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„Isolation and Characterisation of Secondary Metabolites of *Combretum zenkeri*“

verfasst von / submitted by

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List of Abbreviations

AA	acetic acid
AAS	anisaldehyde-sulfuric acid reagent
CC	column chromatography
ELSD	evaporative light scattering detector
EtOAc	ethyl acetate
FA	formic acid
MeOH	methanol
NP	normal-phase system
NP/PEG	natural products-polyethylenglycol reagent
PDA	photodiode array
RP	reversed-phase system
SFE	supercritical fluid extraction
TLC	thin layer chromatography
UV-DAD	ultraviolet-diode array detector

1. Introduction and Aim of the Work

1.1. *Combretum zenkeri* Engl. & Diels

The genus Combretum consists of approx. 20 genera and more than 600 species, which grow worldwide [1, 2]. Several species are used in African and Asian traditional medicine for up to 90 different indications, of which many are related to their anti-infective properties [3]. Despite the broad use, only little research has been published about the constituents and pharmacological effects of these species.

Combretum zenkeri is a scandent shrub or liana, which can measure up to 24 m. It is widely distributed in western Africa and grows in forests, savanna and rocky areas [4]. Its taxonomy is described in table 1.

Table 1: Taxonomy of *Combretum zenkeri* Engl. & Diels [5]

Kingdom:	Plantae
Subkingdom:	Tracheobionta
Superdivision:	Spermatophyta
Division:	Magnoliophyta
Class:	Magnoliopsida
Subclass:	Rosidae
Order:	Myrtales
Family:	Combretaceae
Genus:	Combretum
Species:	*Combretum zenkeri*

In traditional medicine, this species is used in the treatment of malaria and inflammatory diseases. A decoction of the leaves is used as vermifuge, purgative and against hypertension. Chewing its twigs is said to relieve menstrual pain and

also oedemas are treated with *Combretum zenkeri*. Despite its many indications, only little is known about its constituents [6, 7, 8].

First evidence of nephro- and hepatoprotection as well as antioxidant and neuroprotective effects has been deduced from *in vivo* and *in vitro* studies [7, 9, 10]. Despite these efforts, more research is needed to unravel the therapeutic potential of *Combretum zenkeri.*

1.2. Aim of the Work

The aim of this work was the phytochemical characterisation of this species.

The targeted isolation of compounds from bigger amounts of extracts than in previous research as well as their identification by spectrometric and spectroscopic methods were part of this diploma thesis.

2. Material and Methods

2.1. Plant Material

The plant material was collected in the Botanical Garden of the University of Ibadan. Identification was performed by the curator of the Botanical Garden. After authentication the voucher specimen (number FHI/110277) was stored at the Forestry Research Institute of Nigeria (FRIN), Ibadan [11].
A methanolic extract had been produced by soxhlet extraction, dried and stored in a cool and dry place [12]. An amount of 76,7 g was available for further investigation.

2.2. Solvents and Reagents

All used solvents were distilled at least twice. Table 2 provides a list of the reference compounds used in this diploma thesis:

Table 2: Reference compounds

Substances	Purchased from
Abscisic acid	Sigma Aldrich Supelco
Betulinic acid	Phytolab
Caffeic acid	Roth
Isovitexin	Roth
Homoorientin	Extrasynthese
Myricetin	Roth
Myricitrin	Roth
Oleanolic acid	Sigma Aldrich
Orientin	Roth
Quercetin	Roth
Quercitrin	Stock of Department of Pharmacognosy
Robinetin	Roth
Ursolic acid	Sigma
Vitexin	Roth
ß-Sitosterol	Roth

2.3. Extraction Methods

2.3.1. Supercritical Fluid Extraction (SFE)

A MV-10 ASFE system (Waters, USA), operated via Chromescope software, and 25 ml vessels were used for extraction of the methanolic extract. MeOH was added as make-up solvent.

As this method of extraction had never been tested before with *Combretum zenkeri* extracts, a protocol had to be established by preliminary testing with 1 g of the methanolic extract. The values of CO_2 and EtOH (0%, 3%, 6%, 10%) as co-solvent are shown in table 3. The temperature and pressure were 40°C and 200 bar.

Table 3: Parameters of preliminary testing for SFE extraction

Step	Co-Solvent Bottle (1-6)	Co-Solvent Flow (ml/min)	CO2 Flow (ml/min)	Dynamic Duration 1 (min)	Static Duration (min)	Dynamic Duration 2 (min)	Make-up Flow (ml/min)
1	EtOH	0.00	10.00	5.00	5.00	5.00	2.00
2	EtOH	0.00	5.00	2.00	0.00	0.00	2.00
3	EtOH	0.30	9.70	5.00	5.00	5.00	1.70
4	EtOH	0.15	4.85	2.00	0.00	0.00	1.85
5	EtOH	0.00	5.00	2.00	0.00	0.00	2.00
6	EtOH	0.60	9.40	5.00	5.00	5.00	1.40
7	EtOH	0.30	4.70	2.00	0.00	0.00	1.70
8	EtOH	0.00	5.00	2.00	0.00	0.00	2.00
9	EtOH	1.00	9.00	5.00	5.00	5.00	1.00
10	EtOH	0.50	4.50	2.00	0.00	0.00	1.50
11	EtOH	0.00	5.00	2.00	0.00	0.00	2.00

Each extraction step consisted of 3 phases, which were Dynamic Duration 1, Static Duration and Dynamic Duration 2. During Dynamic Duration 1 and Dynamic Duration 2 solvent was continuously pumped through the extraction vessel. During Static Duration, the solvent remained under pressure in the extraction vessel.

A second extraction was performed without co-solvent. MeOH was used as make-up solvent. The extraction cycle was prolonged to 30 minutes (see table 4).

Because of the satisfying results, these parameters were also applied for the extraction of 16 g methanolic extract.

Table 4: Parameters for SFE extraction

Step	Co-Solvent Bottle (1-6)	Co-Solvent Flow (ml/min)	CO2 Flow (ml/min)	Dynamic Duration 1 (min)	Static Duration (min)	Dynamic Duration 2 (min)	Make-up Flow (ml/min)
1	EtOH	0.00	10.00	10.00	10.00	10.00	2.00
2	EtOH	0.00	5.00	2.00	0.00	0.00	2.00
3	EtOH	0.00	10.00	10.00	10.00	10.00	2.00
4	EtOH	0.00	5.00	2.00	0.00	0.00	2.00
5	EtOH	0.00	10.00	10.00	10.00	10.00	2.00
6	EtOH	0.00	5.00	2.00	0.00	0.00	2.00
7	EtOH	0.00	10.00	10.00	10.00	10.00	2.00
8	EtOH	0.00	5.00	2.00	0.00	0.00	2.00
9	EtOH	0.00	10.00	10.00	10.00	10.00	2.00
10	EtOH	0.00	5.00	2.00	0.00	0.00	2.00

2.3.2. Liquid-liquid Extraction

In a preliminary experiment 2 g of the methanolic extract were extracted by partition in two portions of 1 g. After suspension in 50 ml H_2O, partition was performed 2 times with 100 ml $CHCl_3$, each.

For scale-up, approx. 5 g of the methanolic extract were partitioned between 500 ml water and $CHCl_3$. The partition with $CHCl_3$ was repeated twice.

2.4. Chromatographic Methods

The chromatographic methods described below were used for preparative and analytical purposes.

2.4.1. Thin Layer Chromatography (TLC)

The composition of obtained fractions was controlled by TLC. For this purpose, different normal phase and reversed phase systems were used. All pictures of TLCs were taken with a CAMAG TLC Visualizer under use of Vision CATS CAMAL TLC software.

Stationary phases:

1. Silica gel 60 F_{254}-precoated plates, 20 x 20 cm (Merck)
2. HPTLC-pre-coated plates RP-8 F_{254} for nano-TLC (Merck)

Mobile phases:

1. $CHCl_3$-MeOH (97 + 3)
2. $CHCl_3$-MeOH-H_2O (79 + 19 + 2)
3. $CHCl_3$-MeOH (90 + 10)
4. EtOAc-formic $acid_{conc.}$ (FA)-acetic $acid_{conc.}$ (AA)-H_2O (100 + 11 + 11 + 26)
5. EtOAc-MeOH-H_2O (81 + 11 + 8)
6. $CHCl_3$-MeOH (95 + 5)
7. MeOH
8. MeOH-H_2O (95 + 5)

Mobile phases 1 – 6 were applied in normal phase TLC, while mobile phases 7 – 8 were applied in reversed phase TLC. After developing and drying the plates, derivatisation was performed with anisaldehyde-sulfuric acid reagent (AAS) or natural products-polyethylenglycol reagent (NP/PEG).

• AAS:

After spraying with AAS (0,5 ml anisaldehyde, 10 ml acetic $acid_{conc.}$, 85 ml MeOH and 5 ml sulfuric $acid_{conc.}$) the TLC-plate was dried at 105°C for 5 to 10 minutes and analysed under daylight [13].

• NP/PEG:

After spraying with a 1% methanolic solution of NP the plate was dried for several minutes. Then a 5% ethanolic solution of PEG was sprayed on the dry plate and detection performed under UV 366 nm [13].

2.4.2. Column Chromatography (CC)

For fractionation of fraction F6/FC1, column chromatography was used. Silica gel 60 (Merck) served as stationary phase and $CHCl_3$-MeOH mixtures as mobile phase (see table 14, p. 29). 10 ml fractions were collected at a drop rate of 8 drops per minute. Every 10^{th} fraction was analysed by TLC and the fractions were pooled according to similarity.

2.4.3. Flash Chromatography

A puriFlash 4250 system (Interchim, Montulçon, France) was used. Detection was performed by an integrated photodiode array (PDA) detector and an evaporative light scattering detector (ELSD). The fractions were collected with an automatic fraction collector. As stationary phases PuriFlash-30SIHP/120g, PuriFlash-30SIHP/40g and PuriFlash-30SIHP/25g columns were used. Depending on the polarity of the fraction, the mobile phase was a gradient of $CHCl_3$, MeOH and H_2O (see tables 5, 6, 7 and 8, p. 8, 9).

