Effect of provenance, plant part and processing on extract profiles from cultivated European *Rhodiola rosea* L. for medicinal use

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**A B S T R A C T**

The demand for plant material of *Rhodiola rosea* L. (Crassulaceae) for medicinal use has increased recently, amid concerns about its quality and sustainability. We have analysed the content of phenylpropanoids (total rosavins) and salidroside in liquid extracts from 3-year old cultivated plants of European origin, and mapped the influence of plant part (rhizome versus root), genotype, drying, cutting, and extraction solvent to chemical composition. Rhizomes contained 1.5–4 times more salidroside (0.3–0.4% dry wt) and total rosavins (1.2–3.0%) than roots. The qualitative decisive phenylpropanoid content in the extracts was most influenced by plant part, solvent, and genotype, while drying temperature and cutting conditions were of less importance. We have shown that *R. rosea* from different boreal European provenances can be grown under temperate conditions and identified factors to obtain consistent high quality extracts provided that authentic germplasm is used and distinguished between rhizome, roots and their mixtures.

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1. Introduction

*Rhodiola rosea* L. (Crassulaceae) derived medicines are based on Siberian plants, traditional Asian use and primarily Soviet/Russian research and development. The demand for high quality raw material has increased dramatically since it became popular as a herbal remedy worldwide (Saratikov and Krasnov, 1987; Brown et al., 2002). Extracts were originally standardized to their salidroside content, a glycoside based on p-tirosol and found in many *Rhodiola* species but also some other plants such as *Salix* and *Rhododendron* species (TPB 42; Bridel and Beguin, 1926; Thieme et al., 1969). However, the phenylpropanoid glycosides rosarin, rosavin and rosin are much more characteristic of *R. rosea* (plus few closely related (sub)species e.g. *R. rosea* ssp. arctica Boriss. depending on different taxonomic schools), and offer a distinction from other species traditionally used as drug sources including *R. sachalinensis* and *R. crenulata*, via the specified total rosavin content (Ros	extsubscript{tot}), calculated as rosavin, (phenylpropanol β-D-arabinosylpyran (1–6) β-D-glucopyranose) (Kurkin et al., 1985; Ph USSR XI, 1990). Resource shortages of wild *R. rosea* originating from mountainous or Northern boreal areas with short vegetative seasons are not easily solved due to the slow growth of the plant. Roots/rhizomes of usually 5–7 years old plants are required, producing challenges in natural resource management and cultivation. It triggers the risk of adulteration and substitution with other species/subspecies devoid of rosavins (Galambosi, 2006). Common but qualitatively different Asian *Rhodiola* species used as admixtures/substitutes in *R. rosea* labelled products, especially in health shops and on the internet, are the likely reason for zero or low Ros	extsubscript{tot} content detected in independent investigations (Ma et al., 2011). The controlled cultivation of properly authenticated material is therefore indicated, with the potential advantage of producing high quality material with faster biomass development in temperate climates. In addition to Russia, cultivation of *R. rosea* in Asia, America and Europe is increasing (Platikanov and Evstatieva, 2008; Kwesi, 2010). The suitability of growing some European provenances has so far been best investigated by B. Galambosi in Finland, but little has been published on the feasibility in a more temperate climate (Galambosi, 2006; Galambosi et al., 2007, 2009). We therefore cultivated six *R. rosea* provenances under equivalent conditions in south–east England. Plant age, growing conditions and all processing steps were kept consistent between genotypes, which allowed us to study biomass and content of 3-year old plants for inter- and intra-genetic variation avoiding typical confounding risks when using samples from different wild locations. Data
concerning root and rhizome were collected separately since the commercial drug descriptions do not usually specify which is used (Rhodiola rosea rhizoma, Rhodiola rosea radix or Rhodiola rosea rhizoma et radix). Furthermore, we assessed the influence of post-harvest factors (drying, cutting) and the extraction solvent when preparing traditional tinctures. The HPLC/DAD method was complemented with a targeted $^1$H NMR analysis to explore another tool for potential discrimination of drugs and preparations.

2. Results

2.1. HPLC profile and salidroside/Rostot content – influence of the genotype

The phenylpropanoid profile of ethanol 70% extracts was consistent for provenances R1–R5 with a 2.2–3.3 (2.1–3.2)-fold rosavin and a 0.3–0.5 (0.1–0.4)-fold rosarin content compared to rosarin for rhizome (root), respectively (Table 3). R6 deviated with rosavin 4.3 (root 8.6)-fold and rosarin 2.0 (root 2.1)-fold of rosarin. The XRM reference extract had similar rosarin:rosavin ratios but contained relatively more rosarin (Fig. 2). Rosiridin was detectable in all samples (more prominent in the XRM extract), while fresh tinctures showed several other peaks in addition to the reference dry drug; most notably a peak with a salidroside-like spectrum at 16.3 min (Fig. 2). The ratio of salidroside (plus its aglycone tyrosol) and rosavins (Saltot:Rostot ratio) indicates R1–R5 and XRM as containing predominantly phenylpropanoids, and R6 as containing more phenylethanoids (Table 3). Without taking the tyrosol content into account, the salidroside–Rostot ratio is often estimated as 1:3 for rhizoma et radix. Here, R1–R5 rhizomes had a 3.5–9.1 higher Rostot content over salidroside, and in roots, this was 1.2–3.7. R6 contained less Rostot than salidroside in both in the rhizome (0.7) and the root (0.5) (Fig. 2).

