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HPLC profiling and quantification of active principles in leaves of *Hedera helix* L.

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Ivy (*Hedera helix* L., Araliaceae), is an evergreen medicinal and ornamental plant. Depending on leaf polymorphism different shaped ivy leaves were extracted and subsequently analyzed by reversed-phase high performance liquid chromatography (RP-HPLC). Quantitative determination of its most prominent saponins hederacoside C (**1**) and α-hederin (**2**) from different ivy leaf extracts were detected, validated and optimized for quick profiling. The linearity of response, repeatability and reproducibility of the applied RP-HPLC method are reported.

1. Introduction

The genus *Hedera* of the ginseng family (Araliaceae) is represented with 6 species in Europe (Hegi 1975). The common ivy, *Hedera helix* L. is a climbing evergreen woody plant mostly found on different trees, walls, rocks as well as trailing type on the ground, besides Europe also in North and Central Asia and in the Americas. The plant adheres to different surfaces by the support of tiny roots. Flowers are produced in winter or early spring and the flowering branches have different-shaped leaves compared to the non-flowering ones. The polymorphic leaves can be shiny, leathery in different shapes and sizes such as ovate, lanceolate, tri-lobed, ovate-rhomboid or five-lobed (Fig. 1). The plant bears yellow or greenish-yellow flowers in round clusters in the fall. Small fruits develop during the winter and are dark purple or black, sometimes yellow (Hegi 1975, Horz and Reichling 2003, Brendler et al. 2003).

The medicinally important parts are the leaves. In traditional medicine *Hedera helix* was used for a wide number of complaints, especially against bronchitis, whooping cough, arthritis, rheumatism, aches and dysentery. Decoctions of the herb were applied externally against lice, scabies, and sunburn. The sap was applied against headache and earache (Horz and Reichling 2003, Brendler et al. 2003, Wichtl 2002). *Hederae helicis folium* is present in the Deutsche Arzneimittel Codex (DAC 1997) and the Homeopathic Pharmacopoeia (HAB 2000). Actually a new monograph is about to be established for the European Pharmacopoeia (PhEur 2002). The drug is approved by the German Commission E for its efficacy against chronic inflammatory bronchial conditions and productive coughs due to its actions as an expectorant and its spasmolytic effect among children and adults (Blumenthal et al. 1998). In clinical studies, ivy leaf extract given to children with bronchial asthma also showed an improvement of the airflow. Several *in vitro* and *in vivo* experiments were reported covering a wide spectrum of pharmacological activities (Hegi 1975, Horz and Reichling 2003, Brendler et al. 2003, Blumenthal et al. 1998, Büechi and Kähler 2003, Hofmann et al. 2003, Trute et al. 1997, Wagner and Reger 1986, Delmas et al. 2000). However, the leaf and sap can be quite irritating and may cause allergic skin reactions due to saponins and polyacetylenes (Ozdemir et al. 2003).

Phytochemical analysis of ivy can comprise a wide range of compounds such as bidesmodic triterpene saponines (e.g.

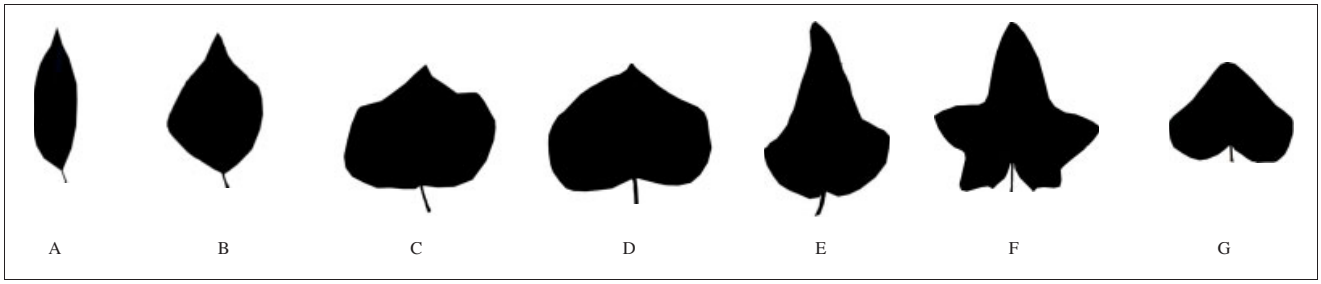


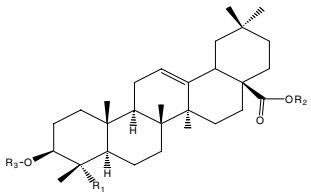
Fig. 1: Different shapes of *Hedera helix*.
A: Lanceolate, B: Lanceloate to ovate, C: Ovate, D: Cordate to ovate, E: Tri-lobed, F: Five-lobed, G: Cordate

