Extracts from *Rhododendron ferrugineum* Do Not Exhibit Grayanotoxin I: An Analytical Survey on Grayanotoxin I within the Genus *Rhododendron*

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- *Rhododendron*
- *Andromeda*
- *Kalmia*
- Ericaceae
- grayanotoxin
- GC-MS

Abstract

For quantitative determination of grayanotoxin I (**1**) in plant material, a GC/MS method was developed after trimethylsilyl derivatisation of the analytes. Forskolin (**5**) was used as an internal standard for quantification. ICH-compliant method validation indicated sufficient specificity, precision, quantitation (15 µg/mL) and detection (5 µg/mL) limits. Regression analysis showed that a non-linear (polynomial) model was preferable to a linear one. For isolation of grayanotoxin I reference material from *Rhododendron ponticum* leaves, an efficient two-step fast centrifugal partition chromatography isolation protocol is described. A survey of 17 different plant species from the genus *Rhododendron* revealed high grayanotoxin I content for *R. catawbiense, R. ponticum, R. degronianum* subsp. *yakushimanum, R.* × *sochadzeae, R. moupinense, R. galactinum*, and *R. mucronatum* var. *ripense*. The content of this compound in leaf material from *R. ponticum* decreased rapidly during drying process. Grayanotoxin I was not detected in different batches of fresh leaves and fruits from *R. ferrugineum*. In contrast to the claims of German health authorities, this traditionally used herb therefore cannot be evaluated as toxic due to the presence of grayanotoxin I.

Abbreviations

BSTFA:	N,O-bis(trimethylsilyl)trifluoroacetamide
FCPC:	fast centrifugal partition chromatography
GT:	grayanotoxin(s)
GT-I:	grayanotoxin-I
IS:	internal standard
SIM:	selected ion monitoring
TMCS:	trimethylchlorosilane
TMS:	trimethylsilyl

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Introduction

The leaves of *Rhododendron ferrugineum* L. (Ericaceae), a subalpine shrub found throughout the Pyrenees and the European Alps, have been traditionally used as aqueous decocts against rheumatism, blood pressure, muscle and metabolic diseases [1]. Recent data indicate antiviral effects against herpes simplex virus type 1 (HSV-1) [2]. However, evaluation by the German regulatory authorities indicated that these claims are not supported by sufficient clinical data [3]. Also potential toxicity due to hydroquinone and andromedotoxin and its derivatives could not be excluded, and a negative monograph was published [3], leading to a complete ban of the phytotherapeutic use of *R. ferrugineum* leaves.

Phytochemical investigation of the leaves from *R. ferrugineum* indicated the presence of triterpenes (ursolic acid, campanulin, friedelin, epifriedelin, α- and β-amyrin [4, 5], phenols (rhododendrin [6], phloracetophenon, and phloracetophenon-4-*O*-glucoside [7]), flavonoids (hyperoside, myricetin-3-*O*-β-galactopyranoside, kaempferol-3-*O*-(6″-*O*-acetyl)-glucoside, quercetin-3-*O*-(6″-*O*-acetyl)-glucoside, quercetin-3-*O*-(6″-*O*-acetyl)-galactoside, quercetin-3-*O*-(3″,6″-*O*-diacetyl)-galactoside, *cis*- and *trans*-taxifolin-3-*O*-α-L-arabinopyranoside [8]), ferruginenes A to C [9], and short chain organic acids. Recently, the detailed composition of the volatile oil and of the flavonoid fraction, including the unusual quercetin-3-*O*-(6″-*O*-(2-methyl-1-oxobutyl))-β-D-glu-

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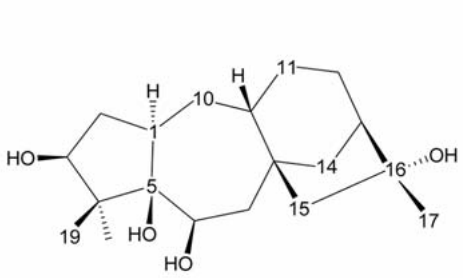
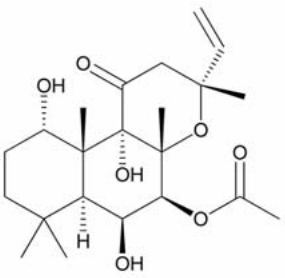


Grayanotoxins I-IV (1-4)



Forskolin (5)

	Abbreviation		10β	10α	14β
Grayanotoxin I	GT-I	1	-CH_3	-OH	-OAc
Grayanotoxin II	GT-II	2	=CH_2		-OH
Grayanotoxin III	GT-III	3	-CH_3	-OH	-OH
Grayanotoxin IV	GT-IV	4	=CH_2		-OAc

Fig. 1 Structural features of grayanotoxins I to IV and forskolin.

copyranoside and 2*R*,3*R*-dihydromyricetin-3-*O*-*β*-L-arabinopyranoside, have been reported [10]. Flavan-3-ols (catechin, epicatechin, gallocatechin, epigallocatechin), oligomeric procyanidins (procyanidins B1 to B7 and C1), cinnamtannin B1, chlorogenic acid, monosaccharides (glucose, fructose), oligosaccharides (sucrose, stachyose, raffinose), fructan and polymeric type II arabinogalactan have been described in the same study as secondary metabolites from the leaves [10]. The presence of arbutin can be exluded [10, 11].

