus were merged to one fraction (**LEUr 48**).

The subfraction **LIV** was further processed by MPLC. Stationary phase SiO2 (0.043–0.060 mm) was used. The gradient elution consisted of EtOAc – MeOH (0 min: 100:0, 60 min: 90:10, 70 min: 90:10) and a washing phase of EtOAc – MeOH (0:100). As result

the collected minor fractions were merged into main fractions **LIV a-d**. The fraction **LIV a** was further separated by RPC method, using a 1 mm thick SiO_2 plate and gradient elution of EtOAc – EtOH (100:0–80:20). The resulting 51 fractions were merged into main fractions **LIV a α- ι**. Subfraction **LIV a δ** was processed by HPLC (LiChrospher RP-C18e, 250×4.6 mm, 5 μm column, elution H_2O – MeOH (55:45–15:85) in ten minutes). As a result, two pure compounds, **LEUr 15** and **LEUr 16** were isolated.

Subfraction **LIV a ε** and **ζ** were purified by an HPLC using Lichrospher RP-C18e, 250×4.6 mm, 5u column elution H_2O – MeOH (99:1 changing to 20:80 in 15 min). As result three pure compounds – experimental ID of **LEUr 17**, **LEUr 19**, and **LEUr 25** – were isolated.

The subfractions of **LIV d** was further separated on an atmospheric pressure column filled with SiO_2 sorbent (0.043–0.060 mm). The gradient elution EtOAc – MeOH (100:0–0:100), resulting fractions **LIV d α** to **δ**. The subfraction **LIV d β** was purified by an HPLC method using a LiChrospher RP-C18e, 250×4.6 mm, 5 μm column (elution with H_2O – MeOH (95:5–70:30 in 20 min), as a result of which a pure compound, **LEUr 24** was collected.

The main fraction **F** was processed by MPLC (SiO_2 (0.043–0.060 mm), eluent: hexane –methylene chloride – MeOH (100:0:0–0:100:0–0:50:50), with a flow rate of 15 mL/min. The resulting fractions were **F I-IX**. The fraction **FVIII** was further purified using an MPLC. As stationary phase SiO_2 (0.043–0.060 mm) was used with gradient elution of CH_2Cl_2 – MeOH – H_2O (100:0:0–100:0:0 to 0:50:50) with 5% increments, with a flow rate of 25 mL/min. The resulting 50 subfractions were merged to six main fractions labelled as **F VIII a-f. F VIII c** was further processed using Sephadex LH20 by using chloroform – MeOH (1:1). Fractions **F VIII c α-ι** were collected.

Subfractions **F VIII c γ** and **δ** were purified using an HPLC method. As column a Kinetex Phenomenex C18, 150×4.6 mm, 5 μm was used. In the case of fraction **γ,** the gradient elution comprised H_2O – MeCN (95:5–0:100) changing in 15 minutes. In the case of **δ** elution of H_2O – MeCN (85:15–0:100) in 15 min was used. As result two pure compounds, **LEUr 45** and **LEUr 46** were obtained.

The isolates of *Rhaponticum carthamoides* were analysed via NMR. NMR spectra were recorded in MeOH-d4 or $CHCl_3$-d4 on a Bruker Avance 600 III spectrometer (^{1}H: 600.13 MHz; ^{13}C: 150.9 MHz) equipped with a 5mm cryo-TXI probe. The peaks of the residual

solvent (dH 3.31; dC 49.00) were taken as reference points. Chemical shifts are expressed in parts per million, and coupling constants (*J*) values are reported in Hz. Data were acquired and processed with the MestReNova v6.0.2-5475 software and were compared to literature data to identify the compounds.

4.3 PHYTOCHEMICAL ANALYSIS OF *RHODIOLA ROSEA*

4.3.1 COMPREHENSIVE STUDY OF *RHODIOLA ROSEA* SAMPLES OF DIFFERENT ORIGIN

The samples described in section (4.1) were ground with Grindomix GM200 (Germany) and sieved. The medium fraction (0.15-0.80 mm) was collected and 5.00 grams of samples were processed according to the method *Peschel et al*. (2016). [53] The samples were extracted with 25.0 mL 70% EtOH (HPLC grade, Molar chemicals, Hungary) to produce a tincture characterised by a drug-extract ratio of 1:5. After 2 hours shaking the samples were soaked for 5 days then shaked for another 30 min. After centrifugation (4500 RPM, 5 min, Rotanta, Hettich) 1.6 mL of the samples were filtered through 0.46 µm syringe filters into HPLC vials and stored at 4°C [53]. The remaining solutions were unified, lyophilized (Hetosicc CD52) and stored in refrigerator at -10°C to maintain their stability. From the unified batch the „Rhodiola stock extract" was used for rotifer analysis.

The HPLC analysis of the samples is based on the method of *Ganzera et al*. [91] The analysis was performed according to *Peschel et al*. (2016) [53] as follows: HPLC analysis was carried out on a Waters® Alliance 2690 HPLC system using Waters® 996 PDA detector. A Phenomenex Luna C18 column (150×4.6 mm, 5µm, 100 Å) was used as a stationary phase, tempered at 40°C. The mobile phase consisted of 25 mM phosphate buffer in aqueous phase adjusted to pH = 7.00 – MeCN (95:5–80:20) changing in 30 min, followed by a 5 min washing phase consisting 0.1% phosphoric acid in MeOH and equilibration with the starting eluent for 10 minutes. The flow rate was set to 1.0 mL/min. Detection was at 275 nm wavelength. For each injection 10 µL sample was injected. Peaks were assigned by spiking the samples with standard compounds and comparison of the UV spectra and retention times.

For the determination of rosavin, cinnamyl alcohol, salidroside and tyrosol external standards were used. Rosavin, rosarin and rosin – peaks equally calculated as rosavin – are summarised to total rosavins (=ROS_{tot}). As a relative parameter we calculated the ratio between ROS_{tot} and CA. As 'total salidroside' (SAL_{tot}= salidroside + aglycon (tyrosol)) the peaks of salidroside and tyrosol were summarised. As relative parameters we calculated ratio between 'total phenylpropenoids' (ROS_{tot} +aglycon CA) and 'total salidroside' (salidroside + aglycon tyrosol) which is abbreviated as PP $_{tot}$/SAL_{tot} (with SAL_{tot} set as 1) [92].

INFLUENCE OF PLANT PART AND PROVENANCE

Three plants each per provenance (Sample set A: see section 4.1) were harvested mid-May of year 9. For comparison between rhizome and root as regards ROS_{tot}, CA, SAL_{tot}, derived ratio markers, and yield parameters we analysed data in 3 ways: (A-I) the average of the whole sample matrix of cultivated authentic R. rosea from 17 provenances (descriptive statistics only), (A-II) means (n = 3, +−s.e.m.) of individual provenances (n= 20) and (A-III) means of the five predefined provenance groups NW-Eur, Alp/Pyr, NE-Eur, Altai, wild Alp [92].

INFLUENCE OF HARVEST SEASON

From sample set B three plants each from 8 provenances were harvested in intervals of 6–7 weeks as follows: mid-May (beginning of the vegetative season directly after flowering), first part of July, mid-August (during fructification and beginning of withering of first-generation stems) and beginning of October (end of vegetative season) in year 6. In the following year, harvest intervals were 8–9 weeks (mid-May, end July, end September). Samples were processed as described in Section 4.3.1. The content of ROS_{tot} and CA (expressed in µg/mL macerate) was determined from rhizome samples using HPLC method described above. For the influence of harvest season we compared: (B-I) harvest date means (±s.e.m.) and range (minimum, maximum) across all provenances within each year with (two-way ANOVA) and without (one-way ANOVA) consideration of provenance differences and (B-II) provenance differences as regards composition (ROS_{tot}, CA, ROS_{tot}-CA ratio) as per harvest date (n = 3, one-way ANOVA) and across all harvest seasons in cultivation year 6 and 7 (n= 3× 7, two-way ANOVA) [92].

STATISTICAL ANALYSIS

N values for ROS_{tot}, CA and SAL_{tot} were calculated as means of HPLC measurement carried out in duplicate. Mean ± s.e.m. of ROS_{tot}, CA, SAL_{tot} as well as derived sum and ratio parameters were calculated for all plants per harvest date (A: n = 60, B: n = 24), per year (B: n = 96 and 72) or over all harvest dates (B: n =168). For provenance comparison means ± s.e.m. of 3 individual plants per genotype and means ± s.e.m of 21 samples per genotype (each from all 7 harvest dates) are used. Significance - indicated as * ($p < 0.05$) or by using different letters - was tested via one-way, two way or three-way ANOVA plus Tuckey's post-test using R-3.2.1 software [92].

