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Phytochemical and Toxicological Research of

Rhododendron ferrugineum L.

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Abbrevations

$CHCl_3$	Chloroform
CO_2	Carbon dioxide
EA	Ethyl acetate
GTX	Grayanotoxin
H_20	Water
MeOH	Methanol
NaCl	Sodium chloride
$NaSO_4$	Sodium sulphate
PBS	Phosphate buffered saline
Rf	Retention factor
RV	Reservoir volumina
SPE	Solid phase extraction
TLC	Thin layer chromatography

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1. Introduction

Figure 1: *Rhododendron ferrugineum*
(Source: http://www.botanikus.de/Gift/alpenrose.html)

The name of *Rhododendron* descends from the Greek: rhodon = rose; dendron = tree [BERG and HEFT, 1991].

1.1 Scientific Classification: [BERG and HEFT, 1991], [ESSERT, 1988]

Division:	*Magnoliophyta*
Class:	*Magnoliopsida*
Order:	*Ericales*
Family:	*Ericaceae*
Genus:	*Rhododendron*
Species:	*R. ferrugineum*

The genus *Rhododendron* belongs to the *Ericaceae* family. There are about 1000 species in the genus of *Rhododendron* [BERG and HEFT, 1991].

1.2 Incidence of *Rhododendrons*

Rhododendron usually grows in the northern hemisphere. Exceptions are the species from north Australia and south Melanesia.
Most of the *Rhododendron* species grow in China, Myanmar, Indonesia, but they can also be found in Europe e.g. *Rhododendron ferrugineum, R. hirsutum, R. kotschy* [HÄNSEL et al, 1994].

There are 7 basic areas of circulation:

a) Himalayan, Western China and Central China
b) Coastal area of China
c) North-east Asia
d) Japan
e) Malay Archipelago
f) Europe
g) America

Rhododendrons have not been found in South America and Africa [BERG and HEFT, 1991].
Rhododendrons prefer mild climate with high air humidity. Most rhododendrons exist in regions with short cold periods but there are also species witch are hardy. In the Alps you can find it above the tree line [TUTIN et al, 1969], [CONERT et al, 1966].
All local rhododendrons are under nature conservancy (A, D, CH, I) [Roth et al, 1994].

1.3 *Rhododendron ferrugineum*

Rhododendron ferrugineum (rusty-leaved alpenrose) is an evergreen plant which can grow up to 0.3 to 1.5 meters tall. The saplings are scaly [MOSER, 1991].
R. ferrugineum has ovate or lanceolate leaves, 2 to 4 cm long. On the upper side the leaves are green, glabrous and sleek. On the underside they are first yellow and later rusty with glandular hairs. The inflorescence is terminal, umbel-like with 6-10 peduncles. The corolla is magenta to dark pink, rarely white. *R. ferrugineum* grows

above the tree line in the Alps, Pyrenees and between the mountains of South Croatia and the Apennines [TUTIN et al, 1969], [CONERT et al, 1966].

A very close relative of *R. ferrugineum* is *R. hirsutum*. The two species generate the hybrid form called *Rhododendron x intermedium* [HALLIDAY, 2001].

1.4 Chemical Composition

1.4.1 Phenolic Compounds

1.4.1.1. Tannins

Tannins are found in vascular plants, primarily within xylem. They react with proteins and accumulate as water-insoluble copolymers [KAUFMANN et al, 1999].

The tannins can be divided into two broad groups:

a) Catechin tannins or condensed proantocyanidines

b) Hydrolysable tannins or gallotannins [HÄNSEL and STICHER, 2007]

Tannins can be the cause of false-positive or false-negative results in biological assays, so they should be eliminated from extracts before testing [SILVA et al, 1998]. The detection of GTXs is also strongly impeded by tannins and/or quercetin [FROHNE and PFÄNDER, 2004].

1.4.1.2 Flavonoids

Flavonoids are naturally occurring polyphenolic compounds containing two benzene rings which are linked together with a heterocyclic pyran or pyrone ring [HÄMÄLÄINEN et al, 2007].

The leaves and flowers of *R. ferrugineum* contain the following 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, *trans*-taxifolin 3-*O*-*α*-arabinopyranoside and *cis*-taxifolin 3-*O*-*α*-arabinopyranoside [CHOSSON et al, 1998].

1.4.1.3 Phenolic Heterosides

Arbutin

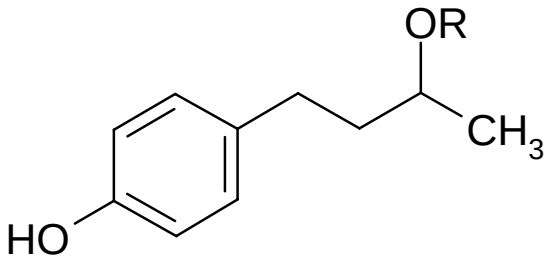
KOSCH reported that there is 0,72% arbutin in the leaves of *R. ferrugineum* [KOSCH, 1939]. Newer references show that arbutin is contained in *R. ferrugineum* [SCHÖNFELDER and SCHÖNFELDER, 2004].

Rhododendrin

Rhododendrin is the glycoside of the arylbutanoid (-)-rhododendrol and is contained in *R. ferrugineum*, this is documented by TLC. It was first isolated by Archangelsky 1901 from the leaves of *Rhododendron chrysantum* [FRANK et al, 1997].

Rhododendrol

Rhododendrol is the aglycon of rhododendrin. Rhododendrin is naturally hydrolyzed to rhododendrol. It was first isolated by Archangelsky 1901 along with rhododendrin from the leaves of *Rhododendron chrysantum* [FRANK et al, 1997].



Rhododendrin (R= -Glc)

Rhododendrol (R= -H)

Figure 3: Chemical structure of rhododendrin and rhododendrol

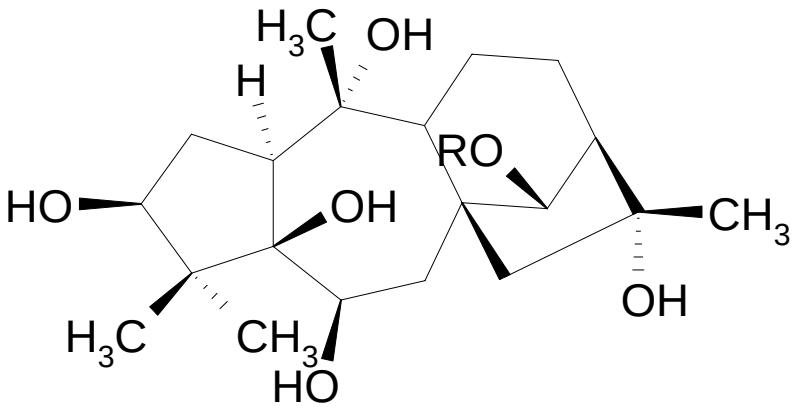
1.4.2 Toxic Diterpenes

Most *Rhododendron* species contain GTXs, also called andromedotoxins, asebotoxins and rhodotoxins [JORDAN, 2006].

GTXs have been also found in *Andromeda*, *Kalmia, Pieris* and *Leucothoe* spp. Today there are more than 30 identified toxins which are deducible from this skeletal structure [FROHNE and PFÄNDER, 2004]. The toxins are found mainly in the leaves but also in the roots, nectar and pollen [HÄNSEL and STICHER, 2007]. Rhododendron-honey could be poisonous and cause intoxications in humans. The first information about honey poisoning is given by a report dating in 401 B. C. [WOOD et al, 1954]. Honey produced in springtime, is more toxic and could contain a higher concentration of GTXs than honey produced in other seasons [GUNDUZ et al, 2006].

The GTXs are diterpenes which are polyhydroxylated cyclic hydrocarbons [KAN et al, 1994]. They are polar, neutral and saturated [KINGHORN et al, 1978]. GTXs can be best isolated in methanol or chloroform. A drawback is that these solvents dissolve a

wide range of compounds. The toxic isomer in many rhododendrons is GTX III. GTX I is also toxic and GTX II is less toxic [WONG et al, 2002].
Grayanotoxin-poisoning seldom occurs in humans. The incidence is higher in sheep and goats. The LD_{50} of grayanotoxin in mice is 1.31 mg/kgKG [HÄNSEL and STICHER, 2007].



Grayanotoxin I (R=$COCH_3$)
Grayanotoxin III (R=H)

Figure 4: Basic structure of GTX I and GTX III

The toxic effect of GTX is the same as the effect of aconitin. GTX binds to the Na^+-channels in membranes and inhibits the deactivation after an action potential. Therefore the permeability of sodium channels increases. The Ca^{2+} inflow will be high because of the continuing depolarisation. The result is a decrease in blood pressure [FROHNE and PFÄNDER, 2004].

Symptoms of poising with GTX are: irritation of mucous membranes of the mouth and gastro-intestinal-tract which causes nausea, vomiting and diarrhoea; dizziness, headache, fever, difficulties of breathing. Intoxications with GTX are rarely fatal [GUNDUZ et al, 2006], [HÄNSEL and STICHER, 2007].

For the research of GTXs it is necessary to use gentle methods because GTX could commute in other forms. A comparison of an old dried extract of *R. ponticum* and an extract of fresh leaves showed that the amount of GTX I was decreased but the amount of GTX II, GTX III and GTX IV was higher [KÜRTEN, 1971].

This shows also that GTX could suffer chemical changes during storage. In older leaf samples or extracts, a series of GTX was found, which were not found in the fresh leaves. Not in every species degradation products originate in the same number. For example many degradation products are reported for *R. ponticum* and *R. catawbiense,* but none for *R. metternichi* and *R. flavum* [KÜRTEN et al, 1971].

1.4.3 Triterpenes

Triterpenes are characteristic compounds of *Ericaceae* family. They are found in the tissues and also in the thick cuticles. The main triterpene in rhododendrons is ursolic acid [HÄNSEL et al, 1994]. Ursolic acid was identified as an active hepatoprotective component and is effective in protecting against induced liver injury in mice. It is chemically and pharmacological similar to oleanic acid [LIU, 1995].

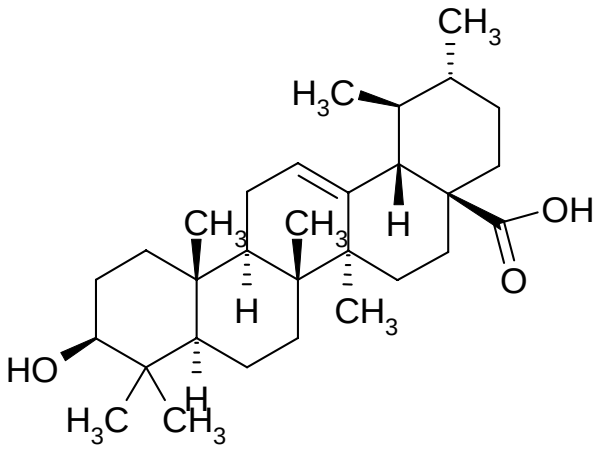


Figure 5: Chemical structure of ursolic acid

Further triterpenes which were found in *R. ferrugineum* are: campanulin, friedelin-type terpenes and α- and β-amyrin [KELLER et al, 1970] [ESSERT, 1988].

1.4.4 Essential Oil

The complex essential oil is contained in the top of the sprouts of *R. ferrugineum* in a concentration at 0.2% [HÄNSEL et al, 1994].

1.4.5 Others

It has been reported that the leaves of *R. ferrugineum* contain organic acids like rhodotann acid and citric acid. In the distillate were found formic acid or acetic acid and butanoic acid [WEHMER et al, 1911].
Calcium, magnesium and potassium were also identified in *R. ferrugineum* [KINZEL and HORAK, 1969].

There is no existing up to date research about the chemical composition of *R. ferrugineum*. The information about the compounds is contradictory and incomplete.

1.5 Rhododendron ferrugineum Usage

The leaves of *R. ferrugineum* are used in traditional medicine against articular gout, concretions, rheumatic ailment, neuralgia, muscular pains, migraine and hypertension. The activity for this field of applications is not scientifically proved. Because the chemical composition has not been well characterized it is inadvisable to ingest the leaves of *R. ferrugineum* as a drug [BAnz, 1990] (see also chapter 1.4.2, page 11).

For traditional medicinal usage dried leaves are made into tea. The maximum dose is 5-6g. Higher doses can cause nausea, diarrhoea or great obnubilation [BAnz, 1990].

R. ferrugineum hom. is a homeopathic preparation which is excluded from the recommendation of non medical use of *R. ferrugineum*. The homeopathic tincture is used to relieve the symptoms of rheumatism, neuralgia and orchitis [SCHÖNFELDER and SCHÖNFELDER, 2004].

2. Objective

The genus of *Rhododendron* belongs to the family of *Ericaceae* [BERG and HEFT, 1991]. Rhododendrons are known as toxic plants and GTXs are accounted for their toxicity. GTXs are secondary metabolites belonging to the diterpene class [KAN et al, 1994].

Rhododendron ferrugineum is one of the European *Rhododendron* species growing in the Alps and Pyrenees. The available literature confirms neither the toxicity of *R. ferrugineum* nor its GTX content. There are no reports about its toxicity in humans, but there is data about its toxicity in animals [BAnz, 1990].

On this basis the present work focuses on the assessment of *R. ferrugineum* extract cytotoxicity and bioassay guided fractionation.

3 Materials and Methods

3.1 Material

3.1.1 Plant Material

Rhododendron ferrugineum L.: leaves collected from Stuhleck (Semmering), Austria in September 2007

3.1.2 Reference Substance

Grayanotoxin III Hemi(ethyl acetate)adduct, 017K1590,
SIGMA-ALDRICH Inc., St. Louis USA

3.1.3 Thin Layer Chromatography

TLC Silica gel 60 F_{254} aluminium sheets 20 x 20 cm, MERCK, Darmstadt, Germany
$CHCl_3$ laboratory quality, dest.
EtOAc laboratory quality, dest.
Propan-2-ol, Prolabo, CE
MeOH laboratory quality, dest.
Aqua dest.
Vanillin purum ≥ 98% (HPLC), 94750, SIGMA-ALDRICH Inc., France
Antimon (III) chloride, B622238, MERCK, Darmstadt, Germany

3.1.4 Column Chromatography

Silicagel 60, grit size 0,063-0,200 mm, MERCK, Darmstadt, Germany

3.1.5 Solid Phase Extraction

Cartridge: "Varian Mega Bond Elut C18"
Bonded Phase C18
Size 6CC

3.1.6 Cell Culture

Cell lines:
- HL-60 promyelocytic leukaemia cell line
- MCF-7 breast cancer cell line

Medium: for HL-60 cells
RPMI 1640 medium, supplemented with:
- 10% heat inactivated foetal bovine serum
- 1% L-Glutamine
- 1% Penicillin/Streptomycin

for MCF-7 cells
D-MEM medium containing:
- 10% fetal bovine serum
- 1% Penicillin/Streptomycin
- 800µg/ml G-418 sulphate

All media and supplements were obtained from "Life Technologies"

All cell types were kept in humidified atmosphere containing 5 % CO_2 at 37°C.