2.1.1. Assay

Liquid ethanolic (70%) rhizome extracts from cultivated plants of European provenance (R1–5) contained 0.4–1.1 mg/ml salidroside and 3.1–4.1 mg/ml Rostot, while corresponding root extracts 0.4–0.7 mg/ml salidroside and 0.8–1.7 mg/ml Rostot (Fig. 3). Despite low intra-genetic variability (maximum r.s.d. 15%, n = 6, for Rostot content in rhizomes) indicating an influence of the genotype, no significant inter-genetic differences have been obtained for the European plants (R1–R5) with 6 samples per genotype when cultivated/processed under the same conditions. The profile and salidroside content of the commercial accession R6 was comparable to the other (0.7 and 0.6 mg/ml in rhizome and root, respectively), apart from the 7.5 (4)-fold lower in Rostot content in rhizomes (roots) (Figs. 2 and 3). The order of genotypes with respect to the Rostot content was consistent for the other two solvents (see below) with minor deviations. Higher intra- and inter-genetic variability was obtained for the root that may be explained by the uncertain borderline between root and rhizome when splitting both parts (see Experimental: processing).

2.2. Rostot content of rhizome and root extracts – influence of drying

With mainly the Rostot content affected by genotype and plant part, we investigated with another batch whether drying and drying temperature may have an additional influence. Rostot was higher in the tinctures obtained from dried material compared to the tinctures from fresh material (Fig. 4). This was the case when focussing on the same used solvent, i.e. comparing the fresh tincture to 40% ethanolic tinctures (DSR 1:5) from the dried drug but also when simulating the effective ethanol concentration in fresh drug-solvent mixtures with 26% ethanol tinctures (DSR 1:7–7.5) from the dried drug.

Higher temperature (50 °C) did obviously not affect negatively Rostot values in the drug. In contrast, the Rostot extraction was in most cases more effective compared to drying at 20 °C. These effects were observed for both rhizome and root (approximately half of the rhizome content) and in principle consistent over the three tested genotypes with three plants each. The overall lower Rostot values of 5-year old plants harvested in November compared to 3 year old plants harvested in March can only be partially explained by the solvent polarity but linked to age and harvest season requiring further investigation.

2.3. Salidroside/Rostot content of rhizome and root extracts – influence of drug fragmentation

The influence of the degree of fragmentation of the drug was investigated by comparing extracts prepared from ground material (particle size <0.4 mm) and comminuted material (particle size between 1.5 and 2.5 mm) for rhizome and root of plants from two genotypes (Fig. 5). While the salidroside content seemed scarcely influenced by the particle size, comminuted material contained less Rostot than the ground material (more pronounced in the rhizome than in the root), although no significant difference was found. The particle size of the starting material may have less influence on the content of resulting extracts than other factors.
tested in this investigation. Ground material seems to be of advantage, while this may not be extrapolated to long term stability without data.

2.4. HPLC profile and salidroside/Rostot content – influence of the extraction solvent

The influence of the extraction solvent on phenylpropanoid profile but also the Salot-Rostot ratio was tested using four provenances. (Table 4, Fig. 6). Regardless differences between genotypes (R6 most deviating), two solvent-related effects were observed: (I) Although rosavin is usually considered as the main phenylpropanoid and is used for calculation of the Rostot content, we found that rosarin becomes relatively more concentrated in extracts of higher polarity, partially even exceeding the rosavin concentration. Reduced rosavin extraction with more polar solvents caused substantially distinctive rosarin–rosavin ratios in rhizome derived extracts from authentic R. rosea provenances 1:0.6–1.2 (ethanol 45%), 1:2.2–2.4 (ethanol 70%) and 1:2.5–4.6 (methanol). (II) The solvent polarity influenced the ratio Salot:Rostot independent from absolute values of these compound groups. In ethanol 45% rhizome extracts Salot is (relatively to Rostot) 1.5–3 times more present than in ethanol 70% (Table 4).

2.4.1. Assay

Across genotypes, salidroside was slightly higher in 45% extracts (rhizome 0.5–0.8 mg/ml, root 0.4–0.5 mg/ml) than in 70% extracts (rhizome 0.4–0.6 mg/ml, root 0.3–0.5 mg/ml) but below quantification limit in methanol extracts (Fig. 7). More pronounced decreased the content in Rostot with higher solvent polarity. Rostot was usually more than double in the rhizome as in the root. The Rostot value in 45% extracts (rhizome 0.8–3.0 mg/ml, root 0.3–1.1 mg/ml) was in all cases significantly different from 70% (rhizome 2.7–5.4 mg/ml, root 1.0–1.7 mg/ml) and methanol (rhizome 2.4–5.9 mg/ml, root 1.2–2.7 mg/ml) extracts but consistently low in all R6 derived extracts (<0.5 mg/ml). Taken together for European provenances in terms of the standard marker compounds, salidroside–rosavin ratios were 1:1.6–3.5 (1:0.7–2.1) in rhizome (root) 45% extracts and 1:4.2–12.0 (1:2.7–3.7) in rhizome (root) 70% extracts, while methanol extracts were comparable to

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**Table 2**

HPLC validation data for salidroside and rosavin.