The literature concerning the presence of toxic diterpenic andromedan derivatives (GT) in leaves of *R. ferrugineum* is not conclusive. This has also been assessed by the German regulatory authorities [3]. Therefore, the present study aims to clarify unambiguously this still open question on the presence of GT in the leaf material by using validated chromatographic methods.

Andromedan diterpens, also called grayanans, are typical toxins from the Ericaceae plant family [12]. The basic C_{20} structure (cf. **Fig. 1**) can be modified with up to 8 hydroxyl groups, partially esterified with acetic-, lactic-, or propionic acid. Glucosides are rare, but known. The obligatory oxygen function at C-3 can be functionalized as C-2,3-epoxy group. The different compounds will decompose to de-esterified products and also to unsaturated compounds after elimination of water, leading to the formation of olefinic derivatives [13]. The structural features of most common GT (GT-I to IV) from Ericaeae are displayed in **Fig. 1**. GT-I (**1**, syn. andromedotoxin, acetylandromedol, rhodotoxin) is assessed to be the main native toxin, while the other GT originate from GT-I by degradation during senescence or drying processes.

GT-I exerts selective effects on voltage-dependent sodium channels by eliminating fast sodium inactivation and causing a hyperpolarizing shift in the voltage dependence of channel activation [14]. For GT intoxication, cholinergic symptoms are described, resulting in incapacitating and sometimes life-threatening bradycardia, hypotension, and altered mental state. Complete heart failure occurs in a significant fraction of patients, and also asystole has been reported frequently [15]. The main source of intoxication with GT is honey (so called bitter or mad honey) from GT-containing nectar, frequently reported from the eastern Black Sea region of Turkey. For review on such frequent intoxications see [16–19]. For review on the actual toxicological assessment of GT and GT-containing products see [16]. For toxicological review on the genus *Rhododendron* see [20].

For quantification of GT, colorimetric methods after derivatisation with antimony salts [21], TLC [22], GC [23], HPLC with RI detection [24], and LC-MS [25] methods have been described, but a recent evaluation indicated [16] that until now no validated methods are known for the routine control of GT in plants and plant-derived products.

Therefore, the present study aimed at developing a validated and sensitive method to quantify GT-I in *R. ferrugineum* leaves and additionally to investigate representative species of the *Rhododendron* genus for the occurrence of GT.

Results and Discussion

For systematic investigation of GT-I content in different species of the genus *Rhododendron*, especially in *R. ferrugineum,* a sensitive and validated method had to be established. As GT-I (**1**, **Fig. 1**) was needed for analytical calibration without being commercially available, it was decided to isolate **1** from fresh leaves of *Rhododendron ponticum* L. for which GT-I is described to be the main naturally occurring GT [26]. Two different isolation procedures were used. Fractionation of dichloromethane soluble compounds, obtained from a hot methanol extract of the leaves material on aluminium oxide stationary phase as described by [27]