Table 5: Method for the fractionation of the methanolic extract

Time (min)	Solvents			Flow rate
0-20	98% $CHCl_3$	2% MeOH		20 ml/min
20-30	98% →95% $CHCl_3$	2% →5% MeOH		20 ml/min
30-40	95% $CHCl_3$	5% MeOH		20 ml/min
40-50	95% →90% $CHCl_3$	5% →9% MeOH		20 ml/min
50-60	90% $CHCl_3$	9% MeOH	1% H_2O	20 ml/min
60-70	90% →80% $CHCl_3$	9% →17% MeOH	1% →3% H_2O	20 ml/min
70-80	80% $CHCl_3$	17% MeOH	3% H_2O	20 ml/min
80-90	80% →70% $CHCl_3$	17% →27% MeOH	3% H_2O	20 ml/min
90-100	70% $CHCl_3$	27% MeOH	3% H_2O	20 ml/min

Table 6: Method for the fractionation of fractions F4/FC1 and F5/FC1

Time (min)	Solvents			Flow rate
0-20	100% $CHCl_3$			20 ml/min
20-40	99% $CHCl_3$	1% MeOH		20 ml/min
40-60	98% $CHCl_3$	2% MeOH		20 ml/min
60-63	98% →95% $CHCl_3$	2% →5% MeOH		20 ml/min
63-68	95% $CHCl_3$	5% MeOH		20 ml/min
68-71	95% →70% $CHCl_3$	5% →27% MeOH	3% H_2O	20 ml/min
71-81	70% $CHCl_3$	27% MeOH	3% H_2O	20 ml/min

Table 7: Method for the fractionation of fraction F9/FC1

Time (min)	Solvents			Flow rate
0-3	100% $CHCl_3$			20 ml/min
3-6	99% $CHCl_3$	1% MeOH		20 ml/min
6-9	98% $CHCl_3$	2% MeOH		20 ml/min
9-12	98% →95% $CHCl_3$	2% →5% MeOH		20 ml/min
12-32	95% $CHCl_3$	5% MeOH		20 ml/min
32-37	95% →90% $CHCl_3$	5% →9% MeOH	1% H_2O	20 ml/min
37-47	90% $CHCl_3$	9% MeOH	1% H_2O	20 ml/min
47-50	90% →70% $CHCl_3$	9% →27% MeOH	1%→3% H_2O	20 ml/min
50-65	70% $CHCl_3$	27% MeOH	3% H_2O	20 ml/min

Table 8: Method for the fractionation of fraction Fb5/FC3

Time (min)	Solvents		Flow rate
0-10	100% $CHCl_3$		20 ml/min
10-20	99% $CHCl_3$	1% MeOH	20 ml/min
20-40	98% $CHCl_3$	2% MeOH	20 ml/min
40-50	97% $CHCl_3$	3% MeOH	20 ml/min
50-60	96% $CHCl_3$	4% MeOH	20 ml/min
60-70	95% $CHCl_3$	5% MeOH	20 ml/min

2.5. Spectrometric and Spectroscopic Methods

2.5.1. Mass Spectrometry (MS)

For low resolution and high resolution mass spectrometry an amaZon speed 3D ion trap instrument and a maXis UHR ESI-Qq-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany) were used. Bruker Compass DataAnalysis 4.1 was used to determine the sum formulas of the detected ions [11].

2.5.2. Nuclear Magnetic Resonance Spectroscopy (NMR)

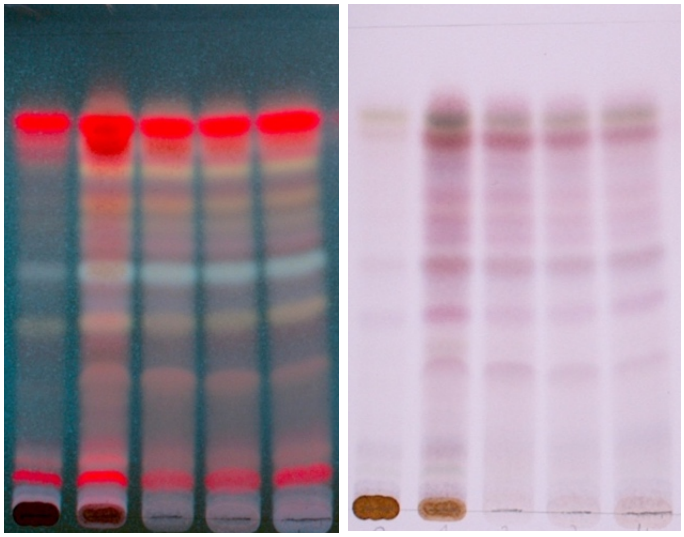
An Avance III HDX 700 MHz NMR spectrometer (Bruker BioSpin, Germany) was used for Nuclear Magnetic Resonance spectroscopy. Processing of all spectra was performed in TopSpin (Bruker BioSpin, Germany) [11].

Temperature at measurement: 298 K
Solvent: fully deuterated chloroform or methanol
Resonance frequency: ^{1}H: 700,40 MHz, ^{13}C NMR: 176,12 MHz

3. Results and Discussion

3.1. Extraction of the Methanolic Extract

The dried methanolic extract was milled and sieved with a sieve no. 710. 1 g of the methanolic extract was extracted by SFE with different amounts of EtOH as co-solvent (parameters see table 3, p. 4). After drying, the composition of the fractions was compared by TLC (see fig. 1).



1	Methanolic extract
2	Fraction (16,7 mg) obtained with CO_2 + 0% EtOH
3	Fraction (7,3 mg) obtained with CO_2 + 3% EtOH
4	Fraction (6,2 mg) obtained with CO_2 + 6% EtOH
5	Fraction (6,3 mg) obtained with CO_2 + 10% EtOH

Fig. 1: TLC of fractions obtained by SFE with different amounts of EtOH as co-solvent

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (97 + 3)

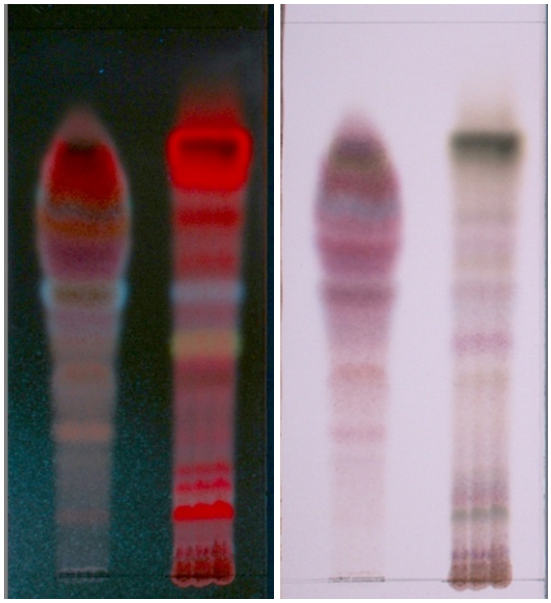
Derivatisation: AAS

Left: UV 366 nm, right: daylight

TLC of the fractions showed that all major apolar compounds including a high amount of chlorophyll were extracted. The polar accompanying substances were removed almost completely. Decreasing amounts of the fractions indicated that the extraction was exhaustive. Due to the similar patterns, all fractions were combined. Another extraction of 1 g methanolic extract was performed without any co-solvent (parameters see table 4, p. 5). This resulted in no detectable impact on the composition of the extract, thus allowing to save resources in subsequent

extractions. Therefore, upscaling to an amount of 16 g of methanolic extract was performed without use of a co-solvent.

Due to technical problems the extraction had to be stopped after three cycles and the remaining methanolic extract was extracted by liquid-liquid extraction. Extraction by partition was tested with small amounts, following portions of 5 g which were extracted with equal amounts of water and chloroform thrice (see ch. 2.3.2., p. 5).



1	SFE extract
2	$CHCl_3$ fraction of extraction by partition

Fig. 2: TLC comparison of extracts obtained by different extraction methods

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (97 + 3)

Derivatisation: AAS

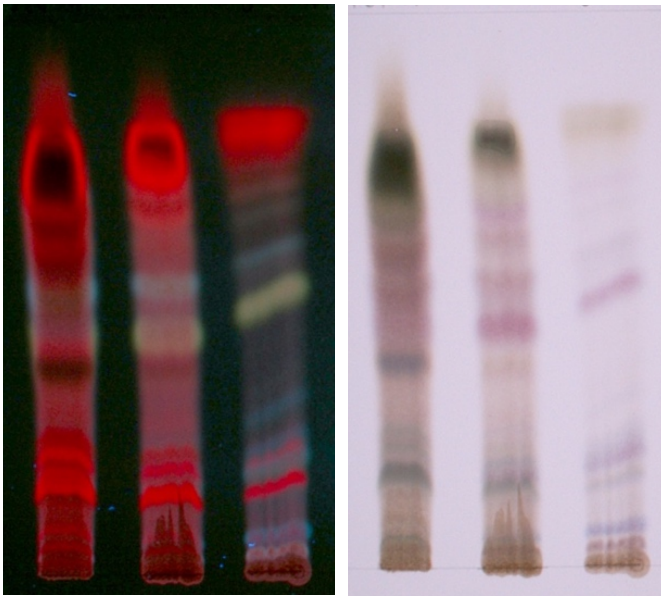
Left: UV 366 nm, right: daylight

The extracts obtained with the different methods were compared by TLC. TLC-analysis clearly showed that the SFE extract contained less chlorophyll than the extract obtained by partitioning (see fig. 2).

In the SFE extract, apolar compounds with an Rf-value higher than 0,5 were found to be enriched, whereas the apolar secondary metabolites of the $CHCl_3$ fraction were difficult to detect due to the high amounts of chlorophyll.

The main compound seen as blue fluorescent band at Rf=0,5 was extracted in both extracts and clearly detectable under UV 366 nm. In contrast, the yellow band at Rf=0,4 was present in the extract obtained by partition, but not in the extract obtained by SFE (see fig. 2, p. 11).