<table>
<thead>
<tr>
<th></th>
<th>R²</th>
<th>Regression equation</th>
<th>Range (mg/ml)</th>
<th>LOD (µg/ml)</th>
<th>LOQ (µg/ml)</th>
<th>r.s.d. (%)</th>
<th>r.s.d. (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salidroside</td>
<td>0.9997</td>
<td>y = 1E + 0.6x–4353.1</td>
<td>0.005–5</td>
<td>2.7</td>
<td>5.6</td>
<td>5.9</td>
<td>9.0</td>
</tr>
<tr>
<td>Rosavin</td>
<td>0.9998</td>
<td>y = 1E + 0.7x–15659</td>
<td>0.0025–10</td>
<td>0.4</td>
<td>1.1</td>
<td>3.7</td>
<td>5.5</td>
</tr>
</tbody>
</table>

a Intra-assay precision within one analytical run 6 injections.

b Between-assay precision at 3 different days (each sample in duplicate).

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**Table 3**

Ratio of key compounds for 70% ethanolic extracts from rhizomes/roots of five European R. rosea provenances (R1–5) and a commercial accession (R6) (mean per genotype, n = 6).

<table>
<thead>
<tr>
<th></th>
<th>Rosarin:rosin</th>
<th>Salidroside:Rostot</th>
<th>Salot:Rostot</th>
<th>Rhizome</th>
<th>Root</th>
<th>Rhizome</th>
<th>Root</th>
<th>Rhizome</th>
<th>Root</th>
<th>Rhizome</th>
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<td>1:4.3</td>
<td>1:1.3</td>
<td>1:1.4</td>
<td>1:0.7</td>
<td></td>
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<tr>
<td>R2</td>
<td>1:2.4:0.5</td>
<td>1:4.9</td>
<td>1:2.2</td>
<td>1:2.6</td>
<td>1:1.6</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>R3</td>
<td>1:2.5:0.3</td>
<td>1:9.1</td>
<td>1:3.7</td>
<td>1:2.8</td>
<td>1:2.3</td>
<td></td>
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<tr>
<td>R4</td>
<td>1:2.3:0.3</td>
<td>1:3.5</td>
<td>1:1.2</td>
<td>1:2.6</td>
<td>1:0.9</td>
<td></td>
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<td></td>
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</tr>
<tr>
<td>R5</td>
<td>1:2.3:0.4</td>
<td>1:7.7</td>
<td>1:2.9</td>
<td>1:2.0</td>
<td>1:1.3</td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>R6</td>
<td>1:4.3:2.0</td>
<td>1:8.6:2.1</td>
<td>1:0.5</td>
<td>1:0.4</td>
<td>1:0.3</td>
<td></td>
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</table>

a Ratio AUC at 254 nm.

b Ratio (mg/ml) salidroside calc. at 275 nm: rosarin + rosavin + rosin calc. at 254 nm.

c Ratio (mg/ml) salidroside + tyrosol calc. as salidroside at 275 nm: rosarin + rosin calc. as rosavin at 254 nm.
70% extracts with respect to RosTot but practically devoid of salidroside.

2.5. Relationship between yield and content

The average yield of root + rhizome of 3-year old plants over all genotypes was fresh 272 ± 173.2 g (min 67.1 g, max 965.8 g) and dried 77.2 ± 53.4 g (min 16.5 g, max 325.1 g). The rhizome yield was higher than the root yield and represented usually two thirds (55–80%) of the drug. The fr. wt / dry wt ratio is around 3.6–3.8:1 for both plant parts, the loss on drying is moderately higher for the root (see Supplementary data Fig. S1). Fig. 8 shows root and rhizome samples from 6 genotypes with respect to the yield (abzissa) and the RosTot value (ordinate) derived from 70% ethanolic extracts. While the generally higher yield and RosTot content in rhizomes is obvious, also the potential selection of high RosTot/high yield plants (R3) and the inferiority of the commercial accession R6 becomes apparent. Nevertheless, correlation tests between salidroside and RosTot ('high content genotype') as well as between yield and salidroside or RosTot Content ('high content/high production genotype') did not reveal a general positive or negative correlation between these characteristics.