Table 1: Plant material and hederacoside C (1), α-hederin (2) contents in Hedera helix extracts

No	Code*	Brief Description	Plant material origin	1 (%)	2 (%)
1	CS	Commercial herbal drug sample (Ready cut in to approx. 5–10 mm^2)	Commercial sample of Caesar & Loretz GmbH, Hilden, Germany (Ch.B.11750152)	5.03	0.83
2	A1	Bright, shiny, green, soft, young leaves (approx. 2–4 cm length and 1.5–2 cm width)	Oxford, UK, June 2003	8.47	0.22
3	A2	Dark green leaves, not soft (approx. 3–6 cm length and 2–3 cm width)	Oxford, UK, June 2003	7.86	0.23
4	B1	Leaves from flowering branch, old, dark green, not soft (approx. 4–7 cm length and 3–5 cm width)	Private Garden, Germany, May 2003	4.72	0.18
5	B2	Leaves from flowering branch, young, light green, soft (approx. 2–6 cm length and 1.5–4 cm width)	Private Garden, Germany, May 2003	4.02	1.62
6	B3	Leaves from non-flowering branch, old, thick, darkish green, not soft (approx. 4–7 cm length and 3–5 cm width)	Private Garden, Germany, May 2003	6.53	–
7	B4	Darkish green, young, soft, climbing leaves (from *Betula*) (approx. 4–7 cm length and 2–5.5 cm width)	University Botanical Garden, Germany, June 2003	2.42	1.76
8	B5	Dark green big, climbing, not soft (from *Betula*) (approx. 5–7.5 cm length and 4–6.5 cm width)	University Botanical Garden, Germany, August 2003	6.33	0.82
9	B6	Dark green, climbing leaves, not soft (from *Betula)* (approx. 4–6.5 cm length and 2–5.5 cm width)	University Botanical Garden, Germany, August 2003	7.07	1.04
10	C	Dark green, whitish veined, not soft (approx. 4–6.5 cm length and 4–7.5 cm width)	Oxford, UK, June 2003	4.81	0.29
11	D	Dark green, not soft, climbing from an hybrid (approx. 4–8 cm length and 6–9 cm width)	University Botanical Garden, Germany, June 2003	8.81	0.73
12	E1	Dark green, whitish veined, not soft leaves (approx. 2.5–4.5 cm length and 3–4.5 cm width)	Private Garden, Germany, May 2003	3.61	0.64
13	E2	Light green thin/soft, veins not distinctive (approx. 2.5–4.5 cm length and 3–4.5 cm width)	Private Garden, Germany, May 2003	4.44	1.71
14	E3	White-green, soft leaves, variegated (approx. 2–5 cm length, 2–5 cm width)	Private Garden, Germany, June 2003	–	2.22
15	E4	White-green, soft leaves, variegated (approx. 4–7 cm length 4–6 cm width)	Vienna, Austria, September 2003	5.15	0.08
16	E5	Leaves without chlorophyll, soft, white-yellowish (approx. 4–7 cm length and 4–6 cm width)	Vienna, Austria, September 2003	3.79	0.66
17	E6	Soft, light green, climbing leaves (from *Betula*) (approx. 2–5.5 cm length and 2–6 cm width)	University Botanical Garden, Germany, June 2003	2.23	4.11
18	E7	Thick, white veins distinctive, dark green, climbing leaves (from *Betula*) (approx. 2–5.5 cm length, 3–7 cm width)	University Botanical Garden, Germany, June 2003	5.72	0.13
19	F1	Light green, whitish veined, soft (approx. 1–5 cm length and 1–4.5 cm width)	Oxford, UK, June 2003	14.63	0.21
20	F2	Light green, soft, trailing leaves (approx. 1–4 cm length and 1–5 cm width)	University Botanical Garden, Germany, June 2003	10.39	0.16
21	F3	Dark green, thick, trailing (approx. 4–8 cm length and 4–10 cm width)	University Botanical Garden, Germany, June 2003	10.03	1.14
22	F4	Dark green, thick, veined distinctive, climbing leaves from a hybrid (approx. 4–5 cm length and 5–6 cm width)	University Botanical Garden, Germany, August 2003	11.76	0.17
23	F5	Darkish green, thick, trailing leaves (approx. 3–4 cm length and 4–6 cm width)	University Botanical Garden, Germany, August 2003	9.84	3.62
24	F6	Light green, young, soft trailing leaves (approx. 3–4.5 cm length and 4–6 cm width)	University Botanical Garden, Germany, August 2003	7.62	6.17
25	F7	Dark green, thick, white veins distinctive, old climbing leaves from a hybrid (approx. 1–3.5 cm length and 1–5 cm width)	University Botanical Garden, Germany, June 2003	11.46	0.08
26	F8	Dark green, veins distinctive, thick climbing from a hybrid (approx. 4–8 cm length and 5–10 cm width)	University Botanical Garden, Germany, June 2003	9.36	0.23
27	F9	Shiny, light green, soft, young climbing leaves (approx. 4–8 cm length and 4–11 cm width)	Private Garden, Germany, June 2003	6.59	2.19
28	G	Light green, soft, young, trailing (approx. 1–4 cm length and 1–5 cm width)	University Botanical Garden, Germany, August 2003	7.78	3.78