4.3.2 ADDITIONAL MARKERS FOR QUALITY CONTROL

ISOLATION OF TWO NEW MARKERS

From the batched lyophilised *Rhodiola* stock extract two additional markers were isolated. The system consisted of a Waters® W600 pump, a W600 controller and a Waters® 2487 dual channel UV detector, controlled by Empower software. As a stationary phase Kinetex XB-C18 column (250×4.6 mm, 5µm, 100 Å) was used. Elution was carried out with H_2O – MeCN (0 min: 83:17 10 min: 72:28 15 min: 50:50) followed by MeOH washing for 5 min and additional 5 min equilibration with H_2O – MeCN (83:17). Two main peaks were detected at 12 and 13 min with UV spectra characteristic to flavonoids. In total forty injections (20 µL each) were carried out and the two peaks were separately collected. The purity of these two fractions (compound 1 and 2) was confirmed by HPLC (comparing with marketed standards described in capital 4.1) and the identity of the evaporated compounds was elucidated by NMR. NMR spectra were recorded in MeOH-d4 on a Bruker Avance 600 III spectrometer (^{1}H: 600.13 MHz; ^{13}C: 150.9 MHz) equipped with a 5mm cryo-TXI probe. As reference points the peaks of the residual solvent (dH 3.31; dC 49.00) were taken. Data were acquired with the MestReNova v6.0.2-5475 software. Chemical shifts are expressed in parts per million, and coupling constants (*J*) values are reported in Hz [90].

ANALYSIS OF THE TWO NEW ANALYTICAL COMPOUNDS

The analytical solutions from the 28 samples and additional six marketed samples (Section 4.1) were made according to the method described in capital 4.3.1.

Rhodiosin (compound 1) and herbacetin (compound 2) were determined from the marketed plant samples using external standard calibration via HPLC equipment conditions and assay (with an extension of running time and detection to 37 min) previously described in section 4.3.1. Peaks showed similar UV/VIS characteristics (compound 1: t_R: ~35.5 min k_{max} 274, 328, 380 nm; compound 2: t_R: ~36.2 min; UV k_{max} 274, 328, 380 nm). For the analysis - the most suitable - 254 nm was chosen. Concentrations of rhodiosin and herbacetin were determined in duplicate from three samples each and expressed in µg/mL macerate. For the evaluation of LOD and LOQ Shimadzu© LabSolutions (version 5.82) software was utilized. Precision was checked at a medium (100 µg/mL) standard concentration on three different days (inter-assay precision) and the same day (intra-assay precision). By spiking of a flavonoid-low extract with 50%, 100% and 150% of the native amounts of rhodiosin and herbacetin, accuracy was checked. Besides the absolute values of rhodiosin and herbacetin (µg/mL), the sum of both flavonoids ($FLAV_{tot}$) and the ratio of flavonoids to phenylpropenoid, as well as the ratio of flavonoids to phenylethanoid compounds (which are usually used for the standardization of *R. rosea*) were also determined. Corresponding data for rosavins expressed as total amount of rosavin, rosarin and rosirin (ROS_{tot}) and their aglycon trans-CA expressed as total phenylpropenoids (PP_{tot} = total rosavins + cinnamyl alcohol), as well as for salidroside and its aglycon (SAL_{tot}, sum of salidroside and tyrosol) were reported previously [53][90][92].

INFLUENCE OF EXTRACTING SOLVENT POLARITY

To determine the effect of the solvent polarity on the extract composition five different extracts were prepared (three parallel each) using aqueous EtOH 30%, 50%, 70% and 90% v/v, respectively for three drug samples of the same provenance (rhizome of a 4-year-old plant, UK cultivation; rhizome and root of a 6-year-old plant, Austrian cultivation). The flavonoid content was expressed in µg/mL. The exhaustiveness of extraction process was checked by repeated extraction (three times). The samples described above were extracted thrice with four different extraction solvents (30% EtOH, 50% EtOH, 70% EtOH, 90% EtOH). After the first extraction (M1) the samples were macerated an additional three times (M2–4) with fresh solvent. Rhodiosin and herbacetin contents (mean ± SD of measurement in duplicate of three samples each) of all four repetitions were compared.

INFLUENCE OF PLANT PART

To characterise plant part differences, three authentic provenances (RR-I, RR-II, RR-III) and a previously identified non-authentic provenance (hybrid R-IV), three 5-year-old plants were split into herb, rhizome and root, dried at 45°C, ground to diameter 0.8–1.5 mm, and then 5 g of each sample was extracted for five days with 25.0 mL 70% EtOH. Contents of rhodiosin and herbacetin were determined and expressed in µg/mL macerate with the method described above (mean ± SD, N=3). [90]

INFLUENCE OF THE DRYING PROCEDURE

To analyse the effect of drying temperature, two 5-year-old plants from five randomly chosen provenances (I–V, 7-year-old plants, cultivated in Austria, harvested in July) were chosen. The samples were split into two groups, rhizome and root. Each of the samples were cut into pieces of 2–6 cm and were halved; one half was dried for 5 days at 45°C and the other one at 65°C also for 5 days using warm air ventilation, thus yielding four root and four rhizome samples at each temperature per provenance. Dry samples were ground and extracted as described in section 4.3.1. Contents of rhodiosin and herbacetin were determined and expressed in µg/mL macerate.

Additionally, to assess the effect of drying duration, two 4-year-old plants from two randomly chosen provenances (VI–VII, 6-year-old plants, Austrian cultivation, harvested in October) were also split into rhizome and root groups. The samples were halved; one half was cut into smaller pieces (1–4 cm) and the other half was cut into bigger chunks (3–8 cm, maximum 1.5 cm thick). The samples were split again into two groups, yielding four root and four rhizome sample groups for both sizes per provenance. The fine-cut samples were dried for ten days, the coarse ones for thirty days, both at moderate air ventilation at 20°C. The dry samples then were ground and extracted as described in section 4.3.1. The content of rhodiosin and herbacetin was determined and expressed in µg/mL macerate (means ± SD, N=4) [90].

INFLUENCE OF DRUG ORIGIN

The four marketed herbal drug samples (drug I–IV), two products (prod I, II) and samples of different provenances (root and rhizome samples from 9-year-old cultivated plants of 18 provenances p01–p18 and two wild alpine plants of unknown age p19-20,

described in section 4.1) were used to assess the influence of drug origin. The samples were extracted as described above in section 4.3.1. In the case of the commercial products, the content of the capsules was directly used for extraction regardless of any other possible ingredients contained. The samples were made in duplicate. We grouped and analysed data as follows: (a) the average of the whole sample matrix of cultivated authentic *R. rosea* from 17 provenances, (b) means (N=3, ± S.E.M.) of all provenances (N=20) and (c) means of five provenance groups: Northwestern European Islands (NW; N=4), Northeastern Europe (NE, N=3), Alps/Pyrenees (ALP/PYR; N=6), Southern Siberia (ALTAI, N=4), plus the two provenances from the Eastern Alps (wild Alp). The experimental design, sampling and analysis have been described previously (*Peschel et al*. 2018) [90], [92].

STATISTICAL ANALYSIS

All samples were prepared in triplicate. The results were calculated as mean ± SD for each treatment/variable factor. The calculation of provenance comparison was carried out I: for all individual plants (total of 51 samples), II: for three individual plants of each genotype (total of 17 samples), III: five geographical groups with divergent N. For geographical region groups, significance was tested for each compound parameter using two-way ANOVA with or without Tukey's post-test (R-3.2.1 software) and results are indicated with different letters for those groups with $p<0.05$ [90].

4.4 PHYTOCHEMICAL ANALYSIS OF *WITHANIA FRUTESCENS*

To determine the optimal extraction solvent, the leaf sample named "Withania A" was used. 1.00 - 1.00 g ground sample was extracted with 10.0 mL of solvent/solvent systems listed in [**Table 1**] in tempered circumstances of 25°C for 10 minutes using an ultrasonic bath. The extracts were evaporated to dryness with the help of a Büchi Rotavapor system (300 mBar, 40 °C). The dry extracts were dissolved in MeOH (HPLC grade) and filtered through 0.45 μm PTFE syringe membrane filter, then analysed with HPLC system comprising of Kinetex 150×4.6 mm, 5μ, RP C-18 column on a Waters 600 HPLC system equipped with DAD detector. Gradient elution was carried out with H_2O – MeOH – EtOH (0 min: 65:17.5:17.5, 25 min: 55:22.2:22.2, 40 min: 0:50:50 ratio; flow rate: 1

mL/min, detection 230 nm). The withaferin A peak was detected at 10 minutes [93]. The results are shown in Section 5.3.