2.6. 1H NMR profile to distinguish plant and preparation dependent differences

The methanol-d4-soluble fractions of a subset of dried tinctures were analysed using 1H NMR. The solvent multiplet at 3.31 ppm was used for calibration and integration/normalization. We compared signals corresponding to aromatic protons (5.8–7.6 ppm), from the sugar moieties (3.9–4.5 ppm) and from the olefinic region (1.4–3 ppm) including those of rosavin and salidroside as described by Ndjoko Isot et al. (2011) (Fig. 9). Differences between rhizome and root (Fig. 9 A, B), extraction solvents (Fig. 9 C, D) and genotypes (Fig. 10) are most obvious quantitatively via key signal intensities such as those of the phenylpropanoids based on the methanol-d4-soluble fractions of a subset of dried tinctures. Although hampered by non-specificity of signals (e.g. glucose and arabinose moieties shared by compounds of both groups plus others),
overlapping or low-intensity, the sugar and olefinic region may provide additional information beyond phenylpropanoid and salidroside content to distinguish plant part, extracts and genotypes.

Principal component analysis of *Rhodiola* NMR data as already been tested for a small set of samples (Ndjoko Ioset et al., 2011) but is still challenging for use in quality dossiers and acceptance by regulators unless chosen algorithms and matrices are tested for robustness-validity or clearly correlated with actual composition data. The targeted integration of selected signals — although summarizing signals of several compounds — may allow a more simple way to characterize specified extracts once sufficient data have accumulated. In a first approach we tested the intensity for proton signals including those of rosavin (R)/salidroside (S) at 7.4, 7.3, 6.37, 7.05, 6.6, 4.05, 2.8, and 2.45 ppm (R 2/6', R 3/5', R 2, S 2/6', S 3/5', S 2a, S 1, unknown, respectively) for correlation with the HPLC determined average content of the tincture. No correlation over the whole sample matrix could be determined showing that different extract types are difficult to compare via collective proton signals. For some subsets of tinctures (e.g. same extraction solvent) the Rostot (HPLC mean) correlated with the signal strength such as at 7.4 ppm (dominated by the 2/6' proton signals for phenylpropanoids) (Fig. 11A). Signal intensities of two selected buckets supposed to include primarily signals of phenylpropanoids or phenylethanoids, can be used to distinguish easily plant part and solvents (of one genotype) as well as genotypes (same plant part and solvent), e.g. the deviation of the provenance R6 from the authentic *R. rosea* plant extracts (Fig. 11B).

### 3. Discussion

#### 3.1. Qualitative suitability of European plants cultivated under temperate conditions

Various reports exist on the rosavin, Rostot and salidroside content in *R. rosea* (Supplementary Table S1). Plant origin, growing site, plant age, extraction solvent and processing methods vary, or are often not precisely stated. Except a few studies with clear distinction between rhizome and root, authors often refer to ‘underground parts’. Equally when specified on products, e.g. when labelled as ‘root’, rhizome plus root may be assumed. Only in some cases is the age of the plant known and a defined harvest season is an exception. Here we provide systematic data for plants from European habitats of the same age grown under the same temperate conditions. The direct HPLC analysis of liquid extracts with minimal sample preparation steps proved to be suitable despite limitations. Based on the consistent drug–solvent ratio of 1:5, estimates can be derived from the composition of liquid extracts to the original drug. Assuming the traditional 70% ethanolic extraction produces close to an optimum yield, dry rhizomes from authentic plants (R1–R5, 3-year old, harvest in March) contained 1.6–2.1% salidroside with corresponding increases in the aglycone tyrosol (rhizome 0.25–0.4%, root 0.2–0.25%) were below average of other investigators and monograph requirements (TPB 42) but are in the range of previous reports when less polar solvents have been used (Table S1). Diversity in salidroside contents from different authors may also be attributed to analytical issues such as a co-eluting peak causing too high integrals when analysed at 205 nm, as previously mentioned. Moreover harvest season, plant age, and plant sex appear to influence salidroside values (Platikanov and Evstatieva, 2008). On the other hand relatively low concentrations of salidroside with corresponding increases in the aglycone tyrosol content, as found in our liquid extracts, suggests decomposition and therefore instabilities within liquid extracts. In summary, our data support cultivation in standard agricultural systems to protect wild

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**Fig. 4.** Rostot (total rosavins; mg/ml) in rhizome and root derived tinctures (ethanol 40% ethanol 26%) derived from the dried (20 °C or 50 °C) or fresh drug (40% ethanol corresponding to 26% ethanol considering water content in the fresh drug) from five-year old plants of three provenances (RII, III, V) harvested in November. Mean (±s.d.) of 3 single plants per genotype and measurement in duplicate.

**Fig. 5.** Salidroside and Rostot (total rosavins; mg/ml) in rhizome and root derived tinctures (ethanol 70%) of two *R. rosea* provenances (R4, R5) either ground or comminuted. Mean (±s.d.) of 3 single plants per genotype and measurement in triplicate.
plant populations. The five European provenances (R1–R5) from very different habitats (Alps, Pyrenees, British Isles, Scandinavia, Iceland) showed, after homogenous cultivation, moderate inter-genetic differences in yield, Rosrot and salidroside content although their significance and consistency has still to be shown over a longer time-frame. So far, comparison between plants of the same genotype as well as relative differences between root and rhizome showed remarkable intra-genetic consistency.