3.2 Plant Material Extraction

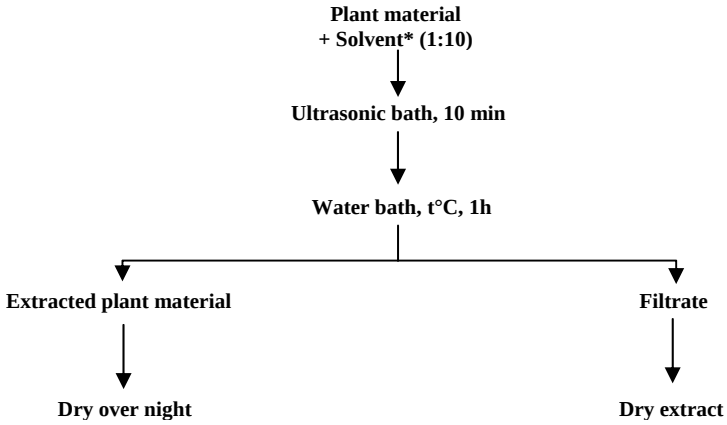
3.2.1 Extraction in Different Polarity Solvents

The dried *Rhododendron ferrugineum* leaves were grounded in a mill with a 0.5 mm sieve. The plant material was extracted with five different polarity solvents: petroleum ether, dichloromethane, ethyl acetate, methanol and water (table. 1).

50g powdered air-dried plant material were extracted under reflux with 500 ml solvent for 1 hour. The layer was filtered and the filtrate was evaporated and stored. The extracted plant material was dried over night and subsequently extracted in the same way with the next solvent (see fig. 6, page 20).

Solvent	**Polarity index**	**Boiling point, °C**	**Solubility in water, % w/w**	**Chemical class extracted**		
Petroleum ether	0,0	40	insoluble	Waxes	Fats	Fixed oils
Dichloromethane	3,1	41	1,6	Alkaloids	Aglycones	Volatile oils
Ethyl acetate	4,4	77	8,7	Alkaloids	Aglycones	Glycosides
Methanol	5,1	65	100	Sugars	Amino acids	Glycosides
Water	9,0	100	100	Sugars	Amino acids	Glycosides

Table 1: Polarity index [HOUGHTON and RAMAN], [CANNELL]



* Petroleum ether, dichloromethane, ethyl acetate, methanol, water

Figure 6: *R. ferrugineum* extraction in different polarity solvents

3.2.2 Extraction According to Wall et al [1996]

100g powdered air-dried plant material were extracted under reflux with 1000 ml solvent for 1 hour. The methanol-layer was filtered and evaporated. The residue was resolved in 300ml methanol-water (9:1) and defatted in a separating funnel with 1200ml hexane. 300ml water were added to the MeOH-H_2O-layer and partitioned with chloroform. After that the $CHCl_3$-layer was washed with 1% NaCl, dried with Na_2SO_4, evaporated and stored. The aqueous-layer was evaporated and stored (see fig. 7).

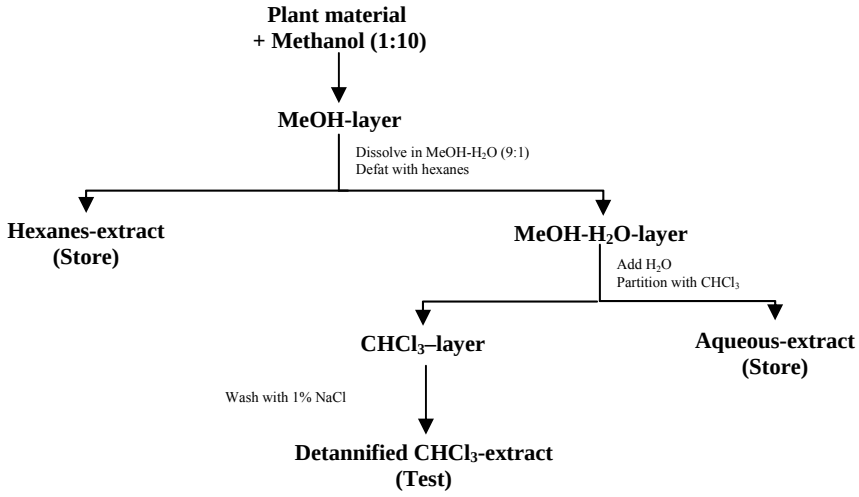


Figure 7: *R. ferrugineum* extraction according to Wall et al [1996]

3.3 Chromatographic Methods

3.3.1 Thin Layer Chromatography

Thin layer chromatography (TLC) is a simple, powerful, fast and cheap analytical method. It involves a stationary phase consisting of a thin layer of adsorbent material, in this case silica gel and a liquid phase [HAHN-DEINSTROP, 2000].

TLC was used for the finger printing of *R. ferrugineum* extracts and for first information about GTX III in the chemical composition of the plant. It was also used for the developing of the right method of separation.

Stationary phase: silica gel 60 F254
Mobile phase:

- ethyl acetate – isopropanol – water (80:24:6)
- ethyl acetate – methanol – water (81:11:8)
- chloroform – methanol – water (90:10:10), lower phase
- chloroform – methanol – water (70:30:10), lower phase
- chloroform – methanol (9:1)
- ethyl acetate saturated with water

Detection:

- 60 % sulfuric acid reagent [KINGHORN et al, 1978]
- vanillin sulfuric acid reagent [WAGNER and BLADT, 1996]
- antimony III chloride reagent [WAGNER et al, 1983]
- white light and UV 365nm

GTX III was used as standard substance.

3.3.2 Column Chromatography

Column chromatography was used to fractionate the extracts obtained by extraction according to Wall et al [Wall et al, 1996].

3.3.2.1 Column Chromatography of $CHCl_3$ Extract

A Ø 2cm column was packed with silica gel 60 in $CHCl_3$-MeOH-H_2O (80:20:10), lower phase at a height of 80 cm.

The plant extract obtained as described in chapter 3.2.2, page 20 was dissolved in methanol, triturated with silica gel 60 and dried. The dried extract-silica-mixture was carefully filled on the top of the column. The mobile phase $CHCl_3$-MeOH-H_2O (80:20:10), lower phase was added and the fractions were collected every 30 minutes. After fraction 105 the mobile phase was changed with $CHCl_3$-MeOH-H_2O (70:30:10), lower phase and after fraction 202 with $CHCl_3$-MeOH-H_2O (50:50:10). The column chromatography operated all in all 8 days. The collected test tubes were checked by TLC and the similar fractions were reunited.

3.3.2.2 Column Chromatography – Fractionation Method for Fraction F3

An Ø 1cm column was packed with silica gel 60 in ethyl acetate saturated with water at a height of 50 cm.

The plant extract obtained as described in chapter 4.8, page 61 was dissolved in methanol, triturated with silica gel 60 and dried. The dried extract-silica-mixture was carefully filled on the top of the column. The mobile phase ethyl acetate saturated with water was added and the fractions were collected every 30 minutes. The column chromatography operated all in all 7 days. The collected test tubes were checked by TLC and the similar fractions were reunited.

3.3.3 Solid Phase Extraction (SPE)

Solid phase extraction is a useful technique for sample preparation to remove solid or semi-solid compounds from a mixture of impurities based on their physical and chemical properties. SPE will be used to concentrate and purify samples for analysis or to isolate analysts of interests from a wide variety of matrices.
[http://www.sigmaaldrich.com/Graphics/Supelco/objects/4600/4538.pdf (2008-05-20)]

SPE was used to separate the fractions obtained by column chromatography.

3.3.3.1 SPE Fractionation Method for Fraction F1

SPE was used after extraction according to Wall et al [1996] and after column chromatography for further separations (see chapter 3.3.2.1, page 22). The SPE was done on C18 cartridges (see chapter 3.1.4, page 17). The cartridge was conditioned with 2 reservoir volumina (RV) methanol and afterwards dried with air for 10 minutes. The fraction which was dissolved in methanol was applied on the cartridge and completely evacuated and collected in a flask (fraction A). The cartridge was eluted successively with 2 RV 90% MeOH (fraction B), 2 RV 80% MeOH (fraction C), 2 RV 70% MeOH (fraction D), 50% MeOH (fraction E) and 4 ml of $CHCl_3$-MeOH (1:1) (fraction F1-2). The six obtained fractions were analysed by TLC. Afterwards the fraction A, B, C, D and E were reunited as F1-1. Both F1-1 and F1-2 were evaporated and tested on cells for cytotoxicity (see chapter 4.6, page 58).

3.3.3.2 SPE Fractionation Method for Fraction F2

SPE was used after extraction according to Wall et al [1996] and after a first fractionation using column chromatography (see chapter 3.3.2.1, page 22). The SPE was done on C18 cartridges (see chapter 3.1.4, page 17). The cartridge was conditioned with 2 reservoir volumina (RV) methanol and afterwards dried with air for 10 minutes. The fraction which was dissolved in methanol was applied on the cartridge and collected in a flask (fraction F2-1). The residual extract on the cartridge was eluted with 2 RV 90% MeOH (fraction A), 2 RV 80% MeOH (fraction B), 2 RV 70% MeOH (fraction C) and 50% MeOH (fraction D). The powder residue on the cartridge was scraped and given in a separate flask. The cartridge was then washed with 4ml of $CHCl_3$-MeOH (1:1) (fraction E) (see chapter 4.7, page 59).

The seven obtained fractions were analysed by TLC (see fig. 46, page 60). Fraction F2-1 and A were evaporated separately and the others (fraction B-E) were reunited as fraction F2-2. Fraction A was fractionated again using the same method in order to separate the two spots visible on TLC. The resulting fractions were added to F2-1 and F2-2 respectively (see fig. 47, page 60). The fraction F2-1 and F2-2 were tested on cells for cytotoxicity (see fig. 48, page 61).

3.3.3.3 SPE Fractionation Method for Fraction F3-6

SPE was used after extraction according to Wall et al [1996] and after column chromatography fractioning of fraction F3 for further separations (see chapter 4.9, page 64). The cartridge was conditioned as described in chapter 3.3.3.1, page 23. Afterwards the fraction F3-6 dissolved in methanol was applied on the cartridge and completely evacuated (fraction F3-6-VL). The cartridge was then eluted with 2 RV 100% MeOH (fraction A), 2 RV 98% MeOH (fraction B) and 4 ml of $CHCl_3$-MeOH (1:1) (fraction C). The four obtained fractions were analysed by TLC. Fraction B and C were reunited as fraction F3-6-1 and evaporated, fraction F3-6-VL was evaporated and stored separate. Fraction B was fractionated again using the same method in order to separate the two spots visible on TLC. The first two resulting fractions were added to F3-6-VL and F3-6-1 respectively. The third resulted fraction was evaporated as F3-6-NL (see fig. 51, page 64). The fraction F3-6-VL and F3-6-1 and F3-6-NL were tested on cells for cytotoxicity (see fig. 52, page 65).

3.4 Proliferation Assay

For the proliferation assay the trypan blue dye exclusion method was used. The trypan blue dye can not permeate intact cell membrane. Dead cells will incorporate the dye and become darker than the viable ones. This contrast was measured with a VI-Cell Cell Viability Analyzer.
[http://www.beckmancoulter.com/Literature/BioResearch/BR-9713B.pdf (2008-05-20)]

3.4.1 HL-60 Human Leukaemia Cell Line

HL-60 cells were seeded at a density of 100 000 cells/ml in T25 flasks. After 24 hours cells were treated with plant extract in increasing concentrations (0.5, 1, 4 mg/ml plant material). The control was treated with the ethanol concentration of the highest extract concentration. The cell number was determined with a cell counter (VI-Cell TM x R Cell Viability Analyzer; Beckman Coulter; Program: VI-Cell x R 2.03) after 24, 48 and 72 hours after extract administration.

3.4.2 MCF-7 Breast Cancer Cell Line

MCF-7 cells were seeded at a density of 10 000 cells/ml in 24-well plates and grown for 24 hours. After 24 hours cells were treated with plant extract in increasing concentrations (0.5, 1, 4 mg/ml plant material). Before counting the MCF-7 cells were washed with PBS (washing buffer), trypsinised and resuspended in medium.
The cell number was determined with a cell counter (VI-Cell TM x R Cell Viability Analyzer; Beckman Coulter; Program: VI-Cell x R 2.03) after 24, 48 and 72 hours after extract administration.

3.4.3 Calculation and Statistic

Calculation of cell division:

$$\frac{(C_{72\,h} + \text{drug}) - (C_{24\,h} + \text{drug})}{(C_{72\,h} - \text{drug}) - (C_{24\,h} - \text{drug})} \times 100$$

$C_{72\,h}$ + drug....cell number after 72 hours of drug treatment

$C_{24\,h}$ + drug....cell number after 24 hours of drug treatment

$C_{72\,h}$ - drug....cell number after 72 hours without drug treatment

$C_{24\,h}$ - drug....cell number after 24 hours without drug treatment

All experiments were made in triplicate and analysed by t-test. The used program was GraphPad Prism 4.0.

4. Results

4.1 Extraction in Different Polarity Solvents

For the first extraction method I used a polarity gradient. Five different polarity solvents were used to separate all compounds: petroleum ether, dichloromethane, ethyl acetate, methanol, water. The plant material (50g) was extracted as described in chapter 3.2.1, page 19. The yield of each extract is listed in table 2.

Solvent	**yield (g)**
petroleum ether	**2.048**
dichloromethane	**2.516**
ethyl acetate	**2.563**
methanol	**9.163**
water	**4.025**

Table. 2: Yield of *R. ferrugineum* extracts in different polarity solvents

The five extracts have been fingerprinted using TLC (see chapter 4.2, page 27).

4.2 Detection Methods

The five extracts have been fingerprinted using TLC. GTX III was used as standard substance.

Small amounts of extract were solubilized in their respective solvent. These solutions were applied on TLC-plates and analyzed using the following solvent systems: ethyl acetate-methanol-water (81:11:8); chloroform-methanol-water (90:10:10), lower phase; chloroform-methanol-water (70:30:10), lower phase; The plates were developed, dried and then sprayed on the one hand with 60% H_2SO_4 (110°C, 5min) and on the other hand

with vanillin sulfuric acid reagent (110°C, 7min). The developed plates were viewed in visible light and under ultraviolet light (365nm) (see fig. 13 – 18, page 33-36).

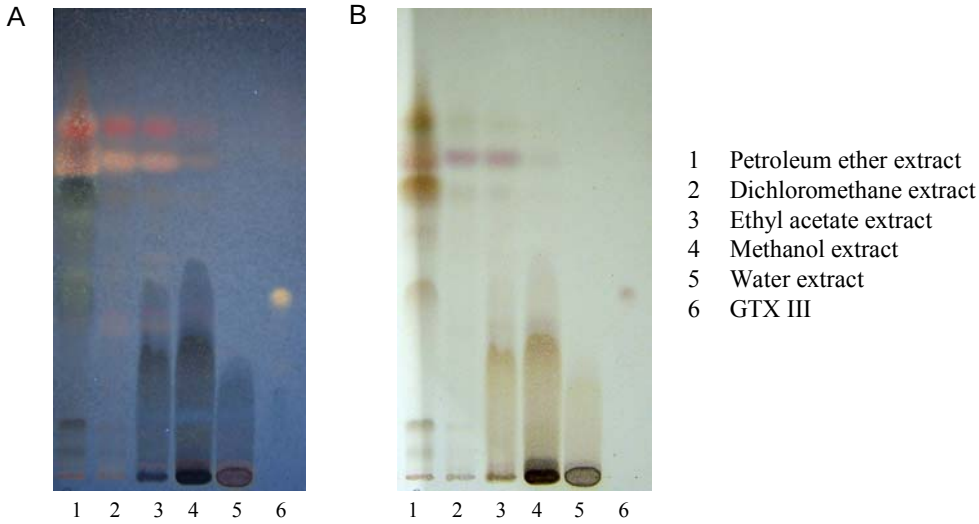
Detection methods previously used to characterize the GTX content were also tried. "Kinghorn et al" utilized the TLC solvent system and detection method of ethyl acetate-isopropanol-water (80:24:6) and 60% H_2SO_4 [KINGHORN et al, 1978]. Keller et al used chloroform-methanol (9:1) as solvent system and antimony III chloride as detection reagent [KELLER et al, 1970].