Table 1. Extraction solvent optimization steps

Extraction solvent		Ratio
1	CH_2Cl_2	100
2	CH_2Cl_2 – MeOH	75:25
3	CH_2Cl_2 – MeOH	50:50
4	CH_2Cl_2 – MeOH	25:75
5	MeOH	100
6	MeOH – H_2O	75:25
7	MeOH – H_2O	50:50
8	MeOH – H_2O	25:75
9	EtOH	100
10	CH_2Cl_2 – EtOH	25:75
11	EtOH – H_2O	75:25

After evaluating the optimal extraction solvent (which was MeOH – H_2O (1:1)), 1.00-1.00 grams of the plant materials (8 twigs, 6 leaves and 4 roots) were extracted and analysed with the method described above. The dry masses of extracts were also measured. The results of the comprehensive analysis of *Withania* plant parts can be found in section 5.3. Additional „Withania E" leaf sample was also macerated to gain *Withania* stock solution for the rotifer viability assay.

According to the preliminary analyses the crude extracts have contained a remarkable amount of withaferin glycosides – especially the root samples –, thus our goal was to establish an optimal and gentle hydrolysis method to further improve the pure withaferin A yield. During the preliminary hydrolysis experiments (results are not shown) 0.5 g of *Withania* leaf extracts (prepared with MeOH – H_2O (1:1)) were hydrolysed for 30 min at 100°C with 20 mL of 10% phosphoric acid, acetic acid, sulphuric acid, and hydrochloric acid. The results of hydrolyses were monitored by TLC (normal phase, CH_2Cl_2 – cyclohexane – MeOH (3.7:1:0.3), detection by spraying with vanillin–sulphuric acid followed by heating at 120 °C for 1 min)

After the preliminary hydrolysis tests a more extensive hydrolysis trial was carried out with the two most effective acids, sulphuric acid and acetic acid. For the trial 20-20 mL of sulphuric acid (1, 2.5, 5.0, 7.5, and 10%) and acetic acid (25, 50, and 100%) were used on a 100°C water bath. Sampling was carried out at 30, 60, and 90 min and 4, 5 and 6 min in the case of sulphuric acid and acetic acid respectively. The samples were analysed with help of HPLC method described above. The results are shown in section 5.3[93].

4.5 ROTIFER ASSAY

The biological assay on bdelloid rotifers was conducted at the Department of Psychiatry, Faculty of Medicine, University of Szeged. Due to their well characterized multiorgan characteristics, rotifers have been widely used as models of aging in *in vivo* toxicological and lifespan models. The effects on rotifer viability of extracts and characteristic active markers of *Panax ginseng*, *Withania frutescens*, *Rhaponticum carthamoides*, and *Rhodiola rosea* were tested *in vivo*. The methods of the animal experiments were previously published in [94].

HPLC ANALYSIS OF THE CRUDE EXTRACTS

The *Panax ginseng* sample was ground with help of Grindomix GM200 (Germany) grinder, then 5.0 g sample vas extracted with 25 mL 50% EtOH with an ultrasonic bath at room temperature for 10 minutes. The extract then was filtered and evaporated to dryness under nitrogen flow, gaining "Ginseng stock extract".

Solutions of redissolved *Rhaponticum carthamoides* (Willd.) Iljin, *Rhodiola rosea* L. and *Withania frutescens* (L.) Pauquy, and *Panax ginseng* C.A. Mey. stock extracts described in above chapters (25 mg/mL), were filtered through 0.45 µm PTFE syringe filters and characterized by HPLC-DAD. HPLC analysis was carried out using a Shimadzu HPLC system utilising a CBM-20A control module connected with LC-20AD pump, DGU-20A5R degasser, SIL20ACH autosampler (tempered to 21°C), CTO-20AC column oven (temperature set to 25°C), and SPD-M20A photodiode array detector modules. For quantification of *W. frutescens*, *P. ginseng*, and *R. carthamoides* the eluent system consisted of 0.1% H_3PO_4 – MeCN. In the case of *W. frutescens* a gradient elution was used with 0.1% H_3PO_4 – MeCN (0 min: 30:70 10 min: 50:50), with a flow rate of 1.8 mL/min. For the evaluation of *P. ginseng*, additional to the method described above

a washing phase of 0.1% H_3PO_4 – MeCN (90:10) was applied for 2 minutes. The flow rate was 1.7 mL/min. For the measurements a Phenomenex Kinetex C18, 150×4.6 mm, 100 Å, 5 μm column, was used. In case of of *R. carthamoides* the elution was as follows 0.1% H_3PO_4 – MeCN (0-1.5 min: 82.5:17.5 6.5 min:77:23), followed by a washing phase with 0:100 for 2 minutes, with a flow rate of 1.5 mL/min. The column used for the analysis was a Phenomenex Kinetex C18, 250×4.6 mm, 100 Å, 5 μm. For the quantification of *R. rosea* as stationary phase a Phenomenex Luna C18, 150×4.6 mm, 100 Å, 5 μm column, was used. The solvent system consisted of 0.01% TFA – MeCN. The flow rate was set to 1.8 mL/min. The gradient started from 0 min. 91:9 6 min. 83:17 8 min. 50:50, followed by a washing phase of 50:50 for 2 minutes. Calibration solution series (5 concentrations each) were made from biologically active markers of the plants. [94]

VIABILITY ASSAY

The culturing, harvesting, and monitoring methods of *Philodina acuticornis* (PA; bdelloid rotifer) have been reported in detail the publication of *Z. Olah et al.* (2017) [86]. According to the current ethical regulations no specific ethical permission was needed to these experiments since they were performed on microinvertebrates. The investigations were carried out in accordance with globally accepted norms: Animals (Scientific Procedures) Act, 1986, associated guidelines, EU Directive 2010/63/EU for animal experiments, and the National Institutes of Health Guide for the Care and Use of Laboratory Animals, 1978. The animal studies also comply with the ARRIVE guidelines. The animals were cultured in a supervised and semisterile environment in standard medium (SM). After a culturing phase (25°C, in standard medium, 12:12 hours of light/dark cycle) the selected rotifers (n=16/well on a 384-well plate, length 220 ± 10 μm and width 60 ± 5 μm) were treated with crude extracts and pure compounds in a final concentration of 100 μM in a 0.1 % DMSO solution. After a 72-hour period without feeding, on the fourth day the monitoring process began in a restricted caloric state, feeding the rotifers with homogenized yeast solution, 50 μg/mL, which is enough for survive but ceases the reproduction. In the monitoring phase morphological properties such as body size index (BSI) and mastax contraction frequency (MCF) were measured. Furthermore, with the help of toxicity and lifespan assay (TSL) the toxicity of the samples was measured. Statistical evaluation was performed with GraphPad

Prism 7.0b software, using one-way ANOVA with post hoc Bonferroni test. Different levels of significance were indicated as follows: $p** \leq 0.01$, $p***\leq 0.001$, and $p**** \leq 0.0001$ [94].

4.6 ASSAY ON GIRK CHANNEL INHIBITION

The GIRK channel inhibition assay was conducted in Rhytmion Ltd, Szeged, Hungary. A preliminary assessment of the biological activity in GIRK channel inhibition assay was also carried out. In this study the dry samples described in section 4.1 were tested. GIRK ion currents were measured using planar patchclamp technology in the whole-cell configuration with a four channel medium throughput fully automated patch-clamp system (Patchliner, Nanion Technologies GmbH, Munich, Germany). Experiments were carried out on HEK-293 (human embryonic kidney, UCL Business Plc., London, Great Britain) cells stably expressing the GIRK1/4 (Kir3.1/3.4) K^+ channels according to a method described earlier [95].

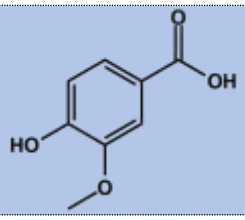
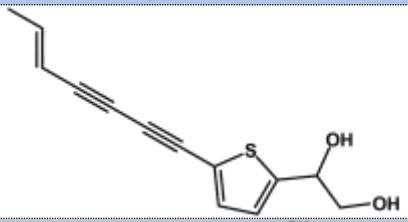
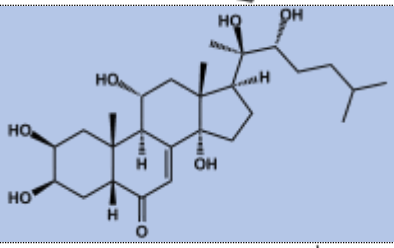
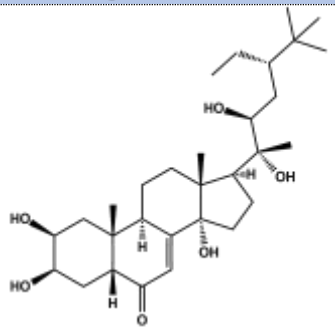
The samples were dissolved in 0.1% DMSO to get three different final concentrations for triplicates. The usual concentrations were 0.400, 1.333 and 4.000 μM for samples with strong activity (such as pure isolates), 30, 100, 300 μg/mL for medium strong and 100, 300 and 750 μg/mL for samples with weak inhibitory potential. Propafenone (1 μM, SigmaAldrich Corporation, St. Louis, USA) was used as a reference compound (71.4 ± 4.6% inhibition).