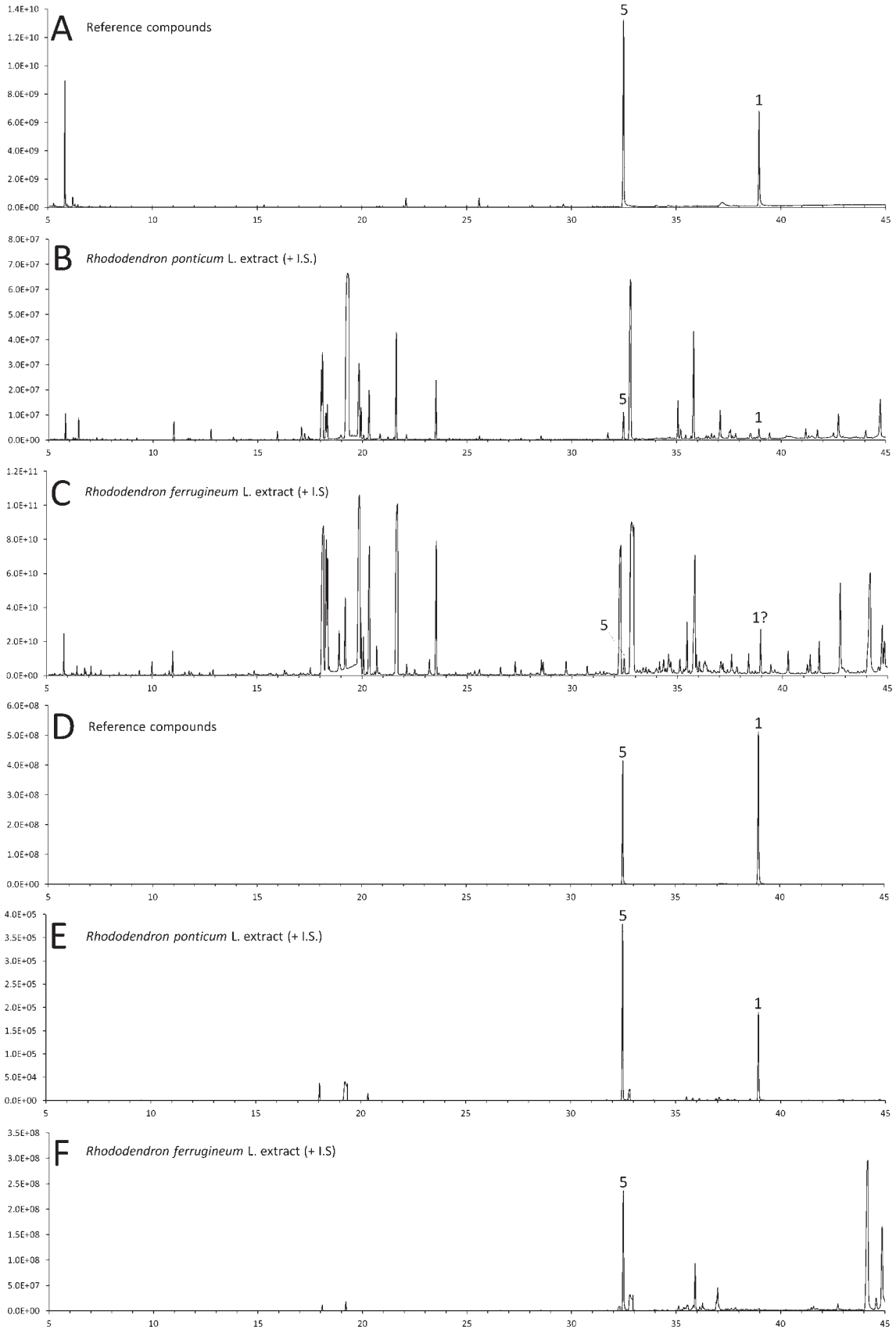


Fig. 2 Representative GC-MS chromatograms of silylated grayanotoxin I (**1**) and internal standard forskolin (**5**) (**A**, **D**) and silylated leaf extracts from *Rhododendron ponticum* L. (**B**, **E**) and *Rhododendron ferrugineum* L. (**C**, **F**) in SCAN-mode (**A** to **C**) and SIM-mode (**D** to **F**). **C** The peak at t_R 39 min (**1**) found in the SCAN-mode can be definitely excluded to be **1** because of a different mass spectrum and absence of specific signals at this retention time in SIM-mode (**F**).

yielded **1**, but in not acceptable purity. For that, a two-step FCPC protocol was developed for the effective fractionation of dichloromethane-soluble compounds: the first step yielded a GT-I containing fraction in the upper phase, which was chromatographed again in a second solvent system so that pure GT-I could be isolated directly from the stationary phase and characterized concerning identity, purity, and content. This time-saving method resulted in good yields of **1** with considerable purity. The protocol is again a good example for the effectiveness of FCPC for rapid preparation of pure compounds as it has recently also been described for other pharmaceutically relevant compounds [28–30].

For quantitation of **1** in plant materials, different methods were investigated: Due to the low UV-absorption of GT, HPLC with UV detection did not work sufficiently. Due to a very low ionization capacity of **1**, the use of LC-MS did also not give promising results. Quantitative TLC with densitometric evaluation gave a clear and well separated spot for GT-I, but systematic evaluation during validation work raised significant problems concerning reproducibility and accuracy.

Sufficient results were obtained by GC-MS analysis of TMS-derivative of **1**: Pre-experiments showed that only a combination of BSTFA, TMCS, and pyridine (10 : 1 : 1, v/v/v) formed a stable and reproducible TMS-derivative. Pyridine was shown to be a mandatory (catalytic) part of the mixture. The silylation process was finished after 3 h at 70 °C and resulted in a single peak in GC-MS analysis (**Fig. 2A**). All attempts with alternative reagents (HMDS, MSTFA, or TMSI), varying volume proportions, or different temperature conditions failed or resulted in more than one silylated product.

The silylation mixture of **1** was analysed by means of high resolution FTMS. The experiment revealed two characteristic adduct ions (*m/z* 708.4152 [M + pyridine + H]$^+$ and *m/z* 651.3547 [M + Na]$^+$) indicating a monoisotopic mass of 628.365 Da ($C_{31}H_{60}O_7Si_3$). Thus, three of five hydroxyl groups of **1** are silylated in the stable end-product. All attempts to vary the silylation protocols to obtain per-silylated **1** failed, and therefore it can be stated that under normal analytical silylation procedures, only the tri-substituted GT-I will be formed. On the other side the formation of this product, named $\mathbf{1}_{TMS}$, was reproducible in all further validation series, and no hints occurred concerning potential over- or undersilylation.

$\mathbf{1}_{TMS}$ could easily be detected during GC-MS (EI) analysis; $\mathbf{1}_{TMS}$ revealed a base peak at *m/z* 73 ([Si(CH$_3$)$_3$]$^+$) and a characteristic signal at *m/z* 298 (probably due to loss of all TMS-groups as well as multiple dehydration processes and elimination of the acetyl group). No peaks for [M]$^{\cdot+}$ or [M-15]$^+$ were detectable.

To improve the quantitation of the $\mathbf{1}_{TMS}$ derivative, the labdan diterpene forskolin **5** (for structural information of this compound, see **Fig. 1**) was chosen as internal standard. The silylation end product $\mathbf{5}_{TMS}$ yielded one single peak in GC/MS (**Fig. 2A**) and was also stable and reproducible under the given conditions.

For $\mathbf{5}_{TMS}$ no signals for [M]$^{\cdot+}$ or [M-15]$^+$ were detectable, but $\mathbf{5}_{TMS}$ showed a characteristic *m/z* at 464 (probably due to elimination of water). The nominal mass of $\mathbf{5}_{TMS}$ was determined to be *m/z* 482, indicating a mono-TMS derivative (see experimental part).