3.2. Authentication

Accession R6, labelled as *R. rosea*, was said to originate from Russia (verbal communication, by supplier) but was low in Rosrot (<0.7% in ethanol 70%) whilst similar to the other plants in terms of salidroside. Neither the HPLC fingerprint nor the NMR spectra revealed other major deviations. It demonstrates that other species may reach the market as *R. rosea*, which is only visible when analysing the Rosrot content unless plants are morphologically compared to authentic material. This is supported by a recent publication showing that out of 18 *R. rosea* root powder samples 9 contained no phenylpropanoids at all or only minimal amounts clearly less than salidroside (Ma et al., 2011).

3.3. Suitability of ratio markers

Our results suggest that authentic *R. rosea* derived rhizomes (roots) deliver ratios of salidroside, phenylpropanoids and presumably rosiridin within certain criteria. As a first approach, we compared the influence of genotype, plant part and solvent on the Salrot/Rosrot ratio and the phenylpropenoid profile. While the first ratio was mainly determined by the plant part but also solvent and genotype, the latter was affected by genotype and surprisingly the extraction solvent, but less by the plant part. Drying and cutting have a limited influence. Plant age, extract age and storage conditions were consistent here but may contribute to profile variation. First data on late-harvested samples indicate a variable season profile, giving the harvest time some importance (not yet published).

HPLC based data by other authors match approximately our rosarin/rosavin/rosin ratios with partially more rosavin dominance (Ma et al., 2011; Ganzera et al., 2001; Altantsetseg et al., 2007). A SPC-TLC densitometry analysis describes completely different ratios for plants from Lithuania and the Altai with 1:0.06:0.5 (1:0.1:2.1) in 40% ethanolic extracts and 1:1.4:0.4 (1:0.5:2.2) in 70% ethanolic extracts, respectively (Kucinskaite et al., 2007). Also Altantsetseg et al. (2007) found moderate solvent-related differences: 1:4:0.1:1 (water), and 1:6:1:1.0 (ethanol 75%). Without data on the pharmacological and clinical difference between the 3 glycosides, their relevance is not yet clear. The additional detection of the mutual hydrolysation product trans-cinnamic alcohol has already been considered by some authors and might be justified chemically as well as pharmacologically (Kurkin et al., 1989; Galambosi et al., 2007; Altantsetseg et al., 2007). With increased data accumulation, marker ratios such as Sal/Rosrot, Salrot/Rosrot, as well as rosarin/rosavin/rosin and salidroside/tyrosol (showing the degree of decomposition) may be used to define thresholds for authenticity and quality control. In the future, other chemical markers such as rosiridin may be chosen, to reflect known pharmacological effects (van Diermen et al., 2009), and genetic authentication may be used to supplement chemical methods (Kylin, 2010).

3.4. Root versus rhizome

The fact that rhizome extracts from the same plant contained usually 2.5–3.5 more Rosrot than the root extracts may partially

### Table 4

<table>
<thead>
<tr>
<th>Accession</th>
<th>Rosrot, mg/ml</th>
<th>Salrot, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>R1</td>
<td>1:1.1:0.4</td>
<td>1:2.5:0.4</td>
</tr>
<tr>
<td>R2</td>
<td>1:2.3:0.4</td>
<td>1:3.0:0.4</td>
</tr>
<tr>
<td>R3</td>
<td>1:3.2:0.3</td>
<td>1:4.3:0.5</td>
</tr>
<tr>
<td>R4</td>
<td>1:3.7:1.0</td>
<td>1:5.2:0.4</td>
</tr>
<tr>
<td>R5</td>
<td>1:4.3:1.3</td>
<td>1:6.7:0.7</td>
</tr>
<tr>
<td>R6</td>
<td>1:5.9:0.5</td>
<td>1:7.1:0.9</td>
</tr>
</tbody>
</table>

* Ratio AUC (254 nm).

* Ratio (mg/ml) salidroside + tyrosol calc. as salidroside at 275 nm: rosarin + rosavin + rosin calc. as rosavin at 254 nm.
more important with the increasing use of 3–5 year old cultivated plants, where both plant parts are much easier to separate and distinguished as it is the case for morphologically more heterogeneous wild material of unknown age (Aiello et al., 2010). Differences in the salidroside content and fingerprint were visible in extracts from root and rhizome, and the 45% ethanolic extracts in particular displayed more non-identified peaks in the root than in the rhizome. We tested extracts for a range of standard flavonoids, flavonoid glycosides and phenolic acids (data not shown) and demonstrated the presence of chlorogenic acid but no substantial flavonoid content, which had been reported by Galambosi (2006) as being greater in the roots. Chemical differences between rhizome and root have partially been described in previous investigations. Platikanov and Evstatieva (2008) reported up to double the salidroside content in rhizomes compared to roots which was more pronounced in male plants than in female with additional influence of the harvest season. Przybyl et al. (2008) found also higher amounts of rosavin and rosarin (but not rosin) as well as salidroside (but not tyrosol) in the rhizome. Taken together, existing data advocate that for standardisation a clear distinction should be made between the root and rhizome or, as is commonly found in commerce, a mixture of both. In our samples, there was a dominant rhizome portion (55–80%, Fig. S1). Similar ratios are also reported by (Galambosi, 2006; Galambosi et al., 2009) and Przybyl et al. (2008) from cultivation in Finland and Poland with variation according to plant age and the harvest technology. In our homogenously cultivated plant selection, a substantially deviating root–rhizome ratio also signalled another species.