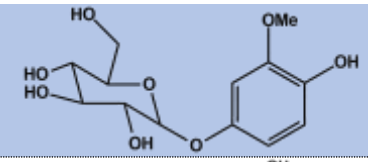
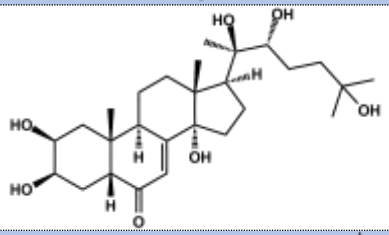
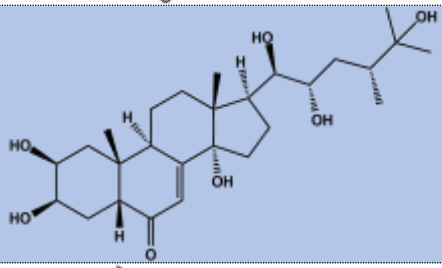
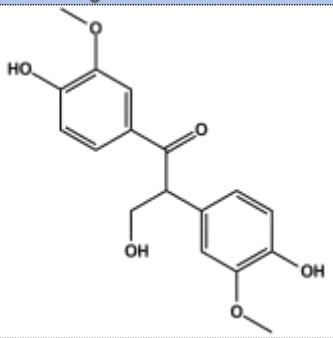
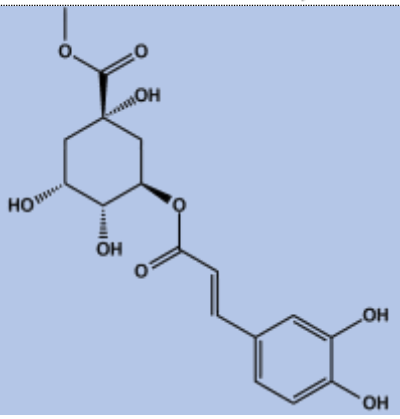
5 RESULTS AND DISCUSSION

5.1 *RHAPONTICUM CARTHAMOIDES*

As result of a series of various methods, such as normal-, medium- and high-pressure chromatography, preparative chromatography with a variety of stationary phases and eluents, combined with modern analytical methods e.g. NMR measurements, in total nine compounds were isolated from the plant. These compounds were identified by comparing their NMR spectra with those reported in the literature as vanillic acid [96], the mixture of *cis* and *trans* isomers of tiophene derivatives [35], ajugasterone C [11], makisterone C [9], tachioside [97], 20-hydroxyecdysone [98], 24-epi-makisterone A [9], evofolin B [99] and chlorogenic acid methyl ester [100]. Results are listed in [**Table 2**].

Table 2. Isolated compounds from *Rhaponticum carthamoides*

Experimental name	Isolated mass (mg)	Compound name	Formula	Literature
LEUr 8	8.37	Vanillic acid		[96]
LEUr 10	4.68			
LEUr 14	9.56	Tiophene derivative (cis-trans mixture)		[35]
LEUr 32	11.79			
LEUr 46	6.25			
LEUr 15	3.37	Ajugasterone C		[11]
LEUr 19	4.33			
LEUr 16	1.64	Makisterone C		[9]

LEUr 17	2.44	Tachioside		[97]
LEUr 24	43.40	20-OH-Ecdysone		[98]
LEUr 25	2.32	24-Epi-makisterone A		[9]
LEUr 45	5.22	Evofolin B		[99]
LEUr 48 (LEUr 27 and 29)	9.93	Chlorogenic acid methyl ester		[100]

Most of the compounds, i.e. as vanillic acid [7], [36]; tiophene derivatives [7], [35]; ajugasterone C [7], [9]–[12], [14]; makisterone C [7], [9], [10], [12]; 20-OH-ecdysone [7], [9]–[12]; 24-epi-makisterione A [7], [9] have been previously reported from this species. The compound tachioside, evofolin B and chlorogenic acid methyl ester have been reported from *R. carthamoides* for the first time by our research group.

The adaptogenic properties of the plant can be explained by the presence of the ecdysteroids including ajugasterone C, makisterone C, 20-hydroxyecdysone and 24-epi-makisterone. The role of substances other than ecdysteroids in the clinical effects of the plant is very dimly understood. Since these substances are common across the plant kingdom and were extracted in small quantities from the plant, it is unlikely that vanillic acid and chlorogenic acid methyl ester play any particular roles. The benzenoid evofolin B, which was first discovered in 1995 [101], has not received much pharmaceutical attention. The *in vitro* superoxide anion generation inhibitory [102], mild *in vitro* lipolytic [103], and weak *in vitro* quinone reductase inducing [104] activities are not directly connected to the therapeutic use of *R. carthamoides*. Tachioside, an aromatic glycoside first discovered in *Berchemia racemosa* [105], has been shown to have tyrosinase [106], moderate alpha-glucosidase [107], and 15-lipoxygenase inhibitory activity [108], as well as antioxidant effects, *in vitro* [109]. The reported actions of some of these compounds, however, make them intriguing for further research and may also present new avenues for *R. carthamoides* research.

5.2 *RHODIOLA ROSEA*

5.2.1 RESULTS OF THE COMPREHENSIVE STUDY OF DIFFERENT *RHODIOLA ROSEA* SAMPLES

DIFFERENCES BETWEEN PLANT PARTS AND PROVENANCES

According to the results the sample set A, which comprised 9 year old plants (see section 4.1) the biomass of rhizomes in average was notably higher than roots, furthermore the ROS_{tot} was in average 1.8 mg/mL (range of 0.5-4.3 mg/mL) and 0.9 mg/mL (range of 0.4-2.5 mg/mL) in the rhizomes and roots respectively. The CA content of the rhizomes was also higher than the roots (0.35 mg/mL compared to 0.26 mg/mL respectively). The rhizomes had higher ratios between rosavins and their aglycons (8.1 ± 1.5:1) and contained on average more phenylethanoids (0.5 mg/mL) compared to the roots (6.2 ± 1.1:1 ratio and 0.3 mg/mL phenylethanoids respectively). According to the results, the difference between phenylethanoids and phenylpropenoids was also notably higher in *R. rosea* than other *Rhodiola* species (6.4 to 5.5 PP_{tot}/SAL_{tot} ratio). [**Table 3**]. The rhizomes

had also significantly higher biomass compared to roots with DW rhizome/root ratio of 1.7:1 [92].

As per region the samples of ALP/PYR and wildALP (rhizomes and roots as well) had notably higher ROStot values compared to NE/NW-EUR and ALTAI provenances. The samples of wildALP also contained higher amount of SALtot and had a higher ROStot/CA ratio than the other samples. Although the samples of NE/NW-EUR and ALTAI had a higher PPtot/SALtot ratio. (**Figure 1**) [92].

Table 3. Phytochemical characteristics of different plant parts

	Rhizome (± s.e.m)	**range (min-max)**	**Roots (± s.e.m)**	**range (min-max)**
ROS_{tot} (µg/mL)	1842 (± 207)	530-4273	980 (± 118)	361-2494
CA (µg/mL)	346 (± 77)	59-1567	257 (± 58)	29-1165
ROS_{tot}/CA ratio (x:1)	8.1 (± 1.5)	1.2-34.3	6.2 (± 1.1)	0.8-18.8
SALtot (µg/mL)	547 (± 123)	112-3079	332 (± 75)	54-1503
PP_{tot}/SAL_{tot} ratio (x:1)	6.4 (± 1.3)	2.1-21.1	5.5 (± 1.1)	1.7-24.8
total DW (g)	75.9 (± 11.6)	7.9-246.0	48.4 (± 7.0)	9.9-136.5
FW/DW ratio (x:1)	4.3 (± 0.2)	2.8-5.7	4.2 (± 0.2)	2.7-7.3
Rhizome/root ratio (x:1)	1.7 (± 0.2)	0.2-2.8	-	-