A further significant improvement of the analytical method was obtained by using SIM-mode (**Fig. 2D** to **F**) with *m/z* 464 for $\mathbf{5}_{TMS}$ and *m/z* 298 for $\mathbf{1}_{TMS}$ with better signal-to-noise ratio and selectivity in comparison to the SCAN-mode (**Fig. 2A** to **C**). Therefore, GC-MS of $\mathbf{1}_{TMS}$ with $\mathbf{5}_{TMS}$ as IS in the SIM-mode was selected for validation and subsequent analysis of plant material.

Validation according to ICH-guidelines indicated the need of non-linear calibration. The working range of the method was 15 to 600 µg GT-I/mL, the limit of quantitation was 15 µg/mL, and the detection limit 5 µg/mL. System precision was determined with RSD 6.7%, and repeatability from 6 independent samples was calculated with RSD 13%. Stability of TMS-ethers in sample solution over 24 h was given.

Under certain conditions, the long-term storage of silylation mixtures resulted in decreasing measuring values. Hydrolysis of TMS-ethers – possibly caused by incoming humidity via the small puncture in the vial-septum – seems to play a central role especially in case of **1**: the ratio [Area **1**/Area **5**] decreased to approx. 60% of the initial value while injecting the solutions repeatedly over a period of 10–15 h. To verify this assumption, we divided the test solution directly after the silylation process in two parts, injected the first immediately and the second, bottled in a sealed vial, after 24 h storage. In this case, good stability and no reduction of the ratio [Area **1**/Area **5**] were observed. Thus, storage of the silylation mixture under moisture-resistant conditions seems to avoid the described degradation of $\mathbf{1}_{TMS}$. Therefore, we decided to use one sealed vial only for one injection and furthermore a long-term storage of freshly prepared silylation-mixtures prior to injection had to be avoided.

For quantification of **1** in *R. ferrugineum*, the whole fresh plants, including soil blocks, were harvested at 3 different places in the Alps (Germany, Austria, and Italy) and transported within one day to the analytical laboratory where leaves and fruits were detached immediately. In all samples investigated, no signals for $\mathbf{1}_{TMS}$ were detectable under the described analytical conditions. From these data, we can exclude the presence of **1** in aerial material of *R. ferrugineum* above the quantitation limit and also the assessment of the German regulatory authorities [3] concerning this GT derivative should be revised. Therefore also the presence of degradation products **2** to **4** in the plant material can be excluded.

In order to investigate representative species of the *Rhododendron* genus on the occurrence of GT-I, fresh leaf material from 17 different defined species was investigated (**Fig. 3**).

The extreme variable genus *Rhododendron* consists of ca. 1000 species on four continents, which are classified in several subgenera, sections, and even subsections. The investigated species from the Rhododendron-Park Bremen belong to major groups within the genus, thereby representing the whole spectrum of variability. The selection includes evergreen and deciduous species, lepidote (= scaly) and elepidote (= non-scaly) species, small, bushy and large, tree-like species as well as azaleas. To avoid any impacts through hybridization, all of the chosen species are part of the German Genebank Rhododendron [31]. Species of this living gene bank have been identified by experts as being "true species" and are since then used as reference specimens.

Very high content of **1** was found for extracts from *R. catawbiense* Michx., a species native to North America, but also found heavily cultured in garden areas in Europe. In accordance with literature all samples from *R. ponticum*, native to Turkey and the Black Sea region, contained GT-I amounts from 0.13% to 0.22%. This will easily explain the high toxicity of this plant and also of honey produced from this species. High values were found also for *R. degronianum* subsp. *yakushimanum* Carrière, native to Japan, and *R. x sochadzeae* Char & Davlianidze, a hybrid between *R. ponticum* × *R. caucasicum*. GT-I values < 0.1% were determined for *R. moupinense* Franch., *R. galactinum* Balf. f. ex Tagg, and *R. mucronatum* var. *ripense* G. Don & E. H. Wilson, all species com-