3.5. Extraction solvent

In line with previous publications, the highest Ros tot concentrations were obtained using moderate polar solvents for extraction, while salidroside yielded more efficiently with hydrophilic solvents (45% ethanol) (e.g. Altantsetseg et al., 2007). An extraction solvent of 30–60% ethanol may offer a compromise that reflects traditional preparations. Sub-optimal yields of both require high value starting materials. Other solutions have already been found for products on the market, such as mixtures of the plant powder or water extracts with extracts >70% ethanol. We have shown that increasing the ethanol portion from 45% to 70% approximately doubled the salidroside–Ros tot ratio due to the slightly lowered salidroside levels but up to 50% higher Ros tot levels. Further reductions in the aqueous concentration of the extracting solvent led to a substantial reduction of salidroside (below detection level in methanol 100%). In view of the qualitative and quantitative data obtained in this study, extracts made using solvent mixtures with substantially different aqueous ratios cannot be considered comparable regarding their concentration of Ros tot or their salidroside–Ros tot ratios, which might be decisive for the therapeutic activity.

3.6. Drug yield and cultivation aspects

Differences in yield between genotypes is apparent (Supplementary Fig. S1), however, their significance remains to be shown over a longer period of time and with a higher sample size. R. rosea is known to have a slow development over the first 1.5 years and the results obtained under temperate southern English conditions are comparable to previous results from Russia, Finland and Canada. From cultivated plants in Russia, Kim (1999) reported rhizome yields of 167.7 g fresh material after 3 years, 422.0 g after 4 years, and 542.6 g after 5 years, while Galambosi (2006) harvested for instance 370 g (root + rhizome) after 3 years and 790 g after 4 years cultivation in the field. Ampong-Nyarko et al. (2011) gave an
average of 58.4 g (25–146 g) dry mass for mixed samples from 3-year old plants from 21 farms in Canada.

For selection purposes a ‘high yield/high content’ and a plant high in both relevant constituent groups (‘high phenolic content in general’) would be of advantage. Our first data provided no correlation between Rostot and salidroside nor between biomass and Rostot or saliroside. We had expected that plants from more extreme climates, when transferred to more moderate conditions...
and with a longer growing season, would experience a ‘diluting effect’, i.e. a higher biomass but lower concentrations of secondary metabolites. Even if this was the case, it was not to an extent that would prevent the production of a suitable pharmaceutical drug.

3.7. Conclusion

In summary, we have shown that *R. rosea* provenances native to European boreal areas are suitable for medicinal purposes when grown under temperate conditions, and that production is feasible in principle as long as only properly authenticated material is used. Consistency of genotype characteristics over a longer period, and efficiency in terms of yield and qualitative superiority of some genotypes still has to be shown with long-term data. The plant part efficiency in terms of yield and qualitative superiority of some genotypes still has to be shown with long-term data. The plant part

4. Experimental

4.1. Seed material, cultivation and plant characteristics

Collected seeds from five wild provenances in Switzerland (R1), Iceland (R2), Shetland (R3), Spain (R4) and Finland (R5) authenticated by Dr. Kump (Linz, Austria) were completed with one sample purchased from a commercial seed supplier (R6, Lot: 80012015100 delivery No. 373081 2006, Jelitto, Schwarmstedt, Germany). Voucher specimens have been deposited at the herbarium of the Biologizentrum der Oberösterreichischen Landesmuseen (Linz, Austria; No: 736649–736656). After seeding in early spring 2006, seedlings were re-potted twice and in November 2006, 30 individual plants per provenance were planted (three blocs with ten individuals each per genotype, 30 cm in and between rows) into experimental plots at University Reading (Berkshire, UK). The six provenances differed in terms of robustness (propagation and survival rate), morphology and biomass production (details not presented in the context of this paper). While geographic variations, in particular the shape of the leaves and the height of the shoots, is known for *R. rosea* from different geographical origins, it soon became obvious that the morphology of stem, leaves, flowers, rhizome and root of provenance R6 deviated from the other five. In addition, the missing characteristic rose-like scent (supposed to derive from the geraniol containing essential oil, see Rohloff, 2002) and red colouration of rhizome cuts plus the variability of characteristics (e.g. colour of the male flowers) and number of growth abnormalities (stem fasciation, androgynous flowers) did not support the identification of R6 as *R. rosea* but more likely as a cross with another *Rhodiola* species (Fig. 1).

4.2. Harvest, yield and processing

Six plants per provenance (randomly two per bloc) were harvested in March, 2009. Male and female plants were not distinguished. After washing, root and rhizome were separated, cut, and dried for 5 d at 40–50 °C (Fig. 1). Fresh and dry weights and their ratios (fr. / dry wt) were recorded. Dry samples were stored in paper bags at temperatures of 12–25 °C. Due to the natural gradient transition from partly underground rhizome into main roots the latter may be still contain adjacent parts of the rhizome up to approximately 15%. However, root and rhizome in 3–5 year old cultivated plants are much easier to distinguish and separate compared to wild plants (Aiello et al., 2010). Further cleaning of roots from rhizome residues was not attempted for three reasons: (1) to maintain the overall yield without losses (2) both organs are normally used together in commercial drugs (3) drug producers when separating manually would use the same cutting approach without further distinction between both parts.