* Codes A–G of *Hedera* extracts are associated to the shapes in Fig. 1
Numbered and cumulative classified plant material with marker components (**1** and **2**) in mg/ml

hederacoside C, α-hederine), volatile oil (e.g. methylethyl ketone), polyines (e.g. falcarinol), steroids (e.g. β-sitosterol), phenols (e.g. rosmarinic acid), and flavonoids (e.g. rutin) (Horz and Reichling 2003, Brendler et al. 2003, Wichtl 2002, Wagner and Reger 1986, Bedir et al. 2000, Toker et al. 1998, Trute and Nahrstedt 1996, Crespin et al. 1994). Although the presence of the alkaloid emetin was reported (Mahran et al. 1975), its occurrence is been questioned (Wagner and Reger 1986).



(1) Hederacoside C $R_1 = CH_2OH$ $R_2 = (\leftarrow 1)$-β-D-D-Glc $(6 \leftarrow 1)$-β-D-Rha
(2) α-Hederin $R_1 = CH_2OH$ $R_2 = H$
$R_3 = (\leftarrow 1)$-α-L-Ara-$(1 \leftarrow 2)$-α-L-Rha

In this study, it is presented that ivy leaves depending on the different leaf shape (Fig.1) and origin occurring in different vegetation phases can have large variations in the yield of the saponins like hederacoside C (**1**) and α-hederine (**2**) in the relevant extracts (Table 1). For this purpose, an optimization study for efficient and effective profiling and fingerprinting of the plant material using reversed-phase high performance liquid chromatography (RP-HPLC) was carried out.

2. Investigations, results and discussion

The DAC monograph on ivy leaves restricts the plant material to contain only young non-flowering, 3 to 5 lobed leaves collected in spring to early summer (DAC 1997). For the quality control, macroscopic, microscopic and a TLC method is suggested only. HAB 2000 requires young shoots before or during the flowering time and describes macroscopic and TLC determination of major compounds similar to DAC. *Hedera helix* is still not represented in the PhEur. However, the monograph is in preparation.

Besides qualitative requirements, quantitative ones like chemical fingerprint analysis also demanded for various aspects like quality control, biological efficacy and safety. From this point of view, in this present study, various *H. helix* samples depending on the leaf type or shape from different geographical origin and growth periods were investigated for fingerprinting of the saponin content to trace possible correlations (Table 1). During profiling of the plant material, to enable fast and efficient quantification, a new analytical method was developed and HPLC conditions were optimized (see Table 2 for gradient).

Table 2: HPLC gradient

Time (min)	Mobile Phase A (%, V/V)	Mobile Phase B (%, V/V)
0–7	80	20
8–21	80→40	20→60
22	40→0	60→100
23–26	0	100
27	0→80	100→20
28–33	80	20

Optimized gradient elution for *Hedera helix* MeOH extracts

Furthermore, the analytical method was validated with respect to linearity, precision, selectivity and specifity. Hederacoside C (**1**) had a retention time of 9.14 min, whereas α-hederin (**2**) was separated at 16.09 min, on a RP-column. The linearity area for the standard substance hederecoside C was 2000–10 000 ng and for α-hederin 800–4000 ng, whereas the limits of detection (LOD) were 200 and 500 ng, respectively. The mean values of the correlation coefficient were 0.9964 and 0.9952, respectively. The RT RSD values for precision were $< 2\%$, the cumulative results can be seen in Tables 3–5. Selectivity for the two compounds was confirmed by DAD peak-purity analysis. The resolution factors of the two peaks from the nearest resolving peaks in *Hedera* extracts was > 2 in all cases.

