Preparative isolation and phytochemical analysis of *Rhaponticum carthamoides*, *Rhodiola rosea* and *Withania frutescens* constituents

Summary of the Ph.D. Thesis

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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 a different stress-related diseases.

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.

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. Later in a study *Israel I. Berkhman* further defined 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. 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. 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. 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. However, the mechanism of actions of these compounds and the full spectrum of metabolites responsible for the adaptogenic effect has not been totally elucidated.

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.

MATERIALS AND METHODS

PLANT MATERIALS

Plant materials of *Rhaponticum carthamoides* was purchased from Herbosus, Espoo (Finland), identified by Zsuzsanna Hajdú. *Rhodiola rosea* root and rhizome samples (in total of 28) were collected across the Eurasian continent, identified by Wieland Peschel. *Withania frutescens* samples of various plant parts such as roots, twigs, leaves and fruits (in total of 80) were either collected or purchased from commercial stores, identified by Wieland Peschel. Due to its well-studied characteristics *Panax ginseng* was used as comprehensive standards for the analyses.

ISOLATION OF COMPOUNDS FROM *RHAPONTICUM CARTHAMOIDES*

For the isolation of new compound from *R. carthamoides* various chromatographic methods were applied, such as atmospheric-, medium- and high-pressure column chromatography, rotational planar chromatography, preparative thin layer chromatography, with a variety of stationary and mobile phases and detection methods. The purified compounds were identified via NMR and MS spectroscopy.

PHYTOCHEMICAL ANALYSIS OF *RHODIOLA ROSEA*

The plant materials were ground and extracted with 70% EtOH to gain a drug-extract ratio of 1:5. The extracts then were filtered through a syringe filters into HPLC vials and stored at 4°C. The analysis were carried out on a Waters HPLC system. Peaks were assigned by spiking the samples with standard compounds and comparison of the UV spectra and retention times. Rosavin, rosarin and rosin are summarised to total rosavins (=ROS_{tot}). As a relative parameter we calculated the ratio between ROS_{tot} and Cinnamyl alcohol (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). The results then were analysed to evaluate the correspondence between plant parts and provenances and influence of harvest season. Additionally two possibly new markers, rhodiosin and herbacetin and the influence of plant part, harvest season, drying temperature and drug origin on their concentrations were also determined from the extracts.

PHYTOCHEMICAL ANALYSIS OF *WITHANIA FRUTESCENS*

To determine the optimal extraction method for *W. frutescens* 1.00-1.00 grams of plant materials were macerated with various extraction solvents/solvent mixtures, then filtered and analysed with HPLC equipped with DAD detector. Additionally a hydrolysis method were also developed applying various acids in different concentrations to maximise the yield of withaferin A.

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 (*Philodina acuticornis*) 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 somnifera, Rhaponticum carthamoides,* and *Rhodiola rosea* were tested *in vivo*. For this the plants mentioned above and their extracts and isolates were used. The viability markers of bdelloid rotifers – body size index, mastax contraction frequency and toxicity lifespan index – were measured after a culturing- and fasting-phase, treating the animals with the extracts and their macerates.