Despite the SFE extraction providing a better enrichment of apolar constituents, the remaining methanolic extract had to be extracted by liquid-liquid extraction due to technical difficulties of the ASFE system, which resulted in tedious and time-consuming maintenance work.



Fraction no.	Extraction cycle	Yield
1	1st cycle	430,6 mg
2	2nd cycle	83,8 mg
3	3rd cycle	29,6 mg

Fig. 3: TLC of extracts from liquid-liquid extraction cycles

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (97 + 3)

Derivatisation: AAS

Left: UV 366 nm, right: daylight

The TLC control proved that threefold partition of the extract between water and chloroform was exhaustive (see fig. 3). Due to high amounts of chlorophyll the secondary metabolites were less visible in the first fraction. Nevertheless, the three fractions were combined and in total, 7,8 g of chloroform extract were obtained.

3.2. Fractionation of the Chloroform Extract

In previous research, fractions of this extract had been purified and secondary metabolites were isolated. Nevertheless, due to the small amounts of some constituents, structure elucidation was not possible for several isolated substances [12]. This work aimed to extend this research by isolating these components as well as other secondary metabolites, to determine their identity using suitable methods.

In order to unravel the apolar compounds, fractionation by flash chromatography was performed. The extract was dissolved in 40 ml $CHCl_3$ and split into 2 portions. To each portion 18 g of silica gel were added and the prepared dry load was loaded on a PuriFlash-30SIHP/120g column for separation. Equilibration was performed with 4 column volumes at starting conditions. Conditions of the method, based on previous research [12], are described in chapter 2.4.3 (see table 5, p. 8).

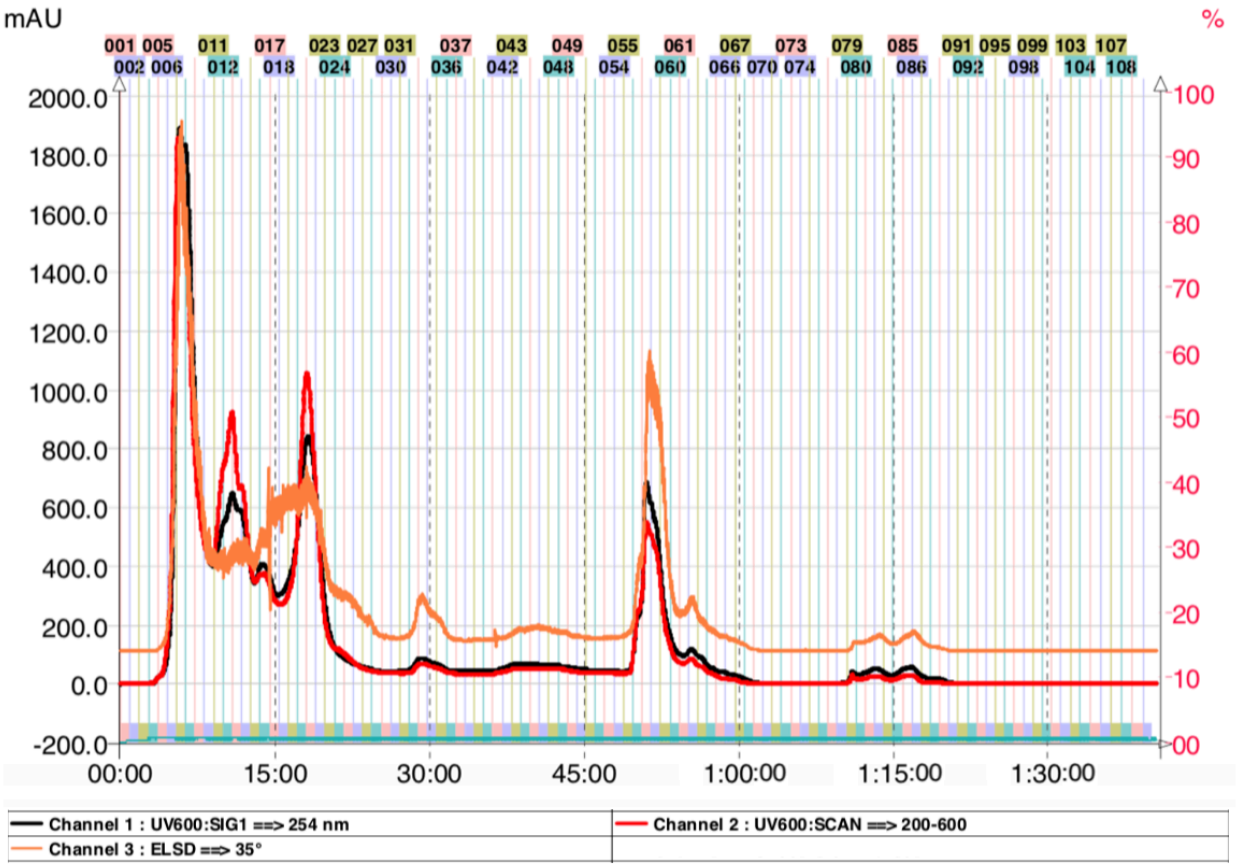


Fig. 4: Flash chromatogram of the first portion of the $CHCl_3$ fraction as detected via ELSD (–), PDA UV scan from 200-600 nm (–) and UV 254 nm (–)

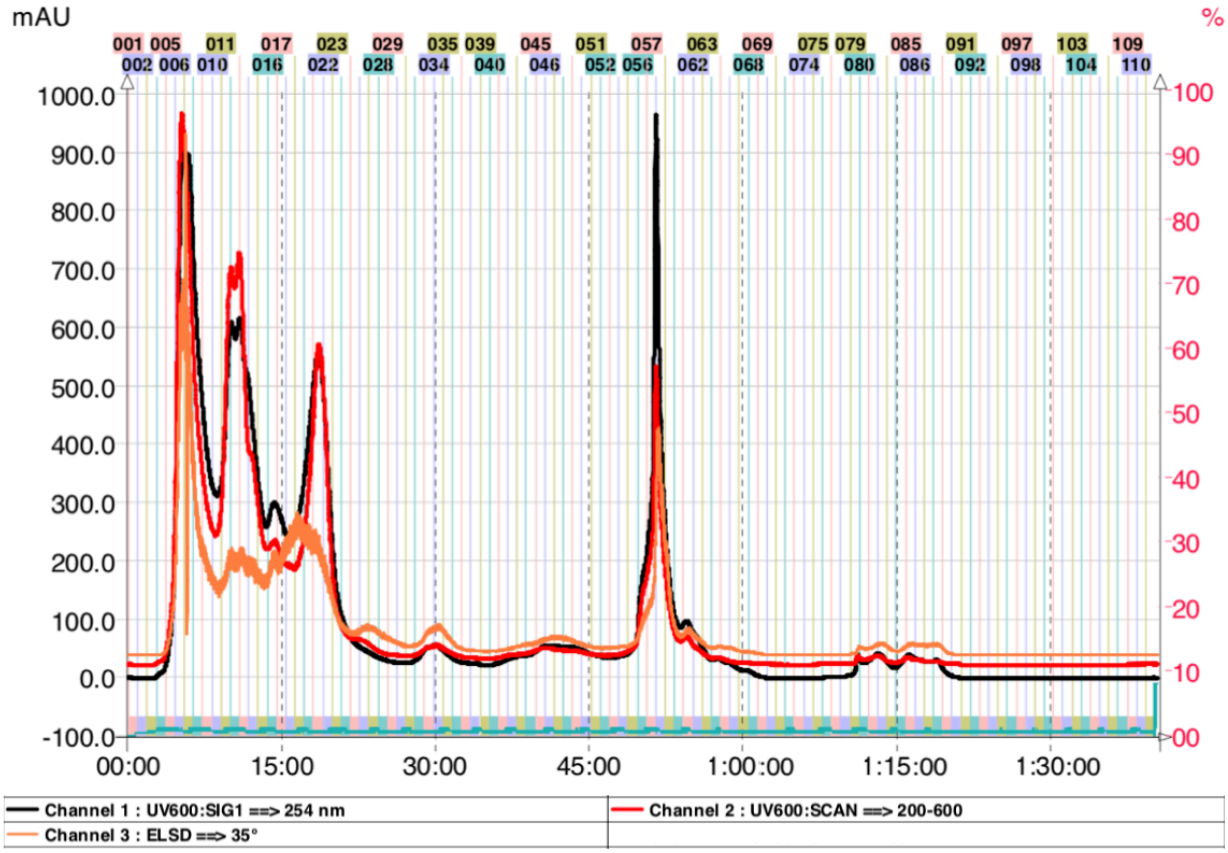


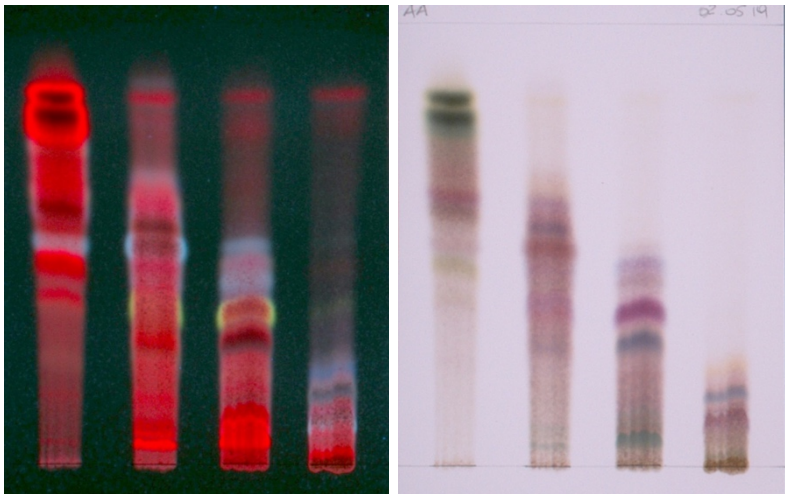
Fig. 5: Flash chromatogram of the second portion of the $CHCl_3$ fraction as detected via ELSD (–), PDA UV scan from 200-600 nm (–) and UV 254 nm (–)

Both detection modes showed that all compounds had been eluted after 1:30:00 h. All fractions were pooled according to the ELSD measurement (see fig. 4, p. 13, fig. 5). The pooled collective fractions are shown below (see table 9, p. 15).

