Development of Biotechnological Protocols in Medicinal Plants Research – Contribution for safeguarding and standardization of raw material for Phytotherapy using *Hypericum* species indigenous to the Balkan region

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Introduction and Aim of the Research Activities

Threats for medicinal plant species are unmonitored trade, over-exploitation, destructive harvesting techniques, as well as habitat loss and habitat changes. As a result, diminution of population sizes, genetic diversity and eventually the extinction of the species could occur.

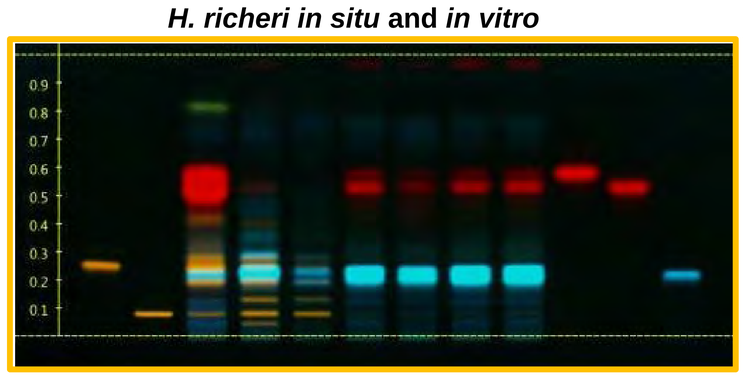
PhytoBalk project - a Bulgarian-Swiss Joint Research Project - strives for

- Biotechnological Protocols for in vitro Cultivation, Standardization and Conservation of valuable medicinal plants indigenous to the Balkan region
- Exploring the practical applicability of biotechnological cultivation techniques for standardized production of medicinal plant raw material
- Hypotheses to be tested: By controlling culture conditions in vitro, it is possible to target the production of plant biomass with desired properties.

Preliminary Results

HPTLC fingerprint comparison of Hypericum sp. from different cultivations

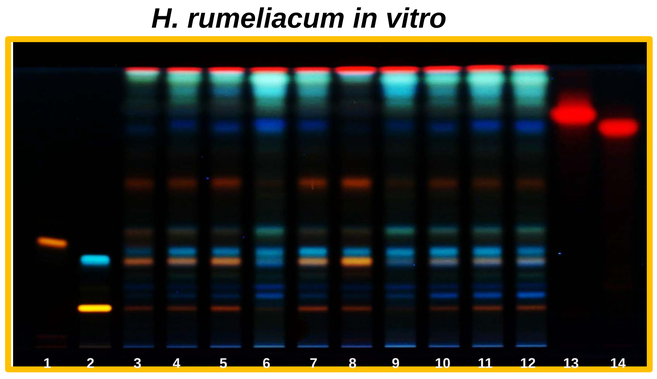
H. richeri in situ* and *in vitro



1 Hyperosid, **2** Rutin
3 *H. richeri in situ* leaves,
4 *H. richeri in situ* flowers,
5 *H. richeri in situ* stems

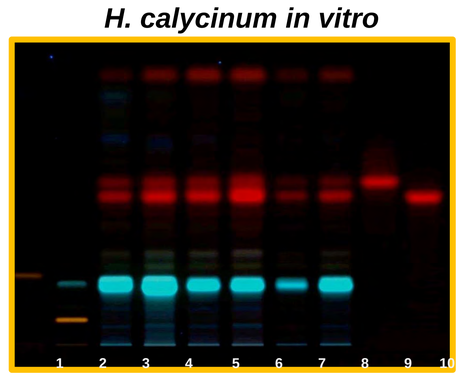
6-9 *H. richeri in vitro samples*
10 Hypericine **11** Pseudohypericine
12 Chlorogenic acid

H. rumeliacum in vitro



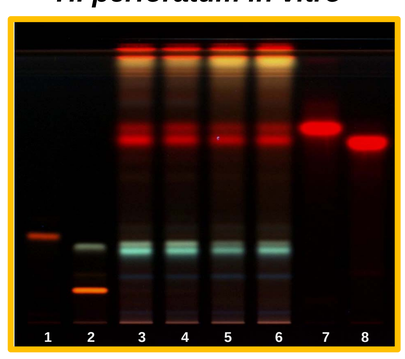
1 Hyperosid, **2** Rutin, Chlorogenic acid
3-12 *H. rumeliacum in vitro* samples
13 Hypericine ,
14 Pseudohypericine