4.2.1 Detection Method According to Kinghorn et al [1978]

In the first step I used "Kinghorn et al" TLC solvent system and detection method of ethyl acetate-isopropanol-water (80:24:6) and 60% H_2SO_4 (see table 3, page 39). In this publication *R. maximum* had been analysed for the content of different GTX derivatives [KINGHORN et al, 1978].

Figure 8: TLC of *R. ferrugineum* extracts

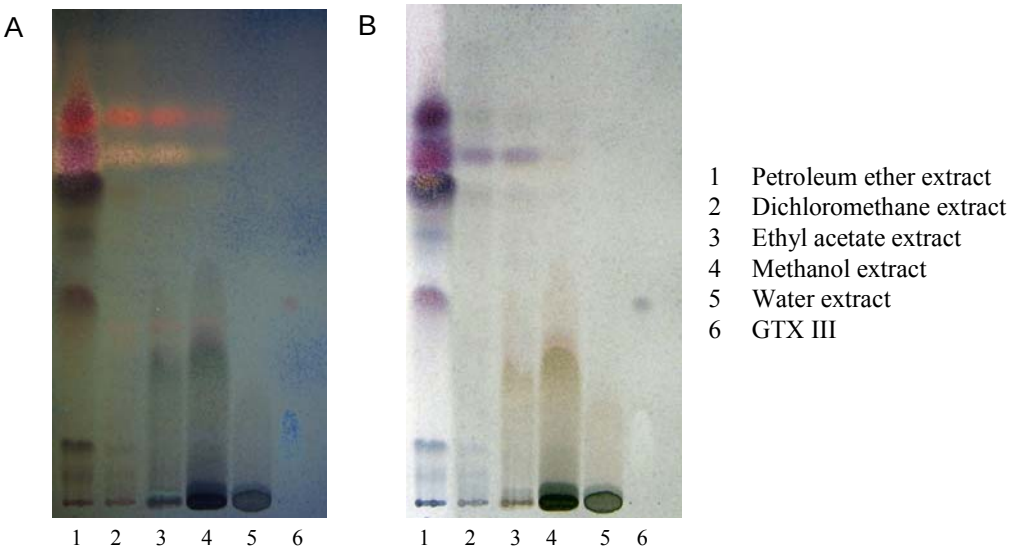
Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-isopropanol-water (80:24:6)
Detection: 60% H_2SO_4, UV 365nm (**A**)
60% H_2SO_4, white light (**B**)



The solvent system ethyl acetate-isopropanol-water (80:24:6) was good for the separation of middle polar components. As seen in fig. 9 it was also performed using another spraying reagent. The detection with 60 % H_2SO_4 proved to be good in making GTX III visible because it fluoresces under UV-light as seen in fig. 8, page 28.

Figure 9: TLC of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-isopropanol-water (80:24:6)
Detection: vanillin H_2SO_4 reagent, UV 365nm (A)
vanillin H_2SO_4 reagent, white light (B)



As seen in fig. 9 GTX III was better visible by spraying with 60 % H_2SO_4 than spraying with vanillin sulfuric acid reagent because it fluoresces under UV-light.

Kinghorn et al described an Rf value of 0.48 for GTX III. After reproducing the experiment twice I obtained an Rf value of 0.45. The Rf values for GTX III are listed in table 3, page 39.

A definite analogy with grayanotoxin III as reference substance couldn't be observed for the *R. ferrugineum* extracts.

4.2.2 Detection Method According to S. auf dem Keller et al [1970]

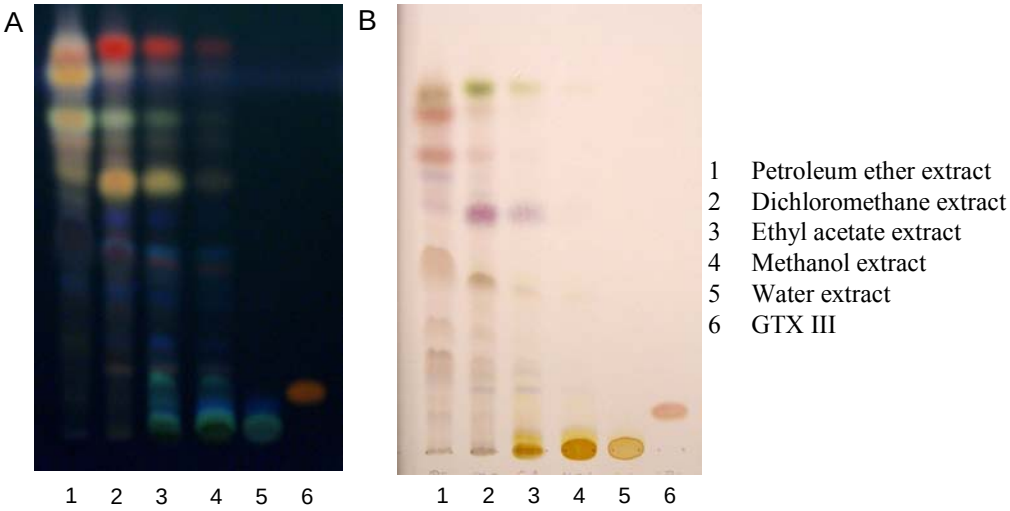
Because vanillin sulfuric reagent and 60 % H_2SO_4 could not detect GTX III in the plant extracts the detection method according to S. auf dem Keller et al using antimony III chloride reagent was also tried.

Keller et al verified GTX III in *Rhododendron ponticum* with chloroform-methanol (9:1) as solvent system, for detection antimony III chloride reagent was used (see fig. 10). Keller et al used TLC -silica gel HF aluminium plates as stationary phase. For the present work silica gel 60 F_{254} aluminium TLC plates were used [KELLER et al, 1970].

Keller et al described an Rf value of 0.04 for GTX III using HF aluminium plates "Riedel" as stationary phase. After reproducing the experiment with silica gel 60 F_{254} as stationary phase I obtained an Rf value of 0.09 (see fig. 10, table 3 page 39).

Figure 10: TLC according to Keller et al [1970] of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol (9:1)
Detection: antimony III chloride reagent , UV 365nm (A)
antimony III chloride reagent , white light (B)



Spraying with antimony III chloride reagent is a good method to make GTX III visible both at visible light and in UV-light. As seen in fig. 11-12, page 32 it was also performed using other solvent systems: ethyl acetate-methanol-water (81:11:8) (see fig. 12, page 32) and chloroform-methanol-water (70:30:10), lower phase (see fig 11) which showed a good separation of GTX III in other tests (see chapter 4.2.3, page 33). The Rf values for GTX III are listed in table 3, page 39.

Figure 11: TLC detection according Keller et al [1970] of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (70:30:10), lower phase
Detection: antimony III chloride reagent , UV 365nm (A)
antimony III chloride reagent , white light (B)

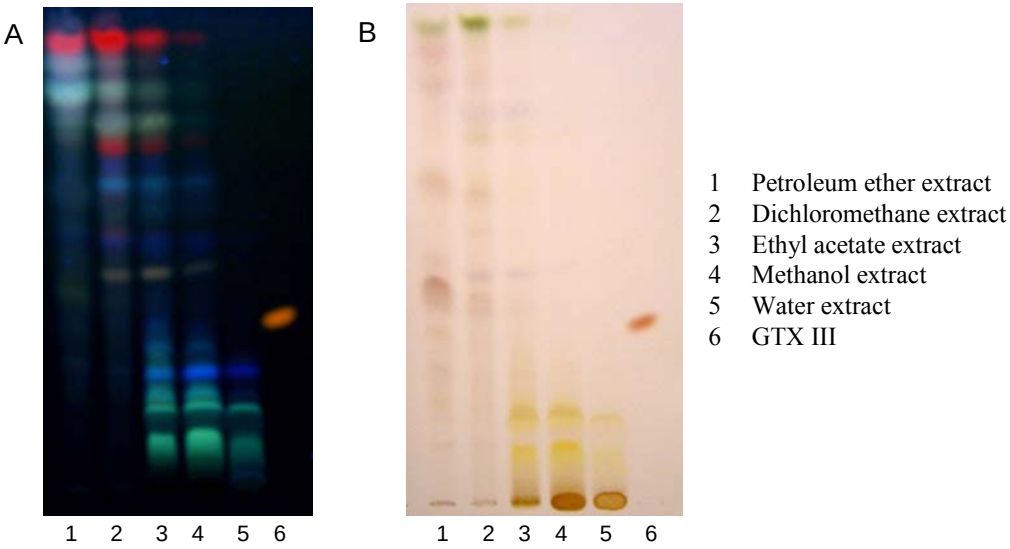
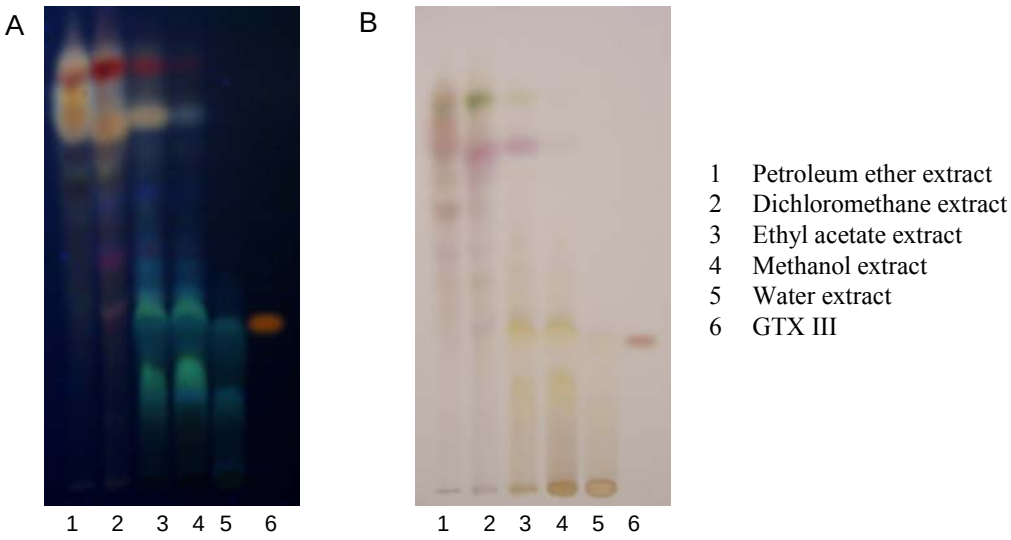


Figure 12: TLC detection according Keller et al [1970] of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-methanol-water (81:11:8)
Detection: antimony III chloride reagent , UV 365nm (A)
antimony III chloride reagent , white light (B)



As seen in fig 11, page 31 and in fig 12 we couldn't make a definite statement about GTX III in *R. ferrugineum.* So the next step was to look for another detection method which maybe makes GTX better visible.

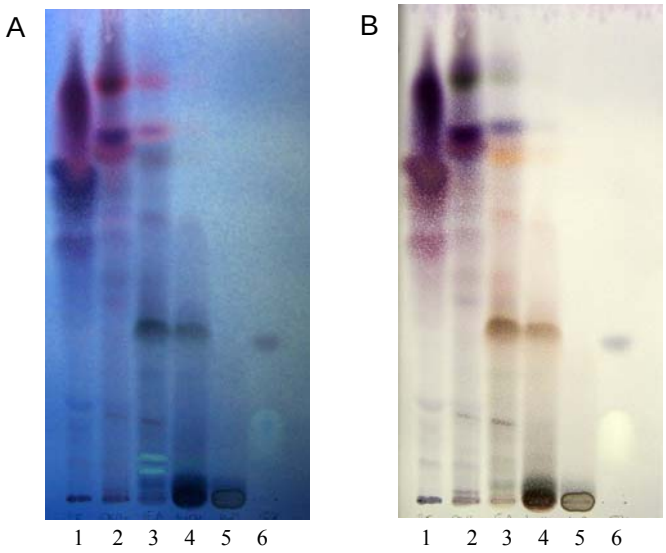
4.2.3 Other Detection Methods

Other detection methods used were vanillin sulfuric acid reagent and 60% H_2SO_4 as detection reagent for TLC, ethyl acetate-methanol-water (81:11:8); chloroform-methanol-water (90:10:10), lower phase and chloroform-methanol-water (70:30:10), lower phase were used as mobile phase.

The solvent system ethyl acetate-methanol-water (81:11:8) was good for the separation of apolar components. GTX III was also better visible by spraying with 60 % H_2SO_4 than spraying with vanillin sulfuric acid reagent because it fluoresces under UV-light as seen in fig. 13-14, page 34.

Figure 13: TLC of *R. ferrugineum* extracts

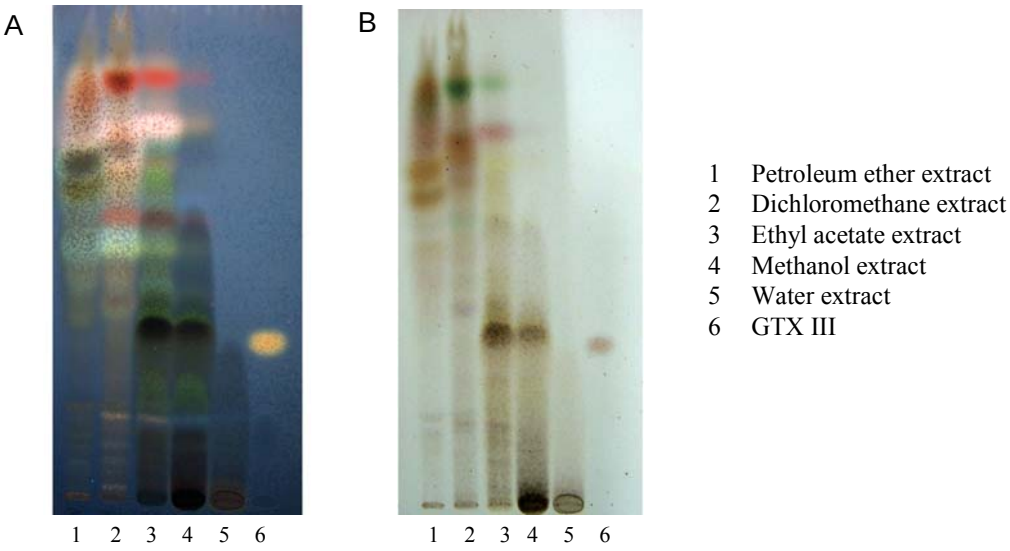
Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-methanol-water (81:11:8)
Detection: vanillin H_2SO_4 reagent, UV 365nm (A)
vanillin H_2SO_4 reagent, white light (B)



1 Petroleum ether extract
2 Dichloromethane extract
3 Ethyl acetate extract
4 Methanol extract
5 Water extract
6 GTX III

Figure 14: TLC of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-methanol-water (81:11:8)
Detection: 60% H_2SO_4, UV 365nm (A)
60% H_2SO_4, white light (B)



The system chloroform-methanol-water (90:10:10), lower phase showed a good separation of apolar components as seen in fig. 15, page 35 but it was not a suitable mobile phase for the characterization of GTX III. As seen in fig. 15 and 16, page 35 GTX III was not transported in this system.