Table 9: Collective fractions of FC1

Collective fraction	Fractions	Yield	Further fractionation
F1	1-4	8,5 mg	–
F2	5-11	2.115,5 mg	–
F3	12-16	940,0 mg	–
F4	17-19	813,4 mg	see chapter 3.2.1., p. 17
F5	20-25	957,1 mg	see chapters 3.2.2 & 3.2.4, p. 20, 25
F6	26-32	258,1 mg	see chapter 3.2.5., p. 28
F7	33-38	230,5 mg	–
F8	39-55	446,6 mg	–
F9	56-60	978,2 mg	see chapter 3.2.3., p. 23
F10	61-65	250,9 mg	–
F11	66-78	93,7 mg	–
F12	79-84	141,8 mg	–
F13	85-91	140,7 mg	–
F14	92-110	14,6 mg	–

To detect secondary metabolites as previously isolated by Wande [12], TLC analysis was performed. In this analysis, blue and pinkish bands were detected under daylight (F4: Rf=0,49/Rf=0,54; F5: Rf=0,35; F6: Rf=0,11/Rf=0,16; F9: Rf=0,50) (see fig. 6, fig. 7, p. 16), which were similar to those of previously isolated substances [12]. Since sufficient amounts were obtained, further purification of fractions F4/FC1, F5/FC1, F6/FC1 and F9/FC1 was performed. Due to the very high amounts of chlorophyll and accompanying substances, fractions F3/FC1, F7/FC1 and F8/FC1 were not further investigated.



F3 F4 F5 F6 F3 F4 F5 F6

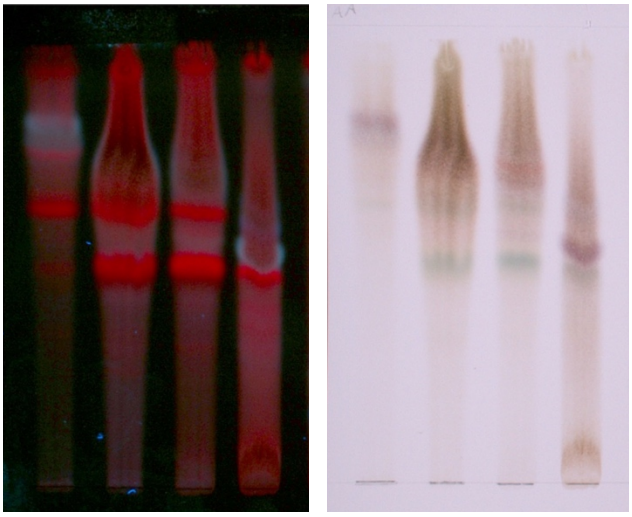
Fig. 6: TLC of fractions F3 – F6 of FC1

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (97 + 3)

Derivatisation: AAS

Left: UV 366 nm, right: daylight



F6 F7 F8 F9 F6 F7 F8 F9

Fig. 7: TLC of fractions F6 – F9 of FC1

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH-H_2O (79 + 19 + 2)

Derivatisation: AAS

Left: UV 366 nm, right: daylight

3.2.1. Fractionation of F4/FC1

Fraction F4/FC1 (813,4 mg) was fractionated in FC2 by flash chromatography with the method described in chapter 2.4.3. (see table 6, p. 8). The aim of this fractionation was the purification and separation of the two detected main compounds at Rf=0,49 and Rf=0,54 (see fig. 6, p. 16). This was achieved by removing chlorophyll and further accompanying substances from these compounds.

The dry load was prepared with approx. 4 g of silica gel. As stationary phase served a PuriFlash-30SIHP/40g column. The fractionation had a duration of 80 minutes and was monitored by ELSD and UV-DAD. 179 fractions were collected (9 ml/fraction) and pooled according to ELSD detection into 17 fractions (see fig. 8, table 10, p. 18).

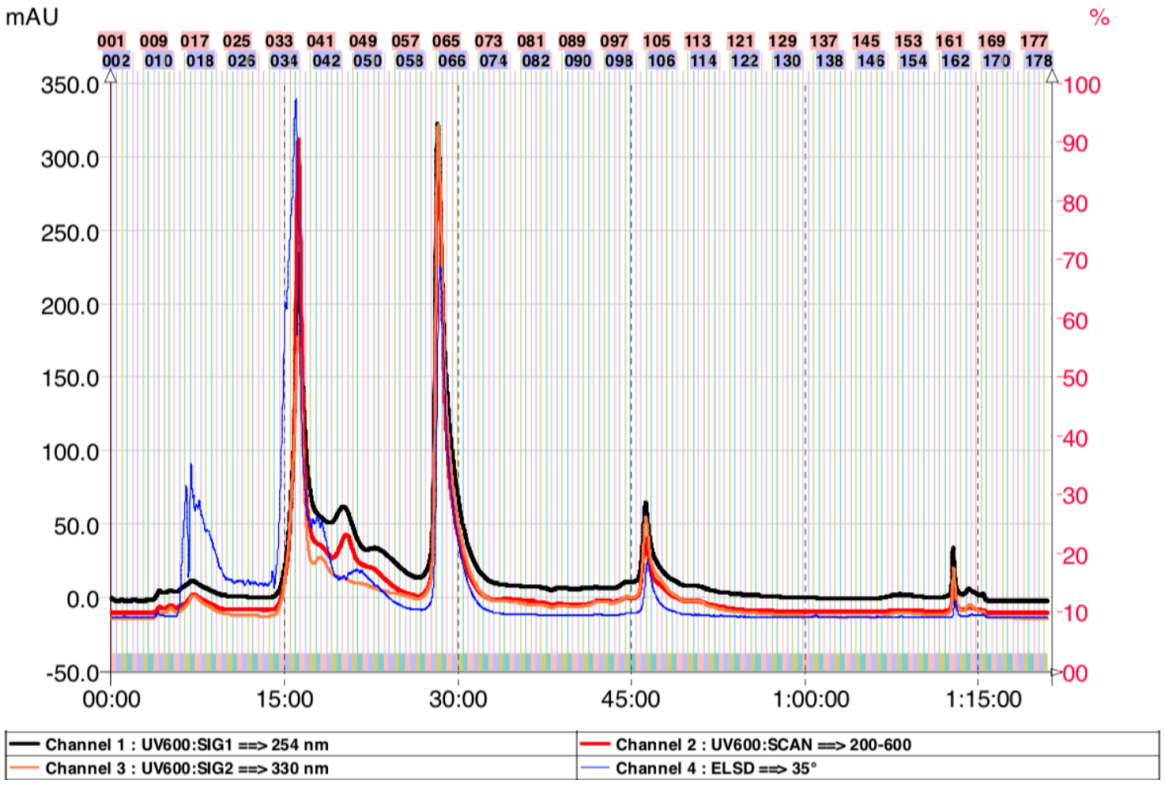


Fig. 8: Flash chromatogram of fraction F4/FC1 as detected via ELSD (–), PDA UV scan from 200-600 nm (–), UV 254 nm (–) and UV 330 nm (–)

Table 10: Collective fractions of FC2

Collective fraction	Fractions	Yield
Fa1	1-13	9,8 mg
Fa2	14-15	32,1 mg
Fa3	16-22	70,0 mg
Fa4	23-31	52,5 mg
Fa5	32-38	23,7 mg
Fa6	39-43	66,4 mg
Fa7	44-49	46,6 mg
Fa8	50-60	45,9 mg

Collective fractions Fa1, Fa9 – Fa17 were not further investigated as they contained high amounts of chlorophyll. In fractions Fa3 and Fa4 the same major compound with small amounts of accompanying substances was detected (see fig. 9). Thus, these two fractions were selected for further studies.

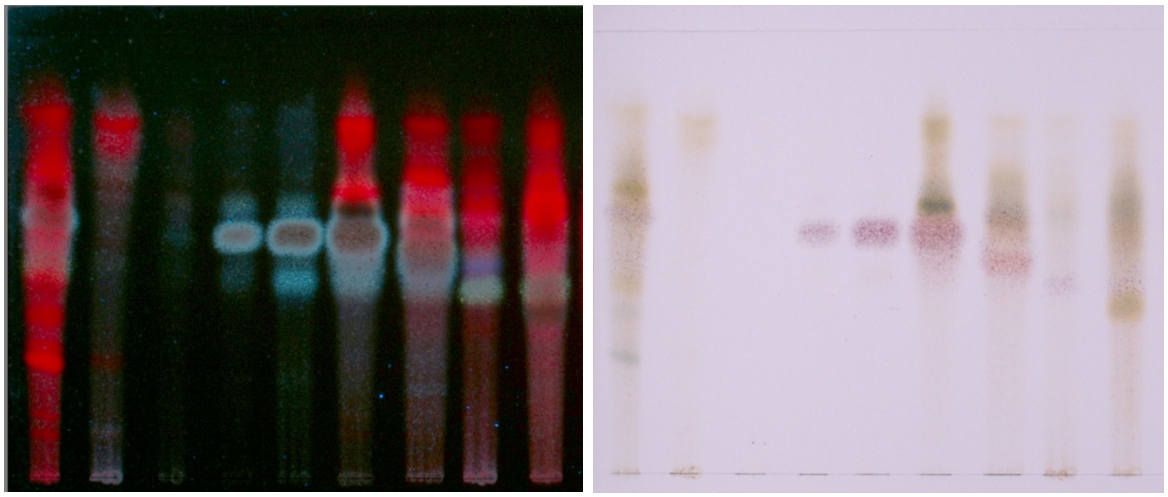


Fig. 9: TLC of fractions of FC2

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (97 + 3)

Derivatisation: AAS

Left: UV 366 nm, right: daylight

Fraction Fa4 was compared with several triterpenic acids and abscisic acid, which are known as secondary metabolites in the genus Combretum. The triterpenic acids showed similar Rf-values and colour after detection with AAS under daylight, but the major compound in fraction Fa4 was slightly more apolar than the authentic references and its fluorescence was clearly different (see fig. 10). In RP-TLC the differences between this compound and the acids were much more pronounced (see fig. 11, p. 20).