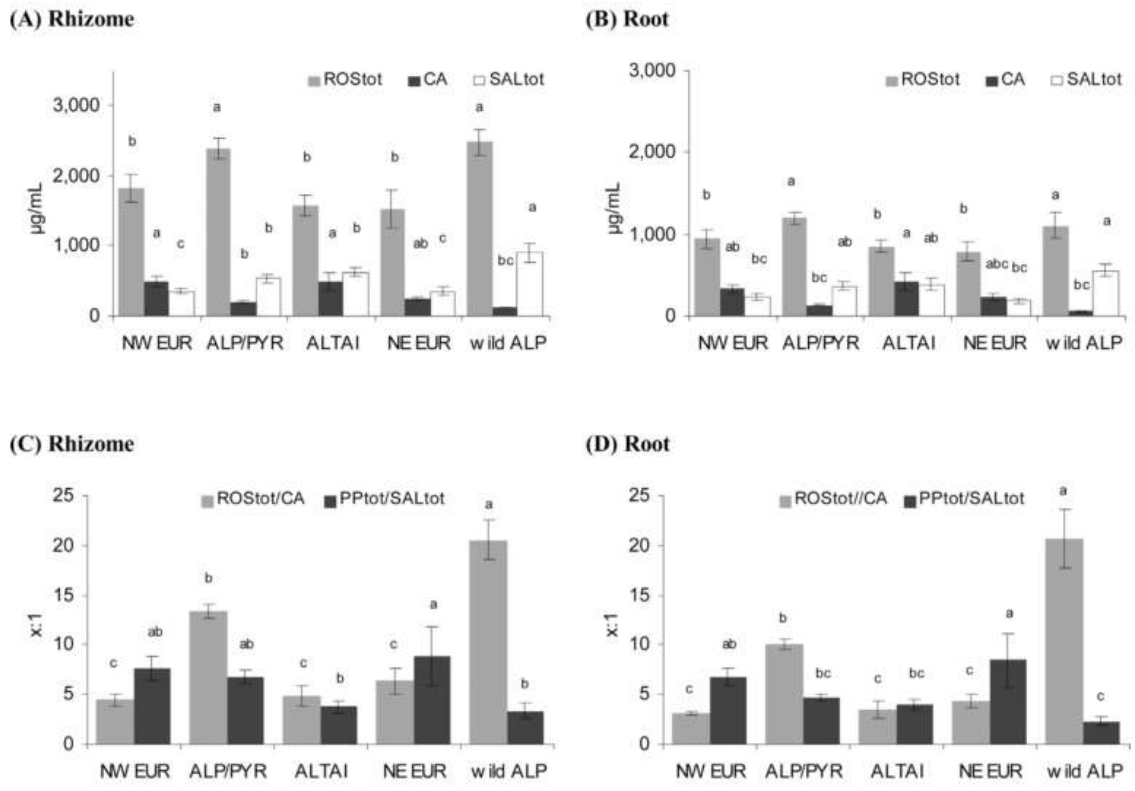


Figure 1. ROS_{tot}, CA and SAL_{tot} contents (**A** and **B**) and ROS_{tot}/CA and PP_{tot}/SAL_{tot} ratio (**C** and **D**) of *Rhodiola* samples of different regions

PHENYLPROPENOID PROFILE AND YIELD OF RHIZOMES AND ROOTS ACCORDING TO HARVEST SEASON

In the trial of sample set B (see section 4.1), across all provenances the 6–7-year-old plants had similarities between the content characteristics, namely the plants contained a higher amount ROS_{tot} when harvested in May than in July to October. It can also clearly be stated that the ROS_{tot} content decreased from year 6 to 7. Since the CA content was similarly influenced by harvest date the ROS_{tot}/CA ratio remained relatively constant. [**Table 4**]. The biomass doubled from May year 6 to September year 7, although the FW/DW ratio remained relatively consistent, thus harvest in the seventh year would be recommended [92].

Table 4. Phenylpropenoid content of different harvest dates

	ROS_{tot}		CA		ROS_{tot}/CA	
Year 6	**mean (± s.e.m.)**	**range**	**mean (± s.e.m.)**	**range**	**X:1**	**range**
May	3123 (±178)	1839 - 4809	356 (±32)	150 - 637	10.0	4.5 - 16.9
Jul	1554 (±119)	557 - 2925	214 (±23)	66- 484	9.0	3.0 - 18.0
Aug	1562 (±91)	335 - 2672	209 (±26)	32 - 537	9.2	3.5 - 17.6
Oct	1470 (±122)	488 - 2534	246 (±41)	88 - 712	8.1	3.0 - 16.2
Year 7	**mean (± s.e.m.)**	**range**	**mean (± s.e.m.)**	**range**	**X:1**	**range**
May	2517 (±164)	1110 - 3741	295 (±24)	107 - 506	9.7	3.2 - 18.6
Jul	1365 (±107)	403 - 2774	225 (±29)	53 - 580	7.9	2.3 - 17.1
Sep	1827 (±125)	592 - 2941	282 (±35)	82 - 642	8.2	3.3 - 22.6

5.2.2 RESULTS OF THE STUDY OF TWO NEW MARKERS OF *RHODIOLA ROSEA*

DETERMINATION OF THE TWO NEW FLAVONOIDS

Compound 1 and 2 (isolation described in section 4.3.2) were identified as rhodiosin and herbacetin, respectively, based on the comparison of their ^{1}H and ^{13}C spectral data with those in the literature [110][111]. Although other flavonoids and phenylethanoids serve as a good quality marker of the plant *R. rosea*, the two new markers could possibly widen the spectra of fingerprint analysis. With the prolongated version of the analysis described by *Peschel et al.* (2016) [53] the two new markers could be detected on baseline separation alongside with the salidroside and rosavins, which can be advantageous in terms of qualitative and quantitative analysis as well. Detection can be carried out in various wavelengths. In our study linearity, precision, LOD, LOQ and accuracy were checked for 254 and 275 nm. LOD was found to be 47.02 µg/mL and 7.60 µg/mL with a signal to noise ratio of >3 and LOQ 156.72 µg/mL and 25.35 µg/mL (signal to noise ratio of >10) for rhodiosin and herbacetin respectively. Recovery rates were 84.66, 89.51 and 93.25% for rhodiosin and 56.42, 64.99 and 75.54% for herbacetin at 50%, 100% and 150%, respectively [90].

SOLVENT POLARITY AND EXHAUSTIVENESS TRIAL

According to the results of the extraction exhaustiveness trial (see section 4.3.20) it is clearly to be stated, that extraction with 70-90% of aqueous EtOH yielded the highest amount of the new flavonoid markers. The extracts prepared with 50% and 30% EtOH contained moderate, but still quantifiable amount of rhodiosin and herbacetin (**Figure 2**). The commercial samples of *Rhodiola* extracts were generally prepared with 40-70% EtOH, which results approx. 75% of exhaustiveness in one run (in terms of rhodiosin/herbacetin and rosavins/salidroside as well), thus the two new compounds could be used as adequate markers for plant characterisation.

INFLUENCE OF PLANT PART

The flavonoid content of herb was rather low (<400 μg/mL, ca. 0.2% of dry weight) compared to the rhizomes and roots (1800-2400 μg/mL, 1.2-1.6 % of dry drug (**Figure 3**)). Irrespectively of the plant part used, the rhodiosin and herbacetin markers were always detectable, thus can be used as analytical markers of further fingerprint determinations [90].