A better solvent system for GTX III was chloroform-methanol-water (70:30:10), lower phase see (fig. 17-18, page 36). This also proved to be a good system for the characterization of middle polar components.

Figure 15: TLC of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (90:10:10), lower phase
Detection: vanillin H_2SO_4 reagent, UV 365nm (A)
vanillin H_2SO_4 reagent, white light (B)

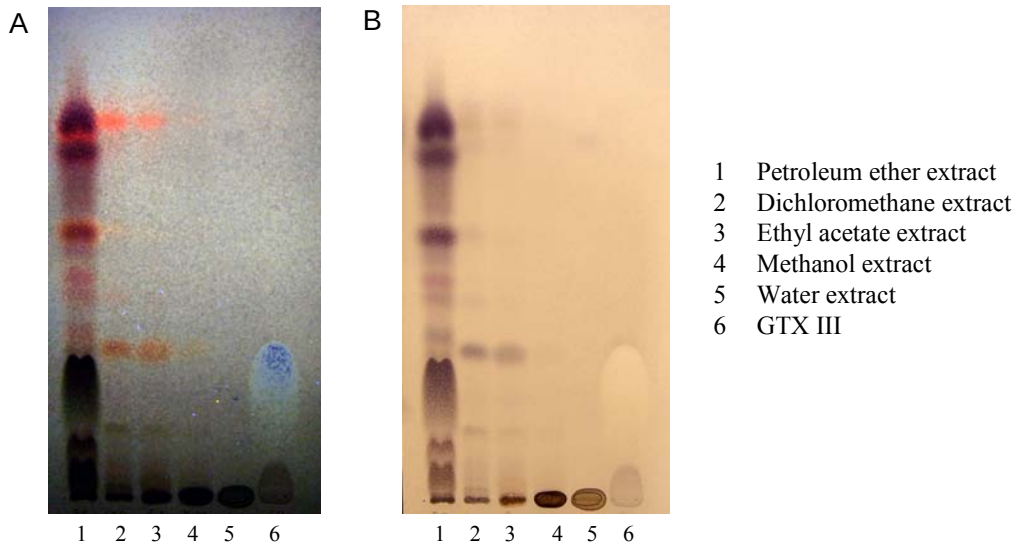


Figure 16: TLC of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F254
Mobile phase: chloroform- methanol-water (90:10:10), lower phase
Detection: 60% H_2SO_4, UV 365nm (A)
60% H_2SO_4, white light (B)

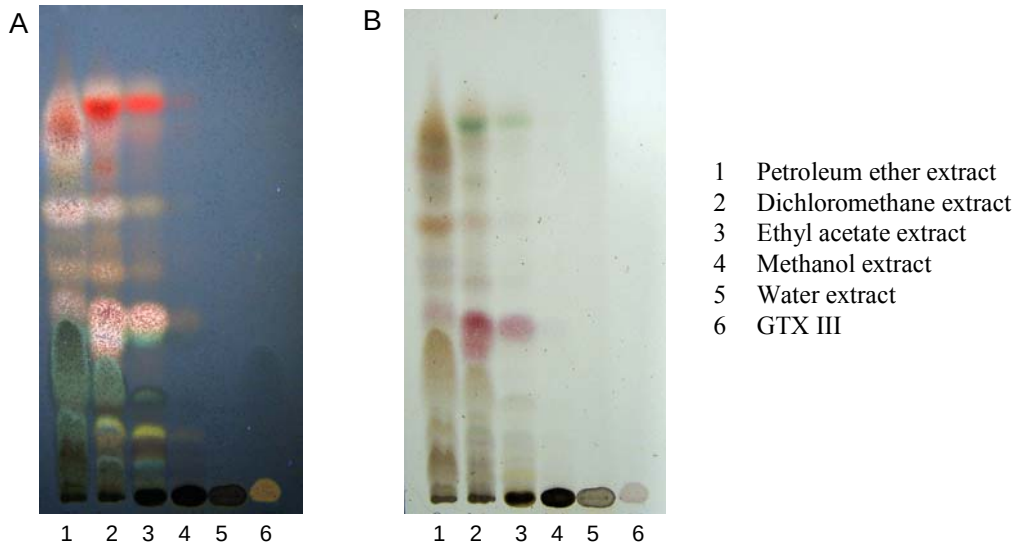
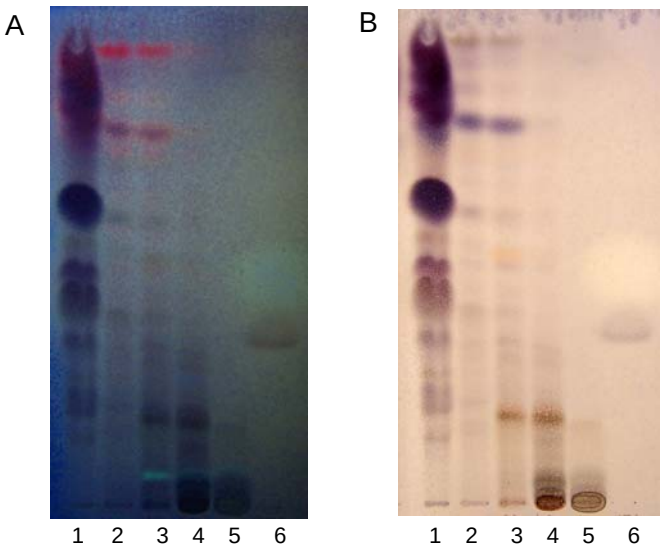


Figure 17: TLC of *R. ferrugineum* extracts

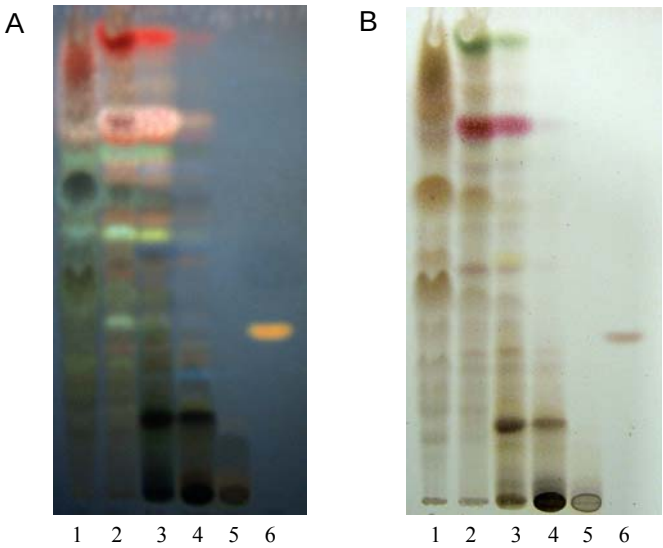
Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methano-water (70:30:10), lower phase
Detection: vanillin H_2SO_4 reagent, UV 365nm (A)
vanillin H_2SO_4 reagent, white light (B)



1 Petroleum ether extract
2 Dichloromethane extract
3 Ethyl acetate extract
4 Methanol extract
5 Water extract
6 GTX III

Figure 18: TLC of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (70:30:10), lower phase
Detection: 60% H_2SO_4, UV 365nm (A)
60% H_2SO_4, white light (B)



1 Petroleum ether extract
2 Dichloromethane extract
3 Ethyl acetate extract
4 Methanol extract
5 Water extract
6 GTX III

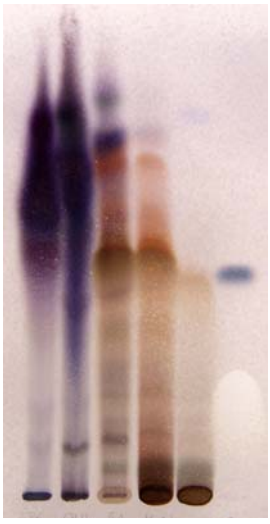
As seen in fig.8 – 18, page 28-36 there is no definite information about GTX III in the different *Rhododendron ferrugineum* extracts after analysis of the plates. No analogue spots to the reference substance were found neither in the visible light nor in UV-light. Furthermore it was found out that the detection with vanillin sulphuric acid is a good method to detect GTX III in visible light, but not in UV-light. The detection with 60 % H_2SO_4 reveals GTX III also in UV-light.

One of the reasons of not detecting GTX III in the extracts could be that the concentration of GTX III in the extract is too low or it is disguised by other components e.g. tannins. Tannins could make the detection of GTX difficult and could also be a reason of false positive results (see chapter 1.4.1.1, page 9).

The next step was to increase the concentration of extract. The used solvent system was ethyl acetate-isopropanol-water (80:24:6) and for detection the plates were sprayed on the one hand with 60% H_2SO_4 (110°C, 5min) and on the other hand with vanillin sulfuric acid reagent (110°C, 7min). The developed plates were viewed in visible light and under ultraviolet light (365nm) (see fig.19-20, page 38).

Figure 19: TLC with higher concentration of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-isopropanol-water (80:24:6)
Detection: vanillin H_2SO_4 reagent, white light

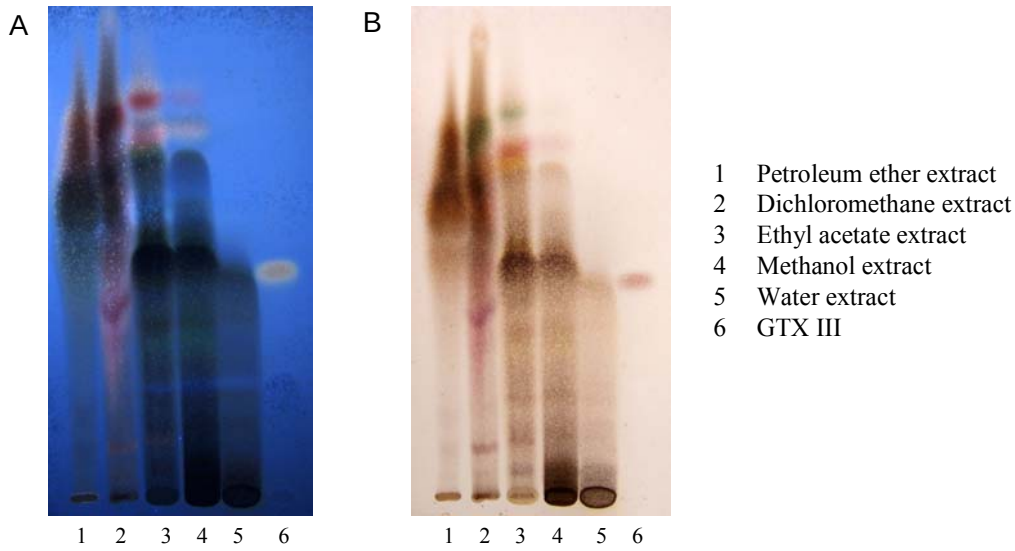


1 2 3 4 5 6

1 Petroleum ether extract
2 Dichloromethane extract
3 Ethyl acetate extract
4 Methanol extract
5 Water extract
6 GTX III

Figure 20: TLC with higher concentration of *R. ferrugineum* extracts

Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-isopropanol-water (80:24:6)
Detection: 60% H_2SO_4, UV 365nm (A)
60% H_2SO_4, white light (B)



As seen in fig. 19, page 37 and fig. 20 there is no definite result after increasing the concentrations of the rhododendron extracts. The next step was to eliminate the tannins which maybe mask the GTX III spot on the TLC (see chapter 4.3, page 40).
Another reason of eliminating the tannins is their possible interference with biological assays.

	Solvent system	Detection	*Rf*	Colour VL	Colour UV 366nm
	E.A.-isopropanol-H_2O (80:24:6)	60% H_2SO_4	0,45	brown-pink	yellow
	E.A.-isopropanol-H_2O (80:24:6)	vanillin sulfuric acid	0,46	blue	blue-orange
	$CHCl_3$-MeOH (9:1)	antimony-III-chlorid	0,09	orange-brown	orange
	E.A.-MeOH-H_2O (81:11:8)	60% H_2SO_4	0,15	brown-pink	yellow
	E.A.-MeOH-H_2O (81:11:8)	vanillin sulfuric acid	0,15	blue	purple
	E.A.-MeOH-H_2O (81:11:8)	antimony-III-chlorid	0,15	orange-brown	orange
	$CHCl_3$-MeOH-H_2O (70:30:10) lower phase	60% H_2SO_4	0,34	brown-pink	yellow
	$CHCl_3$-MeOH-H_2O (70:30:10) lower phase	vanillin sulfuric acid	0,34	purple	brown
	$CHCl_3$-MeOH-H_2O (70:30:10) lower phase	antimony-III-chlorid	0,45	orange-brown	orange
	$CHCl_3$-MeOH-H_2O (90:10:10) lower phase	60% H_2SO_4	0,01	brown-pink	yellow
GTX III detection	$CHCl_3$-MeOH-H_2O (90:10:10) lower phase	vanillin sulfuric acid	0,01	dark blue	brown
GTX III detection Keller et al	$CHCl_3$-MeOH (9:1)	antimony-III-chlorid	0,04	red-brown	not listed
GTX III detection Kinghorn et al	E.A.-isopropanol-H_2O (80:24:6)	60% H_2SO_4	0,48	orange	orange

Table 3: Comparison of the GTX III Rf-results obtained in the present work and by Keller et al [1970], and Kinghorn et al [1978] using TLC analysis

4.3 Extraction Method According to Wall et al [1978]

Tannins are polyphenols which have the property to precipitate proteins through hydrogen bonding. They can therefore give false-positive or false-negative results in biological assays [SILVA et al, 1998] (preparation see chapter 3.2.2, page 20).The extraction method according to Wall et al yields a detannified extract, more suitable for biological assays.

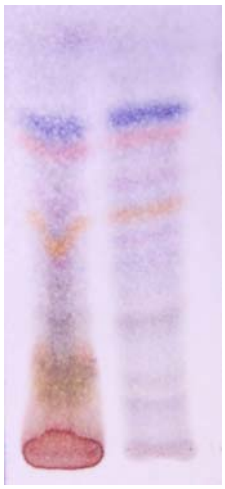
The powdered air-dried plant material (100g) was extracted under reflux with methanol for 1 hour. The methanol-layer was filtered and evaporated. The residue was resolved in a mixture of methanol-water (9:1) and defatted in a separating funnel with hexane. Afterwards water was added to the MeOH-H_2O-layer and partitioned with chloroform. After that the $CHCl_3$-layer was washed with 1% NaCl, dried with Na_2SO_4, evaporated and stored. The aqueous-layer was evaporated and stored (scheme see fig 7, page 20).

When MeOH-H_2O-layer was partitioned with chloroform, an emulsion occurred (see fig. 21). Further on we tried to partition the MeOH-H_2O-layer with ethyl acetate to reduce the inconvenience of an emulsion. Samples of MeOH-H_2O-layer were partitioned with chloroform and ethyl acetate respectively. The ethyl acetate layer was not as detannified as the chloroform layer (see fig. 22, page 41).

Figure 21: Partition of MeOH-H_2O-layer with chloroform

Figure 22: TLC comparison of ethyl acetate and chloroform layers

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (70:30:10), lower phase
Detection: vanillin H_2SO_4 reagent, white light



1 2

1 Ethyl acetate layer
2 $CHCl_3$ layer

Because the ethyl acetate layer was not as detannified as the chloroform layer we decided to use chloroform for partition.