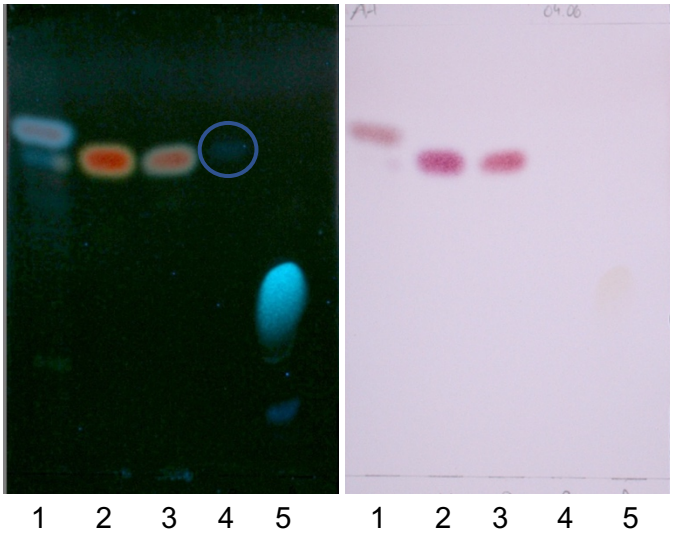


Fig. 10: TLC of fraction Fa4 in comparison to several acids

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (97 + 3)

Derivatisation: AAS

Left: UV 366 nm, right: daylight

1	Fa4
2	Ursolic acid
3	Oleanolic acid
4	Betulinic acid
5	Abscisic acid

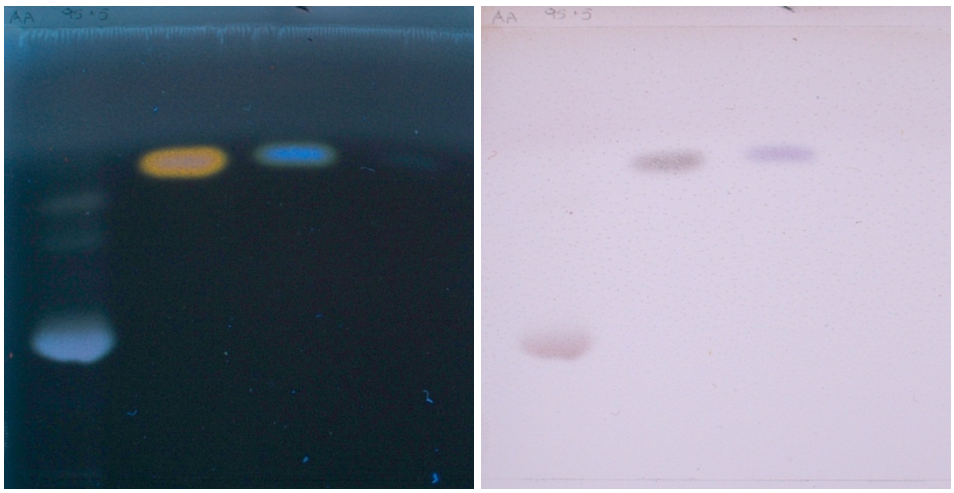


Fig. 11: RP-TLC of fraction Fa4 in comparison to several triterpenic acids

Stationary phase: RP-8 F_{254} for nano TLC

Mobile phase: MeOH-H_2O (95 + 5)

Derivatisation: AAS

Left: UV 366 nm, right: daylight

1	Fa4
2	Ursolic acid
3	Oleanolic acid
4	Betulinic acid

Fraction Fa4 was further analysed by NMR and identified as ß-sitosterol. A TLC comparison with the authentic substance confirmed these results (see fig. 12, p. 21).

1 2

1	Fa4
2	ß-sitosterol

Fig. 12: TLC comparison of fraction Fa4 with ß-sitosterol

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (97 + 3)

Derivatisation: AAS, daylight

3.2.2. Fractionation of F5/FC1

Fraction F5/FC1 (957,1 mg) contained two major compounds, which were detected at Rf=0,28 and Rf=0,35 with bluish and pink colour after spraying with AAS, respectively. The compounds showed a similar polarity as those in fraction F4/FC1, therefore fractionation was performed under the same conditions to obtain separation and purification (see table 6, p. 8). The dry load was prepared by dissolution of the fraction in 14 ml $CHCl_3$ and addition of 4,2 g silica gel. The stationary phase was a PuriFlash-30SIHP/40g column, which was equilibrated with 4 column volumes at starting conditions. Detection was performed with the ELSD and UV-DAD.

191 fractions were obtained (9 ml/fraction) and pooled according to ELSD detection into 20 fractions (see fig. 13, table 11, p. 22). Due to the small amounts, Fb16 – Fb20 were not further investigated.

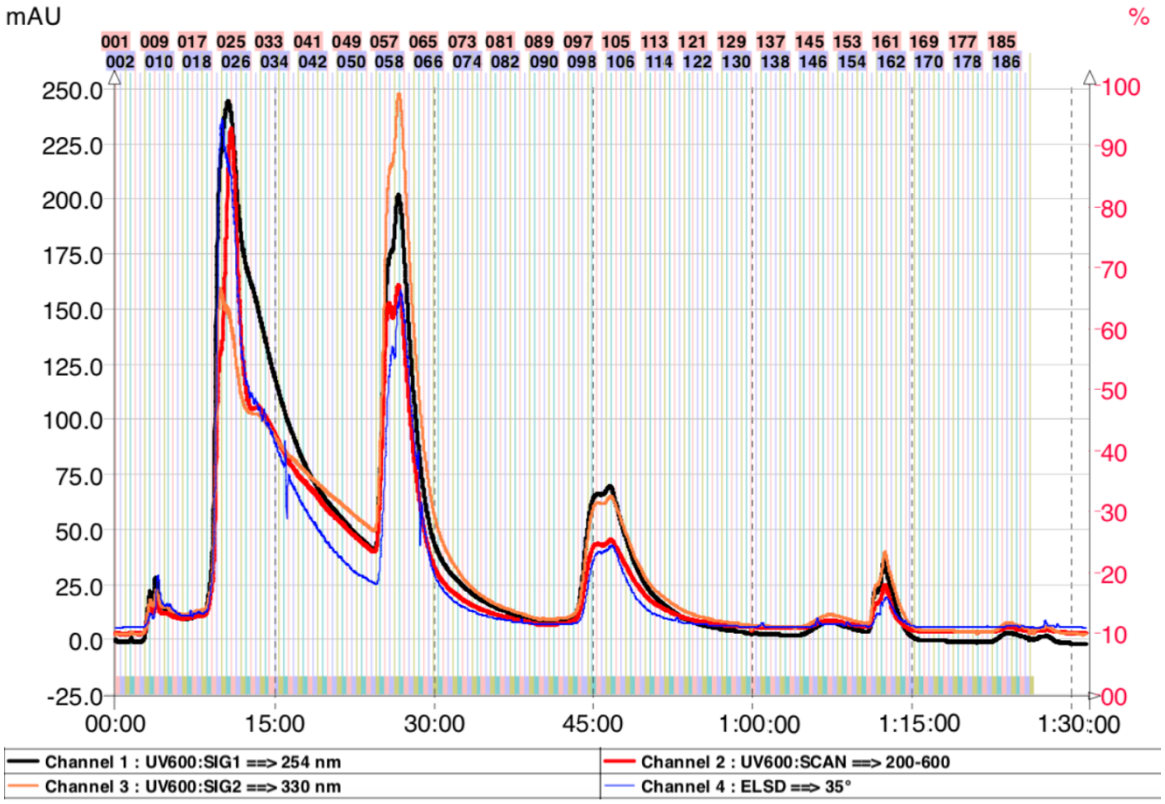


Fig. 13: Flash chromatogram of fraction F5/FC1 as detected via ELSD (–), PDA UV scan from 200-600 nm (–), UV 254 nm (–) and UV 330 nm (–)

Table 11: Collective fractions of FC3

Collective fraction	Fractions	Yield	Combined fractions
Fb1	1-8	8,8 mg	
Fb2	9-10	10,1 mg	see chapter 3.2.4, p. 25
Fb3	11-13	10,8 mg	
Fb4	14-20	21,3 mg	
Fb5	21-28	245,2 mg	
Fb6	29-36	119,6 mg	
Fb7	37-54	77,5 mg	
Fb8	55-58	53,6 mg	
Fb9	59-66	114,2 mg	
Fb10	67-96	69,7 mg	
Fb11	97-101	26,7 mg	
Fb12	102-112	54,9 mg	
Fb13	113-122	16,3 mg	
Fb14	123-143	14,3 mg	
Fb15	144-156	11,8 mg	