H. calycinum in vitro



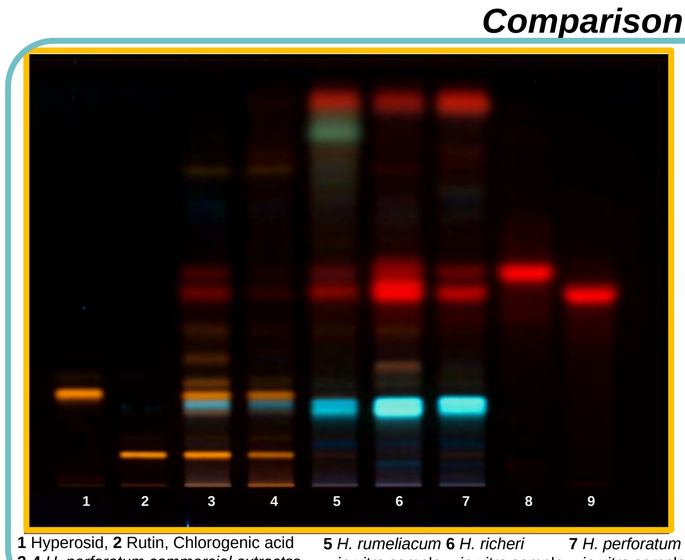
1 Hyperosid, **2** Rutin, Chlorogenic acid
3-8 *H. calycinum in vitro* samples
9 Hypericine ,
10 Pseudohypericine

H. perforatum in vitro



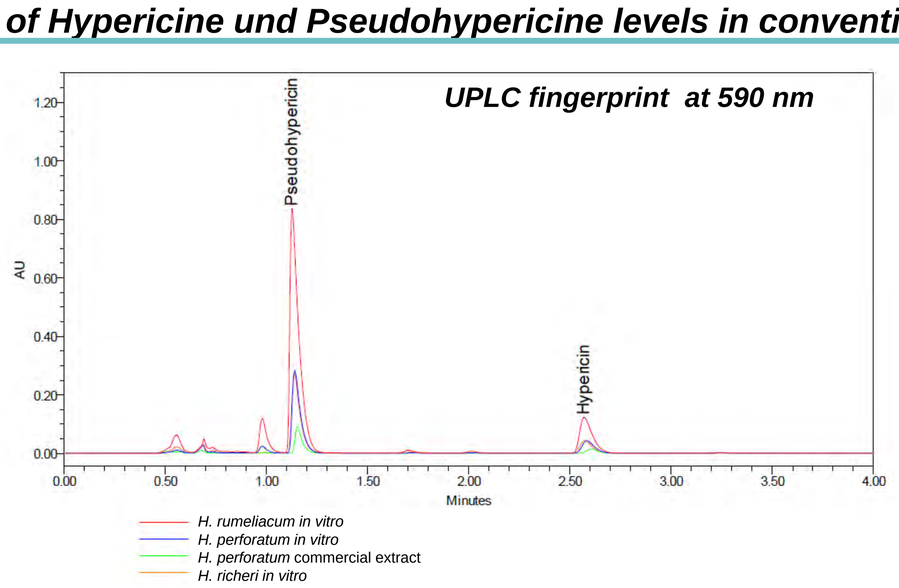
1 Hyperosid, **2** Rutin, Chlorogenic acid
3-6 *H. perforatum in vitro* samples
7 Hypericine ,
8 Pseudohypericine

Comparison of Hypericine und Pseudohypericine levels in conventional and in vitro samples