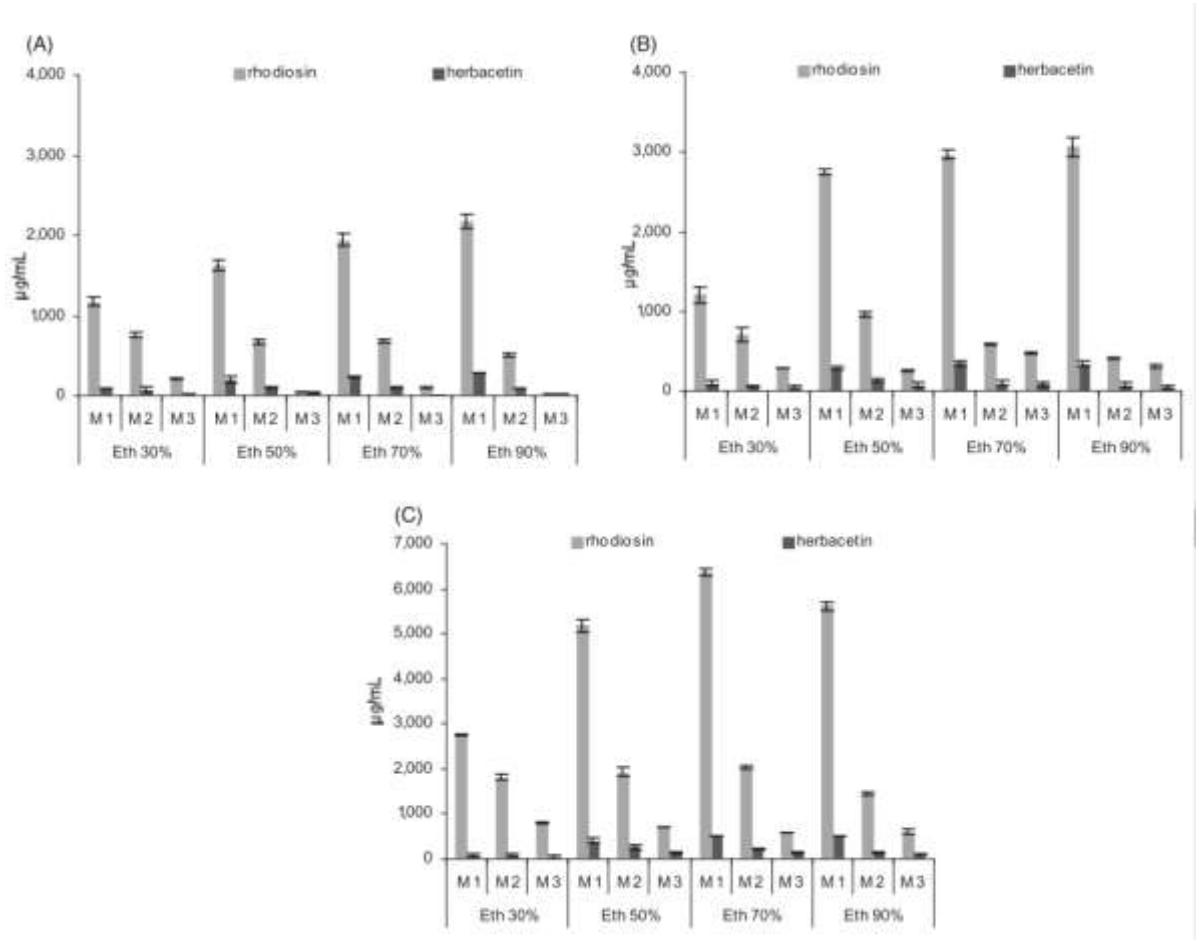


Figure 2. Influence of solvent polarity on rhodiosin and herbacetin contents in rhizome (A, B) and root (C), where M1-M3 are successive maceration steps

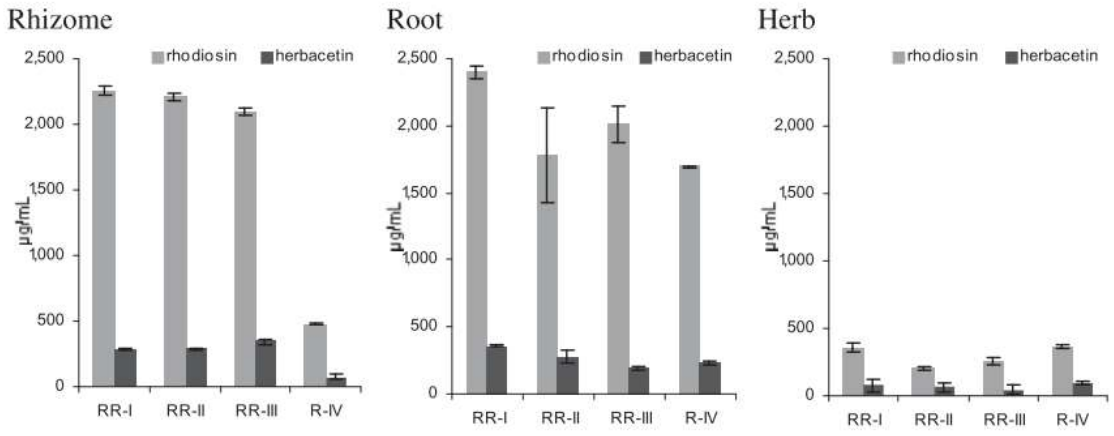


Figure 3. Influence of plant part (extracted with 70% EtOH) on rhodiosin and herbacetin content

INFLUENCE OF DRYING PROCEDURE

According to our data the drying procedure (either duration and temperature) had slightly to no effect on the rhodiosin and herbacetin content and composition of the samples (**Figure 4**). The content was rather influenced by the plant origin.

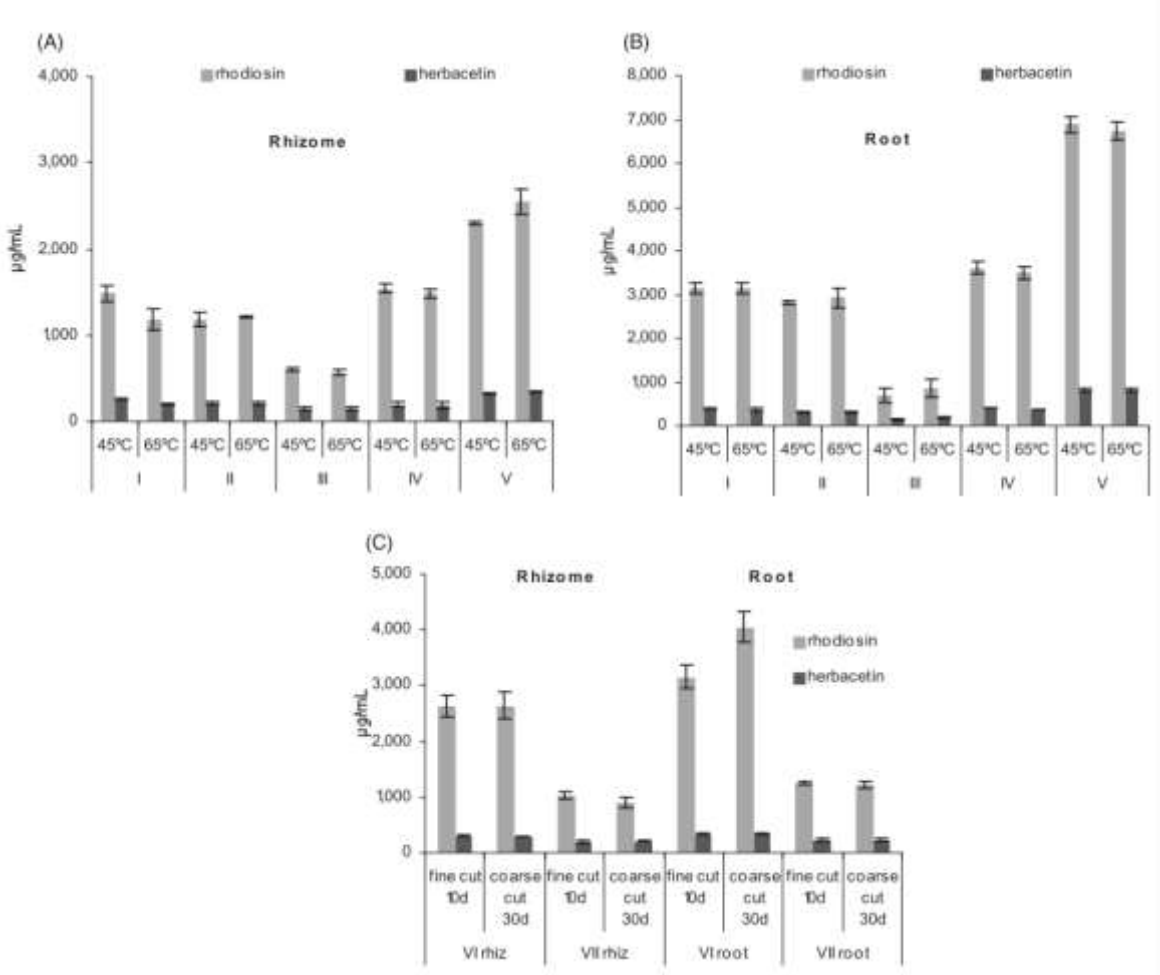


Figure 4. The effect of the drying temperature (**A** and **B**) and duration (**C)** on the herbacetin and rhodiosin content of the samples.

INFLUENCE OF DRUG ORIGIN

The mean flavonoid content of rhizomes and roots was to be found 760-3800 µg/mL and 880-6300 µg/mL respectively. The identified rhodiosin and herbacetin content was also quite constant with a consistent ratio of ca. 10:1. Despite some extreme diverse values, the total flavonoid content was the highest in the sample group originating in Northwestern Europe.

5.3 *WITHANIA FRUTESCENS*

According to our results, the most optimal extraction solvent was MeOH – H_2O 1:1 as showed in [**Table 5**] and (**Figure 5**). With this proportion the best dry extract/withaferin A rate is obtainable. The comparison between root, twig and leaf samples have shown that the leaves have contained the highest amount of withaferin A [**Table 6**]. Thus, although in traditional utilisation the roots are preferable, in industrial conditions to gain pure withaferin A, the leaves could have higher importance.

Table 5. Dry mass and withaferin A gain with different solvent systems.