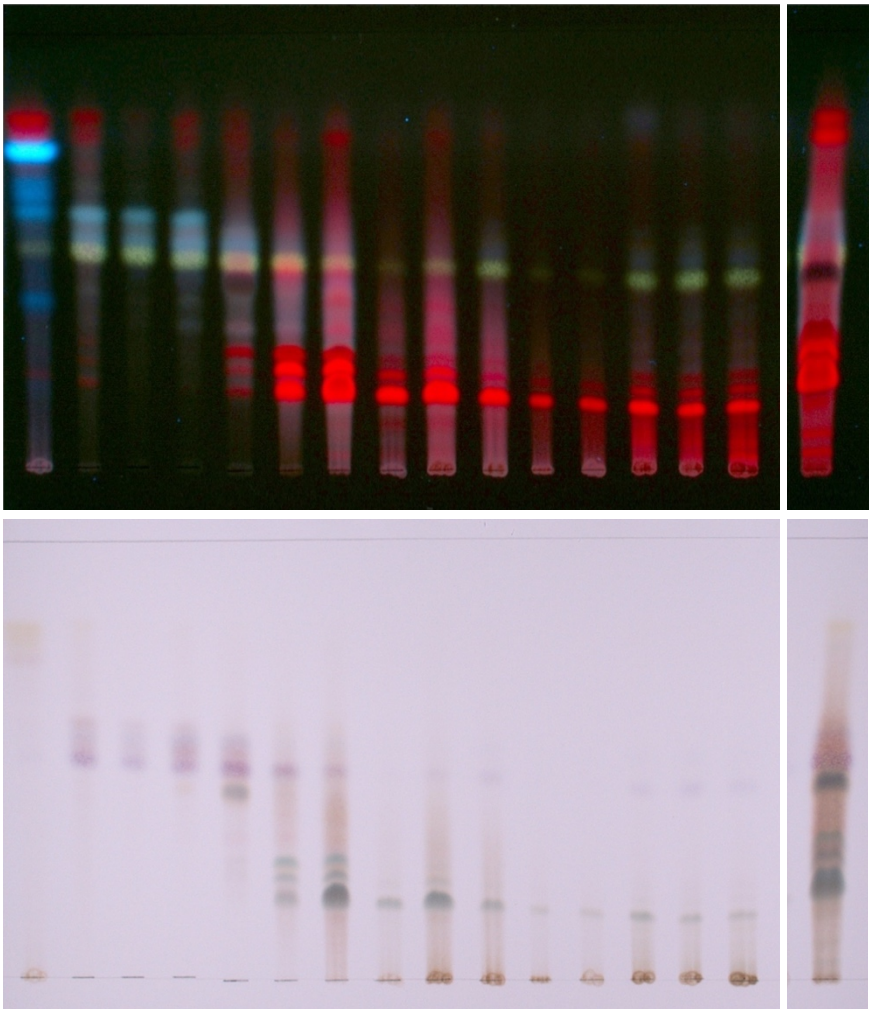


Fig. 14: TLC of fractions of FC3

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (97 + 3)

Derivatisation: AAS

upper: UV 366 nm, lower: daylight

Fractions Fb2 – Fb5 contained the two major compounds of fraction F5/FC1 (see fig. 14) and were combined and fractionated by flash chromatography FC5 (see ch. 3.2.4, p. 26). In fractions Fb6 – Fb15 high amounts of chlorophyll were successfully removed from the secondary metabolites in fractions Fb2 – Fb5. Thus, these fractions were not studied further. The amount of fraction Fb1 was too low for further investigation.

3.2.3. Fractionation of F9/FC1

To purify the main compound, a pinkish band at Rf=0,5 under daylight (see fig. 7, p. 16), fraction F9/FC1 (978,2 mg) was further fractionated by flash chromatography. Due to the higher polarity of the major compound, the separation method had to be adapted accordingly (see table 7, p. 8). The dry load was prepared by dissolution of the fraction in $CHCl_3$ and addition of approx. 4 g silica gel. A PuriFlash-30SIHP/40g column served as stationary phase. Four column volumes $CHCl_3$ were used for equilibration and in 60 minutes, 133 fractions (9 ml/fraction) were collected. They were pooled according to ELSD detection into 11 fractions (see fig. 15, table 12, p. 25).

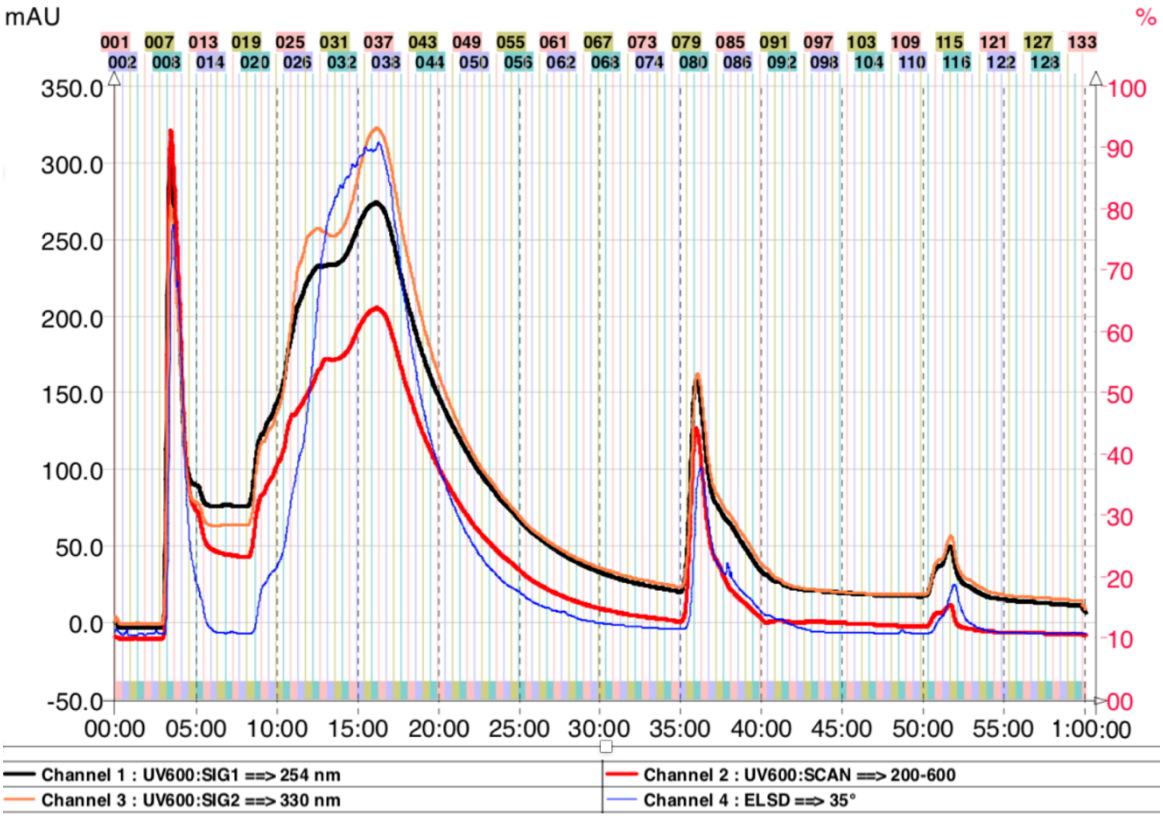
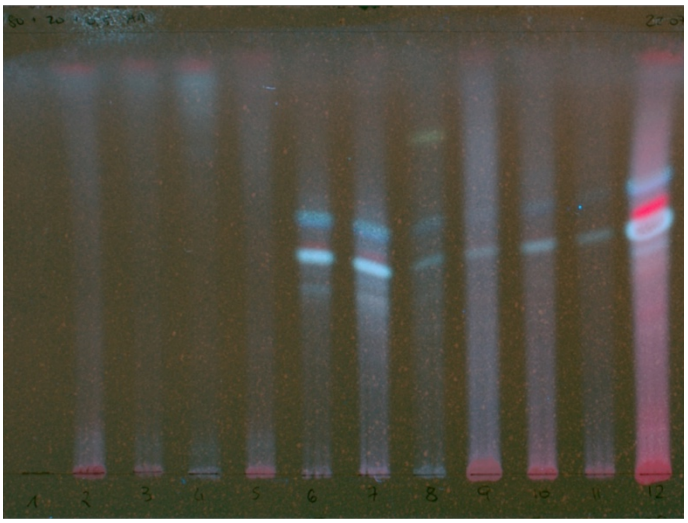


Fig. 15: Flash chromatogram of fraction F9/FC1 as detected via ELSD (–), PDA UV scan from 200-600 nm (–), UV 254 nm (–) and UV 330 nm (–)

Table 12: Collective fractions of FC4

Collective fraction	Fractions	Yield
Fc1	1-6	1,1 mg
Fc2	7-10	66,6 mg
Fc3	11-13	13,9 mg
Fc4	14-18	2,8 mg
Fc5	19-23	24,8 mg
Fc6	24-35	301,4 mg
Fc7	36-49	271,4 mg
Fc8	50-77	89,3 mg
Fc9	78-82	63,2 mg
Fc10	83-111	47,4 mg
Fc11	112-133	48,5 mg



Fc 1 2 3 4 5 6 7 8 9 10 11 F9/FC1

Fig. 16: TLC of fractions of FC4

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH-H_2O (79 + 19 + 2)

Derivatisation: AAS, UV 366 nm

Fractions Fc2 – Fc5 contained two apolar compounds detected at Rf=0,88 and Rf=0,91 and further polar substances at low Rf-values. The strongly fluorescent blue bands in fractions Fc6 and Fc7 at approx. Rf=0,48 and Rf=0,57 indicated high amounts of these main compounds. In fractions Fc8 – Fc11, these blue bands were detected with lower fluorescence, accompanied by polar impurities. A yellow fluorescent band at Rf=0,85 was only detected in fraction Fc8 (see fig. 16).

Further combination and purification was not possible within this thesis due to limited time.

3.2.4. Fractionation of FC5/FC3

Fractions Fb2 – Fb5 (see ch. 3.2.2., p. 21), containing two main compounds at Rf=0,42 and Rf=0,48, were combined (287,4 mg) and further separation was performed by flash chromatography. The aim was to separate these constituents and to remove chlorophyll and other accompanying substances. The method was adapted to the polarity of the combined fractions (see table 8, p. 9).
For the preparation of the dry load the collective fraction was dissolved in $CHCl_3$ and 1 g of silica gel was added. As stationary phase served a PuriFlash-30SIHP/25g column, which was equilibrated with 4 column volumes at starting conditions.

155 fractions were pooled into 19 fractions according to ELSD detection (see fig. 17, table 13, p. 27). Fractions $Fb_5$9 – $Fb_5$19 were not subject of further research as they consisted mainly of polar compounds, which were not investigated in this thesis.