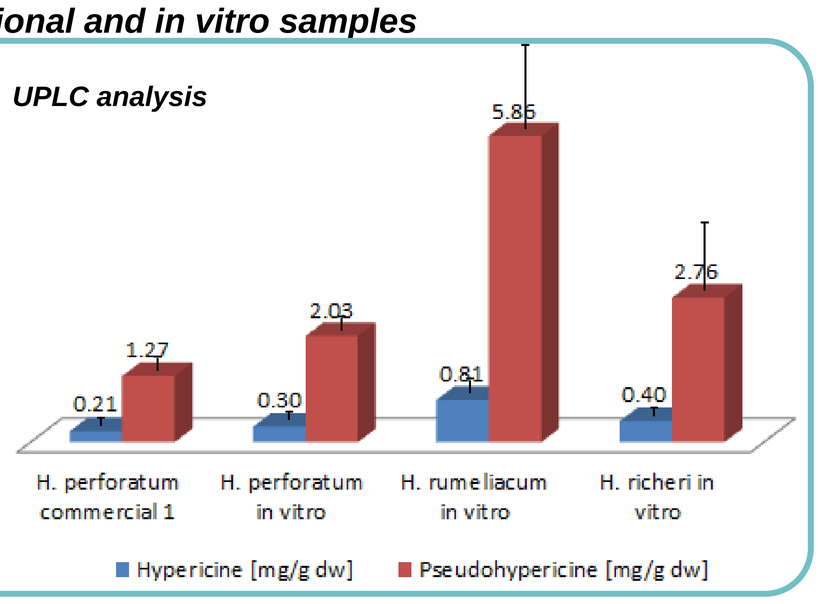


1 Hyperosid, **2** Rutin, Chlorogenic acid
3-4 *H. perforatum commercial extractss*
5 *H. rumeliacum in vitro* sample **6** *H. richeri in vitro* sample **7** *H. perforatum in vitro* sample
8 Hypericine, **9** Pseudohypericine

UPLC fingerprint at 590 nm



UPLC analysis



Discussion and Preliminary Conclusions

- In *in vitro* samples of all tested *Hypericum* species, Flavonoids rather lower, whereas Hypericine and Pseudohypericine levels comparable up to slightly superior to commercial *H. perforatum* extracts except for *H. calycinum*, where almost no Hypericine and Pseudihypericine can be detected
- The development of biotechnological protocols provides innovative tools for medicinal plant research: enabling robustness studies against plant diseases and alternative and controllable production technique for target secondary metabolites.
- *Standardization and Safeguarding:* Conventional cultivation *H. perforatum* might show high variability of monographed Hypericine levels due to environmental factors. Anthracnose threatens yield. Micropropagation with subsequent field cultivation or in vitro production of native material could offer reliable yield with lower content variability
- Current Regulatory Limitations: Non-monographed *Hypericum* species are not an alternative source for pharmaceutical applications. Pure *in vitro* cultivation not yet accepted as production technique for medicinal plant biomass.
- *Sustainability*: Aspect of the conservation of biodiversity and basic research for non-foreseeable future applications guides the scientific activities

Materials and Methods

H. perforatum and H. richeri conventionally cultured plant material

H.richeri was collected from its wild habitat in the Vitosha mountain, Bulgaria. The aerial parts were air dried until constant weight. Two *H. perforatum* dried native commercial extracts were kindly provided for Quality Control purposes from two different Phytopharma companies.

In vitro plant material

Shoot cultures of the different species were initiated as previously described [2, 3]. From each species upper plant parts of the in vitro plantlet from different media variants using different plant growth regulators at varying concentrations have been harvested and air dried prior to extraction

Extraction and Sample preparation

Dried plant material was exhaustively extracted in methanol in ultrasonic bath for 10 min and 2h maceration at 25 °C. Then extracts were filtered and dried *in vacuo*.Extracts were resuspended at a concentration of 10 mg/ml sample in Methanol. Samples were stored at -20°C in the dark prior to analysis.

Dried native extract was resuspended at a concentration of 10 mg/ml sample in Methanol.

HPTLC Analysis

CAMAG HPTLC equipment ATS4, ADC2, TLC Visualizer and Software were used for standardized conditions. HPTLC Silica Gel 60 F_{254} plates 20x10cm (Merck) were used for all experiments.
Method from [1]: Developing distance: 80mm Mobile Phase: ethyl acetate/water/formic acid (90:9:6 v).

HPTLC Analysis (cont.)

References
All references prepared in Methanol: 5µl Rutin 0.05 mg/ml, 10µl Chlorogenic acid 0.01 mg/ml 10µl Caffeic Acid 0.01 mg/ml
Hypericine and Pseudohypericine 30 ug/ml

Phytochemical Detection
Heating for 5min at 110°C prior to subsequent dipping in 5g/l NP in ethylacetate and 50g/l PEG 400 in dichloromethane. Detection at 366 nm.

Radical Scavenging Detection: DPPH

Dipping in 0.05% w/w DPPH in methanol. Detection in white light (transmission).

UPLC Analysis

Method adapted from Ph Eur [1] to UPLC

Waters Acquity HSS T3, 1.8um, 2.1x100mm

T=40°C
F=400ul/min
Runttime=8 min

Isokratic: EtAc 39T / PO4-puffer 0.23mol/l, pH 2.0, 41T / MeOH 160T

Acknowledgements

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References

[1] European Pharmacopeia 7.7 online edition. [2] Danova K et al. (2012) Natural Product Communications, 7: 1–2. [3] A. Mehandzhiyski et al. (2013) Bulgarian Journal of Agricultural Science, 19 (2) 2013, 31–34.

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