It was observed that the hederacoside C (**1**) and α-hederin (**2**) amounts showed broad variations depending on the shape and consequently on the age of the leaves as seen in Table 2. Seven major groups (A–G) have been suggested according to the various shapes of the leaves of *H. helix*, which also can be seen in Fig. 1. For comparison a commercial herbal drug sample (CS) was investigated. Subgroups have also been formed considering the leaf shapes. The chemical composition with regard on the saponins showed major differences. α-Hederin (**2**) amounts in younger flowering branch leaves were remarkably higher than in older ones, in B2, compound **2** was found in 1.62% whereas in B1 it was only 0.18%. Non-flowering branch leaves (B3) contained compound **1** in 6.53%, which was found in flowering branch leaves (B1) in 4.72% amount, compound **2** was not detected in B3.

Hederacoside C contents varied from 0–14.63 mg/ml extract, whereas α-hederin contents varied from 0–6.17 mg/ml in the extracts. Fig. 2 shows also that sample F1 contained the highest amount of **1**, whereas compound **2** was highest in sample F6. All tested samples contained **1** in a higher proportion than **2**, only samples E3 and E6 displayed the opposite ratio.

Table 3: Validation parameters of the HPLC analyses for *Hedera helix* extracts

Compd.	Regression equation y [mg/ml] =	Correlation coefficient	LOD [ng]	Linearity area [ng]
1	2.38261 × PA	0.9964	200	2000–10000
2	4.2542 × PA	0.9952	500	800–4000

limit of detection [ng] (LOD); linearity area for optimization [ng]

Table 4: Repeatability and precision for hederacoside C (1)

Concentration (mg/ml)	Relative standard deviation (%) (n = 3)
0.492	0.55
0.197	0.49
0.099	0.90

Table 5: Repeatability and precision for α-hederin (2)

Concentration (mg/ml)	Relative standard deviation (%) (n = 3)
0.125	0.99
0.083	0.78
0.042	0.83

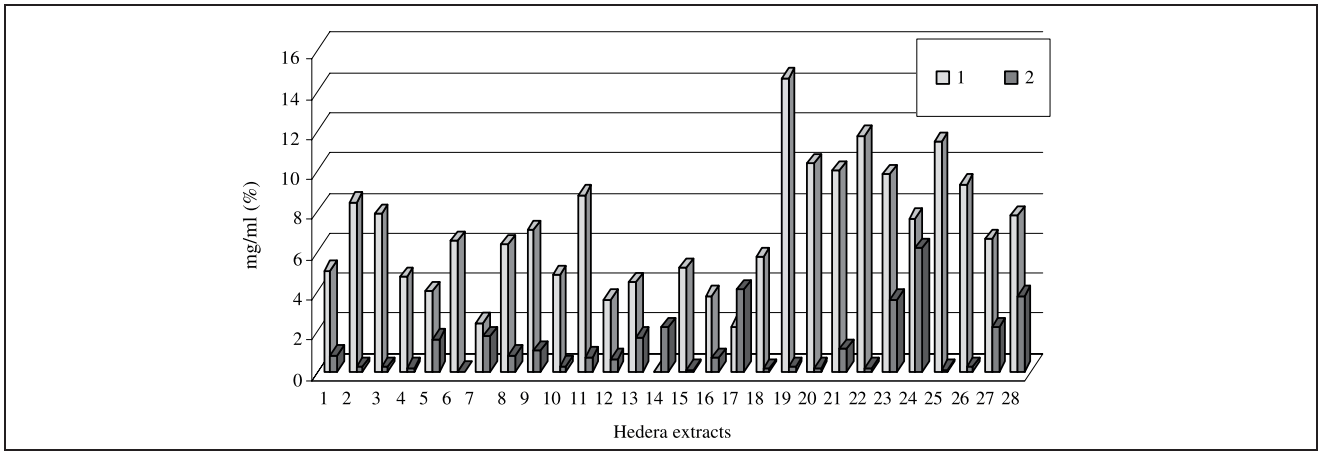


Fig. 2: Percentage of hederacoside C (1) and α-hederin (2) amounts in *Hedera* extracts, Marker compounds of *Hedera helix* in comparison

The fact that seasonal changes in the enzymatic composition in *Hedera* sp. can occur and various growth influencing compounds would also have an impact towards the chemical composition of metabolites in various sites of the plant possibly could explain the variation in the contents of compounds **1** and **2** (Fischer and Feller 1994).