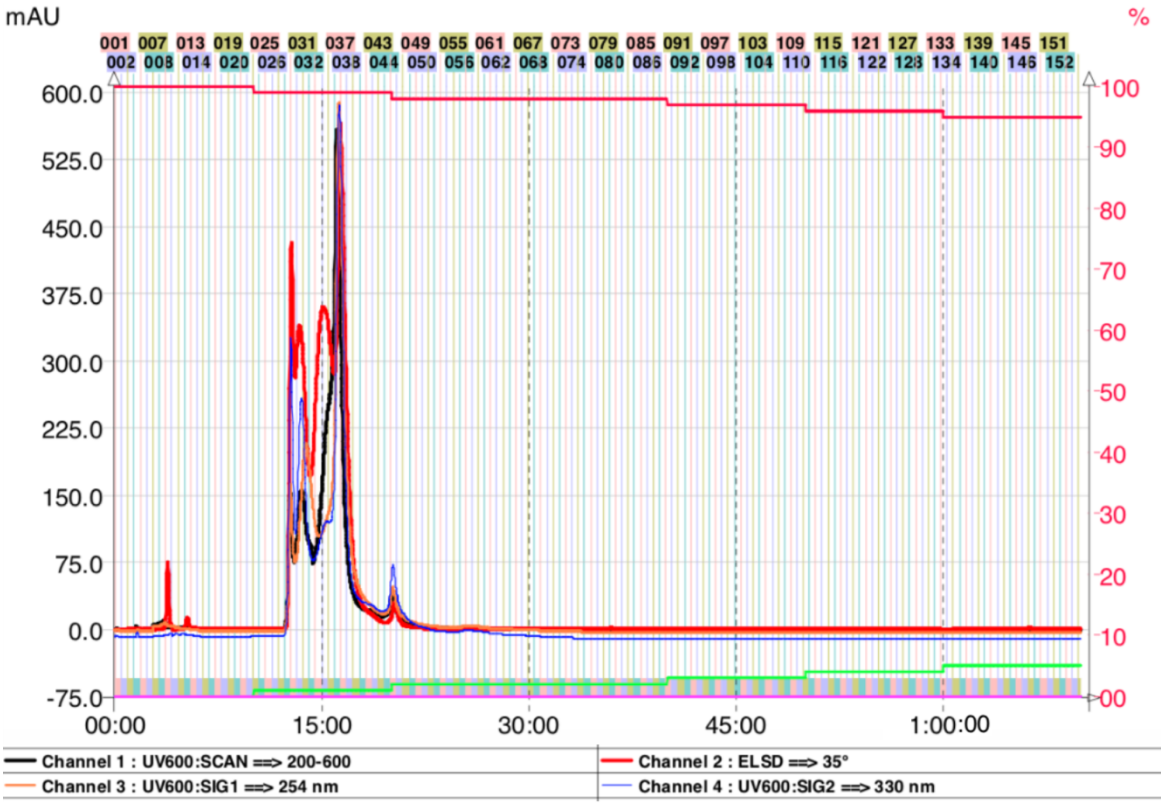


Fig. 17: Flash chromatogram of fractions Fb2 – Fb5 as detected via ELSD (–), PDA UV scan from 200-600 nm (–), UV 254 nm (–) and UV 330 nm (–)

Table 13: Collective fractions of FC5

Collective fraction	Fractions	Yield
$Fb_5$1	1-6	0,7 mg
$Fb_5$2	7-10	7,3 mg
$Fb_5$3	11-13	3,2 mg
$Fb_5$4	14-27	4,6 mg
$Fb_5$5	28-29	31,3 mg
$Fb_5$6	30-31	35,4 mg
$Fb_5$7	32-35	93,0 mg
$Fb_5$8	36-37	67,0 mg

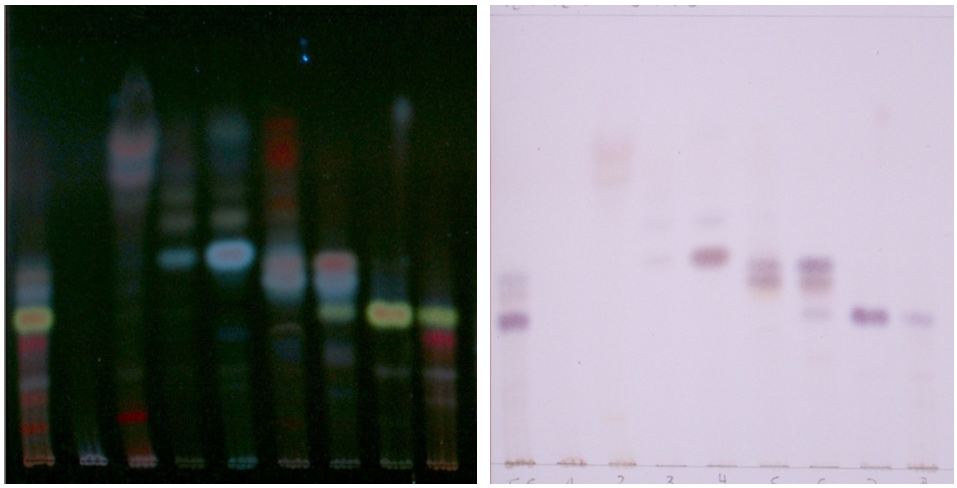


Fig. 18: TLC of fractions of FC5

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (97 + 3)

Derivatisation: AAS

Left: UV 366 nm, right: daylight

Fraction $Fb_5$7 contained a compound which seemed chromatographically uniform in the TLC analysis and was examined by NMR-analysis. This pink band at Rf=0,33 was identified as a mixture of ursolic acid and oleanolic acid, in a ratio of 1:0,5. This mixture of triterpenic acids was the main compound in fraction $Fb_5$8 as well, including further impurities. A small amount of this mixture was also detected in fraction $Fb_5$6, alongside two main constituents with blue and red fluorescence at Rf=0,41 and Rf=0,45 under UV 366 nm. These two compounds were detected in a different ratio as main constituents of fraction $Fb_5$5, too. The main compound of fraction $Fb_5$4, seen as violet band at Rf=0,46 under daylight, was confirmed to be ß-sitosterol by comparison with authentic material (see fig. 18, p. 27). The amounts of fractions $Fb_5$1 – $Fb_5$3 were too low for further analysis. Due to limited time, further purification was not possible within this thesis.

3.2.5. Fractionation of F6/FC1

Due to technical problems of the puriFlash 4250 system, fraction F6/FC1 (258,1 mg) was fractionated by column chromatography (conditions see below). Every 10th fraction was analysed by TLC. Fractions with similar patterns were pooled into 12 fractions (see table 15, p. 30).

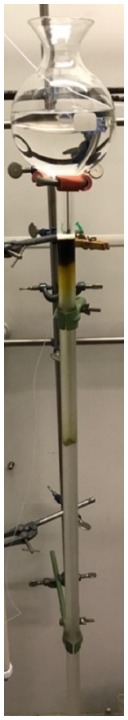


Fig. 19: CC1

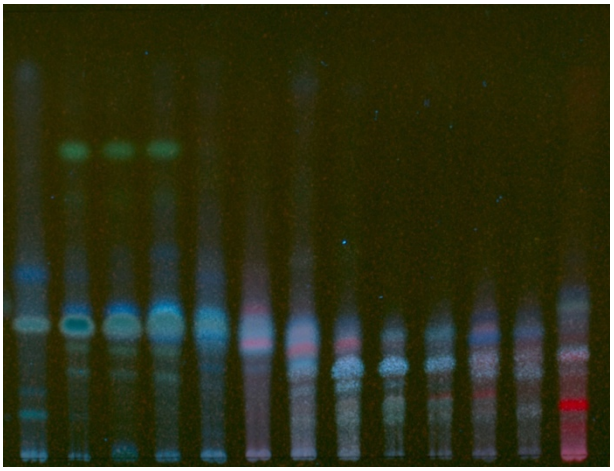
stationary phase: Silica gel 60 (Merck)
mobile phase: $CHCl_3$-MeOH (composition see table 14)
dimensions: d=2 cm, h=93 cm
flow rate: 10 ml / 25 min

Table 14: Mobile phases and fractions of CC1

Mobile phase		Fractions
98% $CHCl_3$	2% MeOH	1-73
97% $CHCl_3$	3% MeOH	74-189
96% $CHCl_3$	4% MeOH	190-218

Table 15: Collective fractions of CC1

Collective fraction	Fractions	Yield
Fd1	1-20	1,8 mg
Fd2	21-40	6,6 mg
Fd3	41-48	3,6 mg
Fd4	49-78	8,8 mg
Fd5	79-128	10,0 mg
Fd6	129-148	10,4 mg
Fd7	149-160	7,4 mg
Fd8	161-167	12,2 mg
Fd9	168-178	26,2 mg
Fd10	179-198	15,6 mg
Fd11	199-208	16,7 mg
Fd12	209-218	17,1 mg



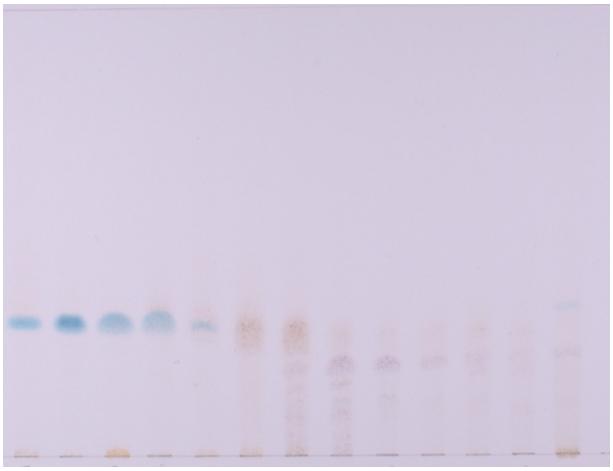
Fd 1 2 3 4 5 6 7 8 9 10 11 12 F6

Fig. 20: TLC of fractions of CC1 under UV 366 nm

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (95 + 5)

Derivatisation: AAS, UV 366 nm



Fd 1 2 3 4 5 6 7 8 9 10 11 12 F6

Fig. 21: TLC of fractions of CC1 under daylight

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: $CHCl_3$-MeOH (95 + 5)