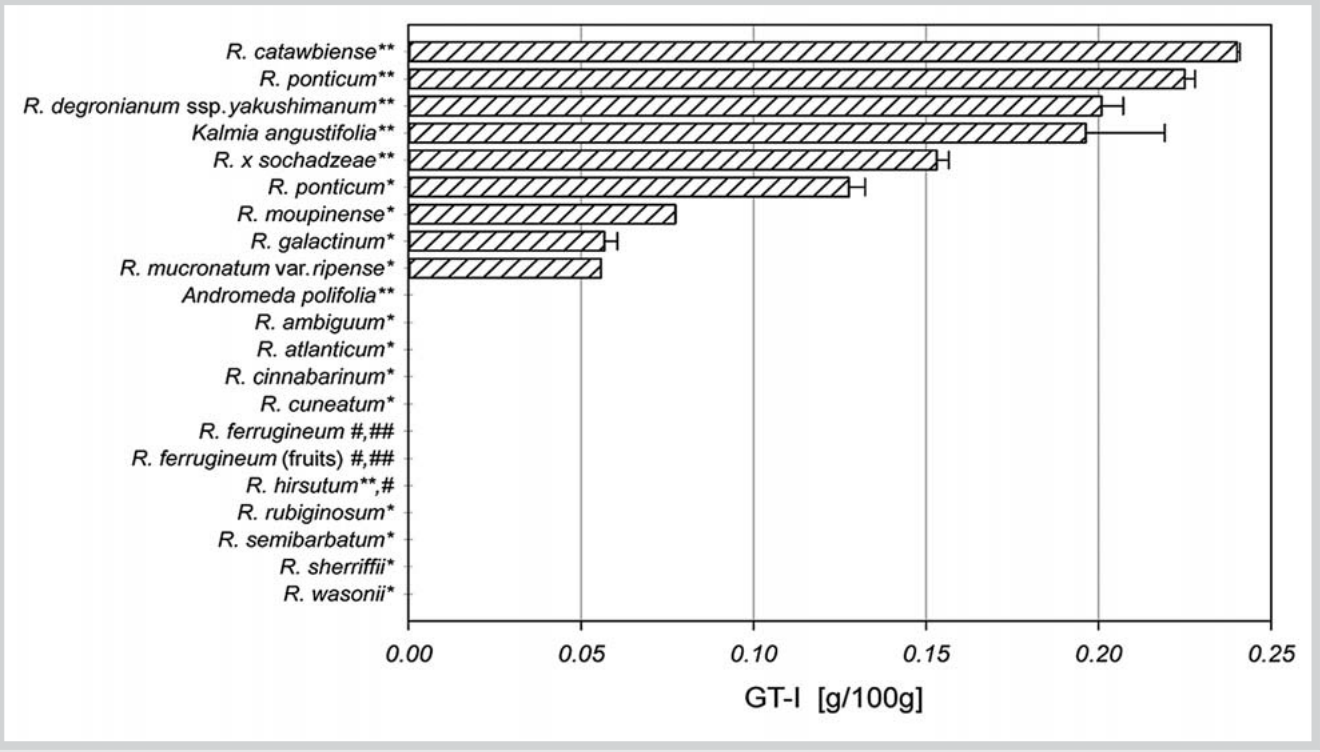


Fig. 3 GC-MS quantification of grayanotoxin I in different species from the genus *Rhododendron, Kalmia angustifolia*, and *Andromeda polifolia*. Bars represent upper and lower results from 2 independent determinations of representative samples. All data are given in g per 100 g fresh leaves. * Cultivated at Rhododendron Park, Bremen; ** cultivated at University of Münster; # wild samples from collection at pre-Alps; ## wild samples from collection in central Alps.

monly found in garden areas in Europe. All other *Rhododendron* species investigated had a GT-I content below the limit of detection (30 ppm). Genera of Ericaceae closely related to the genera *Rhododendron* are *Kalmia* and *Andromeda*. While *Andromeda polifolia* L. did not contain **1**, *Kalmia angustifolia* L. ("sheep-kill") had high content (about 0.2%) of GT-I, which is in accordance with data reported by [32,33].

From these data, it gets clear that the genus *Rhododendron* and related genera do not contain GT-I *per se*. The assessment on potential GT-I related toxicity within a distinct species has always to be done by distinct analytical investigation. For that, the here described method seems to be an efficient tool for a valid decision.

Preliminary experiments [unpublished data] raised concerns on the stability of **1** in the plant material towards different drying conditions. For systematic investigation, representative leaf samples from *R. ponticum* were stored over a 18-days period at room temperature, and GT-I content was quantified at days 0, 2, 4, 6, 8, and 18 under consideration of the respective water content of the plant material. As shown in ◉ **Fig. 4**, **1** was not stable during this drying process and degraded to about 30% related to the initial GT-I content as determined at day 0. The same plant material, which had been lyophilized directly after harvesting showed higher GT-I content of about 55%, related to the content of the freshly harvested leaf material. At this point of the investigation, it got obvious that GT-I seems to degrade quite fast after harvesting and during drying process, and therefore air-dried leaf material from *R. ponticum* stored for a longer time (harvested in Turkey in 2010 and carefully stored at room temperature since then) was investigated: in this case, GT-I content was – as expected – below the limit of detection.

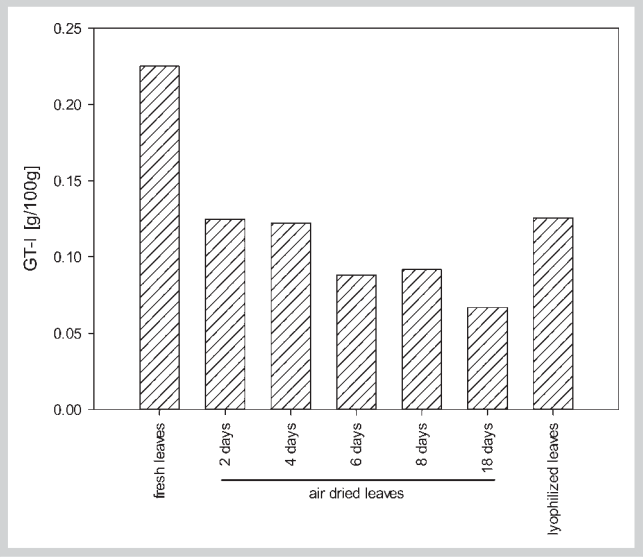


Fig. 4 Influence of drying process on grayanotoxin I content in leaves from *Rhododendron ponticum*. A representative sample of freshly harvested leaves was air-dried over an 18-days period or was lyophilized (rightmost bar). GT-I content was calculated by GC-MS analysis and given in g per 100 g (fresh leaves).

From these investigations, the contradictory data about the distribution of GT in different species of the genus *Rhododendron* [34] have been clarified to a wide extent. Especially in case of *R. ferrugineum* there was need for clarification because a potential toxicity due to GT could not to be excluded, and the publica-

tion of a monograph [3] led to a complete restriction of any phytotherapeutic use of *R. ferrugineum* leaves.