When performing validation studies, the whole analytical procedure including all the steps of sample and extract preparation should be applied as far as possible. Allowed exceptions to the written procedure concerning the number of repetitions as the number of determinations for the various validation characteristics are described in the ICH guidelines (Bakshi et al. 2001, Ermer 2001, ICH 2003). These parameters and issues were considered in the saponin analysis of *Hedera* extracts in this study. As a result, an efficient, fast, and simple method was established to separate the saponins (**1** and **2**) which are thought to be relevant for the pharmacological effects, at least as marker compounds.

However, an open question remains on the minimum amount, relative concentration and ratio of the active components e.g. the saponins. As it is known that α-hederin is a potent haemolytic, cytotoxic, antiexudative saponin (Horz and Reichling 2003, Wichtl 2003, Blumenthal et al. 1998, Wagner and Reger 1986), its content should be as low as possible. Consequently, the amounts and requirements still need thorough dose-activity/dose-response studies both *in vitro* and *in vivo* for whole standardized extracts or to pure active substances of ivy leaves.

Another important conflict issue in the analysis of *Hedera* extracts is the supply and quality of standard substances, which may also influence the acquired amounts of active components. Furthermore, the chromophore insufficiency in the questioned saponines is another drawback in the analysis which also formed a problem as reported earlier by Wagner and Reger (1986) and Crespin et al. (1994). A TLC-densitometric analytical work on *Hedera* saponins is also reported (Barthomeuf et al. 1994).

The extraction conditions of the active constituents were not subject to evaluation and optimization of the present work. Furthermore, it is also known (Elias et al. 1991) that the drying and preparation methods can influence the extract quality of *Hedera* extracts.

Overall, this study shows that *Hedera* extracts can show big variation within each leaf, depending on the shape, vegetation period, place etc. The pharmacology and mechanism of *Hedera* extracts and components still need standardization and further studies.

3. Experimental

3.1. Plant material

Hedera helix L. leaves were collected during different vegetation periods and locations as indicated in Table 1 along with brief descriptions. Fresh leaves were immediately lyophilized to remove the moisture (Bauman, Germany).

3.2. Sample preparation/extraction

Hedera helix leaves were powered and sieved (355). To 500 mg of the powdered leaves 50 ml of MeOH (80%) was added and refluxed for 1 h. After cooling the extract was filtered through cotton into a 100 ml volumetric flask. The residue was again extracted with 30 ml MeOH (80%) under reflux for further 30 min, to exhaust the metabolites from the biological matrix. The second extract was also filtered and combined with the previous filtrate to an exact volume of 100 ml with MeOH (80%). The extracts were stored at + 4 °C and were filtered through 0.2 µm nylon filters (Roth, Germany) prior to HPLC analyses.

3.3. Reference solution

12.3 mg Hederacoside C (Fluka 97151, Germany; purity > 95%), and 5.2 mg α-Hederin (Aldrich 30169-8, Germany; purity > 95%) were dissolved in 25 ml MeOH.

3.4. HPLC-system and conditions

An assembled system consisting of Waters 515 pumps, Waters auto-injector 717 Plus; Gynotek column heater (25 °C); Biotek UV-DAD-Detector; Biotek Kroma System 2000 Software; pre-column, Eurospher-100 C18 (l = 30 mm, Ø = 4,6 mm, particle size 5 µm) Knauer, Germany. Eurospher-100 C18, (l = 12.5 cm, Ø = 4 mm, particle size 5 µm) Knauer, Germany. Flow rate: 1.5 ml/min. Injection volume was 20 µl; and detection of components was at 205 nm.

A gradient mobile phase system was used for the analysis of *Hedera helix* extracts. The gradient was as in Table 2. A: Water: ACN (HPLC-Grade, Baker, Germany): 85% H_3PO_4 (Merck, Germany) (90 : 10 : 0.5) B: ACN.

The cumulative analysis results can be seen in Table 2.

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