ASSAY ON GIRK CHANNEL INHIBITION

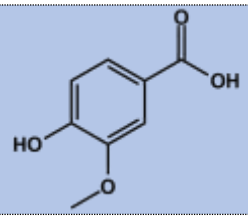
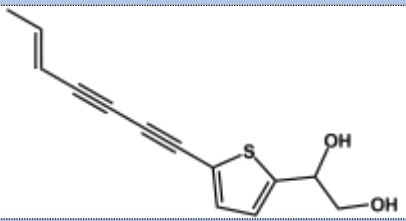
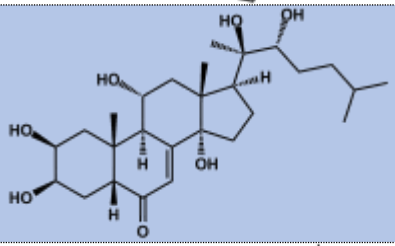
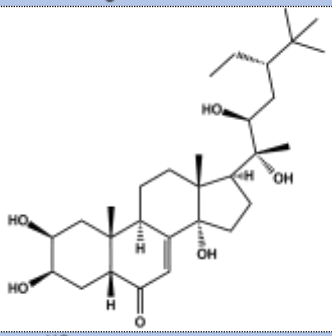
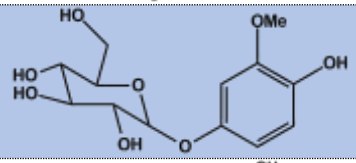
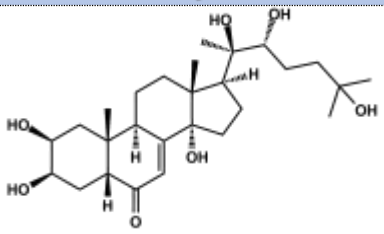
The GIRK channel inhibition assay was conducted in Rhytmion Ltd, Szeged, Hungary. In this study dry extracts of *W. frutescens* (in total of 66) were tested. GIRK ion currents were measured using planar patch-clamp technology in the whole-cell configuration with a four-channel medium throughput fully automated patch-clamp system. Experiments were carried out on HEK-293 (human embryonic kidney) cells. Samples were dissolved in DMSO in three final concentrations for triplicates.

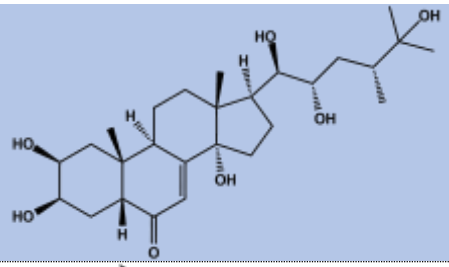
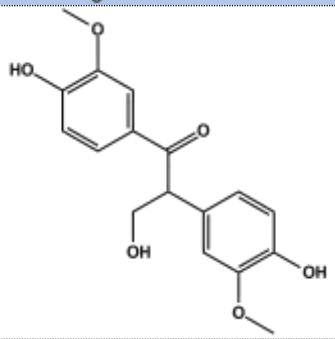
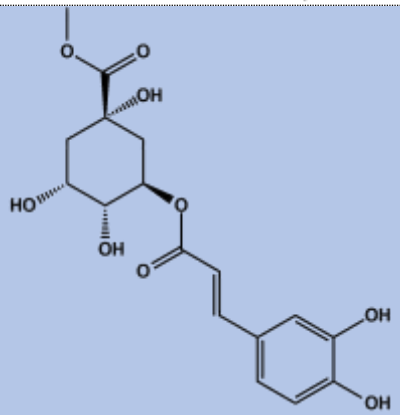
RESULTS AND DISCUSSION

RHAPONTICUM CARTHAMOIDES

As result of the separation series of the *R. carthamoides* nine compounds were isolated, listed in **Table 1.**

Table 1 Compounds isolated from *Rhaponticum carthamoides*

Compound name	Formula
Vanillic acid	
Tiophene derivative (cis-trans mixture)	
Ajugasterone C	
Makisterone C	
Tachioside	
20-OH-Ecdysone	

24-Epi-makisterone A	
Evofolin B	
Chlorogenic acid methyl ester	

Most of the compounds, i.e. as vanillic acid, tiophene derivatives, ajugasterone C, makisterone C, 20-OH-ecdysone, 24-epi-makisterione A, have already been 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 benzenoid evofolin B, which was first discovered in 1995, has not received much pharmaceutical attention. The *in vitro* superoxide anion generation inhibitory, mild *in vitro* lipolytic, and weak *in vitro* quinone reductase inducing activities are not directly connected to the therapeutic use of *R. carthamoides*. Tachioside, an aromatic glycoside first discovered in *Berchemia racemosa*, has been shown to have tyrosinase, moderate alpha-glucosidase, and 15-lipoxygenase inhibitory activity, as well as antioxidant effects, *in vitro*. 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.