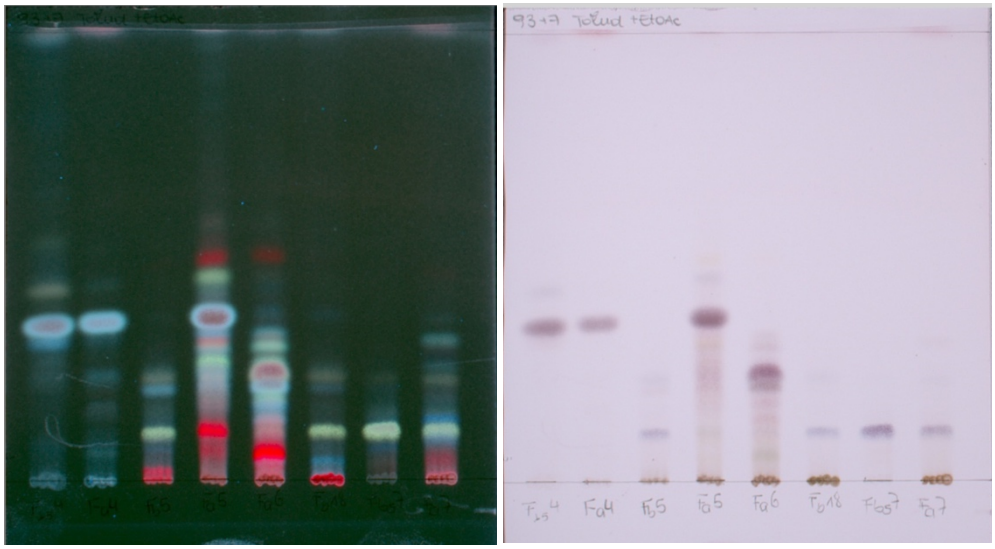
Derivatisation: AAS, daylight

In fractions Fd1 – Fd5 the main compound was visible as blue band at Rf=0,30 after detection with AAS under daylight. Under UV 366 nm, blue and yellow fluorescent impurities were detected in addition to the turquoise fluorescent main compound. In fractions Fd2 – Fd4 the mixture of ursolic acid and oleanolic acid was detected as yellow band at Rf=0,69. A combination of these fractions could be considered to gain a sufficiently high amount for further analysis. Fractions Fd6 – Fd7 were detected as a complex mixture of components and no further purification was performed due to the complexity and insufficient amounts available. A blue fluorescent band at Rf=0,20 was detected as major compound in fractions Fd8 – Fd10, which was visible as violet band under daylight. These fractions could be combined for further purification and isolation of this compound. Fractions Fd11 – Fd12 were a complex mixture of polar substances not promising for the isolation of any single compound (see fig. 20, p. 30, fig. 21).

3.2.6. Comparison of Different Subfractions

Comparison of several subfractions from fractions FC2/FC1, FC3/FC1 and FC5/FC3 was performed by TLC to identify identical constituents.

By TLC, ß-sitosterol was detected as main compound in fractions Fa4, $Fb_5$4 and Fa5. The main compounds of $Fb_5$7, which were identified as a mixture of ursolic acid and oleanolic acid, were present in fractions Fb5, Fb18 and Fa7 as well (see fig. 22).



1 2 3 4 5 6 7 8 1 2 3 4 5 6 7 8

Fig. 22: TLC comparison of subfractions from Fa, Fb and Fb5

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: Toluol-EtOAc (93 + 7)

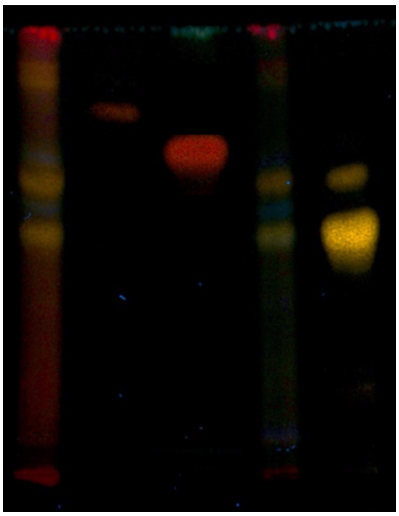
Derivatisation: AAS

Left: UV 366 nm, right: daylight

1	$Fb_5$4
2	Fa4
3	Fb5
4	Fa5
5	Fa6
6	Fb18
7	$Fb_5$7
8	Fa7

3.2.7. Detection of Flavonoids

Due to yellowish, green and orange fluorescent compounds after spraying with NP/PEG, fractions F12/FC1 (141,8 mg) and F13/FC1 (140,7 mg) were analysed by TLC for the occurrence of flavonoids known from other species of the genus Combretum. Nine different flavonoids were chosen, regarding their polarity and their fluorescence after detection with NP/PEG in other TLC analyses.



1 2 3 4 5

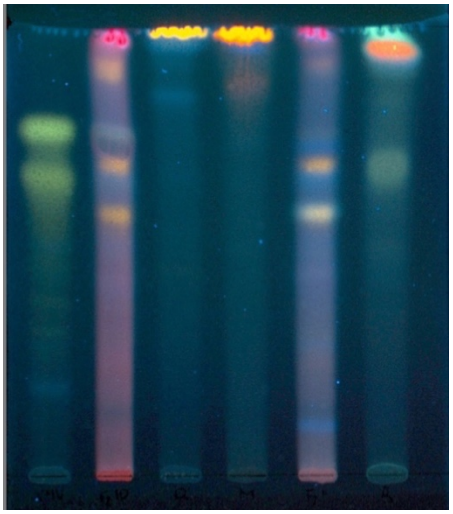
Fig. 23: TLC comparison of F12/FC1 and F13/FC1 with several flavonoids (1)

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: EtOAc-$FA_{conc.}$-$AA_{conc.}$-H_2O (100 + 11 + 11 + 26)

Derivatisation: NP + PEG, UV 366 nm

1	F12/FC1
2	Quercitrin
3	Myricitrin
4	F13/FC1
5	Homoorientin & Orientin



1 2 3 4 5 6

Fig. 24: TLC comparison of F12/FC1 and F13/FC1 with several flavonoids (2)

Stationary phase: silica gel 60 F_{254}-precoated plates

Mobile phase: EtOAc-$FA_{conc.}$-$AA_{conc.}$-H_2O (100 + 11 + 11 + 26)

Derivatisation: NP + PEG, UV 366 nm

1	Vitexin & Isovitexin
2	F12/FC1
3	Quercetin
4	Myricetin
5	F13/FC1
6	Robinetin

Homoorientin (Rf=0,53) and orientin (Rf=0,65) were confirmed as major constituents in fractions F12/FC1 and F13/FC1 due to a comparison with reference substances (see fig. 23, p. 33). Vitexin (Rf=0,65) and isovitexin (Rf=0,75) were confirmed as constituents of these fractions as well (see fig. 24)

For identification of the substance detected as orange band at Rf=0,89 in fractions F12/FC1 and F13/FC1 further investigation is needed, since none of the available references matched.

As a result of this comparison, 4 flavonoids were identified as constituents of *Combretum zenkeri*.

4. Abstract

Plants of the Combretaceae family are used for numerous different indications in Africa and Asia [3]. For instance, twigs of *Combretum zenkeri* Engl. & Diels, a species widely distributed in western Africa, relieve menstrual pain when chewed. A decoction of the leaves of *Combretum zenkeri* is used as vermifuge, purgative and to treat malaria [6].

As this species has hardly been investigated phytochemically, it is not known which compounds are responsible for the antimalaria effect. The aim of this thesis was therefore the isolation and characterisation of secondary metabolites of *Combretum zenkeri* under optimisation of methods for the fractionation.

In previous work, a methanolic extract and its apolar fractions were successfully tested in an antimalaria essay [12]. Thus, in this study polar compounds of this extract were removed with supercritical fluid extraction and liquid-liquid extraction.

The obtained apolar fractions were further separated by flash chromatography and column chromatography. By comparison with authentic substances by TLC, the presence of orientin, homoorientin, vitexin and isovitexin, which had been already detected in Combretaceae, was confirmed.

NMR-analysis confirmed oleanolic acid and ursolic acid as well as β-sitosterol as constituents of *Combretum zenkeri*.

5. Zusammenfassung

Pflanzen aus der Familie der Combretaceae werden sowohl in Afrika als auch in Asien in der traditionellen Medizin für zahlreiche Indikationen verwendet. Das Kauen der Rinde der in Westafrika vorkommenden Art *Combretum zenkeri* Engl. & Diels dient beispielsweise der Linderung von Menstruationsschmerzen. Ein Dekokt der Blätter von *Combretum zenkeri* wird als Anthelmintikum und Abführmittel sowie zur Behandlung von Malaria eingesetzt [6].

Da diese Art phytochemisch wenig erforscht ist, ist nicht bekannt, welche Inhaltsstoffe für die Antimalaria-Wirkung verantwortlich sind. Das Ziel dieser Diplomarbeit war daher die Isolierung und Charakterisierung von Sekundärmetaboliten aus *Combretum zenkeri* sowie die Optimierung von Fraktionierungsmethoden.

Der im Rahmen einer Dissertation hergestellte Methanolextrakt, welcher bereits erfolgreich auf Antimalaria-Wirkung untersucht worden war [12], konnte durch Supercritical Fluid Extraction und Flüssig-Flüssig-Extraktion vom Großteil seiner polaren Bestandteile befreit werden.

Die so gewonnenen apolaren Inhaltsstoffe wurden durch Flash Chromatographie bzw. Säulenchromatographie fraktioniert. In zwei dieser Fraktionen gelang es, durch Vergleiche mit Reinsubstanzen mit einer dünnschichtchromatographischen Analyse Orientin, Homoorientin, Vitexin und Isovitexin als Inhaltstoffe zu bestätigen, welche bereits in der Gattung Combrataceae nachgewiesen worden waren.

Mittels NMR-Analysen konnten auch Oleanolsäure, Ursolsäure und β-Sitosterol als Inhaltsstoffe von *Combretum zenkeri* bestätigt werden.

6. References

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I. Tables and Figures