	Extraction solvent		Dry mass (mg)	Withaferin A content (mg)	Percentage of withaferin A in dry mass (%)
1	CH_2Cl_2	100	9.62	1.72	17.88
2	CH_2Cl_2 – MeOH	75:25	33.41	5.63	16.85
3	CH_2Cl_2 – MeOH	50:50	58.08	7.88	13.57
4	CH_2Cl_2 – MeOH	25:75	85.59	7.93	9.27
5	MeOH	100	69.54	7.68	11.04
6	MeOH – H_2O	75:25	129.91	1.83	1.41
7	MeOH – H_2O	50:50	140.70	20.44	14.52
8	MeOH – H_2O	25:75	157.18	7.92	5.03
9	EtOH	100	76.90	2.39	3.11
10	CH_2Cl_2 – EtOH	25:75	42.83	2.77	6.46
11	EtOH – H_2O	75:25	70.18	5.78	8.24

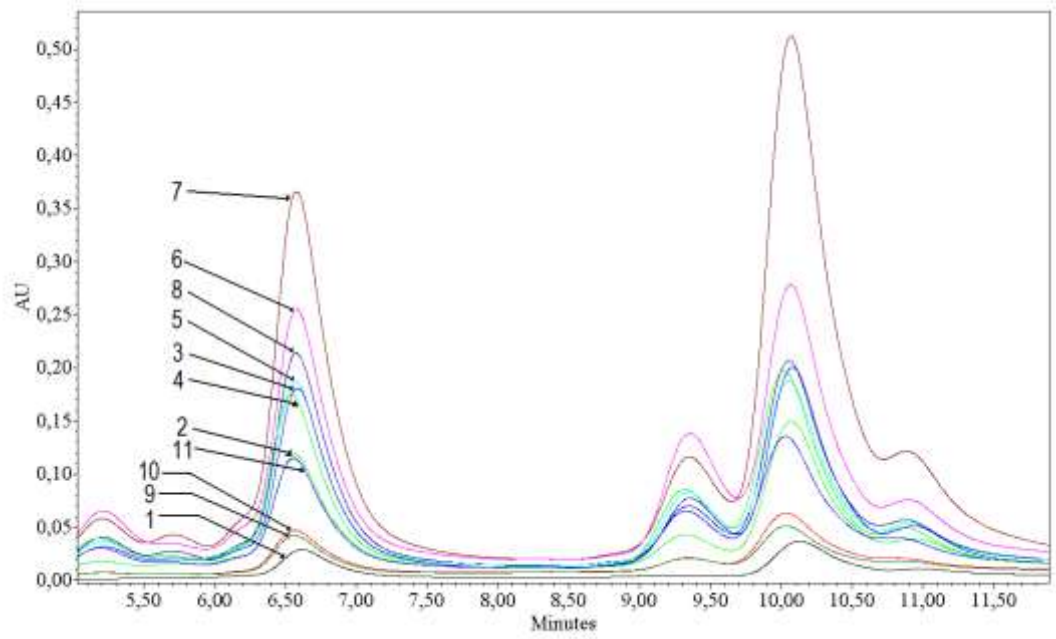


Figure 5. HPLC comparison of various extracts of "Withania A". Extracting solvents: **1**: CH_2Cl_2; **2**: CH_2Cl_2–MeOH 75:25; **3**: CH_2Cl_2–MeOH 50:50; **4**: CH_2Cl_2–MeOH 25:75; **5**: MeOH; **6**: MeOH–H_2O 75:25; **7**: MeOH–H_2O = 50:50; **8**: MeOH–H_2O 25:75; **9**: EtOH; **10**: CH_2CL_2–EtOH 25:75; **11**: EtOH–H_2O 75:25

Table 6. Withaferin A content of different plant parts

	Plant samples	Dry mass (mg)	Withaferin A content (mg)	Average ± SD
Leaf	Withania A	94.53	11.54	7.46 ± 4.96
	Withania B	89.55	4.62	
	Withania C	115.45	4.34	
	Withania D	121.23	4.34	
	Withania E	134.17	15.72	
	Withania F	129.9	4.23	
Root	Withania G	86.05	1.56	1.59 ± 0.06
	Withania H	74.06	1.55	
	Withania I	61.21	1.54	
	Withania K	98.03	1.61	
	Withania L	111.69	1.66	
	Withania M	79.02	1.60	
	Withania N	106.34	1.72	
	Withania O	93.29	1.57	

Stem	Withania P	63.51	1.53	1.53 ± 0.01
	Withania Q	52.05	0.00	
	Withania R	77.11	1.53	
	Withania S	63.46	1.53	
	Withania T	43.79	0.00	

In the hydrolysis trials there was only minor differences in the results. A slight increase in the gained withaferin A yield in correlation with hydrolysis duration is observable. The most effective hydrolysis method seems to be with 1% (V/V) sulphuric acid for 90 minutes [**Table 7-8**].

Table 7. Hydrolysis with sulphuric acid

Acid		Time of sampling (min)	Free withaferin A (mg)
Name	C (V/V%)		
Sulfuric acid	1.00	30	0.81
	1.00	60	0.83
	1.00	90	0.95
	2.50	30	0.84
	2.50	60	0.86
	2.50	90	0.87
	5.00	30	0.86
	5.00	60	-
	5.00	90	-
	7.50	30	-
	7.50	60	0.86
	7.50	90	-
	10.0	30	0.82
	10.0	60	0.84
	10.0	90	0.82

Table 8. Hydrolysis with acetic acid

Acid		Time of sampling(min)	Free withaferin A (mg)
Name	C (V/V%)		
Acetic acid	25	4	0.80
	25	5	0.81
	25	6	0.80
	50	4	0.79
	50	5	0.80
	50	6	0.80
	100	4	0.78
	100	5	0.79
	100	6	0.79

5.4 ROTIFER ASSAY

HPLC ANALYSIS OF THE SAMPLES

The main markers of the crude extracts were determined with the methods described in section 4.5. The extract of *W. frutescens* contained 8.75±0.02 mg/g withaferin A, 0.17±0.01 mg/g withanolide A and 0.17±0.01 mg/ml withanolide B. In the extract of *R. rosea* 8.26±0.013 mg/ml salidroside, 1.78±0.14 mg/g tyrosol, 9.55±0.02 mg/g rosavin and 6.28±0.05 mg/g cinnamyl-alcohol was determined. The extract of *P. ginseng* contained 5.81±0.15 mg/g ginsenoside Rb1. The 20OHe and ajugasterone C content of *R. carthamoides* extract was also quantified and was 30.13±0.03 mg/g and 15.33±0.11 mg/g respectively.

VIABILITY ASSAY

The three viability values, toxicity and survival lifespan (TSL), mastax contraction frequency (MCL) and body size index (BSI) were assessed after three day fasting and the following six day treatment with crude extracts and their active substances. TSL shows the impact of test materials on the specimens' lifespan, meanwhile MCL and BSI are important quantitative markers of viability. In the case of *W. frutescens, R. carthamoides, R. rosea* extracts, and the novel compound tachioside from *R. carthamoides* significant BSI elevation with a slight increase in TSL and MCF were

observed. The pure compounds of rosavin, cinnamyl alcohol, ginsenosid Rb1, withanolide A, withanolide B and withaferin A caused significant decrease in the number of rotifers as well in MCF values. In the case of 20-OH-ecdysone the rotifers have produced just one egg, which they were not able to lay and it hatched inside of the mothers body. This could be explained with the anti-moulting effect the steroid.

5.5 GIRK CHANNEL INHIBITORY ASSAY

In the GIRK channel inhibitory, the leaf samples showed the strongest activity out of the plant samples with IC_{50} values ranging between 11.15 µg/mL and 499.71 µg/mL. The fruits showed moderate activity with IC_{50} values ranging 254.28 µg/mL to 1017.35 µg/mL. The twigs and roots have shown no activity with some exception of the roots with 406.72 µg/mL – 469.14 µg/mL IC_{50}. This can be explained by the fact that roots and rhizomes contain withaferin A in glucosides rather than free constituents, if we suppose that this bioactivity is related to aglycons rather than glycosides. The pure withaferin A showed a concentration dependent inhibition. The results are listed in [**Table 9**].