RHODIOLA ROSEA

COMPREHENSIVE STUDY OF DIFFERENT *RHODIOLA ROSEA* SAMPLES

Comparing the roots and rhizomes in sample set A (4. Appendix), the biomass of latter was notably higher. ROS_{tot} was in average 1.8 mg/mL and 0.9 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 also had higher ratios between rosavins and their aglycons and contained on average more phenylethanoids compared to the roots. According to the results, the difference between phenylethanoids and phenylpropenoids was also notably higher in *R. rosea* than other *Rhodiola* species. Regional differences were also notable. The samples of ALP/PYR and wildALP (rhizomes and roots as well) have 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.

In the trial of sample set B (4. Appendix), 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.

RESULTS OF THE STUDY OF TWO NEW MARKERS OF R. ROSEA

In our study we successfully isolated two compounds, rhodiosin and herbacetin, which could be used as additional markers for quality control of the marketed plant and products containing *R. rosea.* 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. In the trial on effect of solvent polarity 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. The commercial samples of *Rhodiola* extracts were generally prepared with 40-70% EtOH,

which results approx. 75% of exhaustiveness in one run, thus the two new compounds could be used as adequate markers for plant characterisation. The plant part has not significantly influenced the detectable amount of rhodiosin and herbacetin. 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). 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. According to these findings these two compounds might be useful tools in quality control.

WITHANIA FRUTESCENS

According to our results, the optimal extraction solvent was MeOH – H_2O = 1:1. [**Table 2**]. With this solvent mixture the best dry extract/withaferin A rate is obtainable. The HPLC comparison of the root, twig and leaf samples demonstrated that the leaves have contained the highest amount of withaferin A [**Table 3**]. Thus, although in traditional utilisation the roots are preferable, in industrial conditions the leaves might have higher importance to gain pure withaferin A. As a result of the hydrolysis trials, the hydrolysis with 1% (V/V) sulphuric acid for 90 minutes seemed to be the most effective to get the maximal amount of withaferin A aglycone.

Table 2 Dry mass and withaferin A gain in different solvent systems

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

Table 3: HPLC-comparison of various 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	

ROTIFER ASSAY

The assays on rotifers were performed with chemically characterized plant extracts. 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±0.05mg/g cinnamyl-alcohol was determined. The extract of *P. ginseng* contained 5.81±0.15mg/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. The bdelloid rotifers treated with crude extracts have had increased body mass and survivability. Thus, the animals treated with the pure compounds of rosavin, cinnamyl alcohol, ginsenosid Rb1,

withanolide A, withanolide B and withaferin A have suffered a severe decrease in viability properties.

In the case of 20-OH-ecdysone the rotifers have produced just one egg, which they weren't able to lay and it hatched inside of the mothers body. This could be explained with the anti-moulting effect the steroid.

GIRK CHANNEL INHIBITORY ASSAY

In the field of drug research the investigation of the activity of GIRK and hERG channels are becoming an ever increasing role. These 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. Furthermore, it has been shown that melatonin exerts its effect on circadian rhythm partly through GIRK channels. In the GIRK channel inhibitory of *W. frutescens samples*, 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 promising activities of some extracts and the pure compound withaferin A necessitate an in-depth analysis of *W. frutescens* and its main metabolites.

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.

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Z. P. Zomborszki, N. Kúsz, D. Csupor and W. Peschel
Rhodiosin and herbacetin in *Rhodiola rosea* preparations: additional markers for quality control?
Pharmaceutical Biology, Vol 57 (1), pp 295-305 (2019)
IF: 2.971; Q1

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Industrial Crops & Products, Vol 111, pp 446-456 (2018)
IF: 4.191; Q1

L. Mácsai, Zs. L. Datki, D. Csupor, A. Horváth, Z. P. Zomborszki
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Evidence-Based Complementary and Alternative Medicine, Vol 2018, Article ID 3690683, 4 pages
IF: 1.984; Q1

Z. P. Zomborszki, W. Peschel, K. Boros, J. Hohmann and D. Csupor
Developement of an optimized processing method for *Withania frutescens*
Acta Alimentaria, Vol 45 (3), pp 452-456 (2016)
IF: 0.3; Q3

Z.P. Zomborszki, N. Kúsz, J. Hohmann, D. Csupor
Three novel constituents from the roots of *Rhaponticum carthamoides*
Acta Pharmaceutica Hungarica, Accepted for publication
Q4

Notes