In conclusion, the results indicate that GT-I, the genuine and predominant GT derivative, is not present in freshly harvested leaves and fruits from *R. ferrugineum* above the limit of detection (30 ppm). By analogy, potential degradation products (GT-II, GT-III, GT-IV) are neither present. Thus, the still open question on the presence of GT in the leaf material of *R. ferrugineum* was answered using a validated chromatographic GC-MS method.

The data corroborate the fact that GT-I is not stable during common drying processes. Even freeze-dying reduces the amount of GT-I considerably. Thus, GT-I (and its artefacts) may not be detected in long term stored plant material, which may be the reason for the described contradictory literature data in the genus *Rhododendron*.

Furthermore, it was shown that GT-I is detectable in high amounts (≥ 0.15 %) in some species of the genus *Rhododendron,* namely *R. catawbiense, R. ponticum, R. x sochadzeae*, and *R. degronianum* subsp. *yakushimanum.* Leaves of some other *Rhododendron* species (*R. moupinense, R. galactinum*, and *R. mucronatum* var. *ripense*) contain slightly lower contents (0.05–0.1 %).

Materials and Methods

General analytical techniques

If not stated otherwise, chemicals were obtained in analytical quality from VWR.

Plant material

Voucher samples are stored in the archives of the Institute of Pharmaceutical Biology and Phytochemistry, University of Münster, Germany; IPBP registration numbers are given in braces.

Plant material from different sources was used:

[A] Cultivated plants:

- Medicinal Plant Garden of the Institute of Pharmaceutical Biology and Phytochemistry, University of Münster, Germany: *Rhododendron catawbiense* Michx. {IPBP 347}, *R. degronianum* subsp. *yakushimanum* Carrière {IPBP 348}, *R. hirsutum* L. {IPBP 349}, *R. ponticum* L. {IPBP 350}, *R. x sochadzeae* Char & Davlianidze {IPBP 351}, *Andromeda polifolia* L. {IPBP 352}, *Kalmia angustifolia* L. {IPBP 353}. Plants were collected in 2013 by FD, ML and identified by Lars Krüger.
- Rhododendron Park, Bremen, Germany. Numbers in round brackets indicate the accession number in the *German Gene Bank Rhododendron* [31]: *R. ambiguum* Hemsl. (100 007) {IPBP 354}, *R. ponticum* L. (100 393) {IPBP 355}, *R. wasonii* Hemsl. & E.H. Wilson (100 799) {IPBP 356}, *R. cinnabarinum* Hook. f. (100 322) {IPBP 357}, *R. moupinense* Franch. (100 374) {IPBP 358}, *R. rubiginosum* Franch. (100404) {IPBP 359}, *R. atlanticum* (Ashe) Rehder (100013) {IPBP 360}, *R. semibarbatum* Maxim. (100781) {IPBP 361}, *R. cuneatum* W.W. Sm. (100328) {IPBP 362}, *R. sherriffii* Cowan. (100 795) {IPBP 363}, *R. mucronatum* var. *ripense* E.H. Wilson (100 402) {IPBP 364}, *R. galactinum* Balf. f. ex Tagg (100 349) {IPBP 365}. Plants were collected and identified in 2013 by HS.

[B] Wild harvested plants from botanical excursions:

- Pre-Alps, Bavaria, Germany, Rotwand [47°39′1″ N, 11°56′4″ O]: leaves from *R. hirsutum* L. {IPBP 366} and *R. ferrugineum* L. {IPBP 367}. Plants were collected and identified in May 2011 by AH.
- Central Alps, Italy, Hochfeiler 1, Hochfeiler 2 [46°58′21″ N, 11° 43′39″] and Austria, south-western part of Zillertal [47°18′28″ N, 11°52′27″ O]: leaves {IPBP 368/370} and fruits {IPBP 369/371} from *R. ferrugineum* L. Plants were collected and identified in July 2013 by AH.
- Turkey, air dried leaves from *R. ponticum* L. {IPBP 372} were collected and identified in 2010 by Belmar Konuklugil, University of Ankara.

Isolation of grayanotoxin I (1)

GT-I (**1**, ◉ **Fig. 1**) is the main naturally occurring GT derivative in leaves of *Rhododendron ponticum*. As it is not commercially available, it was decided to isolate **1** from fresh leaves of this plant. Two different isolation procedures were used.

Extraction of plant material, according to [27]: Fresh leaves were harvested and stored at − 18 °C. 150 g of powdered plant material were extracted with 400 mL hot methanol for 1 h under reflux. After filtration, solvent was removed by rotary evaporation, and the dried extracts were partitioned between water and dichloromethane. Combined organic phases were taken to dryness to yield extracts *E1* and *E2* (originating from 2 independent extraction procedures). Following, two different isolation procedures for **1** were tested.

Isolation procedure A, based on [27]: Fractionation of E1 was done by column chromatography (stationary phase: neutral Al_2O_3; 500 × 20 mm; mobile phase: chloroform/methanol 9 : 1, v/v; flow: 1.75 mL/min; fraction size: 2.5 min). Fractions 61 to 72, corresponding to 267 to 315 mL of mobile phase, containing **1** were combined according to TLC analysis (see below) to yield 34.2 mg. Further purification was achieved by preparative HPLC (Waters 515 pump, UV detector (Knauer), stationary phase: Eurospher® 100 C18 (Knauer), 7 µm, 250 × 20 mm; mobile phase: CH_3CN/water gradient, 5 : 95 → 100 : 0 in 40 min; flow 9 mL/min; fraction size 0.5 min; detection wavelength 200 nm;) to yield 13.2 mg (yield: 0.009 % referred to fresh leaves). The isolated product was identified by MS and NMR as 1. Purity was insufficient for use as standard for quantification (< 80 %).