The yield of the detannified chloroform extract was 860.5mg. The detannified chloroform extract was compared by TLC with the previous ethyl acetate extract (see chapter 3.2.1, page 19) of *R. ferrugineum* using chloroform-methanol-water (70:30:10), lower phase as mobile phase and detection with 60% H_2SO_4 and vanillin sulfuric acid reagent (see fig. 23-26, page 43-44).

The TLCs show that the extraction method according to Wall et al removes the tannins, while the extract in different polarity solvents yields a tannin containing ethyl acetate extract. There is also no TLC evidence of the presence of GTX III in the ethyl acetate extract and in the detannified chloroform extracts.

Figure 23: Comparison of ethyl acetate extract and detannified chloroform extract by TLC

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (70:30:10), lower phase
Detection: 60% H_2SO_4 , UV 365nm (A)
60% H_2SO_4 , white light (B)
vanillin H_2SO_4 reagent, white light (C)

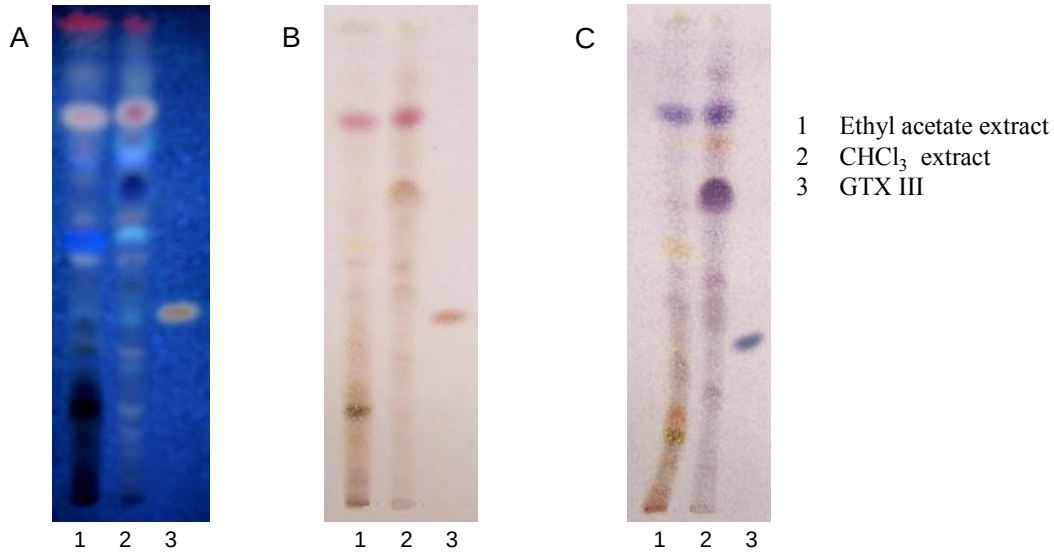
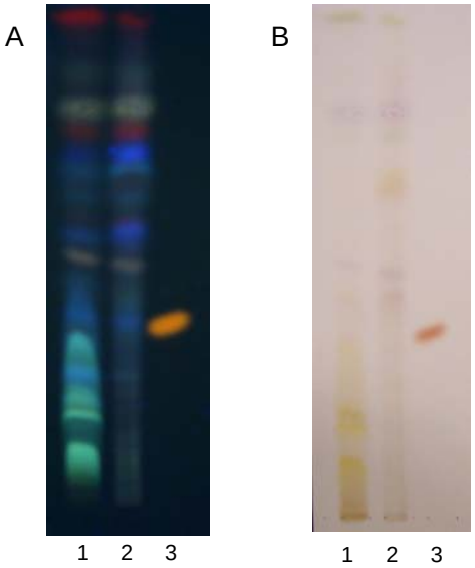


Figure 24: Comparison of ethyl acetate extract and detannified chloroform extract by TLC

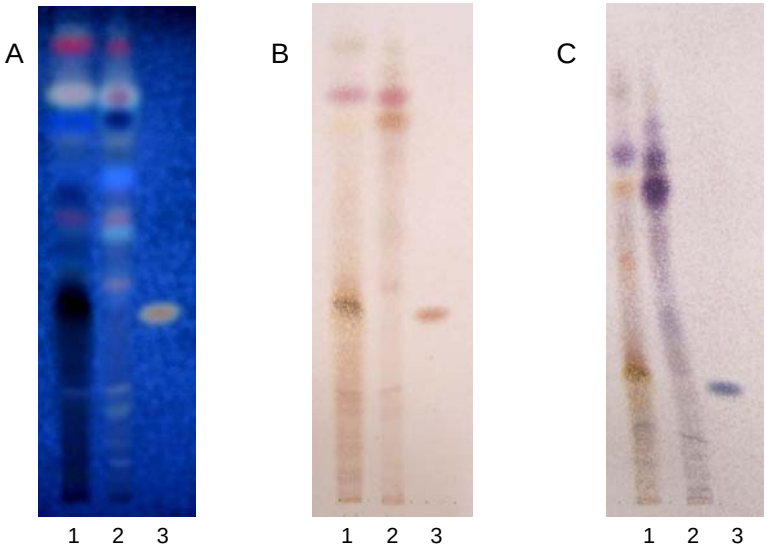
Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (70:30:10), lower phase
Detection: antimony III chloride reagent , UV 365nm (A)
antimony III chloride reagent , white light (B)



1 Ethyl acetate extract
2 $CHCl_3$ extract
3 GTX III

Figure 25: Comparison of ethyl acetate extract and detannified chloroform extract by TLC

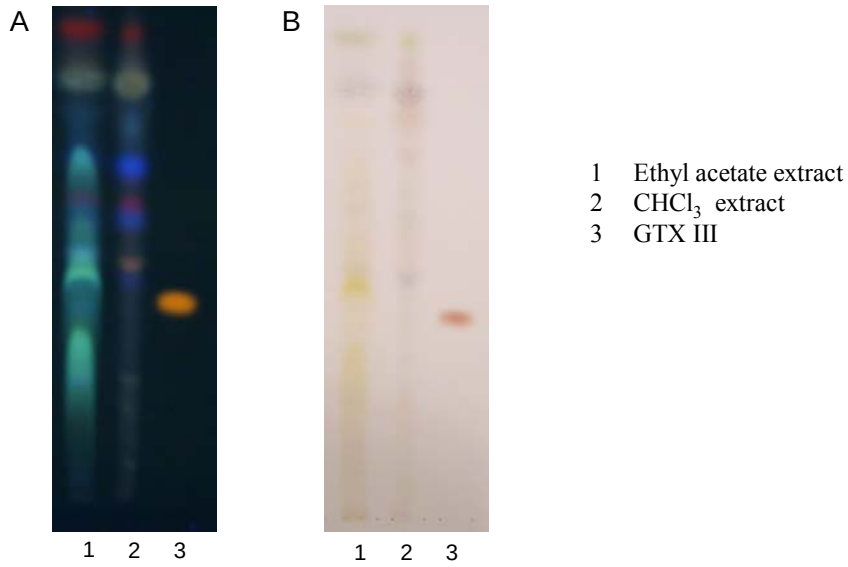
Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-methanol-water (81:11:8)
Detection: 60% H_2SO_4 , UV 365nm (A)
60% H_2SO_4 , white light (B)
vanillin H_2SO_4 reagent, white light (C)



1 Ethyl acetate extract
2 $CHCl_3$ extract
3 GTX III

Figure 26: Comparison of ethyl acetate extract and detannified chloroform extract by TLC

Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-methanol-water (81:11:8)
Detection: antimony III chloride reagent , UV 365nm (A)
antimony III chloride reagent , white light (B)



The extracts obtained using the two different methods have been further characterized by cell based proliferation assay (see chapter 4.4, page 45).

4.4 Proliferation Assay

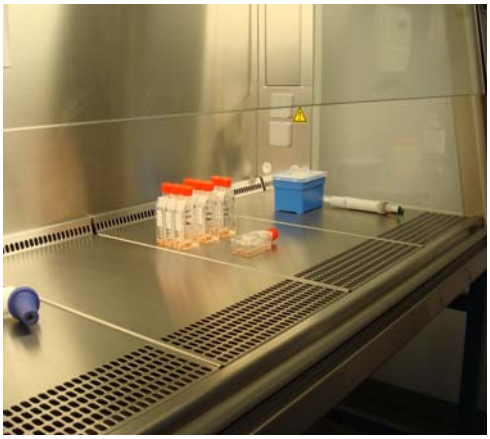


Figure 27: Laminar flow

The ethyl acetate extract which was obtained from extraction in different polarity solvents (see chapter 3.2.1, page 19) and the $CHCl_3$ extract (see chapter 3.2.2, page 20) were tested for cytotoxicity on HL-60 and MCF-7 cell line and their activity was compared. Ethyl acetate extract was chosen because it showed activity in previous tests[1]. Both extracts were dissolved in EtOH for these assays.

4.4.1 Proliferation Assay with *R. ferrugineum* Extracts and HL-60 Cell Line

Logarithmically growing HL-60 human leukaemia cells were incubated with or without different plant material concentrations (0.5 – 4 mg/ml). (See chapter 3.1.5, page 18 and 3.4.1, page 25) The control samples were incubated with the highest EtOH sample concentration (0.04 % EtOH; see also table 4).

3x Control (+EtOH)	
3x R *ferrugineum* E.A. 0.5 mg/ml	3x R *ferrugineum* $CHCl_3$ 0.5 mg/ml
3x R *ferrugineum* E.A. 1 mg/ml	3x R *ferrugineum* $CHCl_3$ 1 mg/ml
3x R *ferrugineum* E.A. 4 mg/ml	3x R *ferrugineum* $CHCl_3$ 4 mg/ml

Table 4: Added extracts to HL-60 cells

The cells were counted after 24, 48 and 72 hours with a cell counter directly. All experiments were made in triplicate and analysed by t-test.

[1] ongoing doctoral thesis Mag. Ruxandra Popescu

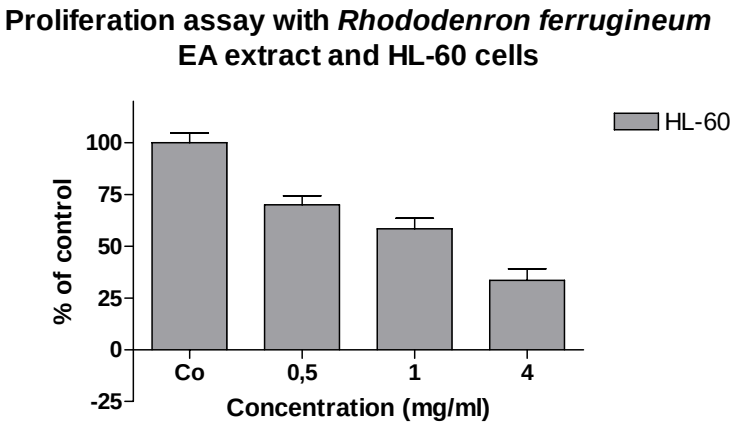


Figure 28: Results of the proliferation assay with HL-60 cells incubated with ethyl acetate extract (before eliminating tannins)

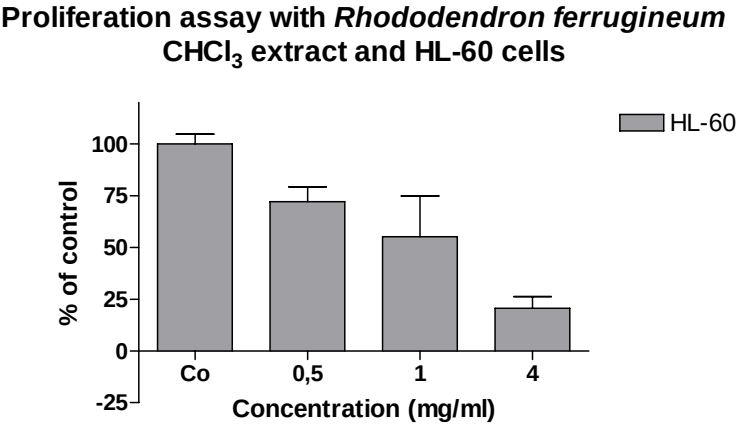


Figure 29: Results of the proliferation assay with HL-60 cells incubated with $CHCl_3$ extract (after eliminating tannins)

Both the ethyl acetate extract and the chloroform extract showed a dose-dependent decrease in the proliferation rate (see fig. 28-29) on HL-60 cells. That shows that activity of the extract exists also after the elimination of the tannins.

4.4.2 Proliferation Assay with *R. ferrugineum* Extracts and MCF-7 Cell Line

MCF-7 breast cancer cells were incubated with or without different plant material concentrations (0.5 – 4 mg/ml, see chapter 3.1.5, page 18 and 3.4.2, page 25). The control samples were incubated with the highest EtOH sample concentration (0.2 % EtOH) (see also table 5).

3x Control (+EtOH)	
3x R *ferrugineum* E.A. 0.5 mg/ml	3x R *ferrugineum* $CHCl_3$ 0.5 mg/ml
3x R *ferrugineum* E.A. 1 mg/ml	3x R *ferrugineum* $CHCl_3$ 1 mg/ml
3x R *ferrugineum* E.A. 4 mg/ml	3x R *ferrugineum* $CHCl_3$ 4 mg/ml

Table 5: Added extracts to MCF-7 breast cancer cells

To determine the effect on the cells, the cells were counted after 24, 48 and 72 hours. Therefore the MCF-7 cells were washed with PBS (washing buffer), trypsinised, added medium to stop the activity of trypsin and determined with a cell counter. All experiments were made in triplicate and analysed by t-test.

As in the case of the proliferation assays with the HL-60 cells, the tests with MCF-7 cells showed a dose-dependent decrease in the proliferation rate (see fig. 30-31, page 48).

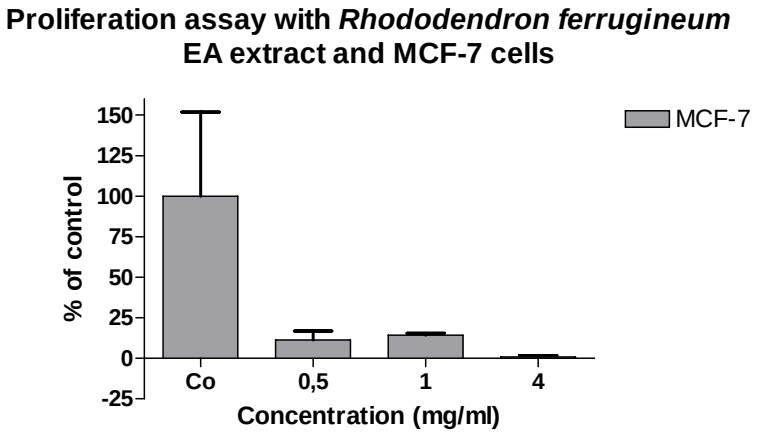


Figure 30: Results of the proliferation assay with MCF-7 cells incubated with ethyl acetate extract (before eliminating tannins)

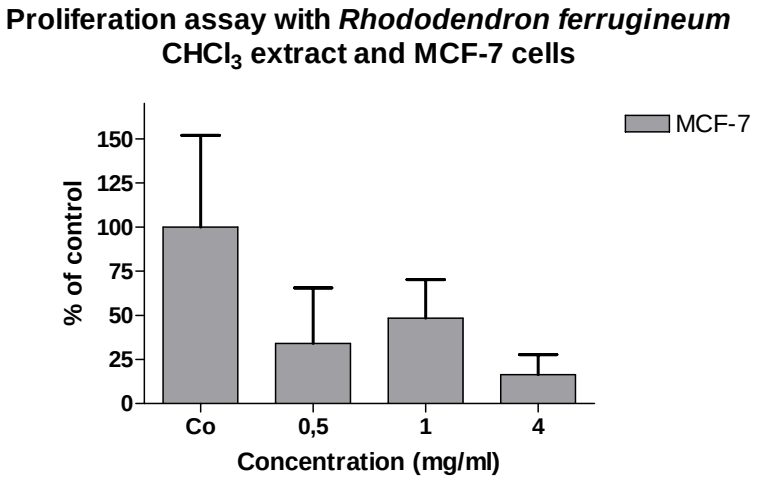


Figure 31: Results of the proliferation assay with MCF-7 cells incubated with $CHCl_3$ extract (after eliminating tannins)

As seen above (fig. 28-30, page 46-48) the rhododendron extracts showed an antiproliferative activity on HL-60 and MCF-7 cells, both before and after eliminating the tannins. The next step was the fractionation of the active extract. Because the activity of the two extracts was similar and because tannins can interfere with biological assays giving false positive results, the detannified chloroform extract was chosen for a further fractionation by column chromatography.