For the drying test, another nine plants from three provenances (RII, RIII, RV) were harvested in November 2011, root and rhizome of each plant split into three parts for drying at room temperature (18–20 °C, 7 d) at 50 °C (5 d) or used fresh (2 d after harvest) for extraction.

4.3. Cutting and extraction

The cut dried drug consisting of 1–8 cm long pieces was powdered using a mill (MF–10 basic, IKA® Werke GmbH & Co. KG, Germany) and a sieve of 0.5 mm mesh diameter. A subset of samples was only comminuted using the same mill, without a sieve,
resulting in pieces of 1.5–5 mm diameter; subsequent comparisons are presented as ‘ground’ versus ‘comminuted’. According to the pharmacopeial standard, to produce a 1:5 ratio between drug and extraction solvent for tinctures, 10 g drug was covered with 50 ml solvent (ethanol 45% v/v, ethanol 70% v/v or methanol, all analytical grade; Fisher, UK) in 50 ml test tubes wrapped in aluminium foil and moderately shaken for 2 h. After 5 d storage at room temperature and another 30 min shaking the tinctures were filtered and stored at 4 °C in the dark. HPLC analysis was performed after 3 months.

For the drying test, fresh samples were cut into 5–10 mm pieces and approximately 100 g of each macerated with ethanol 40% adjusted to a drug solvent ratio (DSR) 1:5 (dry mass) for 20 d at 18–20 °C based on the loss on drying (tr./dry wt ratio). By ascribing this loss on drying (1:3.2–4.2) to mainly loss in water this corresponds to a 26% ethanolic tincture with a drug solvent ratio of approximately 1:7.5 for rhizomes and 1:7 for roots. Dried samples (20 or 50 °C) were equally cut, split and macerated with ethanol 40% (drug solvent ratio 1:5) as well as ethanol 26% (drug solvent ratio 1:7–7.5) resulting overall in 5 rhizome and 5 root tinctures per plant: dried 20 °C/40% ethanol, dried 20 °C/26%, dried 50 °C/40%, dried 50 °C/26%, fresh/40% (26%). This allowed a comparison fresh–dry either focused on the used solvent (ethanol 40%, DSR 1:5) or the effective ethanol concentration during maceration (ethanol 26%, DSR 1:7–7.5).

4.4. HPLC equipment and procedure

A HPLC Waters™ 2695 separation module, with a Waters™ 996 PDA detector controlled by Empower™ software was equipped with an Luna C18(2)100A (150 × 4.6 mm, 5 μm) column (Phenomenex UK, Macclesfield) thermostated at 60 °C. The mobile phase consisted of water (A), and acetonitrile (B), (HPLC grade, Fisher, UK) with a gradient elution from 95A/5B in 30 min to 80A/20B at a flow rate of 1.0 ml/min followed by a 5 min wash and an equilibration period for 5 min. Tincture samples were injected directly (20 μl injection volume) after filtration through 0.45 mm syringe filter (PFF 13 mm, Jaylee Biosciences Ltd, UK). Reference standards (salidroside, rosavin, and a reference extract named ‘XRM’, all LGC Promochem, Teddington, UK) were diluted in ethanol 45%, ethanol 70% and methanol and injected in five different concentrations. Blanks and reference standards were applied after each 12 sample injections. Every sample was injected in duplicate and the complete set analyzed at three different days. The rosavin reference standard was stable over 1 year when stored at 4 °C, while salidroside showed some decomposition after 8 months with an increased tyrosol peak.

4.5. HPLC method development

The direct analysis of tinctures without pre-treatment was based on the method by Ganzera et al. (2001) using water instead of phosphate buffer and acetonitrile solvents. It allowed for the detection of the five marker compounds (salidroside, tyrosol, rosarin, rosavin, rosin, rosiridin) at 205 nm with overall satisfying peak resolution. When injecting hydroethanolic tinctures directly, baseline drifts impeded quantification of all marker compounds at 205 nm. Extra preparatory steps, such as prior removal of tannins, were avoided by recording chromatograms at 254 nm (near absorption maximum of phenylpropanoids) and 275 nm (near absorption maximum of phenylethanoids). This allowed quantification of salidroside and rosavins amended by a qualitative overall fingerprint (rosiridine is only detectable at 205 nm). Notably, at 205 nm salidroside eluted in a mixed peak potentially giving too high integrals, while at 275 nm the two peaks were separated (rt 6.3: λmax 208 nm, rt 6.7 min: λmax 217, 278) (Figs. 2 and 3).

The rosin peak (rt 27.2: λmax 248 nm) was not always clearly separated from another peak (rt 26.8: λmax 210 nm), but at 254 nm the influence of the co-eluting peak was reduced.