Isolation procedure B: Fractionation of E2 was performed by FCPC. The FCPC conditions were as follows: CPC Kromaton®, (Kromaton Technologies), system A: EtOAc/H_2O (1 + 1); system B: heptane/ethyl acetate/methanol/water (1 + 1 + 1 + 1); mobile phases = upper phases; flow: 10 mL·min^{-1}; 1200 rpm, fraction size: 1 min. Fractions were combined according to TLC analysis (TLC system as described below). Separation system A yielded 70 mg of a GT-I enriched fraction. Further purification was subsequently achieved by separation system B. Evaporation of the lower (stationary) phase (200 mL) of separation system B which could easily be pumped out from the FCPC at the end of the run yielded 12.6 mg in 80–90 % purity as proven by NMR spectroscopy (see below). Subsequent column chromatography of this fraction yielded 6.7 mg of pure compound 1 (named GT-I_{RS}). Parameters of CC: silica gel 60 (Merck No. 5554), 0.063–0.200 mm; 500 × 20 mm; step gradient: hexane, ethyl acetate, and methanol.

Reference standards

GT-I_{RS} (**1**): identity of **1** was confirmed by means of LC-ESI-QTOF-MS/MS and ^{13}C-NMR [32]. TLC R_f value: 0.42. The purity of GT-I_{RS} was determined by means of TLC-densitometry (5 to 17.5 µg of GT-I_{RS}) after spraying with 60 % H_2SO_4 and heating. Remission was measured at different wavelengths. The purity was determined to be 89.9 %. Parameters: TLC conditions, see below;

densitometer, Desaga CD60 (Wiesloch); measurement of remission at λ 600, 530, and 254 nm, Kubelka-Munk correction.
GT-III (**3**): andromedol-hemi(ethyl acetate) adduct, Sigma-Aldrich. TLC R_f value: 0.42. Because of its limited purity (< 90%), **3** was only used as TLC-reference standard.
Forskolin (**5**): purity > 98% (according specification of manufacturer), J63292, Lot E21Z044, Alfa Aesar GmbH.

Chromatographic and spectroscopic methods

TLC, according to [35]: Silica gel 60 F_{254}, Merck No. 5554, mobile phase: ethyl acetate-methanol-water (81 + 11 + 8, v/v/v); vol.: 2–10 µL, applied as band; detection: sulfuric acid, 60%, 100 °C, 2 min, UV λ = 366 nm and daylight.
QTOF-MS/MS and high resolution FTMS: LC-ESI-QTOF-MS/MS: Dionex Ultimate 3000 RS liquid chromatography system, Dionex Acclaim RSLC 120 (Thermo Scientific), C18 stationary phase (2.2 × 100 mm), binary gradient (A: water with 0.1% formic acid; B: acetonitrile with 0.1% formic acid), flow: 0.8 mL·min^{-1}: 0 to 9.5 min: linear from 5% B to 100% B; injection volume 1–2 µL; Bruker Daltonics micrOTOF-QII time-of-flight mass spectrometer, Apollo electrospray ionization source in positive mode at 5 Hz, mass range m/z 50–1000. The spectrum of **1** showed characteristic *m/z* values: 847.4068 $[2\,M + Na]^+$, 430.2802 $[M + NH_4]^+$, 395.2429 $[(M–H_2O) + H]^+$, 377.2323 $[(M–2H_2O) + H]^+$, 359.4766 $[(M–3H_2O) + H]^+$.
APCI-QTOF-MS/MS: Direct probe experiments of the TMS derivatives (silylation protocol described below) of **1** and **5** (named $\mathbf{1}_{TMS}$ and $\mathbf{5}_{TMS}$) in APCI mode revealed the grade of silylation. *m/z* values: $\mathbf{1}_{TMS}$ 708.418 $[M + pyridine + H]^+$ and 629.372 $[M + H]^+$ indicating three silylated OH-groups ($C_{31}H_{60}O_7Si_3$); $\mathbf{5}_{TMS}$: 562.318 $[M + pyridine + H]^+$ and 483.274 $[M+H]^+$ indicating one silylated OH-group ($C_{25}H_{42}O_7Si$).
High resolution FTMS: Orbitrap LTQ XL (Thermo Scientific), ESI, positive mode, full ms, 150–2000 Da. Characteristic *m/z* – values of $\mathbf{1}_{TMS}$: 708.4152 $[M + pyridine + H]^+$ ($C_{31}H_{60}O_7Si_3C_5H_5NH$) and 651.3547 $[M + Na]^+$ ($C_{31}H_{60}O_7Si_3Na$).

^{13}C-NMR spectroscopy

Varian 600 Unity Plus. Fraction GT-I_{FCPC} was measured in pyridine-d_5. Reference signal: pyridine-d_5 at δ 123.5 ppm. δ of carbon signals in ppm: 51.39 (C1), 35.83 (C2), 82.61 (C3), 51.76 (C4), 84.45 (C5), 73.73 (C6), 44.11 (C7), 51.03 (C8), 55.62 (C9), 77.94 (C10), 22.45 (C11), 27.36 (C12), 55.04 (C13), 82.82 (C14), 61.18 (C15), 78.55 (C16), 23.97 (C17), 23.39 (C18), 19.80 (C19), 28.30 (C20), 170.35 (C21), 21.22 (C22).