Table 9. IC_{50} values of the tested samples on GIRK channels

Experimental name	Plant code	Plant part	Extraction solvent	IC_{50} (µg/mL)	SD
ZZmet_1	*W. frutescens* A	leaf	MeOH – H_2O (1:1)	44.71	± 0.41
ZZmet_2	*W. frutescens* B	leaf	MeOH – H_2O (1:1)	50.98	± 2.70
ZZmet_3	*W. frutescens* C	leaf	MeOH – H_2O (1:1)	499.72	± 19.91
ZZmet_4	*W. frutescens* D	leaf	MeOH – H_2O (1:1)	105.28	± 3.50
ZZmet_5	*W. frutescens* E	leaf	MeOH – H_2O (1:1)	188.50	± 23.62
ZZmet_6	*W. frutescens* F	leaf	MeOH – H_2O (1:1)	354.01	± 0.47
ZZmet_7	*W. frutescens* L1	leaf	MeOH – H_2O (1:1)	81.26	± 7.50
ZZmet_8	*W. frutescens* L2A	leaf	MeOH – H_2O (1:1)	21.36	± 1.50
ZZmet_9	*W. frutescens* L3	leaf	MeOH – H_2O (1:1)	136.23	± 7.59
ZZmet_10	*W. frutescens* L5B	leaf	MeOH – H_2O (1:1)	28.08	± 0.64
ZZmet_11	*W. frutescens* L7A	leaf	MeOH – H_2O (1:1)	50.09	± 2.62
ZZmet_12	*W. frutescens* L9A	leaf	MeOH – H_2O (1:1)	136.59	± 4.20
ZZmet_13	*W. frutescens* L11B	leaf	MeOH – H_2O (1:1)	71.59	± 9.23
ZZmet_14	*W. frutescens* 2	leaf	MeOH – H_2O (1:1)	18.44	± 0.71

ZZmet_15	*W. frutescens* 7	leaf	MeOH – H_2O (1:1)	9.21	± 0.07
ZZmet_16	*W. frutescens* 12	leaf	MeOH – H_2O (1:1)	64.11	± 7.08
ZZmet_17	*W. frutescens* 16	leaf	MeOH – H_2O (1:1)	50.47	± 1.43
ZZmet_18	*W. frutescens* 31	leaf	MeOH – H_2O (1:1)	68.25	± 9.32
ZZmet_19	*W. frutescens* 35	leaf	MeOH – H_2O (1:1)	319.27	± 71.61
ZZmet_20	*W. frutescens* 40	leaf	MeOH – H_2O (1:1)	102.37	± 12.55
ZZmet_21	*W. frutescens* 45	leaf	MeOH – H_2O (1:1)	324.31	± 21.77
ZZmet_22	*W. frutescens* 63	leaf	MeOH – H_2O (1:1)	85.40	± 8.35
ZZmet_23	*W. frutescens* 74	leaf	MeOH – H_2O (1:1)	29.67	± 1.54
ZZmet_24	*W. frutescens* 81	leaf	MeOH – H_2O (1:1)	77.54	± 8.44
ZZmet_25	*W. frutescens* 82	leaf	MeOH – H_2O (1:1)	176.34	± 20.61
ZZmet_26	*W. frutescens* 85	leaf	MeOH – H_2O (1:1)	210.93	± 28.99
ZZmet_27	*W. frutescens* 86	leaf	MeOH – H_2O (1:1)	283.61	± 15.68
ZZmet_28	*W. frutescens* 89	leaf	MeOH – H_2O (1:1)	447.52	± 39.82
ZZmet_29	*W. frutescens* 93	leaf	MeOH – H_2O (1:1)	264.31	± 8.91
ZZmet_30	*W. frutescens* 108	leaf	MeOH – H_2O (1:1)	13.42	± 0.02
ZZmet_31	*W. frutescens* 110	leaf	MeOH – H_2O (1:1)	12.77	± 0.11
ZZmet_32	*W. frutescens* WF3	leaf	MeOH – H_2O (1:1)	32.09	± 3.04
ZZmet_33	*W. frutescens* WF3	leaf	CH_2Cl_2	11.90	± 1.20
ZZmet_34	*W. frutescens* WF3	leaf	CH_2Cl_2 – MeOH (1:1)	19.20	± 0.77
ZZmet_35	*W. frutescens* WF3	leaf	EtOH	21.71	± 0.56
ZZmet_36	*W. frutescens* WF3	leaf	EtOH – H_2O (1:1)	23.17	± 0.01
ZZmet_37	*W. frutescens* WF4B	leaf	MeOH – H_2O (1:1)	20.96	± 0.10
ZZmet_38	*W. frutescens* WF5A	leaf	MeOH – H_2O (1:1)	17.43	± 0.18
ZZmet_39	*W. frutescens* WF6A	leaf	MeOH – H_2O (1:1)	153.09	± 13.04
ZZmet_40	*W. frutescens* WF7B	leaf	MeOH – H_2O (1:1)	22.27	± 0.69
ZZmet_41	*W. frutescens* WF F-1-V	leaf	MeOH – H_2O (1:1)	41.55	± 2.08
ZZmet_42	*W. frutescens* WF M4VIII	leaf	MeOH – H_2O (1:1)	32.95	± 0.34
ZZmet_F	*W. frutescens* D	leaf	MeOH – H_2O (1:1)	280.37	± 27.11
ZZmet_G	*W. frutescens* WF3	leaf	MeOH – H_2O (1:1)	11.15	± 0.76
ZZmet_43	*W. frutescens* R	twigs	MeOH – H_2O (1:1)	-	-
ZZmet_44	*W. frutescens* T5A	twigs	MeOH – H_2O (1:1)	-	-
ZZmet_45	*W. frutescens* T10B	twigs	MeOH – H_2O (1:1)	-	-
ZZmet_46	*W. frutescens* (N)T12	twigs	MeOH – H_2O (1:1)	-	-

ZZmet_47	*W. frutescens* (N)T28	twigs	MeOH – H_2O (1:1)	-	-
ZZmet_K	*W. frutescens* T10B	twigs	MeOH – H_2O (1:1)	256.57	± 11.73
ZZmet_48	*W. frutescens* M	roots	MeOH – H_2O (1:1)	-	-
ZZmet_49	*W. frutescens* R3	roots	MeOH – H_2O (1:1)	-	-
ZZmet_50	*W. frutescens* R6A	roots	MeOH – H_2O (1:1)	-	-
ZZmet_51	*W. frutescens* (N)R14	roots	MeOH – H_2O (1:1)	-	-
ZZmet_52	*W. frutescens* (N)R32	roots	MeOH – H_2O (1:1)	-	-
ZZmet_A	*R. rosea* extract	roots	MeOH – H_2O (1:1)	406.72	± 16.21
ZZmet_B	*W. frutescens* M	roots	MeOH – H_2O (1:1)	469.14	± 24.58
ZZmet_C	*W. frutescens* (N)R14	roots	MeOH – H_2O (1:1)	-	-
ZZmet_53	*W. frutescens* F4B	fruits	MeOH – H_2O (1:1)	-	-
ZZmet_54	*W. frutescens* F5A	fruits	MeOH – H_2O (1:1)	1017.35	± 55.80
ZZmet_55	*W. frutescens* F7B	fruits	MeOH – H_2O (1:1)	254.28	± 8.34
ZZmet_J	*W. frutescens* F7B	fruits	MeOH – H_2O (1:1)	438.79	± 9.35
ZZmet_E	Withaferin A	isolate	N.A.	0.5321204 =1.13 µM	± 0.046000 =0.0977 µM

The promising activities of some extracts and the pure compound withaferin A necessitate an in-depth analysis of *W. frutescens* and its main metabolites. The overall activities of different extracts are due to the presence of several differently acting compounds. In case of *W. frutescens*, this was the first experiment to assess the GIRK inhibiting activity of the plant. Since *W. frutescens* and the widely applied *W. somnifera* have partly overlapping phytochemical profiles, it can be assumed that the latter also affects these ion channels – however, the characteristic of this effect is not known. Withaferines and withanolides might play a role in such effect, as it was demonstrated by the remarkable activity of withaferin A in our experiments. These results, together with the fact that *W. somnifera*, as an adaptogen has been used traditionally in Asia since ancient times, raise the possibility of GIRK-mediated effects in the overall clinical effect of the plant. Although the roots of the plant did not seem to be active in our experiments, it is not clear how traditional processing methods could influence this activity by changing the composition of the extracts eg. by hydrolysis. It has been shown recently that melatonin exerts its effect on circadian rhythm partly through GIRK channels [112]. This discovery opens new perspectives for the examination of *Withania* metabolites and may lead to the better understanding of the central nervous system effects of this plant.

6 CONCLUSIONS

Although many adaptogenic plants have gained importance and attracted scientific interest in the last decades, there still remains a lot to understand and discover. There might be further applications and mechanics of action to be discovered. In my thesis I have tried – and managed – to widen the scope of view on these valuable plants.

As the result of my studies on the *Rhaponticum carthamoides* we isolated and characterised three, previously undescribed compounds from this species along with 6 already reported constituents.

From *Rhodiola rosea,* we have successfully identified two new marker compounds, which could be used as analytical markers for quality control, either in the case of raw plant samples or marketed products. In addition, we carried out a comprehensive study, in which we analysed the importance of plant origin, plant parts, harvest season and drying procedure on drug composition. These features have a crucial importance in optimization and quality insurance of products based on *Rhodiola* extracts.

In the case of *Withania frutescens,* a relative species of the Asian *Withania somnifera,* we successfully optimised the extraction process and developed a hydrolysis method to maximise the yield of the medicinally important withaferin A. Our results might contribute to the utilization of *W. frutescens,* a plant commonly present Europe. The preliminary assay on GIRK channel inhibition showed promising results, pointing out on the potential role of this activity in the clinical effects of *Withania* species.

Adaptogenic plants are widely used to enhance physical and psychological performance, though their pharmacological profile has not been elucidated in detail. Further co-operative phytochemical-phytopharmacological analysis of these plant extracts may reveal more details on their mechanisms of action.

7 REFERENCES

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