4.5 Column Chromatography of $CHCl_3$ Extract

The column (see chapter 3.1.4, page 17) was packed with silica gel 60 which was elutriated in $CHCl_3$-MeOH-H_2O (80:20:10), lower phase because the $CHCl_3$ system was a good one for separation on the TLC (see chapter 4.1, page 27). Silica gel was used because the stationary phase on the TLC was the same. The plant extract (750mg) and solvent was filled on the top of the column and the valve was opened with a flow rate of 18 drops/min. The collected test tubes were checked by TLC and the fractions with a similar or the same pattern on the TLC plate were reunited (see chapter 3.3.2.1, page 22).

The solvent was changed at first after test tube number 105 in $CHCl_3$-MeOH-H_2O (70:30:10), lower phase and second after test tube number 202 in $CHCl_3$-MeOH-H_2O (50:50:10) to dissolve the polar components from the column. The column chromatography operated all in all 8 days.

Figure 32: Chromatographic column eight hours after starting

Figure 33: First obtained fractions of $CHCl_3$ extract by column chromatography

All in all 11 cumulative fractions were obtained and reunited. The yield of each fraction is listed in table 6.

single fraction 1-13	cumulative fraction 1	**F1**	16.6 mg
single fraction 14-18	cumulative fraction 2	**F2**	196.2 mg
single fraction 19-24	cumulative fraction 3	**F3**	59.9 mg
single fraction 25-32	cumulative fraction 4	**F4**	28.2 mg
single fraction 33-47	cumulative fraction 5	**F5**	40.9 mg
single fraction 48-56	cumulative fraction 6	**F6**	14.9 mg
single fraction 57-77	cumulative fraction 7	**F7**	169.1 mg
single fraction 78-125	cumulative fraction 8	**F8**	34.9 mg
single fraction 126-148	cumulative fraction 9	**F9**	53.6 mg
single fraction 149-163	cumulative fraction 10	**F10**	22.5 mg
single fraction 164-350	cumulative fraction 11	**F11**	289.3 mg

Table 6: Cumulative fractions obtained by column chromatography

4.5.1 Analysis by TLC

All obtained cumulate fractions were analysed by TLC with two different solvent systems:

- $CHCl_3$-MeOH-H_2O (70:30:10), lower phase
- Ethyl acetate – methanol – water (81:11:8)

For detection the following reagents were used (see also chapter 3.1.3, page 17):

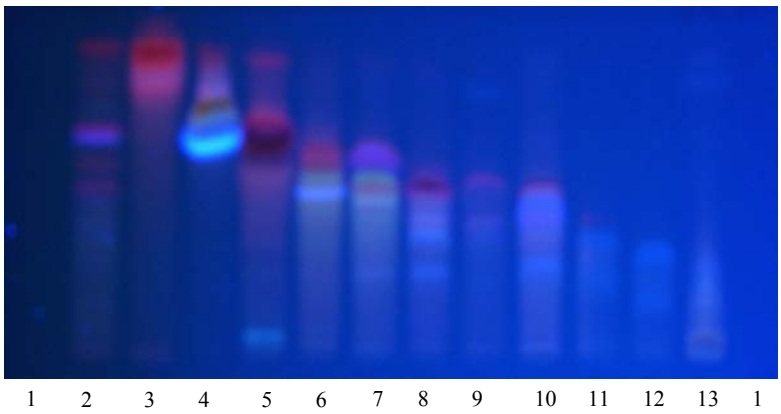
- Vanillin sulfuric acid reagent
- 60 % sulfuric acid reagent

All TLC-plates in each solvent system were scanned without treatment and after spraying with detection reagent (see fig. 34-41, page 52-55)

As seen in fig. 34-35, page 52 the TLC analysis showed a good separation of the compounds but no evidence of the presence of GTX III in the fractions.

Figure 34: TLC of cumulative fractions F1-F11

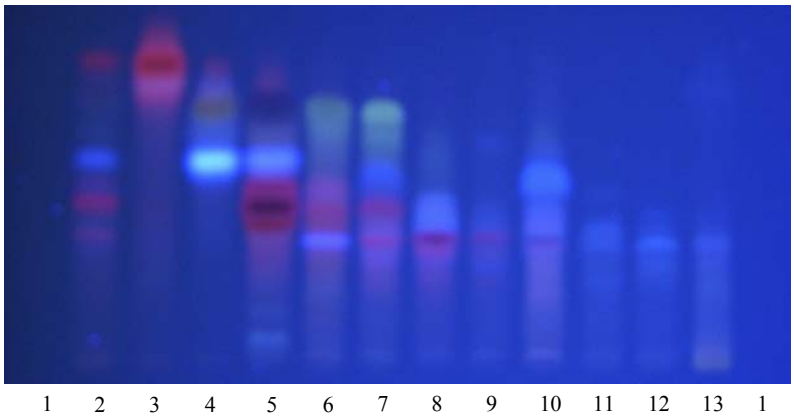
Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (70:30:10), lower phase
Detection: without treatment



1 GTX III
2 $CHCl_3$ extract
3 F1
4 F2
5 F3
6 F4
7 F5
8 F6
9 F7
10 F8
11 F9
12 F10
13 F11

Figure 35: TLC of cumulative fractions F1-F11

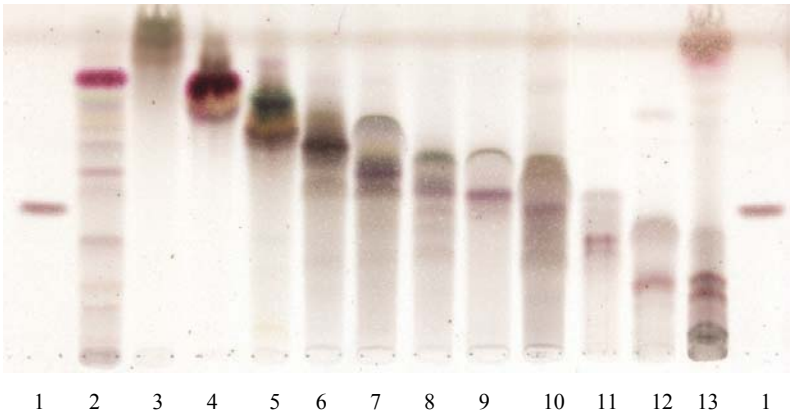
Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-methanol-water (81:11:8)
Detection: without treatment



1 GTX III
2 $CHCl_3$ extract
3 F1
4 F2
5 F3
6 F4
7 F5
8 F6
9 F7
10 F8
11 F9
12 F10
13 F11

Figure 36: TLC of cumulative fractions F1-F11

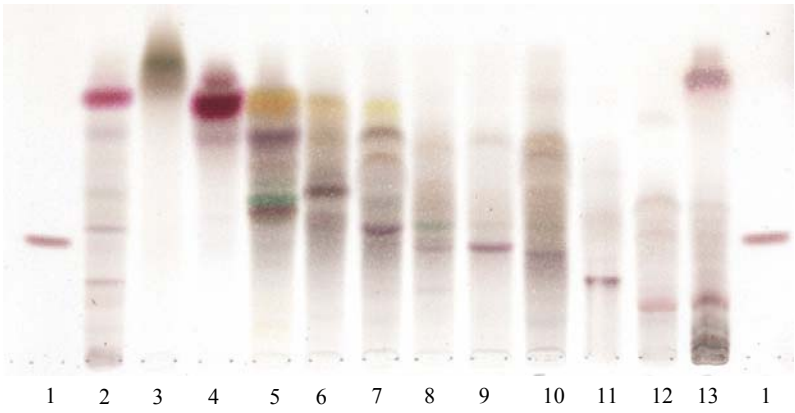
Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (70:30:10), lower phase
Detection: 60% H_2SO_4, white light



1 GTX III
2 $CHCl_3$ extract
3 F1
4 F2
5 F3
6 F4
7 F5
8 F6
9 F7
10 F8
11 F9
12 F10
13 F11

Figure 37: TLC of cumulative fractions F1-F11

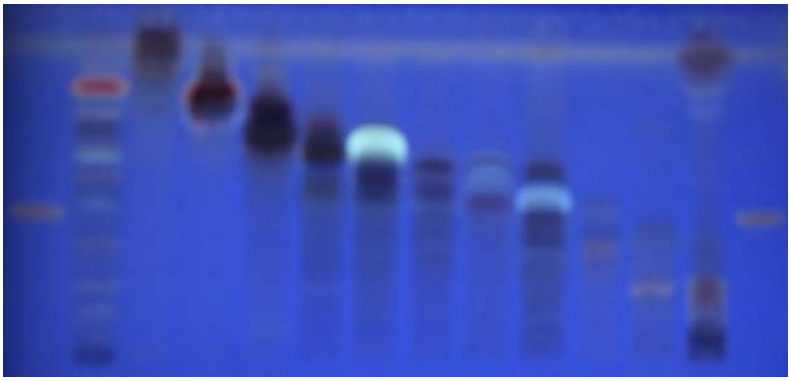
Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-methanol-water (81:11:8)
Detection: 60% H_2SO_4, white light



1 GTX III
2 $CHCl_3$ extract
3 F1
4 F2
5 F3
6 F4
7 F5
8 F6
9 F7
10 F8
11 F9
12 F10
13 F11

Figure 38: TLC of cumulative fractions F1-F11

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (70:30:10), lower phase
Detection: 60% H_2SO_4, UV 365nm

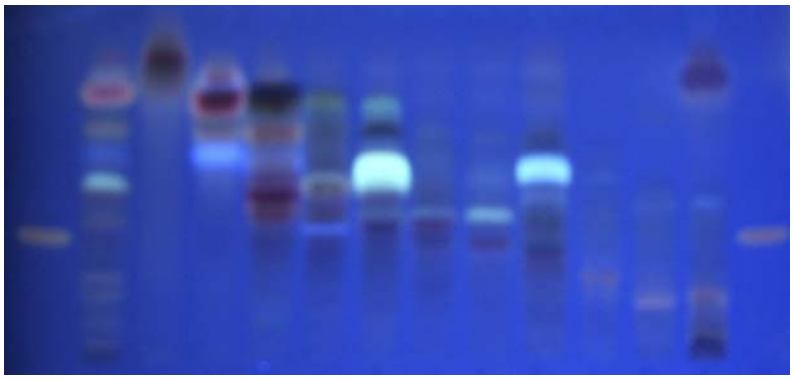


1 2 3 4 5 6 7 8 9 10 11 12 13 1

1 GTX III
2 $CHCl_3$ extract
3 F1
4 F2
5 F3
6 F4
7 F5
8 F6
9 F7
10 F8
11 F9
12 F10
13 F11

Figure 39: TLC of cumulative fractions F1-F11

Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-methanol-water (81:11:8)
Detection: 60% H_2SO_4, UV 365nm

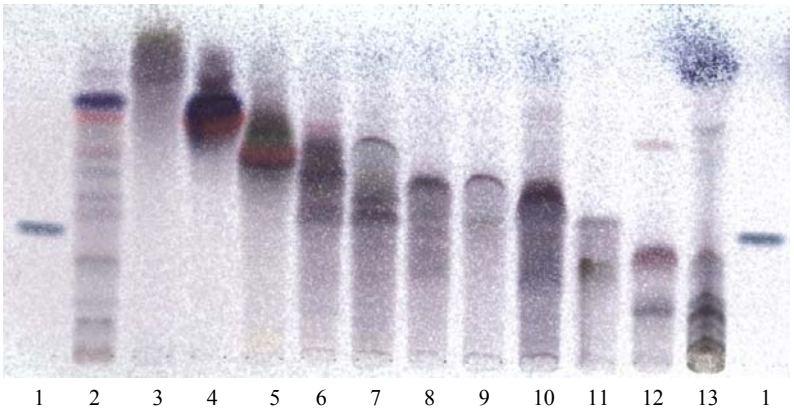


1 2 3 4 5 6 7 8 9 10 11 12 13 1

1 GTX III
2 $CHCl_3$ extract
3 F1
4 F2
5 F3
6 F4
7 F5
8 F6
9 F7
10 F8
11 F9
12 F10
13 F11

Figure 40: TLC of cumulative fractions F1-F11

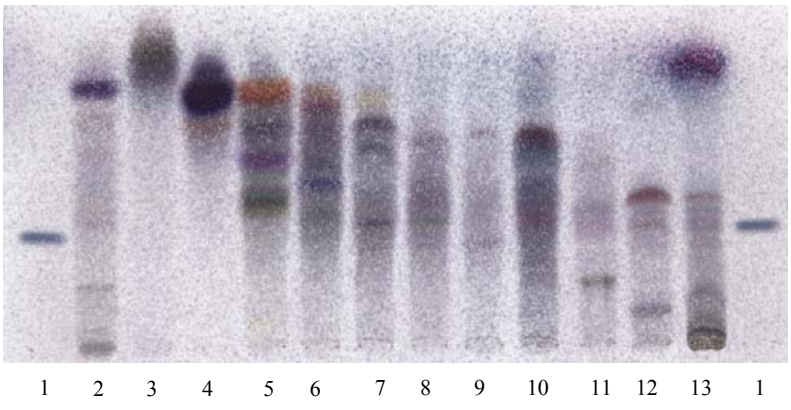
Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (70:30:10), lower phase
Detection: vanillin H_2SO_4 reagent, white light



1 GTX III
2 $CHCl_3$ extract
3 F1
4 F2
5 F3
6 F4
7 F5
8 F6
9 F7
10 F8
11 F9
12 F10
13 F11

Figure 41: TLC of cumulative fractions F1-F11

Stationary phase: silica gel 60 F_{254}
Mobile phase: ethyl acetate-methanol-water (81:11:8)
Detection: vanillin H_2SO_4 reagent, white light



1 GTX III
2 $CHCl_3$ extract
3 F1
4 F2
5 F3
6 F4
7 F5
8 F6
9 F7
10 F8
11 F9
12 F10
13 F11

The next step was to test the fractioned $CHCl_3$ extract on HL-60 cells for the antiproliferative activity.

4.5.2 Proliferation Assays of the Fractions

Logarithmically growing HL-60 human leukaemia cells were incubated with or without extract concentrations of 1 µg/ml (see chapter 3.1.5, page 18 and 3.4.1, page 25). The control samples were incubated with the highest EtOH sample concentration (0.01 % EtOH).