4.6. HPLC fingerprint

The HPLC profiles showed up to 20 peaks with salidroside (UV λmax 274 nm), tyrosol (UV λmax 274 nm), rosin (UV λmax 248 nm), rosavin (UV λmax 248 nm), rosarin (UV λmax 248 nm) and rosiridin (no absorption between 200 and 400 nm) among the predominant ones that were identified using standard references (including spiking of extracts), together with typical UV-spectra and comparison with the literature (Table 1, Fig. 2). Most non-identified peaks had spectra similar to the phenylethanoids salidroside and tyrosol (10.2, 15.2, 16.2, 19.9 min) or no absorption maxima (6.3, 7.6, 26.8 min). Peaks with other spectra (e.g. 21.5 min: λmax 275, 325; 13.5 min: λmax ~261–267 nm) belong presumably to other phenolic glycosides, but did not include spectra typical of flavonoids.

4.7. HPLC assay and validation

Salidroside and tyrosol and their sum Saltot (at 275 nm) and (rosavin, rosin and rosarin, = Rostot, at 254 nm) were quantified by means of external standard (Table 1). The limit of detection measured with single and extract reference standards was 2.7 μg/ml for salidroside and 0.44 μg/ml for rosavin with a signal to noise ratio of 3 or higher. The limit of quantification was 5.6 μg/ml tincture for salidroside and 1.1 μg/ml for rosavin with a signal to noise ratio of 10 or higher. The linearity was determined in the range of 0.005 mg/ml to 5 mg/ml (salidroside) and 0.0025 mg/ml to 10 mg/ml (rosavin) with a linear relationship as provided in Table 2. The precision was checked by 6 repeating injections of standards within one analytical run (r.s.d. 5.9% for salidroside and 3.7% for rosavin) and on three different days with each sample injected in duplicate (r.s.d. 9.0% for salidroside and 5.5% for rosavin). The recovery tested thrice by adding salidroside/rosavin to a low content root tincture was between 87.1% and 122.0% for rosavin and between 96.8% and 111.7%.

Three ratio markers potentially relevant for analytical and pharmacological comparison of extracts were calculated for some comparisons: the rosavin/rosiridin ratio (with rosarin set as 1; =phenylpropanoid ratio), the ratio between salidroside and total rosavins (Rostot = Σ rosarin, rosavin, rosin, rosiridin) and the ratio between total salidroside (Saltot = salidroside + tyrosol) and total rosavins (Saltot: Rostot) (Table 1).

4.8. Statistical analysis

Salidroside and Rostot values (HPLC) represent mean ± s.d. (n 3–6). When appropriate, pair-wise comparisons of specific treatments to the control treatment according to the experimental design were performed and relevant control treatments indicated in the figure captions. All results were statistically evaluated for significance with GraphPad® (InStat 3) statistics software by one-way ANOVA followed by the Bonferroni’s post-test. Significant differences from selected control or treatment are shown as *P < 0.05. Correlations between yield and Rostot and salidroside content were tested using Pearson’s correlation test.

Supplementary data: Total yield data were calculated as mean ± s.e. of 6 individual plants per genotype (inter-genetic variance), significance between genotype groups was tested using Student ANOVA/MRT and indicated using different letters when p < 0.05. Intra-genetic variation between individual rhizome and root samples (fr. wt and dry wt) were expressed as ±r.s.d.
4.9. $^1$H NMR sample preparation, equipment and processing

For a subset of 38 tinctures covering all variations of the factors plant part, genotype, solvent, cutting, 1 ml of each tincture were transferred into a 5 ml amber vial and dried under nitrogen until reaching a constant weight. The residue was dissolved in 1 ml methanol-$d_4$ using 3 min sonication. Eight hundred μl were transferred to the NMR tube and left to stand for 2 h before using the supernatant for analysis.

NMR spectra were recorded on Bruker AVANCE 400MHZ spectrometer ($^1$H, 400 MHZ) equipped with a multinuclear probehead with z-gradient. The TopSpin 3.1 software was used for spectra acquisition and processing. The standard 1D $^1$H NMR spectra are acquired with 30° pulse length, relaxation delay of 2 s. The numbers of scans were 64. The spectra were recorded at 300 K. Spectra were calibrated and normalised on the methanol-$d_4$ solvent peak.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.phytochem.2012.10.005.

References


Bridel, M., Beguin, C., 1926. Isolation of rutinoside, asparagine and a new glycoside, hydrolysable by emulsion, salidroside from $S$alix $t$riandra $L$. $S$eacons $A$cad $S$cience 183, 321–323.


Kim, E.F., 1999. Rodiol$\text{a}$ roso$\text{v}$aya ($Zo$loto$\text{y}$ kore$\text{n}$y) i biologiceskie osnovy vvedeniya yeyo v kultury. Dissertation Al$\text{t}$ai State University, Barnaul.


TPB 42: Temporary Pharmacopoeia Ordinance No 42 ‘Rosavidinum’. Ministry of Health of the USSR, Authority for introduction of new medicines and medical devices, Pharmacopoeia committee, Severzev WA, Denisov IN.