Quantification of 1 in plant material

Plant material (e.g., leaves) was grounded in a mortar by use of liquid N_2. An aliquot (0.9–1.1 g) was exactly weighted and extracted with 10 mL methanol in a 15 mL falcon-tube. The suspension was ultra-sonicated for 90 min and centrifuged at 6500 × *g* for 5 min. An aliquot of 500 µL was used for subsequent silylation. 500.0 µL of the extract and 100.0 µL of an IS-stock solution (containing 0.5 mg·mL^{-1} of **5** in methanol) were taken to dryness under a stream of nitrogen gas. 25.0 µL pyridine, 25.0 µL TMCS (Machery & Nagel), and 250.0 µL BSTFA (Machery & Nagel) were added. The mixture was heated in a sealed vial for 3 h at 70.0 °C on a hotplate. The temperature was controlled with an adjustable contact thermometer.

GC-MS analysis of silylated grayanotoxin I in plant extract

1 µL of the silylation-mixture was analysed in a 6890 N GC System with Agilent 5973 mass selective detector (single quad.) (Agilent Technologies). Mobile phase: He; stationary Phase: HP-5MS (30 m × 0.25 mm × 0.25 µm); temp. gradient: 100 °C to 310 °C (5 °C/min), 15 min isothermal; EI – 70 eV; SCAN mode and SIM mode (for quantitative determination). SIM-mode: 0–34 min: m/z = 464; 35–45 min: m/z = 298. The retention times were as follows: $\mathbf{5}_{TMS}$ = 32.5 min, $\mathbf{1}_{TMS}$ = 39.0 min. The following relevant mass fragments of the silylated compounds **1** and **5** were recorded:
$\mathbf{1}_{TMS}$: *m/z* (%), 520 (1), 460 (2), 430 (7), 388 (7), 370 (12), 358 (11), 299 (11), 298 (40), 255 (8), 207 (16), 183 (8), 157 (9), 145 (15), 143 (13), 133 (11), 117 (20), 75 (48), 73 (100), 43 (25).
$\mathbf{5}_{TMS}$: *m/z* (%), 464 (58), 436 (3), 354 (3), 353 (4), 331 (6), 279 (14), 237 (14), 219 (15), 209 (13), 191 (42), 177 (20), 165 (22), 163 (19), 129 (32), 123 (36), 109 (26), 107 (21), 95 (32), 81 (31), 73 (78), 43 (100).

Analytical validation, according to [36]

Identity: Peak identification of 1 and 5 was ensured by the respective GC/MS-spectra and by co-injection with reference standards.
Non-linear calibration: Stock solutions (in methanol): **1** (GT-I_{RS}) 0.50 mg·mL^{-1}; **5** (IS) 0.51 mg·mL^{-1}. Concentrations in measured samples (after silylation, with consideration of purity of GT-I_{RS}): **1**: 15–600 µg·mL^{-1}; IS **5**: 340 µg·mL^{-1} in each case. Analysis of data showed that a polynomic (quadratic) regression was preferable to a linear one. This was also supported by Mandel's fitting test [37]; calibration curve [y = area **1**/area **5**; x = conc. **1**/conc. **5**]: $y = 0.5084\,x^2 – 0.1631\,x + 0.0258$ ($R^2 = 0.9988$).
Range: 15 to 600 µg·mL^{-1} of 1 in sample solution (the volume of silylation mixture was 300 µL in each case).
Quantitation limit (LOQ), based on the calibration curve:
15 µg·mL^{-1} of 1 in sample solution (equal to 90 mg per kg plant material).
Detection limit (LOD), based on signal-to-noise approach:
5 µg·mL^{-1} of 1 in sample solution (equal to 30 mg per kg plant material).
Precision: Repeatability (system precision) was determined to be 6.7%. The intra-assay precision was 13.7% (% RSD), determined under the same conditions performed with double injections of 6 independent sample preparations.

Drying experiments

Air drying: Some representative leaves of *Rhododendron ponticum* were harvested in the Medicinal Plant Garden of the Institute and stored in the lab at room temperature under protection from direct daylight. In order to determine the average loss of water, all individual leaf weights were measured at day 0 (= initial fresh weight), 2, 4, 6, 8, and 18 (= dry weight) and averaged for each measuring day. The mean values of weight loss (WL) were as follows: day 2: 40.3% WL (27% RSD, n = 22), day 4: 56.6% WL (19% RSD, n = 20), day 6: 66.7% WL (10% RSD, n = 19), day 8: 69.3% WL (10% RSD, n = 15), and day 18: 71.5% WL (9% RSD, n = 14). Relative water contents (RWC) were as follows: day 2: 46.3%; day 4: 24.6%, day 6: 11.1%, day 8: 7.6%, day 18: 4.7%. At each examination day a random leaf sample was taken from the population, grounded in a mortar by use of liquid N_2 and analysed by means of GC/MS as described above. In order to be able to compare the results with fresh leaves, the amount of **1** was cal-

culated based on consideration of the respective amount of water loss at each measuring day.

Freeze drying: In a parallel experiment, a collection of some representative leaves were freeze dried. The loss of water was determined to be 75%.

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Conflict of Interest

The authors declare no conflicts of interest.

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