The cells were counted after 24, 48 and 72 hours with a cell counter directly. All experiments were made in triplicate and analysed by t-test.

The results showed no significant effect of the 11 fractions (see fig. 42). The next step was to increase the concentration from 1 to 20µg/ml (see fig. 43, page 57).

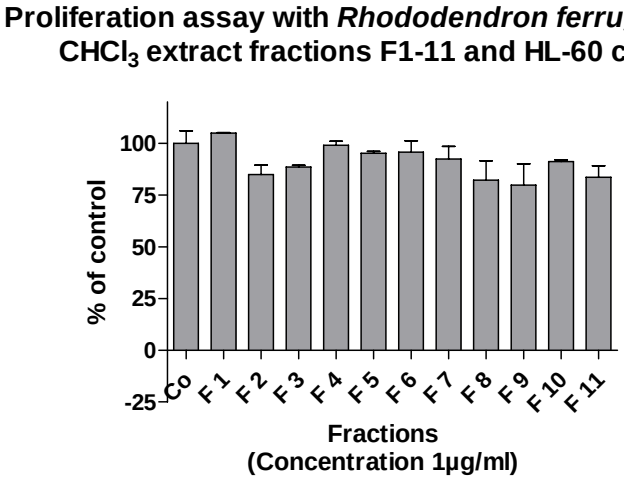


Figure 42: Results of the proliferation assay with HL-60 cells incubated with the fractioned $CHCl_3$ extract (concentration 1 µg/ml)

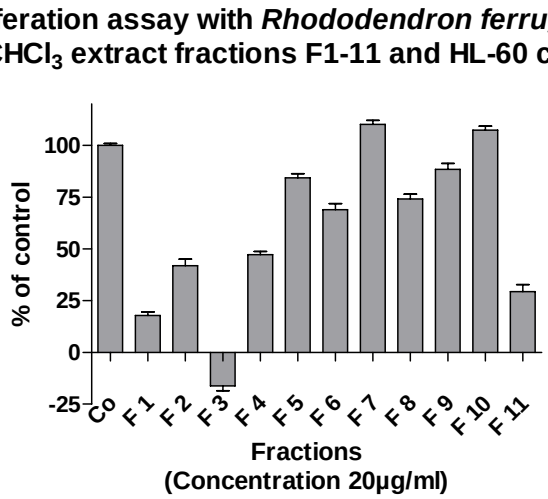


Figure 43: Results of the proliferation assay with HL-60 cells incubated with the fractioned $CHCl_3$ extract (concentration 20µg/ml)

With a concentration of 20µg/ml extract the $CHCl_3$ fractions showed different activities in the proliferation assay.

As seen in figure 43 the most active fractions are F3 and F1. For further research fractions F1, F2 and F3 were chosen (see chapter 4.6-4.8, page 58-61).

4.6 Fractionation of Cumulative Fraction F1

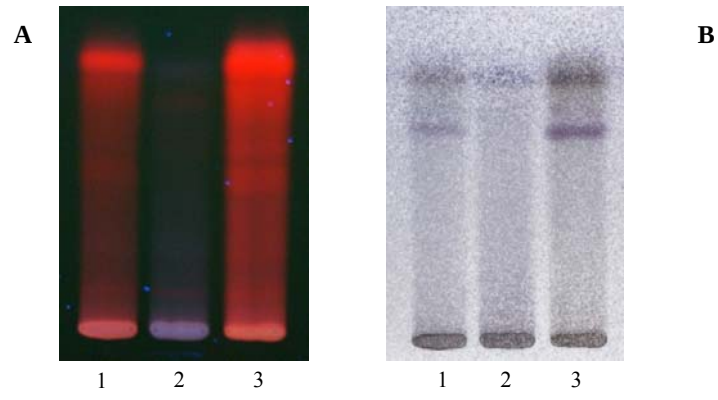
For further separation of fraction F1 after column chromatography SPE was used (see chapter 3.3.3.1, page 23). The two obtained fractions were analysed by TLC (see fig. 44).

The SPE was done on C18 cartridges (see chapter 3.1.4, page 17). The cartridge was conditioned with methanol. The fraction which was dissolved in methanol was applied on the cartridge. The cartridge was eluted successively with methanol from 90% to 50% and finally with $CHCl_3$-MeOH (1:1). The collected fractions were checked by TLC and the fractions with a similar or the same pattern on the TLC plate were reunited. Both obtained fractions F1-1 (2.40mg) and F1-2 (0.63mg) were evaporated and tested on cells for cytotoxicity (see fig. 45, page 59).

Figure 44: TLC of fraction F1separeted by SPE

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (90:10:10), lower phase
Without chemical treatment, UV 365nm (A)
Detection: vanillin H_2SO_4 reagent, white light (B)

1 F1 (total)
2 F1-1
3 F1-2



The two obtained fractions F1-1 and F1-2 were analysed by proliferation assay on HL-60 cells as described in chapter 3.4.1, page 17. A concentration of 10µg/ml extract was used. After 24, 48 and 72 hours the cells were counted. The results of the proliferation assay are shown in fig. 45, page 59.

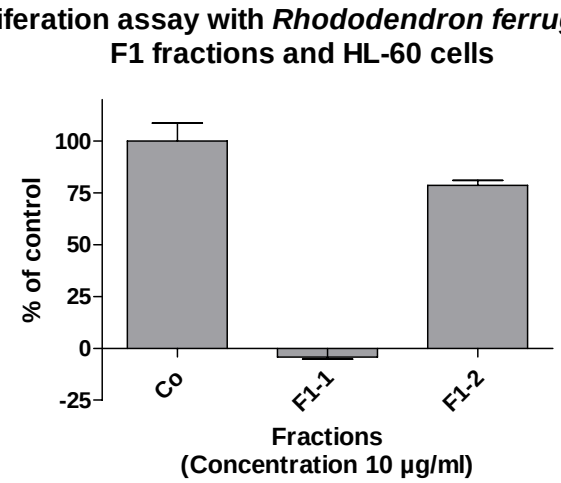


Figure 45: Results of the proliferation assay with HL-60 cells incubated with the fractionated cumulative fraction F1 (concentration 10µg/ml extract)

As you can see in figure 45 the F1-1 fraction shows a high activity on HL-60 cells. As revealed by TLC (see fig. 44, page 58) apolar constituents must be responsible for the cytotoxic activity of fraction F1-1.

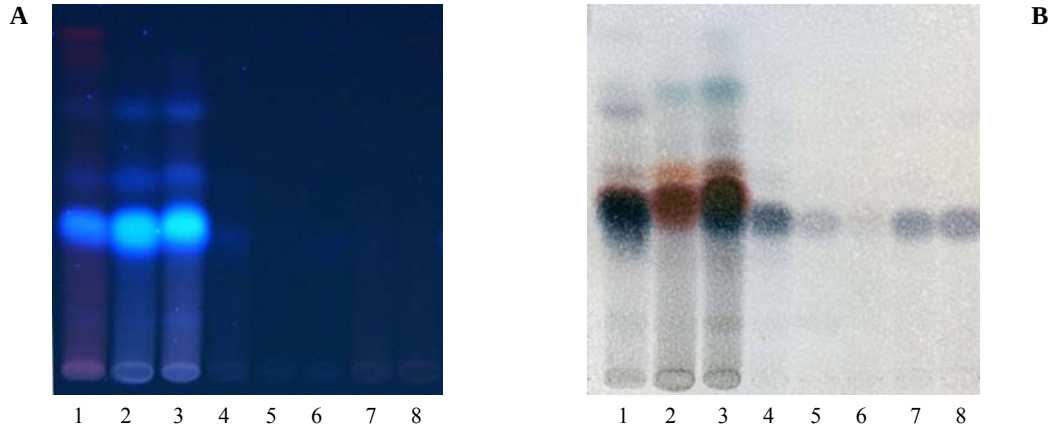
4.7 Fractionation of Cumulative Fraction F2

For further separation of fraction F2 SPE was used (see chapter 3.3.3.2, page 24). The extract on the cartridge was eluted with 2 RV 90% MeOH, 2 RV 80% MeOH, 2 RV 70% MeOH and 50% MeOH and finally washed with 4ml of $CHCl_3$-MeOH (1:1). Seven fractions were obtained during the first step of SPE, which were analysed by TLC (mobile phase: $CHCl_3$-MeOH-H_2O 90:10:10, lower phase) shown in figure 46, page 60. Fractions with identical spots were reunited.

As seen in figure 47, page 60 the two main components from F2 (see fig. 46, page 60) were separated in two fractions: F2-1 (21.88mg) and F2-2 (54.93mg) and tested on HL-60 cell line (see figure 48, page 61).

Figure 46: TLC of the fractioned F2 after the first SPE

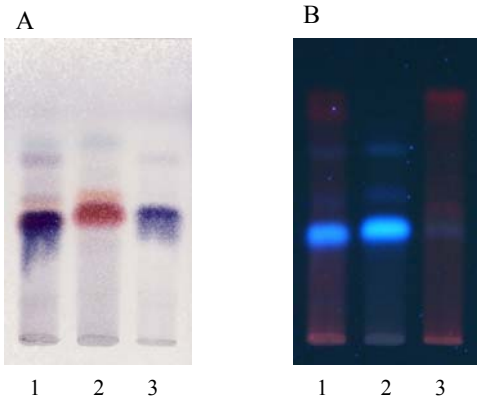
Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (90:10:10), lower phase
Without chemical treatment, UV 365nm (A)
Detection: vanillin H_2SO_4 reagent, white light (B)



1 F2 (total) 2 F2-1 3 fraction A 4 fraction B
5 fraction C 6 fraction D 7 fraction E 8 powdery residue

Figure 47: TLC of F2-1 and F2-2 fractions

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (90:10:10), lower phase
Without chemical treatment, UV-366 nm (B)
Detection: vanillin H_2SO_4 reagent, white light (A)



1 F2 (total)
2 F2-1
3 F2-2

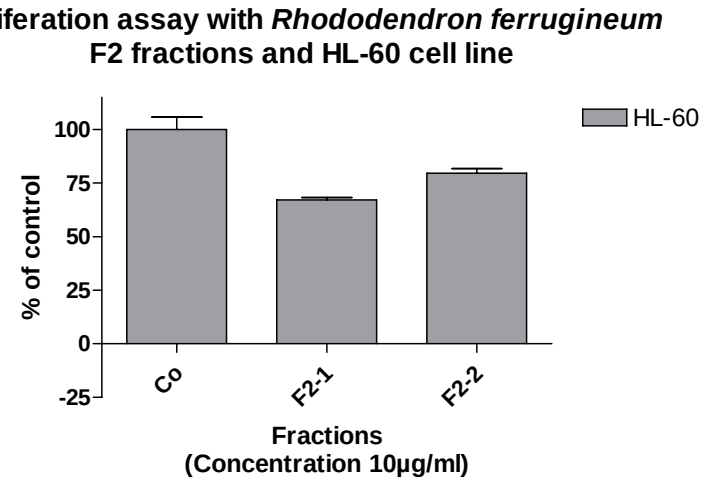


Figure 48: Results of the proliferation assay with HL-60 cells incubated with the fractionated cumulative fraction F2 (concentration 10µg/ml extract)

As seen in figure 48 the more active fraction is F2-1 but both have a lower inhibitory effect than fraction F2. In fraction F2-1 there are four visible spots under UV-light and under white light with a main component that is blue-fluorescent under UV light and red in visible light after detection with vanillin sulphuric reagent (see fig. 47, page 60).

4.8 Fractionation of Cumulative Fraction F3

Column chromatography was used for the separation of fraction F3 (See chapter 3.3.2.2, page 22). The column was packed with silica gel 60 which was elutriating in ethyl acetate saturated with water at a high of 50 cm. The plant extract and solvent was filled in the column and the valve was opened with a flow rate of 3ml/30 min. The collected test tubes were checked by TLC and the fractions with a similar or the same pattern on the TLC plate were reunited. The column chromatography operated all in all 7 days.

Seven cumulate fractions were obtained and reunited. The yield of each fraction is listed in table 7.

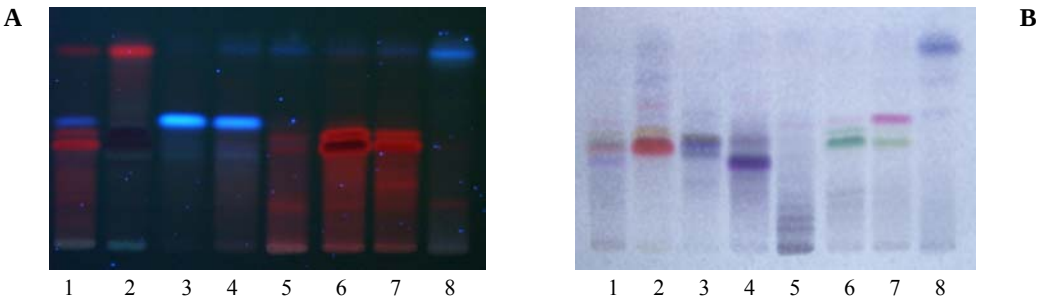
single fraction 1-9	cumulative fraction 1	**F3-1**	25.00mg
single fraction 10/11	cumulative fraction 2	**F3-2**	10.10mg
single fraction 12-20	cumulative fraction 3	**F3-3**	4.50mg
single fraction 21-30	cumulative fraction 4	**F3-4**	4.80mg
single fraction 31-35	cumulative fraction 5	**F3-5**	2.80mg
single fraction 46-52	cumulative fraction 6	**F3-6**	3.00mg
single fraction 53-325	cumulative fraction 7	**F3-7**	23.10mg

Table 7: Cumulative fractions of fraction F3 obtained by column chromatography

All cumulative fractions (F3-1 to F3-7) were analysed by TLC (see fig. 49) and were also tested for their antiproliferative activity on HL-60 cells (see fig. 50, page 63).

Figure 49: TLC of the fractionated cumulative fraction F3 after column chromatography

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (80:20:10), lower phase
Without chemical treatment, UV 365nm (A)
Detection: vanillin H_2SO_4 reagent, white light (B)



1 F3 (total) **2** F3-1 **3** F3-2 **4** F3-3
5 F3-4 **6** F3-5 **7** F3-6 **8** F3-7

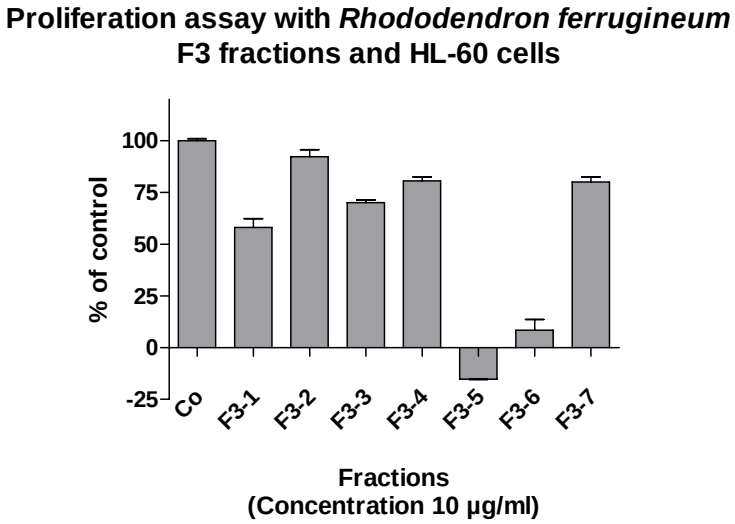


Figure 50: Results of the proliferation assay with HL-60 cells incubated with the fractionated cumulative fraction F3 (concentration 10µg/ml extract)

As seen above in fig. 50 fraction F3-5 and F3-6 were the most active fractions. Both had the same two main components so we decide to use F3-6 for further analysis because we had more extract available. F3-6 was further separated. The separation of fraction F3-6 is described in chapter 4.9, page 64.

4.9 Fractionation of Cumulative Fraction F3-6

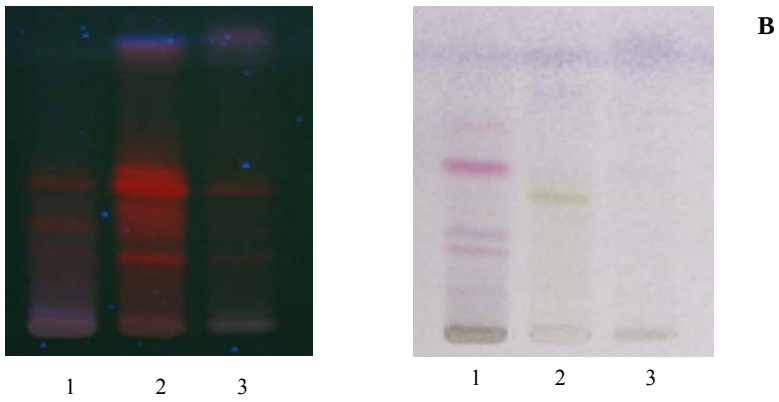
As seen in fig. 50, page 63 the most active fractions are F3-5 and F3-6. F3-6 was further fractioned. SPE was used and conditioned as described in chapter 3.3.3.3, page 24. The fraction F3-6 dissolved in methanol was applied on the cartridge and completely evacuated. The cartridge was then eluted with 2 RV 100% MeOH, 2 RV 98% MeOH and finally with $CHCl_3$-MeOH (1:1).

As seen in figure 51 the two main components from F3-6 were separated in three fractions: F3-6-VL (1.06mg), F3-6-1 (0.84mg) and F3-6-NL (1.63mg) and tested on HL-60 cell line (see fig. 52, page 65).

Figure 51: TLC of the fractionated cumulative fraction F3-6

Stationary phase: silica gel 60 F_{254}
Mobile phase: chloroform-methanol-water (80:20:10), lower phase
Without chemical treatment, UV 365nm (A)
Detection: vanillin H_2SO_4 reagent, white light (B)

1 = F3-6-VL
2 = F3-6-1
3 = F3-6-NL



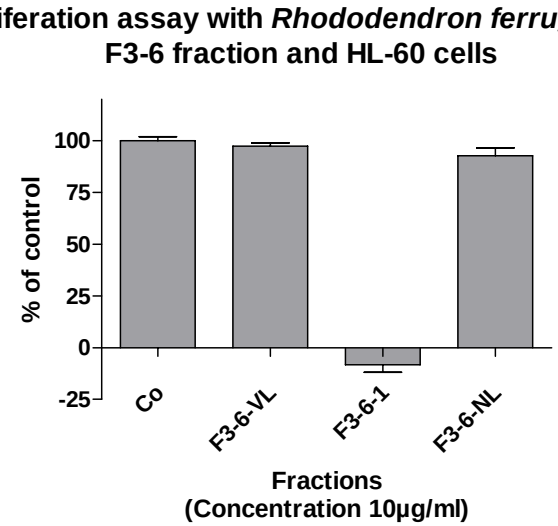


Figure 52: Results of the proliferation assay with HL-60 cells incubated with the fractionated cumulative fraction F3-6 (concentration 10µg/ml extract))

As seen in fig. 52 the most active fraction is F3-6-1. The components separated in F3-6-1 are responsible for the activity of F3-6 on the HL-60 cells. The TLC characterization of fraction F3-6-1 showed a high content in chlorophyll and an apolar blue-fluorescent component under UV light see (fig. 51, page 64).

Separation of fractions F1-1 and F3-6-1 and compound identification was not possible due to the low amount of the respective fractions. For further analysis a higher amount of plant material is necessary to start with.

5. Discussion

Rhododendron is a genus of plants belonging to the family of *Ericaceae* which also grows in Austria [BERG and HEFT, 1991]. Rhododendrons are known as toxic plants and for their toxicity GTXs are accounted. GTXs belong to the diterpene class [KAN et al, 1994] and have been identified in species such as *Andromeda*, *Kalmia* and *Leucothoe* [HÄNSEL and STICHER, 2007].

However there is no literature which clearly confirms the toxicity of *R. ferrugineum* and no reports about intoxications after *R. ferrugineum* usage in humans. Available data about *R. ferrugineum* poisoning refers to animal intoxications [HÄNSEL et al, 1994].

Studies on the GTX content have been done on several *Rhododendron* species, yet no recent literature has investigated *R. ferrugineum*. GTX III and GTX I are the most studied grayanotoxins and have a range of pharmacological activities causing hypotension, respiratory depression, and excitatory activity in muscle spindle afferents [KINGHORN et al, 1978].

In the present work it was analysed if GTX III is present in *R. ferrugineum* and the cytotoxicity of *R. ferrugineum* extracts was characterized using bioassay guided fractionation.

For the plant material extraction two different methods have been used and afterwards compared: The first method is based on the subsequent extraction in five different polarity solvents: petroleum ether, dichloromethane, ethyl acetate, methanol, water (see chapter 3.2.1, page 19 and chapter 4.2.1, page 38). The second method described by Wall et al in Phytomedicine 1996 aims at the elimination of tannins from the extract (see chapter 3.2.2, page 20 and chapter 4.3 page 40). The plant material is extracted in methanol, resolved in methanol-water, defatted with n-hexane and partitioned with chloroform.

The five extracts obtained by the first extraction method and the detannified chloroform extract have been characterized by TLC using GTX III as reference substance and different solvent systems and detection reagents.

Solvent systems and detection methods previously described in the literature have been used in the present work to investigate if the GTX III is present in *R. ferrugineum* extracts.

"Keller et al [1970]" verified GTX III in *Rhododendron ponticum* by TLC with chloroform-methanol (9:1) as mobile phase and antimony III chloride as detection reagent. This method was reproduced exactly to Keller's specifications. Keller et al achieved a retention factor (Rf) for GTX III of 0.04 using HF aluminium plates "Riedel" as stationary phase. With silica gel 60 F_{254} as stationary phase I obtained an Rf value of 0.09 (fig. 10 in chapter 4.2.2, page 30).

Antimony III chloride proved to be a good detection reagent for GTX III. Therefore I used the same detection reagent with other solvent systems, too (see figure 11-12, page 32). A definite analogy with GTX III as reference substance couldn't be observed for the *R. ferrugineum* extracts.

In another step I used "Kinghorn et al [1978]" TLC solvent system and detection method of ethyl acetate-isopropanol-water (80:24:6) and 60% sulfuric acid (see table 3, page 39). In this publication *R. maximum* had been analysed for the content of different GTX derivatives [KINGHORN et al, 1978]. Again, a definite analogy with GTX III as reference substance couldn't be observed for the *R. ferrugineum* extracts.

For the cytotoxicity assessment a cell based proliferation assay with HL-60 human promyelocytic leukaemia (see chapter 4.4.1, page 45) and MCF-7 human breast adenocarcinoma cell lines (see chapter 4.4.2, page 47) was used. Research from the ongoing PhD thesis of Mag. Ruxandra Popescu showed a high antiproliferative activity of the ethyl acetate *R. ferrugineum* extract. For this reason the ethyl acetate extract has

been further analysed for the cytotoxic activity in comparison with the detannified chloroform extract.

Because tannins can interfere with biological assays giving false positive and negative results [SILVA et al, 1998] and because the activity of the rhododendron extract was not influenced by the elimination of tannins the detannified chloroform extract was chosen for the bioassay guided fractionation.

The antiproliferative activity of the *R. ferrugineum* extract did not diminish after the elimination of tannins (see fig. 28 – 31, page 46-48). The detannification also yields a pure extract with a higher amount of active compounds.

The extract was first fractionated by column chromatography using ($CHCl_3$-MeOH-H_2O 80:20:10, lower phase) as mobile phase (see chapter 4.5, page 49). The obtained fractions were characterized by TLC (see fig. 34 – 41, page 52-55) and tested for their antiproliferative activity. Three out of the total eleven fractions showed a significant decrease of the HL-60 proliferative rate, namely fraction F1, F2 and F3 (see fig. 43, page 57).

The three fractions were further separated. Fraction F1 and F2 were fractionated by SPE using methanol and water in different ratios. Fraction F1 yielded two fractions F1-1 and F1-2 of which fraction F1-1 proved to maintain the antiproliferative activity (see fig. 45, page 59).

Fraction F2-1 and F2-2 were separated from fraction F2, each of them showing to have one main component. Neither of the two fractions of F2 significantly decreased the proliferation rate (see fig. 48, page 61).

For the fractionation of the most active fraction F3, column chromatography with ethyl acetate saturated with water as mobile phase was used. The seven new fractions were tested again for their activity on HL-60 cells (see fig. 50, page 63).

Fraction F3-5 and F3-6 preserved the activity of fraction F3. The two active fractions have a similar fingerprint with two main components as seen by TLC (see fig. 49, page 62). Further on fraction F3-6 was chosen for SPE separation which yielded three fractions. Fraction F3-6-1 enclosed the components responsible for the activity of fraction F3. TLC analysis of fraction F3-6-1 revealed a high content in chlorophyll and an apolar blue-fluorescent component under UV light (see fig. 51, page 64).

The TLC analysis of the *R. ferrugineum* extracts before and after removing the tannins and also after the first column chromatographic separation revealed no identical spots with the GTX III spot. This data concludes with utmost probability that there is no GTX III in the *R. ferrugineum* extract or that the amounts are under the detection limit. This doesn't exclude the possibility of *R. ferrugineum* containing other GTXs than GTX III. More than 30 compounds deducible from the skeletal structure of GTXs are known [FROHNE and PFÄNDER, 2004].

In conclusion the ethyl acetate and detannified chloroform extracts of *R. ferrugineum* decreased the proliferation rate and viability of HL-60 human promyelocytic leukaemia and MCF-7 human breast adenocarcinoma cell lines.

The most active fractions of the detannified chloroform extract were fraction F1-1 and F-3-6-1. The cytotoxic activity of fraction F1-1 and F-3-6-1 on HL-60 cell line appears to be due to other apolar and middle polar compounds than GTX III.

Further separation and identification of the active substances must occur in other investigations. A higher amount of plant material should be extracted for the isolation and structure elucidation of the compounds responsible for the cytotoxicity of *R. ferrugineum.*

6. Summary

The *Rhododendron* genus belongs to the family of the *Ericaceae* and encloses more than 1000 species [BERG and HEFT, 1991]. Many *Rhododendron* species are known as toxic plants just as many *Ericaceae* species. The toxicity is ascribed to GTXs, substances which belong to the class of the diterpenes [WONG et al, 2002].
Rhododendron ferrugineum grows also in Austria. The chemical composition of the species is little investigated, as well as its toxicity.

The statements about the existance or absence of GTXs in *R. ferrugineum* are contradictory. On the one hand there are no reports about symptoms of intoxication in humans after traditional-medical application; on the other hand, case reports of poisoned animals e.g. sheep by *R. ferrugineum* exist which suggest the presence of GTXs or similar compounds [BAnz, 1990].

To find out whether *R. ferrugineum* is toxic or not, and if so which substances could be responsible for the toxicity, I have examined *R. ferrugineum* extracts first by TLC. For this GTX III [1] was used as reference substance, as well as different solvent systems and detection reagents (see 3.1.3, page17).

Because tannins could mask the GTX III detection on the TLC or can also give false positive or negative results in biological assays, the second step was to eliminate the tannins from the extract.

Hence, the ethyl acetate extract (before separation of the tannins) as well as the $CHCl_3$-extract (after elimination of the tannins) were tested on HL-60 and MCF-7 cell lines for their cytotoxicity.

[1] This was the only available GTX at that time.

Afterwards the active detannified $CHCl_3$ extract was fractionated to separate the substances which are responsible for the cytotoxic effect. Subsequently the obtained fractions (F1 - F11) were once more tested on cells for their antiproliferative activity and the most active factions (F1, F2 and F3) were further separated with the help of SPE and column chromatography.

At the end of my work I have received two factions (F1-1, F3-6-1) containing the substances responsible for the cytotoxicity of the *R. ferrugineum* extract. Further separation and identification of the active substances must occur in further investigations.

7. Zusammenfassung

Die Gattung *Rhododendron* zählt zur Familie der *Ericaceae* und umfasst über 1000 Arten [BERG and HEFT, 1991]. Über viele *Rhododendron*-Arten ist bekannt, dass sie, ebenso wie viele *Ericaceaen* –Arten, toxisch sind. Die Toxizität wird Grayanotoxinen (GTX) zugeschrieben, eine Substanz der Klasse der Diterpene [WONG et al, 2002]. *Rhododendron ferrugineum* wächst unter anderem auch in Österreich und ist eine wenig erforschte Art, die Inhaltsstoffe, vor allem die toxischen sind weitgehend unbekannt. Die Aussagen über das Vorhanden sein oder Fehlen von Grayanotoxinen in *R ferrugineum* sind widersprüchlich. Einerseits gibt es keine Berichte über Vergiftungserscheinungen beim Menschen nach der volkmedizinischen Anwendung, andererseits existieren Fallberichte über Vergiftung beim Weidevieh durch *R. ferrugineum*, welche auf ein Vorhanden sein von Grayanotoxinen oder ähnlichen Substanzen schließen lassen [BAnz, 1990].

Um herauszufinden, ob *R. ferrugineum* toxisch ist oder nicht, und wenn ja von welcher Substanz die Toxizität ausgeht, habe ich die hergestellten Extrakte von *R. ferrugineum* zuerst dünnschichtchromatografisch untersucht. Dafür wurde GTX III [1] als Referenzsubstanz verwendet sowie unterschiedliche Fließmittel und Sprühreagenzien. (Siehe 3.1.3, Seite 17)

Da Gerbstoffe sowohl die Detektion von GTX III verhindern als auch verantwortlich für falsch positiv oder negative Ergebnisse bei biologischen Assays sein könnten, wurden in einem zweiten Schritt die Gerbstoffe aus dem Extrakt entfernt. Daher wurden der Ethyl Acetat-Extrakt (vor Abtrennung der Gerbstoffe) sowie der gerbstofffreie $CHCl_3$-Extract an HL-60 und MCF-7 Zellen auf ihre Zytotoxizität getestet.

[1] Das zu diesem Zeitpunkt einzige am Markt verfügbare GTX

Anschließend wurde der $CHCl_3$-Extrakt wegen der gezeigten Zytotoxizität fraktioniert, um die Substanz, der diese Wirkung zugeschrieben werden kann, von Begleitsubstanzen abzutrennen. Im Anschluss wurden die erhaltenen Fraktionen (F1 - F11) erneut an Zellen getestet und die aktivsten Fraktionen (F1, F2 und F3) mit Hilfe von SPE und Säulenchromatographie erneut aufgetrennt und gereinigt.

In den somit erhaltenen zwei Fraktionen (F1-1, F3-6-1) waren die Substanzen mit Zytotoxizität enthalten. Die weitere Auftrennung und Identifizierung der aktiven Substanzen muss in weiteren Untersuchungen erfolgen.

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